The Role of PKMζ in Reconsolidation and in the Maintenance of Memory in Male and Female Rodents

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Table of Contents

List of Figures **7**

Abstract **8**

Résumé **11**

Acknowledgements **15**

Contribution to Original Knowledge **17**

Contribution of Authors **18**

Commonly Used Abbreviations **19**

Chapter 1: Introduction **21**

1.1. Dynamic Theories of Memory Dynamics 22

1.1.1. Consolidation Theory 22

1.1.2. Reconsolidation Theory 24

1.1.3. Reconsolidation in Humans 27

1.2. Memory Destabilization 29

1.2.1. When Does Destabilization Occur? 29

1.2.2. Initiating Memory Destabilization 31

1.2.3. Intracellular Mechanisms of Memory Destabilization 33

1.2.4. Protein Degradation in Memory Destabilization 35

1.3. Memory Reconsolidation 36

1.3.1. Mechanisms of Reconsolidation 36

1.3.2. The Reconsolidation Window 39

1.4. AMPAR Exchange During Destabilization and Reconsolidation 40

1.5. Protein Kinase M ζ 45

1.5.1. PKMζ Expression and Regulation 45
1.5.2. PKMζ and Memory Maintenance 48
1.5.3. Challenges to the PKMζ Memory Maintenance Hypothesis 51
1.5.4. Sex-Differences and PKMζ 56
1.6. Fear Conditioning and the Basolateral Amygdala 58
1.7. Summary and Rationale 61
Chapter 2: Memory destabilization reduces synaptic PKMζ within one hour after retrieval 64
2.1. Preface……..... 65
2.2. Abstract…….. 67
2.3. Introduction…. 68
2.4. Materials and Methods 70
2.4.1. Animals……..... 70
2.4.2. Surgery……..... 70
2.4.3. Infusions……..... 71
2.4.4. Fear conditioning..... 72
2.4.5. Sacrificing……..... 73
2.4.6. Subcellular fractionation 74
2.4.7. Western Blotting..... 74
2.4.8. Statistical analyses..... 75
2.5. Results……..... 76
2.5.1. Memory retrieval reduces synaptic PKMζ 76
2.5.2. Reduction in PKMζ is destabilization-dependent..... 76

2.5.3. Proteasome activation is necessary for post-retrieval decrease of PKMζ 76
2.6. Discussion……..... 77
2.7. Figures…… ..... .80
Chapter 3: Memory reconsolidation requires de novo synthesis of PKMζ 84
3.1. Preface……..... 85
3.2. Abstract……..... 87
3.3. Introduction……. 88
3.4. Materials and Methods 90
3.4.1. Animals……..... 90
3.4.2. Surgery……..... 90
3.4.3. Infusions……..... 91
3.4.4. Fear conditioning..... 92
3.4.5. Sacrificing……..... 94
3.4.6. Subcellular fractionation 94
3.4.7. Western Blotting..... 95
3.4.8. Statistical analyses..... 96
3.5. Results……..... 97
3.5.1. Reconsolidation increases synaptic PKMζ..... 97
3.5.2. Synthesis of PKMζ is necessary for reconsolidation 97
3.5.3. Acute inhibition of PKMζ synthesis does not impair stable memory..... 98
3.5.4. Preventing destabilization protects memory from amnesic effect of PKMζ-antisense……..... 99
3.6. Discussion……..... 100

3.7. Figures……..... 104

Chapter 4: ζ-stat is a PKMζ-specific inhibitor that disrupts auditory fear memory in male, but not female, mice 109

4.1. Preface……..... 110

4.2. Abstract……..... 112

4.3. Introduction…… ..... ……113

4.4. Materials and Methods 115

4.4.1. Animals……..... 115

4.4.2. Surgery……..... 116

4.4.3. Auditory fear conditioning..... 116

4.4.4. Drug Infusions..... 117

4.4.5. Statistical analysis..... 117

4.5. Results……..... 118

4.5.1. ζ-stat impairs memory in wild-type but not PKMζ-null males..... 118

4.5.2. ζ-stat does not disrupt memory maintenance in female mice, but ZIP does……..…..... 118

4.6. Discussion……..... 119

4.7. Figures……..... 123

Chapter 5: Discussion 126

5.1. Summary……..... 127

5.2. Implications……..... 128

5.2.1. Dynamic Regulation of PKMζ..... 128

5.2.2. KIBRA……..…..... 130

5.2.3. Modeling PKMζ Dynamics..... 131

5.2.4. Clarifying the Role of PKMζ in Reconsolidation 132

5.2.5. Memory Maintenance in Female Mice 135

5.3. Limitations……..... 136

5.4. Future Directions 139

5.5. Conclusion……. 142

References..... 144

List of Figures

Figure 2.1: Memory retrieval reduces synaptic PKMζ 81

Figure 2.2: APV prevents post-retrieval reduction in PKMζ 82

Figure 2.3: β-lac prevents post-retrieval reduction in PKMζ 83

Figure 3.1: Reconsolidation increases synaptic PKMζ 105

Figure 3.2: Post-retrieval infusion of PKMζ-antisense impairs long-term memory 106

Figure 3.3: Infusion of PKMζ-antisense 24h post-retrieval does not impair memory ... 107

Figure 3.4: Pre-retrieval APV prevents amnesic effect of PKMζ-antisense 108

Figure 4.1: ζ-stat impairs memory in wild-type but not PKMζ-null male mice 124

Figure 4.2: ZIP, but not ζ-stat, disrupts memory maintenance in female mice 125

Abstract

Synaptic connections that underlie memory in the brain must balance between long-term stability and plasticity. Maintenance of stable long-term memories is an active process that requires the persistent activity of the protein kinase C isoform, PKMζ. However, memories can become temporarily unstable and plastic. After recall, memory destabilization renders memories labile for a few hours before they re-stabilize through the process of reconsolidation. What happens to maintenance processes, and PKMζ in particular, during this period of lability and how they are restored during reconsolidation is not known. PKMζ is believed to maintain memories by preventing endocytosis of GluA2-containing α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptors (AMPARs) from the postsynaptic density. However, during destabilization these GluA2-AMPARs are internalized and then reinserted around the time of reconsolidation. This suggests that the PKMζ maintenance mechanism may be transiently suspended during destabilization and restored during reconsolidation. Here we investigated the role of PKMζ in memory destabilization and reconsolidation.

In Chapter 2, we studied whether memory destabilization leads to a loss of PKMζ expression at the synapse. We studied changes in PKMζ in rats after retrieval of a long-term auditory fear conditioning memory. Using Western blotting, we quantified levels of PKMζ in basolateral amygdala (BLA) synaptosomes and found that PKMζ is depleted from the synapse within 1 hour after retrieval of this memory. Activation of *N*-methyl-D-aspartate receptors (NMDARs) is necessary for this reduction in PKMζ because inhibiting them with DL-2-amino-5-phosphonopentanoic acid (APV) prevented the decrease of PKMζ. We also discovered that activation of the ubiquitin-proteasome

system after retrieval is required for PKMζ expression to decrease in BLA synapses. Therefore, our results indicate that memory destabilization leads to a loss of synaptic PKMζ within 1 hour after retrieval.

Given that destabilization of a long-term auditory fear memory promotes a loss of PKMζ in the BLA, we determined in Chapter 3 whether reconsolidation results in an increase of PKMζ expression at BLA synapses. Indeed, we found that, following reconsolidation, expression of PKMζ is increased 24 hours after retrieval compared to its low during destabilization. We found that this increase requires the synthesis of new PKMζ because post-retrieval infusion of PKMζ-antisense impaired long-term memory retention. This is in contrast to the memory maintenance phase (ie. 24 hours after retrieval when reconsolidation is complete), when blocking synthesis of PKMζ had no effect on retention of the auditory fear memory. Finally, in line with the results of Chapter 2, we found that activation of NMDARs is necessary to render long-term memory retention vulnerable to PKMζ-antisense. This outcome suggests that without memory destabilization, reactivated memories will not require significant synthesis of new PKMζ in order to persist.

While not often addressed in neuroscience research, there are numerous differences between male and female animals. Several studies imply that PKMζ is no exception and that this protein may serve different functions in males and females. That is, they show that manipulations of PKMζ seem to disproportionately affect memory of males compared to females. In Chapter 4, we investigated whether male and female mice maintain long-term memory with PKMζ. Considering the nonspecific binding of ζ inhibitory peptide (ZIP), we first tested whether another inhibitor, 8-hydroxynaphthalene-

1,3,6-trisulfonic acid (ζ-stat), presented with higher selectivity for PKMζ. We found that this molecule impaired memory maintenance in wild-type but not PKMζ-null males, suggesting high specificity to inhibit PKMζ. In light of this outcome, we used ζ-stat to test whether PKMζ is required for long-term memory maintenance in female mice. We discovered that, in contrast to male mice, ζ-stat did not impair long-term memory in wild-type female mice; ZIP, however, did. These data suggest that PKMζ does not maintain long-term memory in female mice, but a different process might be involved that is sensitive to ZIP. How long-term memory is maintained in female mice remains unknown, although our data suggest that PKMζ is not involved.

