Brain Research Bulletin 105 (2014) 36–45

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Review

Does PKM(zeta) maintain memory?

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ARTICLE INFO

Article history:
Received 12 August 2013
Received in revised form
16 September 2013
Accepted 19 September 2013
Available online 26 September 2013

Keywords:
Protein kinase Mzeta
PKM
ZIP
Memory maintenance
Epigenetics
Restabilization

ABSTRACT

Work on the long-term stability of memory has identified a potentially critical role for protein kinase Mzeta (PKMζ) in maintaining established memory. PKMζ, an autonomously active isoform of PKC, is hypothesized to sustain those changes that occurred during memory formation in order to preserve the memory engram over time. Initial studies investigating the role of PKMζ were largely successful in demonstrating a role for the kinase in memory maintenance; disrupting PKMζ activity with ζ-inhibitory peptide (ZIP) was successful in disrupting a variety of established associations in a number of key brain regions. More recent work, however, has questioned both the role of PKMζ in memory maintenance and the effectiveness of ZIP as a specific inhibitor of PKMζ activity. Here, we outline the research both for and against the idea that PKMζ is a memory maintenance mechanism and discuss how these two lines of research can be reconciled. We conclude by proposing a number of studies that would help to clarify the role of PKMζ in memory and define other mechanisms the brain may use to maintain memory.

Contents

1. Introduction

Understanding the neural mechanisms that support long-term memory formation and storage is a fundamental goal of neuroscience. Historically, a large majority of this research has focused on identifying the molecular components of long-term memory formation and, accordingly, a broad understanding of the memory acquisition process has gradually emerged. Until recently, far less work was dedicated to finding the neural mechanisms that support the long-term maintenance of established memories. The discovery of the putative memory maintenance molecule protein kinase Mzeta (PKMζ) shifted attention from induction of memory to its maintenance. How memories are maintained and updated over time is now a topic of considerable interest.

The idea that memory could be actively maintained by enzymatic activity was initially proposed by Francis Crick (1984), who hypothesized that enzymes could actively replace synaptic proteins without changing a synapse's overall activity state. From this perspective, changes that occur during learning could be maintained through persistently active kinases that continuously replace synaptic proteins as they degrade. Early work in the sea snail *Aplysia* identified a number of kinases that show this type of

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http://dx.doi.org/10.1016/j.brainresbull.2013.09.005

persistent activity following synaptic stimulation, including PKA (cAMP-dependent protein kinase A), CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), and PKC (protein kinase C) (Schwartz and Greenberg, 1987; Schwartz, 1993). Blocking these proteins, however, affected the development of intermediate-term facilitation, rather than the maintenance of long-term potentiation (Sutton and Carew, 2000). Nonetheless, research searching for a persistently active "maintenance" enzyme continued to target PKC, which is known to be cleaved *in vitro* to a persistently active PKM form in which the catalytic portion of the molecule exists in the absence of the inhibitory domain (Inoue et al., 1977; Takai et al., 1977). A thorough analysis of the expression of each PKC isoform subsequently revealed that the only PKM found in the mammalian hippocampus matched the PKCζ isoform (Sacktor et al., 1993; see Sacktor, 2008 for review). PKMζ was therefore identified as the isoform of PKC that had the potential to be persistently active during memory maintenance.

Initial research on PKMζ in memory suggested that the molecule acts as a general maintenance mechanism capable of actively sustaining a variety of established associations throughout the brain. More recent work, however, has questioned both the role of PKMζ in memory maintenance and the specificity of the pharmacological agent used to inhibit PKMζ in these studies. Here, we present the evidence both for and against the idea that PKMζ is a general neural mechanism for long-term memory maintenance and outline future studies that will be useful in revealing how established memories are maintained over time.

2. Protein Kinase Mzeta

PKMζ is an atypical isoform of protein kinase C (PKC) that is believed to actively maintain those cellular changes that occur during memory formation in order to preserve the memory engram over time. In vitro work has demonstrated that PKMζ is both necessary and sufficient to maintain established long-term potentiation (LTP) (Ling et al., 2002), the putative cellular mechanism for memory. A wealth of work from Todd Sacktor's lab at the SUNY Downstate Medical Center has succeeded in characterizing the structure, synthesis, and mechanisms of PKMζ (see Sacktor, 2012 for review). A standard PKCζ molecule consists of an amino-terminal regulatory domain connected to a carboxy-terminal catalytic domain by a small hinge region. Under resting conditions, a pseudosubstrate sequence on the regulatory domain of the PKCζ molecule binds to the catalytic domain, effectively inactivating the molecule until stimulation occurs and temporarily relieves the autoinhibition of the molecule (Newton, 2003; Nishizuka, 1988; Sossin, 2007). PKMζ is unique in that, in mammals, it is transcribed from an internal promoter on the PKCζ gene, so PKMζ mRNA only contains information about the catalytic portion of the molecule (Hernandez et al., 2003; Muslimov et al., 2004). Translation of PKMζ mRNA produces a protein identical to the catalytic portion of a PKCζ molecule without any of the regulatory region. Once synthesized, therefore, PKMζ is constitutively active without requiring second messenger binding. This constant activity is believed to allow PKMζ to actively maintain the facilitated synaptic connections that represent the memory engram in the brain (Pastalkova et al., 2006; Sacktor, 2008, 2011).

The most commonly used inhibitor of PKMζ is myristoylated zeta-pseudosubstrate inhibitory peptide (ZIP), a synthetic peptide that mimics the pseudosubstrate sequence of PKCζ (Ling et al., 2002; Sajikumar et al., 2005; Serrano et al., 2005). Hypothetically, ZIP should specifically bind and inactivate only those molecules with a compatible binding region for the peptide. As the forebrain expresses high levels of PKMζ and only minor amounts of PKCζ (Hernandez et al., 2003), PKMζ should largely be the target of ZIP infusions. More recent studies have recognized that the other atypical PKC isoform, PKCλ/ι is also a target of the ZIP peptide and may contribute to some of ZIP's effects on LTP and memory (Ren et al., 2013; see discussion section for details).