Taken together, our data demonstrate a dynamic regulation of PKMζ expression during memory destabilization and reconsolidation. We provide support for the hypothesis that PKMζ maintains long-term memory, with the important caveat that this may not be the case in female animals. Thus, this work provides important insights into the neurobiology of memory maintenance and plasticity, pointing to several areas of inquiry for future research to pursue.

Résumé

Les connections synaptiques qui sont à la base de la mémoire gardent un équilibre entre la stabilité et la plasticité à long-terme. Le maintien des souvenirs à long-terme nécessite l'activité constante de l'isoforme PKMζ de la protéine kinase C. Cependant, les souvenirs peuvent devenir temporairement instables et malléables. Suite au rappel, la déstabilisation des souvenirs les rend labiles pour quelques heures, avant d'être re-stabilisés à nouveau via le processus de reconsolidation. On ne connaît pas ce qui advient des processus de maintien de la mémoire, et de la protéine PKMζ en particulier, pendant cette période d'instabilité et comment ces processus sont rétablis pendant la reconsolidation. On croit que PKMζ maintiendrait les souvenirs en prévenant l'endocytose des récepteurs de l'acide propionique alpha-amino-3-hydroxy-5-methyl-4-isoxazole contenant la sous-unité GluA2 (GluA2-AMPARs) au niveau de la densité postsynaptique. Toutefois, lors de la déstabilisation, ces GluA2-AMPARs sont internalisés et ensuite réinsérés aux environs de la période de reconsolidation. Ceci suggère que les mécanismes de maintien de la mémoire médiés par PKMζ peuvent être momentanément suspendus lors de la déstabilisation, puis rétablis au cours de la reconsolidation. Ici nous avons examiné le rôle de PKMζ dans la déstabilisation et la reconsolidation.

Au chapitre 2, nous avons étudié si la déstabilisation des souvenirs entraine une réduction de l'expression de PKMζ au niveau synaptique. Nous avons étudié chez le rat les changements des niveaux de PKMζ suite au rappel d'un souvenir de peur auditive conditionnée. À l'aide d'analyses par Western blot, nous avons quantifié les niveaux de PKMζ contenus dans des synaptosomes extrait de l'amygdale basolatérale (BLA). Nous

avons trouvé que les réserves synaptiques de PKMζ s'appauvrissent dans l'heure suivant le rappel de ce type de souvenir. L'activation des récepteurs N-methyl-D-aspartate (NMDARs) est nécessaire pour observer cette réduction de PKMζ puisqu'inhiber ces récepteurs avec l'acide DL-2-amino-5-phosphonopentanoïque (APV) empêche la baisse des niveaux de PKMζ. Nous avons aussi découvert que, suite au rappel, l'activation du système ubiquitine-protéasome est requise pour diminuer l'expression de PKMζ dans les synapses de la BLA. Par conséquent, nos résultats indiquent que la déstabilisation de la mémoire mène à une réduction de PKMζ au niveau synaptique dans l'heure suivant le rappel.

Étant donné que la déstabilisation d'un souvenir de peur auditive favorise la diminution de PKMζ dans la BLA, nous avons déterminé au chapitre 3 si la reconsolidation résulte en l'augmentation de l'expression de PKMζ aux synapses de la BLA. En effet, nous avons découvert que, suivant la reconsolidation, l'expression de PKMζ est augmentée 24 heures après le rappel alors qu'elle est faible pendant la déstabilisation. Nous avons constaté que cette augmentation requiert la synthèse de nouvelle PKMζ puisque l'infusion post-rappel d'un antisens spécifique à PKMζ a modifié la rétention de souvenirs à long-terme. À l'opposé, bloquer la synthèse de PKMζ lors de la période de maintien de la mémoire (c.-à-d. 24 heures suivant le rappel, lorsque la reconsolidation est complétée) n'a eu aucun effet sur la rétention à long-terme des souvenirs de peur auditive. Finalement, en lien avec les résultats obtenus au chapitre 2, nous avons trouvé que l'activation des NMDARs est nécessaire pour rendre le maintien à long-terme des souvenirs vulnérable à l'antisens de PKMζ. Ce résultat suggère que,

sans la déstabilisation de la mémoire, la synthèse de nouvelle PKMζ ne sera pas requise de façon significative pour que les souvenirs réactivés perdurent.

Bien que très peu abordé en neuroscience expérimentale, il y a de nombreuses différences entre les animaux mâles et femelles. Plusieurs études supposent que PKMζ ne déroge pas à la règle et que cette protéine pourrait avoir différentes fonctions chez les mâles et les femelles. En effet, elles montrent que les manipulations effectuées sur PKMζ semblent affecter disproportionnellement la mémoire des mâles comparé à celle des femelles. Au chapitre 4, nous avons vérifié si les souris mâles et femelles maintiennent les souvenirs grâce à PKMζ. Considérant la liaison non-spécifique du peptide inhibiteur ζ (ZIP), nous avons d'abord testé si un autre inhibiteur, l'acide 8-hydroxynaphthalène-1,3,6-trisulfonique (ζ-stat), présentait une plus grande sélectivité pour PKMζ. Nous avons constaté que cette molécule altérait le maintien des souvenirs chez les souris sauvages (wildtype), mais pas chez les souris mâles déficientes en PKMζ (PKMζ-null), ce qui suggère une grande spécificité pour inhiber PKMζ. À la lumière de ces résultats, nous avons utilisé ζ-stat pour tester si PKMζ est requise pour le maintien à long-terme des souvenirs chez les souris femelles. Nous avons découvert que contrairement aux souris mâles, ζ-stat n'a pas altéré les souvenirs chez les souris femelles wildtype. Par contre, ZIP s'est montré efficace chez les deux sexes. Ces données suggèrent que PKMζ ne maintient pas la mémoire à long-terme chez les souris femelles, mais plutôt qu'un processus différent et sensible à ZIP pourrait être impliqué. La façon dont la mémoire à long-terme est maintenue chez les souris femelles est encore inconnue, bien que nos résultats laissent croire que PKMζ ne serait probablement pas impliquée.

Considérées dans leur ensemble, nos données démontrent une régulation dynamique de l'expression de PKMζ lors de la déstabilisation et de la reconsolidation de la mémoire. Nos résultats appuient l'hypothèse que PKMζ maintient la mémoire à long-terme, sous réserve que cela ne puisse pas être le cas chez les femelles. Ainsi, cet ouvrage apporte d'importantes connaissances en neurobiologie de la plasticité et du maintien de la mémoire, menant à de nombreuses questions de recherches à explorer pour le futur.

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Contribution to Original Knowledge

- Memory destabilization causes a reduction of synaptic PKMζ protein in the BLA within 1 hour after retrieval.
- This reduction is transient and requires both NMDAR and proteasome activation.
- In the BLA, reconsolidation requires synthesis of new PKMζ.
- Acute infusion of PKMζ-antisense will not disrupt memory storage when the infusion occurs 24 hours after retrieval.
- In the absence of NMDAR activation, PKMζ-antisense does not affect memory retention when infused immediately after retrieval.
- ζ-stat impairs maintenance of auditory fear conditioning memory in male wild-type but not PKMζ-null mice.
- ζ-stat does not impair memory in female wild-type mice.

Contribution of Authors

This work originated with a project developed and implemented by Dr. Virginia Migues and was initially supervised by Dr. Karim Nader. During the final stage, this project was mainly supervised by Dr. Oliver Hardt. Both supervisors provided input on experimental design and Dr. Hardt provided advice with data analysis throughout.

In Chapters 2 and 3, I conducted most cannulation surgeries with occasional help from Karine Gamache. I handled all animals, prepared drugs for infusions, carried out behavioural experiments, and sacrificed rats myself. Where infusions occurred, they were completed with the help of Karine Gamache, Josue Haubrich, Jane Zhang, or Carmelo Milo. I also scored all freezing behaviour from these experiments and analyzed the data myself. Finally, I completed subcellular fractionation and Western blots myself.

Chapter 4 grew out of a collaboration between Dr. Karim Nader and Dr. Todd Sacktor of SUNY Downstate. Dr. Sacktor proposed testing ζ-stat in wild-type and PKMζ-null mice in auditory fear conditioning. PKMζ-null mice were obtained from Dr. Wayne Sossin and genotypes were validated by the lab of Dr. Arkady Khoutorsky. Experiment 4.1 was designed in consultation with Dr. Sacktor. Data from this experiment are to be included in an upcoming manuscript that is currently in preparation. The idea to test ζ-stat in female mice was sparked by Dr. Jeffrey Mogil however the experiment's design came from Dr. Oliver Hardt and myself. In Chapter 4, I conducted all surgeries, handled all mice, carried out each behavioural experiment, prepared all drugs, infused all mice, and sacrificed all animals. Data from Experiment 4.1 were scored by Karine Gamache whereas I scored data from Experiment 4.2.

Karine Gamache graciously translated the résumé.

Commonly Used Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid
AMPAR	AMPA receptor
GluA1-AMPAR	AMPAR containing GluA1 subunit
GluA2-AMPAR	AMPAR containing GluA2 subunit
CP-AMPAR	Calcium-permeable AMPARs (GluA2-lacking)
APV	DL-2-amino-5-phosphonopentanoic acid
Arc	Activity-regulated cytoskeletal-associated protein
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
C/EBPβ	CCAAT enhancer binding protein β
C/EBPδ	CCAAT enhancer binding protein δ
CaMKII	Calmodulin-dependent kinase II
CBP	CREB-binding protein
CREB	cAMP-responsive element binding protein
CPP	Condition Place Preference
ERK/MAPK	Extracellular signal-regulated kinase/mitogen-activated protein kinase
ICAP	[4-(5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl] methyl dihydrogen
KIBRA	Kidney/brain protein
KO	Knockout
LTP	Long-term potentiation

NAc	Nucleus accumbens
NMDA	*N*-methyl-D-aspartate
NMDAR	NMDA receptor
GluN2A-NMDAR	NMDAR containing GluN2A subunit
GluN2B-NMDAR	NMDAR containing GluN2B subunit
NSF	*N*-ethylmaleimide- sensitive factor
ODN	Oligodeoxynucleotide
PBS	Phosphate buffered saline
PICK1	Protein interacting with C kinase 1
Pin1	Protein interacting with NIMA 1
PKA	Protein kinase A
PKC	Protein kinase C
PKCζ	Protein kinase C ζ
PKCι/λ	Protein kinase C ι/λ
PKMζ	Protein kinase M ζ
PKMι/λ	Protein kinase M ι/λ
PSD	Post-synaptic density
UPS	Ubiquitin-proteasome system
Zif268	Zinc finger-containing transcription factor 268
	Also known as: early growth response protein 1 (EGR-1)
ZIP	ζ inhibitory peptide
β-lac	Clasto-lactacystin beta-lactone
ζ-stat	8-hydroxynaphthalene-1,3,6-trisulfonic acid

Chapter 1: Introduction

1.1. Dynamic Theories of Memory Dynamics

Although the conscious experience of memory is about recalling the past, its primary purpose is guiding future behaviour. Information from past experience is often categorized as semantic memory (knowledge of impersonal facts) or episodic memory (recollections of personal experiences; Renoult, Irish, Moscovitch, & Rugg, 2019; Tulving & Donaldson, 1972). Importantly, one's knowledge of ideas or associations is liable to change when presented with more accurate information. That episodic memories are just as pliable—while deeply uncomfortable— is relatively uncontroversial (Loftus, 2003). Perhaps more unnerving, the vividness of a memory has little to do with its accuracy (Day & Ross, 2014; Talarico & Rubin, 2003). Even those with exceptional memory are vulnerable to remembering false information (Patihis et al., 2013). One interesting theory suggests that perceptions evolved primarily to be useful rather than truthful (Hoffman, Singh, & Prakash, 2015). Likewise, memory seems to have evolved to adaptively guide future behaviour rather than to accurately recall the past.

1.1.1. Consolidation Theory

One way to incorporate new information into existing knowledge is through the consolidation of new memories. Nearly the entire 20th century of memory neuroscience operated within the paradigm of consolidation theory (McGaugh, 2000). Broadly, the theory states that short-term, unstable memories consolidate into long-term memories in a time-dependent process and that, after consolidation, memories are relatively stable (Glickman, 1961; McGaugh, 1966). Thus, according to this account, disruption of consolidation mechanisms shortly after learning will impair long-term memory but the same treatment will not affect memory later on.

Research into long-term memory consolidation proceeded exclusively in living animals until the discovery of long-term potentiation in brain slices (Bliss & Lømo, 1973). In long-term potentiation (LTP), high-frequency electrical stimulation of a presynaptic neuron leads to enduring potentiation of postsynaptic responses. Findings from LTP research show considerable overlap with findings obtained in freely moving animals (Dong et al., 2015; Doyère, Debiec, Monfils, Schafe, & Ledoux, 2007; Hong et al., 2013). That is, many manipulations that impair long-term potentiation in vitro can also impair memory consolidation in vivo. This overlap suggests that LTP-like mechanisms may mediate long-term memories in the brain (Lisman, Cooper, Sehgal, & Silva, 2018; Malenka & Nicoll, 1999; Teyler & Discenna, 1984).

Research in LTP and freely moving animals revealed numerous proteins and pathways necessary to stabilize long-term memory. For instance, it is generally accepted that consolidation involves protein synthesis, transcription factors like cAMP-responsive element binding protein (CREB), *N*-methyl-D-aspartate receptors (NMDARs), remodeling of the actin cytoskeleton, calmodulin-dependent kinase II (CaMKII), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptors (AMPARs), mitogen-activated protein kinase (MAPK), and brain-derived neurotrophic factor (BDNF), to name just a few (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998; Cammarota et al., 2002; Duvarci, Nader, & Ledoux, 2005; Kandel, 2012; Lee, Everitt, & Thomas, 2004; Malinow & Malenka, 2002; McGaugh, 2000; Rodrigues, Schafe, & Ledoux, 2001; Rudy, 2015; Silva, Kogan, Frankland, & Kida, 1998). Importantly, memories are only vulnerable to disruptions of these molecular processes for a few

hours after initial learning. This transient susceptibility to amnesic agents implies that, after consolidation, memories are relatively fixed and unchangeable.

1.1.2. Reconsolidation Theory

A significant challenge to consolidation theory came with the publication of two papers showing retrieval could render memories vulnerable to amnesic treatment (Nader, Schafe, & Le Doux, 2000; Przybyslawski, Roullet, & Sara, 1999). While traditional consolidation theory suggests memories become stable shortly after learning, these papers showed that pharmacological agents could disrupt seemingly stable memories after retrieval. They also showed that this vulnerability was time-dependent, similar to the consolidation of new memories (McGaugh, 1966, 2000), suggesting that memories must reconsolidate after retrieval. For example, Nader and colleagues showed that after retrieval of a consolidated, long-term auditory fear conditioning memory, rats infused with anisomycin in the basolateral amygdala (BLA) showed impaired performance on a subsequent test (Nader et al., 2000). Importantly, this amnesic effect only occurred after retrieval. That is, the memory of rats that did not undergo reactivation was not vulnerable to anisomycin. Further, anisomycin only impaired memory if it was given shortly after retrieval and had no effect six hours later. However, this was not the first demonstration that post-retrieval manipulations can disrupt memory. In 1968, one group showed that electroconvulsive shock after re-exposure to a conditioned stimulus (CS) could impair memory (Misanin, Miller, & Lewis, 1968). The work of Nader and colleagues was especially important, though, because 1) it identified a key mechanism for reconsolidation, protein synthesis, and 2) it showed that the effect was specific to the BLA, at least for auditory fear conditioning memory

(Nader et al., 2000). Together, these findings launched a renewed interest in reconsolidation to study how long-term memories can change after retrieval (Finnie & Nader, 2012; Nader & Hardt, 2009; Tronson & Taylor, 2007).

It is now generally accepted that after retrieval certain memories are temporarily destabilized, vulnerable to change, and must be reconsolidated shortly thereafter. This post-retrieval plasticity has been demonstrated in many species including crabs (Barreiro, Suarez, Lynch, Molina, & Delorenzi, 2013; Suárez, Smal, & Delorenzi, 2010), *Aplysia* (S. Lee et al., 2012), snails (Gainutdinova et al., 2005), mice (Huynh, Santini, & Klann, 2014; Lux, Masseck, Herlitze, & Sauvage, 2015; Rao-Ruiz et al., 2011), rats (De Oliveira Alvares et al., 2013; Debiec, Bush, & LeDoux, 2011; Lee & Flavell, 2014; Nader et al., 2000), and humans (Agren et al., 2012; Schwabe, Nader, & Pruessner, 2014; Soeter & Kindt, 2015). Further, reconsolidation has been demonstrated in many different brain areas and memory tasks: auditory fear conditioning (Ben Mamou, Gamache, & Nader, 2006; Duvarci & Nader, 2004; Milton et al., 2013; Nader et al., 2000), contextual fear conditioning (Frankland et al., 2006; Lee & Flavell, 2014; Rao-Ruiz et al., 2011), conditioned taste aversion (Garcia-delaTorre, Perez-Sanchez, Guzman-Ramos, & Bermudez-Rattoni, 2014), and conditioned place preference (Ren et al., 2013; Xue et al., 2013) to name a few. Given this broad reach across many species, brain regions, and memory tasks, destabilization and reconsolidation seem to be fundamental components of memory processing.