Using ZIP to inhibit PKMζ activity, molecular cascades both up- and downstream of the molecule have been identified. Upstream, a number of signaling cascades that are activated during memory formation have also been identified to drive synthesis of PKMζ. Molecules important for both learning and PKMζ translation include CaMKII (Ca^{2+}-calmodulin-dependent protein kinase II), PKA (protein kinase A), MAPK (mitogen-activated protein kinase, also called ERK), PI3 K (phosphoinositide 3-kinase), and mTOR (mammalian target of rapamycin). This overlap suggests that activation of these signaling cascades during a learning event promotes the rapid translation of PKMζ required for maintenance. Indeed, PKMζ mRNA contains a long 5′ UTR that allows its synthesis to be carefully regulated at the synapse (Hernandez et al., 2003). Translational repression, possibly through miRNA regulation of the PKMζ transcript, allows stores of PKMζ mRNA to be deposited in dendrites (Muslimov et al., 2004), so that stimulation of the synapse during a learning event could trigger immediate synthesis of the maintenance molecule (Sacktor, 2008).

Once PKMζ is locally synthesized, it actively maintains established potentiation at the synapse. One identified mechanism of PKMζ is to enhance α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking to the synapse (Yao et al., 2008). A direct infusion of PKMζ into hippocampal pyramidal neurons doubles the number of postsynaptic AMPA receptors (Ling et al., 2002), contributing to the increased synaptic conductance characteristic of LTP. PKMζ seems to increase the number of AMPA receptors at the synapse through a trafficking mechanism, as PKMζ modifies levels of *N*-ethylmaleimide-sensitive fusion protein (NSF), which interacts with the GluR2 subunit of AMPA receptors to augment their trafficking to the postsynaptic membrane (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998). Blocking the interaction between NSF and AMPA receptors prevents the potentiation normally observed when PKMζ is perfused into cells (Yao et al., 2008). Additionally, PKMζ is known to interact with other proteins that are involved in regulating the trafficking of GluR2 to the synapse, including kidney and brain expressed protein (KIBRA) (Buther et al., 2004; Yoshihama et al., 2009) and protein interacting with PKC1 (PICK1) (Yao et al., 2008). Finally, Migues and colleagues (2010) have demonstrated PKMζ may play a role in blocking endocytosis of GluR2-containing AMPA receptors. Blocking internalization of GluR2 with the peptide $GluR2_{3Y}$ was sufficient to prevent the impairments in LTP maintenance caused by ZIP (Migues et al., 2010). Together, these studies suggest that PKMζ may maintain memories by enhancing the number of GluR2-containing AMPA receptors at the synapse through both enhanced trafficking and reduced endocytosis. Indeed, recent work has suggested that GluR2-containing AMPA receptors are largely calcium impermeable and expression of these receptors at the synapse may mark a synapse that is currently in a stable form that is resistant to disruption (Clem and Huganir, 2010; Hong et al., 2013; Rao-Ruiz et al., 2011; Shi et al., 2001). More recent work has indicated that PKMζ may support enhanced PSD-95 levels at the synapse, as overexpression of PKMζ in hippocampal neurons produces increased PSD-95 clustering at the synapse and ZIP application disrupted these enhancements (Shao et al., 2012). Currently, all known actions of PKMζ appear to be postsynaptic; whether PKMζ also functions presynaptically is unclear.

3. Initial studies: inhibiting PKMζ with ZIP disrupts memory maintenance

After it was convincingly demonstrated that PKMζ is critical for maintaining established LTP, the next step was to test whether

Table 1
Studies showing positive effects of manipulating PKMζ during memory maintenance.

Structure	Task	Animal	Inhibitor	Controls	Drug timing	Test timing	Effect	Reference
Global	OA	Drosophila	Chelerythrine or DN (transgenic)	Veh, wt	Before ACQ	24 h post-ACQ	Memory impaired	Drier et al., 2002
	OA	Drosophila	*Enhanced* PKMζ (transgenic)	Wt, no hs	30 m or 1 h post-ACQ	24 h after hs	Memory enhanced	Drier et al., 2002
	Sensitization	Aplysia	ZIP or chelerythrine	Veh, scrZIP	1 day post-ACQ	1 day post-INJ	Memory impaired	Cai et al. (2011)
AMY	AA	Rat	ZIP	scrZIP	1 day post-ACQ	7 days post-INJ	Memory impaired	Gamiz & Gallo, 2011
	Auditory FC	Rat	ZIP	Veh, scrZIP	1 day post-ACQ	2 h or 24 h post-INJ	Memory impaired (cued and context fear); Rescued by $GluR2_{3Y}$ treatment	Serrano et al., 2008; Kwapis et al., 2009; Migues et al., 2010
	Context FC	Rat	ZIP	Veh	1 day or 7 days post-ACQ	2 h post-INJ	Memory impaired	Kwapis et al., 2012
	IA	Rat	ZIP	Veh, scrZIP	1 day post-ACQ	2 h post-INJ	Memory impaired	Serrano et al., 2008
	CPP^{M}	Rat	ZIP	Veh, scrZIP	1 day post-test 1	1 day post-INJ	Memory impaired	He et al. (2011)
	CPA^{M}	Rat	ZIP	Veh	1 day post-test 1	1 day post-INJ	Memory impaired	He et al. (2011)
	FPS	Rat	ZIP	Veh, scrZIP	1 day or 7 days post-ACQ	2 h, 2 days, 10 days, or 15 days post-INJ	Memory impaired when tested at 2 h or 2 days post-INJ; no impairment when test delayed (see Table 2)	Parsons and Davis (2011a,b)
DH	APA	Rat	ZIP, chelerythrine	Veh, scrZIP	1 day post-ACQ	2 h post-INJ	Memory impaired	Pastalkova et al. (2006), Serrano et al. (2008)
	WC^{D}	Rat	ZIP	Veh	15 day after diazepam	2 h post-INJ	Memory impaired	Monti et al. (2012)
	MWM	Rat	ZIP	Veh, scrZIP	1 day post-ACQ	2 h post-INJ	Memory impaired	Serrano et al., 2008
	OLM	Rat	ZIP	scrZIP	1, 6, or 34 days post-ACQ	1 day post-INJ	Memory impaired; rescued by $GluR2_{3Y}$ treatment	Hardt et al., 2010; Migues et al., 2010
	RAM	Rat	ZIP	Veh, scrZIP	1 day post-ACQ	2 h post-INJ	Memory impaired	Serrano et al. (2008)
	TEC	Rat	ZIP	scrZIP	1 day post-ACQ	2 h post-INJ	Memory impaired	Madronal et al., 2010
DL Striatum	IB: Habit	Rat	ZIP	scrZIP	1 day post-ACQ	2 days post-INJ	Memory for habit-based responding impaired	Pauli et al. (2012)
DM Striatum	IB: Contingency	Rat	ZIP	scrZIP	1 day post-ACQ	2 days post-INJ	Memory for contingency impaired	Pauli et al. (2012)
NAcc core	$CPP^{C,\ M,\ FF}$	Rat	ZIP	Veh	1 day post-ACQ	1, 3, 7, or 14 days post-INJ	Memory impaired	Shabashov et al. (2012); Li et al. (2011)
	AMPH LS	Rat	ZIP	Veh	1 day post-ACQ	30 m post-INJ	Memory Impaired	Song et al. (2013)
Nacc shell	CPP^{C}	Rat	ZIP	Veh	1 day post-ACQ	3 days post-INJ	Memory weakened; normal at test 1, extinguished rapidly	Shabashov et al. (2012)
pPIR	Olfactory FC	Rat	ZIP	scrZIP	1 day or 1 month	2 days post-INJ	Remote memory impaired	Sacco and Sacchetti (2010)
2° AC	Auditory FC	Rat	ZIP	scrZIP	1 day or 1 month	2 days post-INJ	Remote memory impaired	Sacco and Sacchetti (2010)
2° OC	Visual FC	Rat	ZIP	scrZIP	1 day or 1 month	2 days post-inj	Remote memory impaired	Sacco and Sacchetti (2010)
SMC	Sensorimotor reaching	Rat	ZIP	Veh	Immediately post-test	4 days post-inj	Memory impaired	von Kraus et al. (2010)
IC	CTA	Rat	ZIP or DN (viral)	Veh, scrZIP, Control Vector	3 day, 1 month, or 3 months post-ACQ	2 days, 7 days, or 1 month post-INJ	Memory impaired	Shema et al. (2007, 2009, 2011)
	CTA	Rat	*Enhanced* PKMζ (viral OE)	Control vector	5 day pre-ACQ or 5 days post-ACQ	2 days post-test or 7 days post-INJ of virus	Memory enhanced	Shema et al. (2011)
IL mPFC	CPP^{M} EXT	Rat	ZIP	Veh	1 day post-test 1	1 day post-INJ	EXT memory impaired	He et al. (2011)
	CPA^{M} EXT	Rat	ZIP	Veh	1 day post-test 1	1 day post-INJ	EXT memory impaired	He et al. (2011)

AMY: amygdala; DH: Dorsal Hippocampus; DL: dorsolateral; DM: dorsomedial; NAcc: Nucleus accumbens; pPIR: posterior piriform cortex; 2° AC: secondary auditory cortex; 2° OC: secondary occipital cortex; SMC: sensorimotor cortex; IC: insular cortex; IL mPFC: infralimbic medial prefrontal cortex; OA: odor avoidance; AA: active avoidance; FC: fear conditioning; IA: inhibitory avoidance; CPP: conditioned place preference; CPA: conditioned place aversion; ^{M}: morphine; ^{C}: cocaine; ^{FF}: high-fat food; FPS: fear-potentiated startle; APA: active place avoidance; WC^{D}: context-mediated diazepam withdrawal; MWM: Morris water maze; OLM: object location memory; RAM; radial arm maze; TEC: trace eyeblink conditioning; IB: instrumental behavior; AMPH LS: amphetamine-induced locomotor sensitization; ZIP: zeta-pseudosubstrate inhibitory peptide; scrZIP: scrambled ZIP; Veh: vehicle; DN: dominant-negative PKMζ; OE: Overexpression; ACQ: acquisition; INJ: injection; EXT: extinction.

memories also require this protein for long-term stability. The first studies investigating PKMζ's role in memory maintenance were overwhelmingly positive, showing that inhibiting PKMζ activity with either chelerythrine (a PKC inhibitor with a high affinity for PKM isoforms at low doses) or ZIP (the peptide designed to specifically inhibit PKMζ) was sufficient to disrupt a fully consolidated memory (see Table 1). The general protocol for testing whether a memory requires PKMζ for maintenance was similar in all of these studies: at least one day after the learning event (after the consolidation window is closed), PKMζ would be inhibited in the brain region believed to store the association. The animals' memory would then be assessed either within a few hours of the injection or the following day to determine whether blocking PKMζ had any effect.