The finding that memories can be impaired after retrieval generated some criticism over the years. These criticisms include that the amnesic agents could be lesioning the target tissue (Rudy, Biedenkapp, Moineau, & Bolding, 2006), that

reconsolidation blockade impairs retrieval but not memory storage (Lattal & Abel, 2004; Power, Berlau, McGaugh, & Steward, 2006), that reconsolidation blockade enhances extinction learning (Fischer, Sananbenesi, Schrick, Spiess, & Radulovic, 2004), and that certain memories do not undergo destabilization and reconsolidation (Taubenfeld, Milekic, Monti, & Alberini, 2001).

These issues have been addressed in the literature explicitly (Nader & Hardt, 2009). Briefly, Nader and Hardt argue that new learning following reconsolidation blockade demonstrates that the region remains functional and not significantly damaged by amnesic treatments. Since non-reactivated animals do not show impairments given the same pharmacological agents, this further suggests that they do not destroy tissue per se (Debiec, LeDoux, & Nader, 2002; Jobim et al., 2012; Nader et al., 2000).

Next, a group of findings showed that hippocampal memories believed to be impaired by reconsolidation blockade could show spontaneous recovery during future tests (Lattal & Abel, 2004; Power et al., 2006). However, other research has found no spontaneous recovery following successful reconsolidation blockade (Duvarci & Nader, 2004; Jobim et al., 2012; Lee et al., 2004; Xue et al., 2013). Numerous studies also show that reconsolidation blockade reduces brain correlates of long-term memory like synaptic varicosities, evoked field potentials, and others (Agren et al., 2012; S. Chen et al., 2014; Doyère et al., 2007; S. Lee et al., 2012). These findings suggest that reconsolidation blockade induces a memory storage impairment rather than a retrieval impairment.

These two lines of evidence also support the claim that reconsolidation blockade is not enhancing extinction. Common reconsolidation-blocking agents, like anisomycin,

actually seem to disrupt extinction rather than enhance it (Suzuki et al., 2004; Suzuki, Mukawa, Tsukagoshi, Frankland, & Kida, 2008).

Finally, not all memories are vulnerable to typical reconsolidation blockade treatments after retrieval (Milekic, Pollonini, & Alberini, 2007; Taubenfeld et al., 2001; Wang, de Oliveira Alvares, & Nader, 2009). This finding may be due to a variety of reasons including the specifics of the experimental design and boundary conditions. It may be that certain memories do destabilize and reconsolidate but are not vulnerable in the specific brain region being manipulated (Einarsson & Nader, 2012; Milekic et al., 2007; Nader et al., 2000; Taubenfeld et al., 2001). That is, experiments must be designed to target the appropriate brain structure during destabilization of a given memory. For instance, one report found that remote contextual fear memories are vulnerable to systemic injections of anisomycin but not infusions in the hippocampus (Frankland et al., 2006, but see Debiec et al., 2002; Einarsson & Nader, 2012). However, they found that recent memories are vulnerable to hippocampal anisomycin, suggesting that the site of reconsolidation likely changes over time. These findings suggest that, while memories can undergo destabilization and reconsolidation, there are boundary conditions that limit when and where this plasticity can occur.

1.1.3. Reconsolidation in Humans

The prospect of modifying or weakening specific memories has the potential to create new clinical treatments for human patients. Numerous experiments have demonstrated that it is, in fact, possible to disrupt reconsolidation in humans (Agren et al., 2012; Kindt & Soeter, 2018; Kindt, Soeter, & Vervliet, 2009; Schiller et al., 2010). In many cases, experimenters have utilized the β-adrenergic antagonist, propranolol (Kindt

& Soeter, 2018; Kindt et al., 2009; Soeter & Kindt, 2015), a drug commonly used to treat conditions such as hypertension and certain anxiety disorders in human patients. In animal studies, propranolol has been used to disrupt reconsolidation of fear memories in rodents (Debiec et al., 2011; Debiec & Ledoux, 2004; Ortiz, Giachero, Espejo, Molina, & Martijena, 2014). Unlike protein synthesis inhibition, blocking β-adrenergic receptors seems to weaken the emotional component of the memory while leaving other associations intact (Cogan, Shapses, Robinson, & Tronson, 2018). For instance, in one study, human subjects with a spider phobia were exposed to a tarantula for 2 minutes (memory reactivation). Next, the participants took propranolol, or a placebo (Soeter & Kindt, 2015) to block reconsolidation processes. Participants who had received propranolol showed increased approach behaviour to the spider, compared to the placebo group, and this improvement was sustained for at least one year. Interestingly, research using fMRI shows that reconsolidation blockade can even reduce activation in relevant brain areas like the amygdala (Agren et al., 2012; Björkstrand et al., 2016, 2017). Thus, reconsolidation blockade weakens both the behavioural performance and the neural response to fear stimuli.

Perhaps most importantly, reconsolidation blockade can improve the condition of patients with post-traumatic stress disorder (PTSD; Brunet et al., 2008). In one study, patients received propranolol or a placebo after retrieval of a traumatic event. One week later, those that received propranolol showed decreased heart rate and skin conductance after retrieving the event again, compared to the week before. While these data show promise, this study had a relatively small sample size (19 subjects) and several subsequent experiments have failed to show a similar effect with propranolol or

other drugs (Wood et al., 2015). However, a more recent study found that repeated memory reactivation with propranolol—once a week for six weeks—could successfully reduce self-reported PTSD symptoms that remained low even six months later (Brunet et al., 2018). These findings suggest that reconsolidation-based treatments can work, but more research is needed to determine the correct treatment parameters.

1.2. Memory Destabilization

1.2.1. When Does Destabilization Occur?

Under what conditions a memory becomes labile is an area of active research. As stated above, the age of a memory is an important predictor of whether it will destabilize (and in what region) following retrieval (Frankland et al., 2006; Milekic & Alberini, 2002; Wang et al., 2009). That is, certain memories destabilize only when retrieval occurs within days after training but not several weeks later (Milekic & Alberini, 2002). Other memories are only vulnerable beyond one month after learning (Wang et al., 2009). Still others show no time-dependent boundary condition (Einarsson & Nader, 2012; Nader et al., 2000). A paper by Wang and colleagues provides one explanation to reconcile these variations in metaplasticity (Wang et al., 2009). They first showed that the strength of an auditory fear memory serves as a boundary condition. Weak fear memories can destabilize one day after training whereas strong memories cannot. If strong memories are retrieved 30 or 60 days after training, however, they will be vulnerable to reconsolidation blockade. This discrepancy seems to be mediated by the presence of NMDARs containing the GluN2B subunit (GluN2B-NMDARs), which are crucial for destabilizing memory after retrieval (Ferrer Monti et al., 2016; Milton et al., 2013; Zhang, Haubrich, Bernabo, Finnie, & Nader, 2018). They found that one day after

training, plastic memories have more synaptic GluN2B-NMDARs and strong, fixed memories have fewer GluN2B-NMDARs. By 60 days post-training, when strong memories become plastic again, both strongly- and weakly-trained animals have comparable GluN2B-NMDAR expression. Thus, boundary conditions may in part reflect the makeup of synaptic receptors.

These boundary conditions provide clues to a possible adaptive function of memory destabilization and reconsolidation. A prominent theory suggests that this post-learning plasticity serves to update memories to maintain their relevance in a changing environment (Exton-McGuinness, Lee, & Reichelt, 2015; Finnie & Nader, 2012; Lee, 2009). This updating process may involve the use of prediction error to signal that the existing memory is inadequate and something new should be learned (Sevenster, Beckers, & Kindt, 2013). Inactivation of the ventral tegmental area (VTA), a central region for signalling prediction error via dopamine release, can prevent memory destabilization (Reichelt, Exton-McGuinness, & Lee, 2013). In fact, inhibition of dopamine receptors in the BLA prevents labilization after retrieval (Merlo et al., 2015). Further, merely changing the timing of a tone-shock pairing (i.e., shocking the animal 10 seconds after the onset of the tone compared to 30 seconds) is sufficient to trigger destabilization of an auditory fear conditioning memory (Díaz-Mataix, Ruiz Martinez, Schafe, Ledoux, & Doyère, 2013). Other work shows that new contextual information can be incorporated into a memory through the reconsolidation process (De Oliveira Alvares et al., 2013; Lee, 2010). This allows animals to respond appropriately to new, relevant environments without merely generalizing their response to all contexts (De

Oliveira Alvares et al., 2013). Thus, the presentation of novel information seems capable of triggering plasticity in existing memories.