In a number of species (drosophila, aplysia, mice, and rats) and throughout the brain (including hippocampus, amygdala, and the insular cortex), it was demonstrated that blocking ZIP could impair memory storage (see Table 1). Following a positive finding in Jerry Yin's lab showing that chelerythrine application could reverse odor avoidance memory in fruit flies (Drier et al., 2002), a seminal study by Eva Pastalkova and colleagues (2006) demonstrated that ZIP could erase both *in vivo* LTP in the hippocampus and memory for an aversive active avoidance task housed in the same structure. Within a few years, studies had shown ZIP-induced memory impairments in the amygdala for fear conditioning (Kwapis et al., 2009, 2012; Migues et al., 2010; Serrano et al., 2008), inhibitory avoidance, and active avoidance (Gamiz and Gallo, 2011), in the hippocampus for the Morris water maze (Serrano et al., 2008), radial arm maze (Serrano et al., 2008), object location memory (Hardt et al., 2010) and trace eyeblink conditioning (Madronal et al., 2010), in the insular cortex for conditioned taste aversion (CTA) (Shema et al., 2007; Shema et al., 2009), and in the nucleus accumbens for drug memory (Crespo et al., 2012).

For the first time, a logical, simple mechanism had been described to maintain memory: PKMζ was responsible for actively maintaining memories in the structures that stored these associations throughout the brain. Before long, however, research began to deviate from this path, suggesting that the mechanisms for long-term memory storage may not be as simple as a single protein actively maintaining established synaptic potentiation.

4. Conflicts arise: PKMζ may not always maintain memory

4.1. Does PKMζ maintain all types of memory?

There were a few discrepancies among the early studies on PKMζ's role in memory storage that were difficult to reconcile within the framework laid out by the literature (see Table 2). First, a number of studies failed to find effects for ZIP infusions into regions of the brain that are known to participate in the storage of certain associations. For example, two different studies showed that intra-hippocampal ZIP infusions had no effect on an established context fear association (Kwapis et al., 2009; Serrano et al., 2008) despite a wealth of literature suggesting that the hippocampus plays a key role in context fear memory (e.g. Anagnostaras et al., 1999; Kim and Fanselow, 1992; Matus-Amat et al., 2004; Rudy and O'Reilly, 1999). One explanation for this lack of effect was that the hippocampus does not play a role in storing the memory for context, per se, but only participates in its acquisition. Alternatively, it was also suggested that PKMζ may not play a role in storing general background information, such as contextual or procedural information (Serrano et al., 2008), but only stores foreground or discrete associations. Other studies have shown that ZIP is not effective in disrupting taste familiarity in the insular cortex (Shema et al., 2007) or procedural information for

Table 2
Studies indicating PKMζ may not maintain all memories throughout the brain.

Structure	Task	Animal	Inhibitor	Problem	Explanation	Reference
DH	Context FC	Rat	ZIP	Context fear memory not impaired by ZIP	DH not required for context fear memory storage OR PKMz only maintains *precise* information (not background/procedural information)	Serrano et al. (2008); Kwapis et al. (2009)
	Object recognition	Rat	ZIP	Object recognition memory not impaired by ZIP	The hippocampus may not store memory for object identity	Hardt et al. (2010)
	Cocaine CPP	Rat	ZIP	Memory for cocaine context intact despite ZIP infusion	Similar to context fear conditioning findings; context memory may not be maintained in DH	Shabashov et al. (2012)
NAcc core	Withdrawal CPA^{M}	Rat	ZIP	Memory for withdrawal context intact despite ZIP	Aversive context memory may not be maintained in NAcc.	Li et al. (2011)
NAcc shell	CPP^{M}	Rat	ZIP	Memory for morphine-paired context intact despite ZIP	Cocaine-paired ontext memory may not be maintained in DH. Memory may be weakened by ZIP but not detected on Test 1 (see Shabashov et al., 2012).	Li et al. (2011)
BLA	FPS	Rat	ZIP	Memory permanently impaired if test came 2 h or 2 days post-INJ. No impairment observed if initial test given 10 or 15 days post-INJ	ZIP-induced impairments may require retrieval of the memory within a certain time window post-INJ. See text for in-depth discussion.	Parsons and Davis (2011a,b)
Global	Trace FC; MWM	Mouse	PKCζ KO; cKO	Neither memory not impaired by global knockout of PKCζ/PKMζ	PKMζ may not be necessary for memory maintenance; possible compensatory maintenance effects activated by PKMζ KO before training. See text for in-dept discussion.	Volk et al. (2013)
	Auditory FC, object recognition, object location, and CPP^{C}	Mouse	PKCζ KO	Memory intact for all paradigms tested despite PKMζ knockout. ZIP impaired CPP^{C} memory in PKMζ knockout animals.	See above. PKMζ may not be necessary for memory maintenance; possible compensation due to pre-training knockout of PKMζ. ZIP may be inhibiting other molecules, as it is effective in PKMζ knockout mouse.	

the Morris water maze in the hippocampus (Serrano et al., 2008). Together, these findings suggest that PKMζ may not indiscriminately store all associations throughout the brain; background information may require a different storage mechanism.

4.2. Is PKMζ involved in memory maintenance or reconsolidation?

It is more difficult to explain the findings of Parsons and Davis (2011a), who showed that ZIP was only effective in impairing memory when the initial memory test was given within a few days of injection. Using olfactory fear-potentiated startle, the researchers convincingly demonstrated that infusions of ZIP were sufficient to permanently disrupt olfactory fear memory when tested either 2 h, 1 day, or 2 days after drug infusion. When the initial test was delayed to either 10 or 15 days after infusion, however, memory was not impaired by ZIP infusion (Parsons and Davis, 2011a). This suggests that the memory must be retrieved within a certain time window after ZIP infusion to observe the memory-impairing effects of PKMζ inhibition. Why this time window exists, however, is unclear. The timecourse of ZIP degradation was more recently identified and the peptide is fully cleared from brain tissue within 24 h after an intracranial infusion (Kwapis et al., 2012). Thus, the time window for ZIP's effects on memory impairment does not match that of its degradation from brain tissue; memory impairments are still observed at 2 days after infusion, a timepoint well after ZIP has been fully cleared from the brain. One possibility is that ZIP may temporarily disrupt PKMζ synthesis so that PKMζ levels are disrupted even after the peptide is eliminated. As a positive feedback loop exists between PKMζ activity and new PKMζ synthesis (Kelly et al., 2007; Sacktor, 2011; Westmark et al., 2010), it is possible that disrupting PKMζ activity has a lingering impact on PKMζ levels even after the peptide is removed. From this perspective, it is possible that if the memory is retrieved while PKMζ levels are disrupted, the memory is unable to restabilize and is lost, leading to a permanent disruption of the memory. PKMζ levels would likely recover within a few days, however, so that memories tested 10 or 15 days after ZIP infusion would be retrieved in the presence of normal PKMζ levels that would allow for sufficient restabilization and storage. If this is true, however, it suggests that ZIP's ability to disrupt memory maintenance requires retrieval of that memory while PKMζ levels are disrupted. PKMζ may not be a memory "maintenance" mechanism, therefore, but a "restabilization" mechanism.

Not all researchers have observed this time window of ZIP efficacy, however (see Table 1; Gamiz and Gallo, 2011; Shema et al., 2007). Using conditioned taste aversion, Shema and colleagues have shown that ZIP is effective in disrupting memory even when the initial test occurs a full month after ZIP injection into the insular cortex (2007). Similarly, Gamiz and Gallo (2011) found that memory for active avoidance is disrupted when the initial test occurs seven days after ZIP is infused into the amygdala. Although these studies suggest that ZIP is effective regardless of when the memory is first tested, it is possible that the time window of ZIP's efficacy changes based on either the memory or the brain structure being investigated. Future studies should determine whether other associations, particularly those stored outside the amygdala, are affected by ZIP even when the initial test is delayed.

Others have argued that these results are because Parsons and Davis (2011a) used a low dose of ZIP (Nader, 2011; Sacktor, 2012; but see Parsons and Davis, 2011a). This seems unlikely, however, as Parsons & Davis (2011a) used the standard concentration of ZIP, 10nmol/ζl, the same dose used in a large majority of the initial research on PKMζ (Kwapis et al., 2009; Kwapis et al., 2012; Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2007). The major difference is that Parsons and Davis (2011a) infused a volume of 0.5 μl per hemisphere into the amygdala, as compared to a volume of 1.0 μl infused into various structures by other labs (Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2007; but see Kwapis et al., 2009). While the *total* amount of drug infused into brain tissue differed between the studies, the concentration was identical, so only the spread of the drug should differ. As research has demonstrated that a volume of 0.5 μl generally covers the amygdala for a variety of compounds (Allen et al., 2008; Parsons et al., 2006), including ZIP (Kwapis et al., 2012) it can be concluded that this was an appropriate volume of drug to use. Larger volumes sacrifice specificity, as the drug likely infuses into nearby structures in addition to the amygdala. Therefore, the dose used by Parsons and Davis (2011b) was sufficient and comparable to the dose used in most other studies.

4.3. Does ZIP really inhibit PKMζ?

To further complicate matters, recent work has suggested that ZIP-induced memory impairments may not due to PKMζ inhibition per se. A detailed analysis of the spread of ZIP following its intracranial infusion at 10 nmol/μl estimated that its concentration was approximately 100 μM after correcting for spread dilution and loss of drug through the cannulae tract (Lisman, 2012; Wu-Zhang et al., 2012; but see Yao et al., 2013). *In vitro* studies have demonstrated that at this concentration, ZIP is not only effective in inhibiting PKMζ activity, but also blocks the activity of other kinases, specifically CaMKII, which has also been implicated in memory storage (e.g. Cao et al., 2008; see Sanhueza and Lisman, 2013 for review).

Additionally, two recent studies using genetic knockout mice to remove different exons of the PKCζ/PKMζ gene found that removal of active PKMζ had no effect on LTP or memory (Lee et al., 2013; Volk et al., 2013). Volk and colleagues (2013) used both a traditional global knockout and a conditional floxed knockout targeting exon 11 of the catalytic domain of PKMζ to block endogenous PKMζ before LTP induction or learning. Though knockout animals had no noticeable PKMζ expression, they showed normal LTP induction and maintenance. Further, these animals showed normal learning and memory in two different hippocampal tasks: associative trace fear conditioning and spatial Morris water maze learning. Surprisingly, bath application of ZIP to hippocampal slices 60 min after LTP induction reversed established LTP in both the wild type and knockout animals, suggesting that ZIP reverses established LTP through a PKMζ-independent mechanism. Using a slightly different knockout, in which exon 9 of the PKCζ/PKMζ gene is removed, Lee and colleagues (2013) similarly observed normal learning and memory for cued fear conditioning, novel object recognition memory, object location memory, and cocaine conditioned place preference (CPP) memory in the knockout animal. Consistent with the results of Volk et al. (2013), they also showed that ZIP injection into the nucleus accumbens disrupted memory for cocaine-related CPP in both the wild type and knockout animals. Together, these results indicate that ZIP may reverse memory through a mechanism besides PKMζ.

The results of Volk (2013) and Lee (2013) suggest that ZIP inhibits an unidentified enzyme (or set of enzymes) that normally supports LTP and memory maintenance in the absence of PKMζ. What could this mystery enzyme be? One possibility is that ZIP inhibits the atypical PKCλ/ι, which contains the same pseudosubstrate binding region as PKMζ/PKCζ and should therefore be a target of ZIP. Volk and colleagues (2013) demonstrated that PKCλ/ι does not form a truncated, constitutively active PKM product following LTP induction and they observed no difference in total PKCλ/ι expression levels following LTP. This does not entirely rule out the possibility that PKCλ/ι is playing a role in maintaining LTP and memory in PKMζ knockout animals, however, as the researchers did not measure activity of the PKCλ/ι molecule following LTP. Indeed, preliminary research from the Sacktor laboratory has found compensatory increases in PKCλ/ι phosphorylation when PKMζ is

knocked out constitutively (Tsokas et al., 2012). Further, recent work by Ren and colleagues (2013) indicates that PKCλ/ι may indeed play a key role in LTP expression or maintenance, as selective inhibition of the lamda PKC molecule reverses LTP with 20 min in hippocampal slices. Future studies will be needed to determine whether PKCλ/ι is involved in maintaining memory, however.