1.2.2. Initiating Memory Destabilization

Destabilization of a memory after retrieval requires several molecular processes (Finnie & Nader, 2012; Flavell, Lambert, Winters, & Bredy, 2013). Interestingly, these processes seem to be somewhat dissociable. That is, labilization can occur in the absence of overt retrieval. Expression of a learned behaviour seems to require activation of AMPARs, whereas memory destabilization does not (Barreiro et al., 2013; Ben Mamou et al., 2006; Garcia-delaTorre et al., 2014; Milton et al., 2013). For instance, one study found that inhibiting AMPARs before retrieval with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) impaired freezing behaviour to a conditioned tone stimulus. Moreover, anisomycin administration after retrieval impaired long-term memory, suggesting that the memory was still destabilized, even in the absence of overt retrieval (Ben Mamou et al., 2006).

Instead, memory destabilization requires the activation of other receptors. As stated above, prediction error signalling via dopamine release from the VTA seems to be crucial for triggering destabilization (Merlo et al., 2015; Reichelt et al., 2013). In the amygdala specifically, inhibition of either D1 or D2 dopamine receptors will prevent destabilization (Merlo et al., 2015). Activation of cannabinoid-1 receptors (CB1Rs) is also required to labilize a memory (Kim, Moki, & Kida, 2011; Lee & Flavell, 2014; Suzuki et al., 2008). For instance, blocking CB1Rs before retrieval prevents the amnesic effect of MK-801, which can typically block reconsolidation (Lee & Flavell, 2014). On the other hand, CB1R agonists can promote destabilization (Lee & Flavell, 2014). Research

shows that other receptors are also necessary for destabilization: L-type voltage gated calcium channels (LVGCCs; Kim et al., 2011; Suzuki et al., 2008), β-adrenergic receptors (Lim et al., 2018), NMDA receptors (Ben Mamou et al., 2006; Flavell et al., 2013), and muscarinic acetylcholine receptors (mAChRs; Stiver et al., 2015; Stiver et al., 2017). Notably, inhibition of any one of these receptors is sufficient to prevent destabilization. These findings seem to imply that the activation of all of these receptors is required to render memory labile after retrieval.

In particular, activation of NMDARs is the most well-studied mechanism of initiating memory destabilization (Flavell et al., 2013; Zhang et al., 2018). The NMDAR partial agonist, D-cycloserine (DCS), can promote labilization in memories that do not normally destabilize following retrieval (Bustos, Giachero, Maldonado, & Molina, 2010; Espejo, Ortiz, Martijena, & Molina, 2016). General NMDAR antagonists, like DL-2-Amino-5-phosphonopentanoic acid (APV), can prevent destabilization when infused directly in the brain before retrieval. Used in this way, APV can prevent the amnesic effect of anisomycin on reconsolidation (Barreiro et al., 2013; Ben Mamou et al., 2006). However, APV can also impair reconsolidation when infused after retrieval (Torras-Garcia, Lelong, Tronel, & Sara, 2005). Research has since found that specific NMDAR subunits are necessary for different components of this process. GluN2B-NMDARs are necessary to destabilize a memory, not for reconsolidation, whereas GluN2A-NMDARs are necessary for reconsolidation, not destabilization (Milton et al., 2013; Yu, Huang, Chang, & Gean, 2016). Administration of GluN2B-NMDAR antagonists can, therefore, prevent reconsolidation blockade (Ben Mamou et al., 2006; Lopez, Gamache, Schneider, & Nader, 2015) or memory modulation (Ferrer Monti et al., 2016), but

GluN2A-NMDAR antagonists cannot (Yu et al., 2016). As previously mentioned, weak auditory fear memories show more GluN2B-NMDARs compared to strong ones, which coincides with their susceptibility or resistance to memory destabilization (Wang et al., 2009). Overexpressing GluN2A-NMDARs can artificially increase the ratio of GluN2A/GluN2B-NMDARs and is sufficient to prevent normally-plastic memories from undergoing destabilization (Holehonnur et al., 2016). This differential expression of GluN2B-NMDARs may even help explain the difficulty in translating reconsolidation blockade treatments to human PTSD patients (Wood et al., 2015).

1.2.3. Intracellular Mechanisms of Memory Destabilization

Following receptor activation, several intracellular cascades facilitate memory destabilization. Through these processes, synapses are made labile before being restabilized through reconsolidation later on.

Reorganization of the cytoskeleton is an essential component of memory plasticity (Lamprecht, Margulies, & Farb, 2006; Rehberg, Bergado-Acosta, Koch, & Stork, 2010). One interesting study found dynamic changes in Rac1, a regulator of actin polymerization, through destabilization and reconsolidation of cocaine-cue memories in the nucleus accumbens (NAc; Wright et al., 2019). The authors found decreased activity of Rac1 during memory destabilization which returned to normal by six hours following retrieval when reconsolidation is believed to have occurred (Nader et al., 2000). They found that expression of a constitutively active Rac1 mutant prevented destabilization, presumably by preventing actin polymerization. Interestingly, they used a photoactivatable Rac1 mutant and found that activation of Rac1 while the memory is still labile seems to lock the spine in a weakened state, resulting in impaired performance.

Therefore, it seems that decreased Rac1 activity is essential for destabilization and that Rac1 activation stabilizes the cytoskeleton once synaptic reconsolidation has occurred.

CaMKII activation is another well-studied component of learning and memory as well as destabilization, specifically (Vigil & Giese, 2018). GluN2B-NMDARs, which are essential for initiating destabilization, bind to CaMKII, and this interaction facilitates long-term potentiation (Barria & Malinow, 2005). Of note, CaMKII binds with much less affinity to GluN2A-NMDARs, which may explain why neurons with a higher GluN2A/GluN2B-NMDAR ratio are more resistant to destabilization following retrieval (Holehonnur et al., 2016). It may even be that CaMKII, itself, can increase GluN2B-NMDARs at the PSD, which might further promote destabilization (Vigil & Giese, 2018).

Destabilization and reconsolidation seem to involve a balance between activation and inhibition of CaMKII (Vigil & Giese, 2018). One report found that overexpression of CaMKII during retrieval impaired both recall and retention of auditory and contextual fear memories (Cao et al., 2008). Others have found that retrieval increases expression of the endogenous inhibitor CaMK2N1 and that knockdown of CaMK2N1 after retrieval impairs long-term memory expression in later tests (Vigil, Mizuno, Lucchesi, Valls-Comamala, & Giese, 2017). On the other hand, inhibiting CaMKII activity prevents reconsolidation blockade, suggesting that CaMKII is necessary first to destabilize memory (Jarome, Ferrara, Kwapis, & Helmstetter, 2016). These findings suggest that after retrieval, neurons must balance between activation of CaMKII (through GluN2B-NMDAR activation) and inhibition (via CaMK2N) to control destabilization and reconsolidation.

1.2.4. Protein Degradation in Memory Destabilization

An important consequence of CaMKII activation in memory labilization is the activation of the ubiquitin-proteasome system (UPS) to degrade proteins (Jarome et al., 2016). Elevated proteasome activity and ubiquitination have been observed after retrieval in several studies (Jarome et al., 2016; Lee et al., 2008; Ren et al., 2013; Werner, Milovanovic, Christian, Loweth, & Wolf, 2015). Increased polyubiquitination has been observed as early as 15 min post-retrieval in some areas (Ren et al., 2013). However, others report no increase at this time point (Lee et al., 2008), suggesting that polyubiquitination occurs at different rates in different structures. In the amygdala, increased activity of the UPS requires CaMKII-dependent phosphorylation of the proteasome subunit Rpt6 (Jarome et al., 2016). Of course, polyubiquitination precedes degradation of the proteins themselves. For instance, polyubiquitination of the synaptic protein Shank occurs in the hippocampus within one hour after retrieval, but Shank degradation does not occur until two hours post-retrieval (Lee et al., 2008). Similarly, retrieval of cocaine-induced conditioned place preference (CPP) memory increases the ubiquitination of N-ethylmaleimide sensitive fusion protein (NSF) within 15 minutes in the NAc core (Ren et al., 2013). However, it takes another one to two hours for the proteasome-dependent reduction in NSF to occur.

Only memories that destabilize show polyubiquitination after retrieval (Lee et al., 2008). While it does not trigger the labilization process per se, proteasome activity seems to be necessary for destabilizing the memory. For instance, infusion of the proteasome inhibitor, clasto-lactacystin beta-lactone (β-lac), prevents the amnesic effect of anisomycin on reconsolidation (Lee et al., 2008; Lee, 2008). Thus, without prior

protein degradation, protein synthesis inhibitors do not disrupt reconsolidation. This effect occurs in several species including mice (Lee et al., 2008; Sol Fustiñana, de la Fuente, Federman, Freudenthal, & Romano, 2014), rats (Ren et al., 2013; Mikaela L. Stiver et al., 2017), crabs (Sol Fustiñana et al., 2014), and *Aplysia* (Lee et al., 2012) suggesting that the role of degradation in destabilization is well-conserved. Importantly, protein degradation is necessary for destabilization in general, not just for weakening the retrieved memory. Without proteasome activation, post-retrieval manipulations that enhance memory strength are ineffective as well (Lee, 2008; Sol Fustiñana et al., 2014).

1.3. Memory Reconsolidation

1.3.1. Mechanisms of Reconsolidation

Once destabilized, a memory must soon be re-stabilized through reconsolidation in order to persist as long-term memory. This reconsolidation process shares a number of molecular requirements with consolidation of new memories. Both require activation of NMDARs (Ben Mamou et al., 2006; Kim et al., 2011; Rodrigues et al., 2001), CREB and gene transcription (Fukushima et al., 2014; Kida et al., 2002; Kim et al., 2011; Villain, Florian, & Roullet, 2016), PKA (Kandel, 2012; Tronson, Wiseman, Olausson, & Taylor, 2006), extracellular signal-regulated kinase/MAPK (ERK/MAPK; Duvarci et al., 2005; Giese & Mizuno, 2013; Emiliano Merlo, Milton, & Everitt, 2018), and mTOR-dependent protein synthesis (Jobim et al., 2012; Parsons, Gafford, & Helmstetter, 2006) to name just a few. As previously mentioned, dynamic spine morphology has been observed during memory destabilization and reconsolidation. For instance, Rac1 activity is essential to stabilize the actin cytoskeleton during reconsolidation (Wright et al.,

2020). In addition to these intracellular mechanisms, reconsolidation also requires ongoing neuronal excitability. Either by enhancing inhibitory neurotransmission (Bustos et al., 2010; Espejo et al., 2016; Reichelt et al., 2013) or optogenetic inhibition of neurons (Lux et al., 2015), reducing postsynaptic excitability disrupts reconsolidation and leads to long-term memory impairment.

Consolidation and reconsolidation are dissociable, with differential requirements of specific signalling molecules. Several reports suggest that zif268, an immediate early gene, is especially necessary for reconsolidation but not consolidation (Bozon, Davis, & Laroche, 2003; Lee, 2010; Lee et al., 2004). Zif268-null mice show intact object recognition memory after training but their long-term memory after reactivation is impaired (Bozon et al., 2003). Synthesis of brain-derived neurotrophic factor (BDNF), but not zif268, is necessary for consolidation of contextual fear in the hippocampus but not zif268 (Lee et al., 2004). The same report found that inhibiting zif268 synthesis during retrieval impairs reconsolidation, whereas preventing translation of BDNF did not. Using microarray technology, Barnes and colleagues identified numerous other genes that are differentially regulated between consolidation and reconsolidation (Barnes, Kirtley, & Thomas, 2012). These findings argue for a dedicated focus to studying reconsolidation mechanisms rather than simply extrapolating all findings from consolidation research.

The most well-replicated finding in the reconsolidation literature is that administration of protein synthesis inhibitors after retrieval can disrupt re-stabilization (Duvarci et al., 2005; Gainutdinova et al., 2005; Jobim et al., 2012; Lee et al., 2012; Nader et al., 2000; Suárez et al., 2010). One criticism of this literature is the heavy

reliance on anisomycin which is known to significantly alter neurotransmitter release as well as contribute to cell death through apoptosis (Canal, Chang, & Gold, 2007; Curtin & Cotter, 2002; Iordanov et al., 1997; Rudy et al., 2006). Thus, more recent research has tended to utilize other protein synthesis inhibitors like rapamycin, which may or may not have similar effects as anisomycin (Huynh et al., 2014; Jobim et al., 2012; Li et al., 2013; Lopez et al., 2015).

In order to more specifically inhibit the translation of individual mRNAs, researchers have used antisense oligodeoxynucleotides (ODNs). These nucleotides are synthesized in a sequence-specific manner in order to block the translation of a target mRNA (Dias & Stein, 2002). This method allows one to determine which specific proteins must be synthesized in a given context. In regard to reconsolidation, antisense ODNs have been used to determine that synthesis of several proteins is necessary for reconsolidation: zif268 (Barnes et al., 2012; Lee, 2010; Lee et al., 2004; Maddox, Monsey, & Schafe, 2011) C/EBPβ (Milekic et al., 2007), C/EBPδ (Arguello et al., 2013), and Arc (Maddox & Schafe, 2011).

Although most research has focused on impairing long-term memories after destabilization, memory strengthening can also occur. While inhibition of β-adrenergic receptors can impair fear memories (Debiec & Ledoux, 2004; Soeter & Kindt, 2015), β-adrenergic receptor agonists can enhance such memories (Debiec et al., 2011). Similarly, auditory fear memory can be enhanced with a post-retrieval infusion of a PKA activator or impaired using a PKA inhibitor (Tronson et al., 2006). Furthermore, the same memory can undergo multiple periods of destabilization-reconsolidation. With

each repeated reactivation session, PKA activation can increasingly strengthen the memory.

1.3.2. The Reconsolidation Window

Destabilization seems to occur immediately after retrieval, rendering the memory transiently labile before it is restabilized during reconsolidation. This plasticity period, between the onset of destabilization and the end of reconsolidation, is sometimes referred to as the “reconsolidation window” (Debiec et al., 2011; Ferrer Monti et al., 2016; Jarome et al., 2012; Wright et al., 2020). Precisely how long the reconsolidation window remains open is not clear and is likely to vary for different manipulations and different brain regions. For instance, one report found downregulation of GluA1-3 AMPAR subunits in the hippocampus within one hour of retrieval. By four hours after retrieval, only GluA1 subunit levels had returned to baseline (Rao-Ruiz et al., 2011). On the other hand, Hong et al. found increased CP-AMPAR expression at the synapse within 5 minutes after retrieval that returned to baseline within 3 hours (Hong et al., 2013). Given this variability, it seems useful to limit this discussion to a single brain structure. Thus, the reconsolidation window of the BLA specifically will be described here.

Within 5 minutes after retrieval, there is an exchange of AMPARs at the PSD, which returns to baseline by 3 hours post-retrieval (Hong et al., 2013). Others have also found that phosphorylated GluA1-AMPARs remain elevated at 1 hour after retrieval (Holehonnur et al., 2016; Jarome et al., 2012). Zif268 expression increases by 30 minutes after retrieval and returns to baseline by 1-hour post-retrieval (Jarome et al., 2012). Proteasome activity is increased in the amygdala within 90 minutes of retrieval

(Jarome et al., 2016), although the polyubiquitination of proteins should precede the actual degradation. For instance, retrieval triggers the ubiquitination of NSF in the NAc within 15 minutes, but the actual decrease in protein expression does not occur until around 1 hour after retrieval (Ren et al., 2013). Infusion of C/EBPδ-antisense in the BLA 5 hours after retrieval is sufficient to disrupt reconsolidation (Arguello et al., 2013). There was no effect when the infusion occurred 12 hours post-retrieval, suggesting the antisense ODN does not impair memory maintenance. Infusion of general protein synthesis inhibitors like anisomycin or rapamycin can impair reconsolidation in the BLA when infused immediately after retrieval but not 6 hours later (Jarome et al., 2012; Nader et al., 2000). Thus, the reconsolidation window is generally believed to be closed within 6 hours. What to make of these findings? It seems likely that during lability there are a series of semi-independent, overlapping mechanisms that begin and end at different times. They are semi-independent because blocking any one of them seems to disrupt reconsolidation. Furthermore, as time passes since the induction of destabilization, a smaller number of targets will remain vulnerable to disruption.

1.4. AMPAR Exchange During Destabilization and Reconsolidation

A central component of memory destabilization and reconsolidation is the movement of AMPARs at the PSD. During destabilization, GluA2-AMPARs are internalized from the membrane and replaced with GluA2-lacking AMPARs. Within hours, these GluA2-lacking AMPARs are removed and GluA2-AMPARs return to the PSD (Hong et al., 2013). This AMPAR exchange seems to be fundamental to post-retrieval plasticity.