4.4. Problems with the scrambled ZIP control peptide

One additional finding of both the Volk and Lee studies is that the scrambled peptide that is often used as an inactive control for ZIP is both an effective inhibitor of PKMζ activity (Lee et al., 2013; Volk et al., 2013) and is able to reverse LTP maintenance in hippocampal slices (Volk et al., 2013). This is consistent with the finding of at least one previous study that showed partial reversal of fear conditioning memory following scrambled ZIP infusion into the amygdala (Kwapis et al., 2009). As the scrambled peptide itself appears to inhibit the maintenance mechanism for both LTP and memory, it is not the most appropriate comparison for ZIP; in order to determine if memory is impaired, ZIP animals should be compared to an animal that shows normal levels of learning and memory, such as a vehicle-infused animal. Studies that compare only ZIP and scrambled ZIP animals therefore need to be interpreted cautiously (see Table 1).

5. Reconciling the conflicting research: Does PKMζ maintain long-term memory?

Although these recent studies complicate what was once a clear and convincing argument that PKMζ is the "memory maintenance" molecule, they do not conclusively rule out the possibility that PKMζ normally acts to maintain memory. Most obviously, both of the studies using PKMζ knockout animals disrupt PKMζ expression well before LTP induction or learning of the behavioral task (Lee et al., 2013; Volk et al., 2013). In these studies, LTP induction or memory acquisition occurred in the absence of PKMζ. It is possible that disruption of PKMζ activity triggered a compensatory mechanism that allowed for normal maintenance of LTP and memory despite PKMζ knockout. In order to conclusively determine that PKMζ is not necessary for memory maintenance, it is imperative that a conditional knockout is used to test whether disrupting PKMζ *after* consolidation has an effect on established memory maintenance. Currently, with knockouts that occur well before learning, the researchers are not able to dissociate between the learning and maintenance phases of the association. As ZIP has been shown to disrupt memory up to 3 months following a learning event (Shema et al., 2009), there is sufficient time for a conditional knockout to be induced between the learning and test phases to test whether a genetic knockout of PKMζ after learning is sufficient to disrupt an established memory.

Additionally, this research is unable to account for the results of Shema and colleagues (2011), who demonstrated that viral overexpression of PKMζ in the insular cortex either before or 7 days after CTA learning is able to enhance long-term memory for the task. There are a couple of possible explanations to reconcile these two findings. First, as previously mentioned, PKMζ may maintain memory under normal circumstances, but a compensatory mechanism may support memory maintenance in the absence of functional PKMζ. Alternatively, it is possible that PKMζ overexpression activates some downstream mechanism that is also a nonspecific target of ZIP that remains to be identified. In either case, it seems that PKMζ does promote long-term memory.

Thus, although recent work on PKMζ demonstrates instances in which the protein may not be required for memory maintenance, it is likely that PKMζ does play a role in memory maintenance under normal learning conditions. In light of the findings of Parsons and colleagues (2011a), PKMζ's role may be to restabilize an updated memory, instead of actively maintaining an established memory in the absence of retrieval. Additional tests will need to be done to determine the precise length of this post-infusion time window of ZIP's effectiveness in order to identify why it exists.

6. Future directions

Perhaps it is not surprising that the neural mechanisms underlying memory maintenance appear to be more complicated than originally described. The molecular cascades supporting the induction, reconsolidation and extinction of memories are far more complex than the PKMζ-centric model that has been described to date for general memory maintenance. Indeed, it appears that in the absence of PKMζ, other mechanisms can compensate and maintain memory, suggesting the existence of unidentified components of the memory maintenance machinery. Whether these mechanisms also maintain memory in the presence of intact PKMζ is currently unclear.

More work is needed to conclusively determine the role of PKMζ in memory maintenance or retrieval and to identify what is being inhibited by ZIP (besides PKMζ) to block memory in PKMζ knockout animals. One critical study that needs to be conducted in the near future should test whether a conditional knockout of PKMζ that is induced *after* learning is effective in reversing a memory acquired in the presence of functional PKMζ. Until this study is completed, it is impossible to determine whether the effects of Volk et al. (2013) and Lee et al. (2013) are the result of compensatory mechanisms maintaining memory in the absence of functional PKMζ. The results of this study will affect the direction of future memory maintenance research. If the conditional knockout shows impairments in memory maintenance that are similar to the effects of ZIP, this will validate the wide field of work supporting the idea that PKMζ maintains memory. If the conditional knockout fails to block the maintenance of memory, however, this will suggest that PKMζ is not the major mechanism maintaining memory and other mechanisms will need to be explored.