Most AMPARs in the adult brain are composed of either the GluA1 and GluA2 subunits or GluA2 and GluA3 subunits, although GluA1 homomers do play a role as well (Diering & Huganir, 2018). Uniquely, GluA2-containing AMPARs (GluA2-AMPARs) do not inwardly rectify (Malinow & Malenka, 2002). That is, membrane current can pass through GluA2-AMPARs equally well in either direction whereas inwardly rectifying GluA2-lacking AMPARs overwhelmingly pass current inwardly. This difference in rectification allows for the monitoring of postsynaptic AMPAR movement by measuring changes in the rectification index. As inwardly rectifying, GluA2-lacking AMPARs reach the synaptic membrane, the rectification index increases. Further, these GluA2-lacking AMPARs are calcium-permeable (CP-AMPARs) whereas GluA2-AMPARs are impermeable to calcium (CI-AMPARs; Diering & Huganir, 2018). Thus, the relative distribution of these AMPARs at the PSD profoundly impacts the membrane dynamics during synaptic transmission.

Accordingly, CP-AMPARs seem to play a unique role in learning and plasticity (Clem & Huganir, 2010; Malinow & Malenka, 2002; Rumpel, LeDoux, Zador, & Malinow, 2005). These CP-AMPARs are not generally found at the PSD. Instead, they seem to exist at perisynaptic sites, ready to be quickly incorporated into the postsynaptic membrane during LTP induction (Diering & Huganir, 2018). Rapid phosphorylation and insertion of CP-AMPARs can be seen during both new learning and immediately after retrieval (Clem & Huganir, 2010; Hong et al., 2013; Jarome et al., 2012). The synaptic presence of these CP-AMPARs roughly corresponds to periods of enhanced plasticity. For instance, one report found that timing a reconsolidation manipulation to peak concentrations of CP-AMPARs improved its efficacy (Clem & Huganir, 2010).

Specifically, they used the reconsolidation update procedure wherein extinction occurring within one hour after retrieval seems to impair long-term memory (Clem & Huganir, 2010; Monfils, Cowansage, Klann, & Ledoux, 2009; Schiller et al., 2010). Clem and Huganir found that this intervention could impair memory 1- but not 7-days after learning, which coincided with data showing elevated CP-AMPAR expression at 1- but not 7-days after retrieval. Other work showed that blocking CP-AMPARs after retrieval impairs reconsolidation (Hong et al., 2013). How long CP-AMPARs remain at the PSD varies. After initial learning, they are enriched at the PSD within 5 minutes, persist there for at least 24 hours, and their expression returns to baseline within one week after learning (Clem & Huganir, 2010; Hong et al., 2013). This time-course is compressed after retrieval when CP-AMPARs are inserted within 5 minutes and removed within 3 hours (Hong et al., 2013).

Activity-dependent insertion of CP-AMPARs seems to require the removal of GluA2-AMPARs first. Blocking endocytosis of GluA2-AMPARs after retrieval prevents insertion of CP-AMPARs and limits memory destabilization (Hong et al., 2013; Rao-Ruiz et al., 2011). Similarly, mutant mice lacking protein interacting with C kinase-1 (PICK1), which is important for GluA2-AMPAR recycling (Lin & Huganir, 2007), also show low insertion of CP-AMPARs and impaired CP-AMPAR-mediated plasticity (Clem, Anggono, & Huganir, 2010). That GluA2-AMPARs must first be removed for CP-AMPARs to be inserted could reflect a limited number of AMPAR slots at the synapse that must be made available in order for CP-AMPAR-mediated plasticity to occur (Opazo, Sainlos, & Choquet, 2012).

While CP-AMPARs insertion occurs in an activity-dependent manner, GluA2-AMPARs regularly move in and out of the PSD in addition to activity-dependent trafficking (Diering & Huganir, 2018; Scholz et al., 2010). These AMPARs reside in intracellular stores through the action of PICK1 (Citri et al., 2010; Lin & Huganir, 2007). NSF contributes to the dissociation of PICK1-GluA2 (Hanley, Khatri, Hanson, Ziff, & Louis, 2002), which may allow for their insertion into the extrasynaptic membrane. From there, GluA2-AMPARs can laterally diffuse into the postsynaptic density where they are trapped by local proteins (Huganir & Nicoll, 2013; Opazo et al., 2012). The maintenance of GluA2-AMPARs at the PSD will be discussed further below but requires interactions with several different proteins including GRIP1/GRIP2, BRAG2, and AP2 (Diering & Huganir, 2018; Lee, Liu, Wang, & Sheng, 2002; Scholz et al., 2010). Eventually, GluA2-AMPARs laterally diffuse from the PSD where they undergo endocytosis in clathrin-coated pits.

While the recycling of GluA2-AMPARs is ongoing, activity-dependent endocytosis is also possible. Synaptotagmin-3 (Syt3), for instance, can internalize GluA2-AMPARs in the presence of calcium following neuronal stimulation (Awasthi et al., 2019). This Syt3 mechanism may represent one pathway through which GluA2-AMPARs leave the PSD to allow CP-AMPARs to be inserted during plasticity. As mentioned above, CP-AMPARs do not remain at the PSD indefinitely and are eventually replaced with GluA2-AMPARs. These GluA2-AMPARs seem to be primarily recycled receptors rather than new ones (Lin & Huganir, 2007). In summary, CP-AMPAR insertion is important during periods of plasticity but these receptors are later replaced with GluA2-AMPARs.

New learning results in a persistent increase in AMPARs at the PSD (Dong et al., 2015). After the internalization of CP-AMPARs, an enrichment of newly inserted GluA2-AMPARs maintains synaptic transmission and memory storage (Hong et al., 2013). Thus, the maintenance of GluA2-AMPARs at the postsynaptic density is essential for the persistence of long-term memory. Manipulations that cause internalization of GluA2-AMPARs lead to significant memory loss (Dong et al., 2015). For instance, disrupting the GluA2-NSF interaction in the hippocampus causes endocytosis of these receptors and impairs both object location and contextual fear memories (Migues, Hardt, Finnie, Wang, & Nader, 2014). Similarly, infusions of the protein kinase C (PKC) inhibitor, ζ inhibitory peptide (ZIP), in the BLA lead to internalization of GluA2-AMPARs and amnesia of an auditory fear conditioning memory (Migues et al., 2010). Importantly, blocking the endocytosis of these AMPARs with the peptide $GluA2_{3Y}$ prevents the amnesic effect of these manipulations. Endocytosis of GluA2-AMPARs also seems to mediate memory loss through forgetting (Migues et al., 2016). In this case, blocking their removal with $GluA2_{3Y}$ prolongs the life of the memory by seemingly blocking active forgetting. The concentration of GluA2-AMPARs may, therefore, be closely related to the strength of the memory. Other work has found that GluA2-AMPAR levels in postsynaptic fractions do indeed correlate with memory strength (Hara et al., 2012; Migues et al., 2010; Sebastian, Vergel, Baig, Schrott, & Serrano, 2013). That is, stronger memories seem to have higher concentrations of GluA2-AMPARs.

In order for long-term memory to persist, GluA2-AMPARs must be persistently maintained at the synapse. Pharmacologically blocking the maintenance of GluA2-AMPARs during memory storage leads to their endocytosis, and they do not return once

the pharmacological agent has been removed (Migues et al., 2014, 2010). This suggests that, after consolidation, homeostatic mechanisms at the synapse promote the internalization of GluA2-AMPARs but not their insertion back into the membrane. Despite these mechanisms, long-term memories can persist for years and even decades. This begs the question, by what mechanism can the cell actively oppose these persistent pro-internalization processes? The protein PKMζ has gained considerable attention as a molecule that may maintain long-term memory by countering AMPAR internalization (Kwapis & Helmstetter, 2014; Ling et al., 2002; Sacktor, 2011, 2012).

1.5. Protein Kinase M ζ

1.5.1. PKMζ Expression and Regulation

PKMζ is an atypical PKC isoform transcribed from an internal promoter within the *PRKCZ* gene (Hernandez et al., 2003). This gene also codes for full-length PKCζ, although the expression of this protein is nearly absent in the forebrain (Hernandez et al., 2003). On the other hand, PKMζ protein is seemingly only expressed in the brain (Hernandez, Oxberry, Crary, Mirra, & Sacktor, 2014; Muslimov et al., 2004). Within neurons, this protein and its mRNA are enriched at dendritic spines (Hara et al., 2012; Hernandez et al., 2014; Muslimov et al., 2004) although PKMζ is also found in the nucleus (Hernández et al., 2014; Ko et al., 2016). There are two dendritic targeting elements (DTEs) that target PKMζ mRNA to the dendrite—one element is necessary to transport the mRNA to the dendrite, the other is necessary for localization at the dendritic tip (Muslimov et al., 2004).