In order to identify the function of PKMζ in memory, future studies should also develop and use novel inhibitors of the kinase. As every inhibitor has shortcomings (such as ZIP blocking proteins besides PKMζ and genetic knockouts possibly activating compensatory mechanisms), a conclusive role for PKMζ will only be identified through converging studies using a range of techniques to inhibit the molecule. Other methods of blocking PKMζ include antisense oligodeoxynucleotides targeted to PKMζ mRNA, which could prevent new translation of PKMζ, and TAT-conjugated peptides designed to inhibit either PKMζ itself or its downstream targets. Some preliminary work from Todd Sacktor's lab has indicated that an oligodeoxynucleotide targeted to PKMζ mRNA administered pre-training to the hippocampus was sufficient to disrupt memory consolidation for an active place avoidance task (Tsokas et al., 2012). When published, this finding, which is largely consistent with the memory impairments observed with ZIP, will strengthen the conclusion that PKMζ primarily functions as a memory maintenance molecule. TAT-conjugated proteins, on the other hand, could be used to block PKMζ by inhibiting the molecule (or its downstream targets) in a manner similar to ZIP. Fusing TAT to a protein or peptide sequence allows the molecule to pass through the cell membrane and access intracellular signaling cascades (Becker-Hapak et al., 2001). To this end, TAT could be fused to peptide sequences that inhibit PKMζ or its downstream targets and directly infused into a brain region in order to locally inhibit intracellular PKMζ. Although ZIP already contains a myristoylated N-terminal, which allows it to cross the cell membrane,

comparing the results of ZIP to a similar TAT-conjugated peptide sequence would ensure that the effects of ZIP are not simply due to myristoylation of the N-terminal. Further, TAT-fused peptides designed to block downstream targets of PKMζ could be used to test the mechanisms of PKMζ, as well. PKMζ is known to interact with a number of proteins involved in regulating GluR2 trafficking to the synapse, including NSF, KIBRA, and PICK1 (Buther et al., 2004; Yao et al., 2008; Yoshihama et al., 2009). Inhibiting these downstream molecules, possibly through TAT-fused peptide infusions, could test the proposed mechanisms of PKMζ activity, as well.

What other molecules might actively maintain established memory? It is clear that mechanisms besides PKMζ contribute to memory maintenance, as long-term memory is intact in PKMζ knockout animals (Lee et al., 2013; Volk et al., 2013). There are a few candidate molecules to target in future studies of memory maintenance. First, it would be fruitful to comprehensively determine what kinases besides PKMζ are inhibited by ZIP infusion. Although ZIP is generally considered to be a PKMζ inhibitor, the pseudosubstrate sequence mimicked in the peptide is identical in PKMζ, PKCζ and the other atypical isoform of PKC, PKCλ/ι (Bosch et al., 2004; Jiang et al., 2006; Ren et al., 2013; Standaert et al., 2001). As PKCλ/ι is found in the brain (Hernandez et al., 2003; Naik et al., 2000; Oster et al., 2004), infusions of ZIP are likely to inhibit its activity in addition to the activity of PKMζ. Indeed, a recent study demonstrated that while low doses of ZIP (0.5 μM) selectively inhibit PKMζ *in vitro*, a slightly higher concentration (2.0 μM) also disrupts PKCλ/ι activity (Ren et al., 2013). As the standard concentration of ZIP used in behavioral studies is far higher than this (10 μM), it is likely that a majority of the studies using ZIP to inhibit PKMζ also inhibited PKCλ/ι. By comparing low, PKMζ-specific doses of ZIP to slightly higher doses intended to inhibit both PKMζ and PKCλ/ι, Ren and colleagues (2013) were also able to determine that PKCλ/ι appears to support early LTP expression in hippocampal slices. PKCλ/ι is therefore a key target of ZIP that may support memory maintenance in the absence of PKMζ. Although it is tempting to conclude that the memory impairments observed following ZIP infusion in a number of the initial studies on PKMζ are *actually* due to PKCλ/ι inhibition, this is not necessarily the case. First, it is unclear how PKCλ/ι could continuously sustain an established memory, as it lacks a constitutive isoform that could actively maintain synapses in their potentiated state (Volk et al., 2013). Second, PKMζ overexpression has been demonstrated to enhance memory (Shema et al., 2011), suggesting that PKMζ indeed plays a role in memory stability. Future studies should directly test whether PKCλ/ι plays a specific role in memory maintenance or expression by blocking the molecule, possibly with siRNA or antisense oligonucleotide, during the maintenance phase of memory. Further, research should focus on clarifying how PKMζ and PKCλ/ι work in concert to maintain LTP and memory. Finally, it is possible that other kinases are affected by the relatively high doses of ZIP used in behavioral studies; CaMKII, for example, is known to be inhibited by high concentrations of ZIP *in vitro* and may be non-specifically inhibited by ZIP infusions in a number of the behavioral studies discussed in this review. As ZIP can have nonspecific effects on other molecules, it is unclear whether its effects on memory and LTP maintenance are indeed due to PKMζ inhibition.

Other mechanisms may be important in maintaining memory, as well. One compelling idea is that epigenetic mechanisms may participate in long-term memory maintenance. Epigenetics, changes in the chromatin structure that regulate transcriptional access to DNA, can produce long-lasting changes in gene expression that might serve as a mechanism for memory stability. Accordingly, a number of recent studies have focused on the role of epigenetic changes in memory formation and storage (for review, see Barrett and Wood, 2008; Jarome and Lubin, 2013; Zovkic et al., 2013). Changes in methylation have been identified as a possible memory maintenance mechanism, partially because these changes are stable over time. Histone lysine methylation, for example, which can either activate or repress gene expression depending on the number of methyl groups associated with a specific lysine residue, has been shown to be dynamically regulated following context fear conditioning (Gupta et al., 2010; Gupta-Agarwal et al., 2012). Importantly, long-term changes in histone H3 lysine 4 trimethylation (H3K4me3) have been observed following learning; H3K4me3 is significantly decreased in the entorhinal cortex 24 h after context fear conditioning (Gupta-Agarwal et al., 2012). At least some epigenetic changes are therefore long-lasting and could play a role in memory maintenance. Additionally, some forms of DNA methylation are self-perpetuating; DNMT1, for example, appears to actively maintain at least some existing methylation patterns (Law and Jacobsen, 2010; Santos et al., 2005; Zovkic et al., 2013). These persistent changes could explain how synaptic potentiation is maintained over long periods of time; epigenetic control of transcription could ensure continuous gene expression to replenish degrading proteins at the synapse. It is currently unclear whether other epigenetic mechanisms, such as histone acetylation and nucleosome remodeling are involved in memory maintenance. As these chromatin modifications appear to regulate the induction of memory (for review, see Barrett and Wood, 2008; Vogel-Ciernia and Wood, 2012) and blocking histone deacetylation promotes the formation and persistence of memory following a weak training event (e.g. Guan et al., 2009; Levenson et al., 2004; McQuown et al., 2011), it is logical to expect some involvement of these epigenetic mechanisms in the maintenance of existing memory. Whether PKMζ or PKCλ/ι are targeted by these epigenetic mechanisms to promote memory maintenance remains to be determined.

It is also possible that structural changes at the synapse support long-term memory maintenance (Lamprecht and LeDoux, 2004). Research has shown that dynamic actin filament rearrangement appears to underlie the maintenance of LTP in hippocampal slices. Inhibiting actin rearrangement in hippocampal slices with actin assembly inhibitors (cytochalasin D, cytochalasin B, and latrunculin A) can reverse established LTP without affecting basal synaptic transmission (Krucker et al., 2000). Rearrangement of the actin cytoskeleton in dendritic spines might support memory maintenance, as well; changes in spine size have also been associated with fear conditioning (Ostroff et al., 2010). It is likely that these structural changes at the synapse and the increases in PKMζ observed following LTP are not mutually exclusive processes. Instead, increases in PKMζ are probably related to the dynamic changes in the cytoskeleton that seem to support long-term LTP and memory maintenance. Indeed, AMPA receptors play an important role in both PKMζ-related memory maintenance and cytoskeletal changes observed during L-LTP. PKMζ is known to increase AMPA receptor trafficking to the synapse to actively maintain potentiation (Yao et al., 2008). Work on synapse morphology has similarly shown that blocking AMPA receptors in cultured neurons triggers the removal of mature dendritic spines *in vitro* (McKinney et al., 1999). Together, these studies suggest that increases in PKMζ and cytoskeletal rearrangement work in concert to maintain LTP. Following a learning event, the cytoskeleton is rearranged to produce strengthened connections in activated synapses, including changes in AMPA receptor expression at the postsynaptic density. These changes are then actively maintained through the activity of PKMζ, which works to maintain a sufficient number of AMPA receptors at the postsynaptic density to maintain the facilitated synaptic connection.

Recent work has suggested that ZIP may disrupt the reconsolidation, rather than the maintenance of memory, as Parsons & Davis (2011a) only observed disruptions when the memory was tested within a certain time window after ZIP infusion. This brings up the possibility that ZIP infusions disrupt the

reconsolidation process, rather than the maintenance of memory. Memory retrieval is believed to induce a temporary period of lability that allows an existing association to be updated (Finnie and Nader, 2012). From this perspective, following memory retrieval, the synapses storing the engram are destabilized, characterized by a period of deconstruction and protein degradation that makes the synapses malleable (Jarome et al., 2011; Lee, 2008; Lee, 2010; Lee et al., 2008). This is followed by a period of restabilization that re-solidifies the synaptic connections and ultimately stabilizes and stores the updated association. PKMζ may act during this restabilization phase, helping to resolidify the synaptic connections that support the memory. Indeed, one identified action of PKMζ is upregulation GluR2-containing AMPA receptors at functional synapses (Migues et al., 2010; Yao et al., 2008). Recent studies have indicated that GluR2-containing AMPA receptors, which are impermeable to calcium, are expressed when memory is in a stable phase and exchanged for calcium permeable AMPA receptors when the memory is in a labile phase (Clem and Huganir, 2010; Hong et al., 2013). Accordingly, PKMζ could restabilize memory after retrieval by increasing GluR2 at synapses.

The results of Parsons & Davis (2011a) may represent a failure of the memory to restailize in the absence of sufficient PKMζ. From this perspective, when the memory is tested, it destabilizes normally, but it may fail to properly restabilize without adequate PKMζ levels. In order to test whether PKMζ is a restabilization mechanism, rather than a storage mechanism, two studies are in order. First, research will need to determine the exact length of the window of ZIP's efficacy following infusion. Parsons & Davis (2011b) demonstrated that ZIP disrupted memory when the initial memory test occurred 2 days post-infusion but not when the test was delayed to 10 days after infusion. Thus, at some point between 2 and 10 days, the ZIP infusion is no longer effective. Information about exactly how long this time window lasts will aid in the identification of a function reason for the window's existence. Second, the hypothesis that ZIP is disrupting memory restabilization needs to be empirically tested. This would be a relatively simple test; as memory restabilization mechanisms are only required when the memory is destabilized through the retrieval process, blocking destabilization of the memory trace should prevent any effects of blocking restabilization. If ZIP prevents restabilization, a pre-retrieval infusion that blocks the destabilization process (such as an inhibitor of either NMDA receptors or protein degradation (Ben Mamou et al., 2006; Choi et al., 2010; Jarome et al., 2011)) should prevent ZIP from impairing memory, even when tested shortly after infusion. On the other hand, if ZIP disrupts memory maintenance, destabilization of the memory trace should not be required for these effects and ZIP should disrupt memory regardless of the pre-retrieval infusion.

Memory maintenance research is currently in an interesting phase. We have begun to identify major players contributing to the long-term stability of memory, but we are also beginning to see that memory maintenance is more complicated than originally described. Whether PKMζ turns out to be the mechanism that maintains memory or whether it will be identified as a restabilization mechanism, it is clear that our understanding of memory maintenance is currently incomplete and several compelling lines of research have been triggered by this discussion. It will be interesting to follow how our understanding of memory maintenance progresses as these questions are answered.

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