Under basal conditions, there is little translation of PKMζ mRNA in the dendrite (Bal et al., 2016). This may result in part from BC1 RNA, which is a regulator of dendritic translation that co-localizes with PKMζ mRNA in dendrites (Muslimov et al., 2004). However, the dominant hypothesis is that protein interacting with NIMA 1 (Pin1) actively represses dendritic translation, including PKMζ mRNA, under basal conditions (Westmark et al., 2010). Glutamatergic signalling seems to disrupt the enzymatic activity of Pin1, leading to increased protein synthesis. Further, Pin1-KO mice show roughly 200% more PKMζ than wild-type animals. Following LTP induction, the application of ZIP is sufficient to lower dendritic translation (Westmark et al., 2010), suggesting that PKMζ engages in positive feedback to promote its own synthesis. This positive feedback may be through direct phosphorylation of Pin1 by PKMζ. The two proteins co-precipitate and PKMζ is capable of phosphorylating Pin1 at Ser16, which inhibits its enzymatic activity (Westmark et al., 2010).

PKMζ has a structure that enables it to be persistently active. The full-length PKCζ protein contains a regulatory domain that is attached to the catalytic domain by a hinge region. Because PKMζ is transcribed from an internal promoter within an intronic region of *PRKCZ*, it only contains a fraction of the hinge as well as the full C-terminal catalytic domain (Hernandez et al., 2003). Thus, PKMζ is constitutively active. It is this shorter, persistently active PKM structure that distinguishes it from PKCζ. While a PKM version of the closely related atypical PKC, PKCι/λ, exists, its presence in the brain is not very pronounced and rather difficult to detect (Hernandez et al., 2003). This paucity of PKMι/λ implies that the overwhelming source of atypical PKM in neurons is PKMζ. Phosphoinositide-dependent protein kinase-1 (PDK1) seems to phosphorylate PKMζ at

T410 whereas T560 is an autophosphorylation site (Kelly, Crary, & Sacktor, 2007). Importantly, phosphorylation of PKMζ does not seem to be activity-dependent, although the expression of PKMζ protein is subject to increase after stimulation (Kelly, Crary, et al., 2007). Thus, it seems that PKMζ is phosphorylated shortly after synthesis.

Following in vitro stimulation that induces LTP or new learning in vivo, synthesis of new PKMζ increases in the dendrite (Hsieh et al., 2016; Osten, Valsamis, Harris, & Sacktor, 1996; Palida et al., 2015; Sacktor et al., 1993). Translation of PKMζ requires multiple signalling molecules and pathways including actin polymerization, PI3 kinase, CaMKII, ERK/MAPK, PKA, mTOR, and other PKCs (Kelly, Crary, et al., 2007; Kelly, Yao, Sondhi, & Sacktor, 2007). The increase in PKMζ protein does not occur during early LTP but can be observed as soon as 30-40 minutes after tetanization of hippocampal cells in vitro (Osten et al., 1996; Sacktor et al., 1993). Once synthesized, PKMζ protein levels remain elevated for at least 3 hours after tetanization (Tsokas et al., 2016). This is in contrast to most other PKCs which show only transient elevation after tetanic stimulation (Osten et al., 1996). Similarly, increases in PKMζ protein can occur within 2.5 hours after learning and persist for at least one month (Hsieh et al., 2016). These data suggest that PKMζ plays a role in late LTP and later stages of memory consolidation.

In addition to being constitutively active, PKMζ is also protected from degradation by Kidney/Brain protein (KIBRA). Levels of KIBRA and PKMζ correlate in cells and the two proteins co-precipitate (Vogt-Eisele et al., 2014). KIBRA binds to the catalytic domain of PKMζ and PKCζ but not to other classical or novel PKC isoforms (Büther, Plaas, Barnekow, & Kremerskothen, 2004). PKMζ can phosphorylate KIBRA at Ser975

and Ser978, although this does not seem to affect their interaction (Büther et al., 2004; Vogt-Eisele et al., 2014). More specifically, KIBRA seems to bind exclusively to phosphorylated PKMζ (Vogt-Eisele et al., 2014). It is not clear whether binding to KIBRA requires phosphorylation of new PKMζ or if this phosphorylation occurs shortly thereafter. Nonetheless, there is little unphosphorylated PKMζ, suggesting it degrades quickly, but KIBRA-bound PKMζ is protected from this fate (Vogt-Eisele et al., 2014). Further evidence shows that while PKMζ can be ubiquitinated and degraded by proteasome activation, overexpression of KIBRA prevents this (Vogt-Eisele et al., 2014). Other research shows that KIBRA overexpression enhances LTP while decreasing KIBRA expression impairs LTP and long-term memory (Heitz et al., 2016; Makuch et al., 2011; Vogt-Eisele et al., 2014). Thus, KIBRA seems to be an essential regulator of PKMζ and long-term memory.

1.5.2. PKMζ and Memory Maintenance

Considerable research attention focused on whether PKMζ is a molecular lynchpin of memory maintenance. While a number of tools have been used to examine the role of PKMζ, many experiments utilized ζ inhibitory peptide (ZIP) to disrupt PKMζ activity. ZIP contains the pseudosubstrate sequence of the ζ catalytic domain and can thereby disrupt the activity of PKMζ (Laudanna, Mochly-Rosen, Liron, Constantin, & Butcher, 1998; Ling et al., 2002; Pastalkova et al., 2006; Yao et al., 2013). Typically, ZIP is applied after memory consolidation to show that it can disrupt maintenance of long-term memory rather than consolidation or reconsolidation. This approach has been used in many tasks and brain regions to conclude that PKMζ may be essential for long-term memory maintenance (Gámiz & Gallo, 2011; Hardt, Migues, Hastings, Wong, &

Nader, 2010; Kwapis, Jarome, Gilmartin, & Helmstetter, 2012; Kwapis, Jarome, Lonergan, & Helmstetter, 2009; Pastalkova et al., 2006; Serrano et al., 2008; von Kraus, Sacktor, & Francis, 2010). Results from studies using ZIP have also lead to the suggestion that PKMζ or homologous PKMs maintain memory in several different species: snails (Solntseva, Kozyrev, & Nikitin, 2015), *Aplysia* (Cai, Pearce, Chen, & Glanzman, 2011; S. Chen et al., 2014), mice (Ko et al., 2018; Mei, Nagappan, Ke, Sacktor, & Lu, 2011), and rats (Hardt et al., 2010; Pastalkova et al., 2006; Serrano et al., 2008). Experiments with ZIP provide evidence that memory maintenance is vulnerable to disruption even three months after learning (Shema, Hazvi, Sacktor, & Dudai, 2009). Furthermore, ZIP is capable of disrupting persistent pain in rodents, suggesting that PKMζ may play a role in maintaining pain as well (Laferrière et al., 2011; Nasir et al., 2016).

As mentioned, LTP results in persistent elevation of GluA2-AMPARs at the PSD, which must be maintained for the memory to survive. PKMζ works to counteract the homeostatic processes that promote the internalization of GluA2-AMPARs (Migues et al., 2010; Sacktor, 2011). One way to test whether a protein is necessary for GluA2-AMPAR maintenance is by using the $GluA2_{3Y}$ peptide. It is a short peptide that mimics the tyrosine-rich (3Y) section of the GluA2 tail that binds proteins for GluA2 trafficking and regulation (Yu, Wu, Liu, Ge, & Wang, 2008). Infused $GluA2_{3Y}$ presumably outcompetes full-length GluA2 subunits for binding to proteins that internalize these AMPARs. Thus, if PKMζ maintains memory via GluA2-AMAPRs, then inhibiting its activity should lead to amnesia which is rescued by $GluA2_{3Y}$. In fact, ZIP does cause internalization of GluA2-AMPARs and amnesia, but co-infusion of ZIP and $GluA2_{3Y}$

leaves memory intact (Li et al., 2011; Migues et al., 2010). Similarly, viral expression of shRNA specific to PKMζ disrupts LTP and inhibitory avoidance memory. However, the infusion of $GluA2_{3Y}$ rescues LTP and long-term memory in this model (Dong et al., 2015). Other experiments support the role of PKMζ in GluA2-AMPAR trafficking. Overexpression of PKMζ or perfusion of PKMζ in vitro increases the number of AMPARs at active synapses but not silent ones (Ling, Benardo, & Sacktor, 2006; Shao, Sondhi, van de Nes, & Sacktor, 2012). Further, the expression of an active PKMζ mutant limits the lateral movement of GluA2-AMPARs on the plasma membrane (Yu et al., 2017). This effect was found at both synaptic and extrasynaptic sites suggesting that PKMζ disrupts the pathways that lead to diffusion and endocytosis of these AMPARs. Thus, the prevailing theory suggests that PKMζ might maintain long-term memory through the regulation of GluA2-AMPARs.

NSF-GluA2 binding seems to be a prerequisite for PKMζ maintenance of memory and LTP. Similar to ZIP, blocking NSF-GluA2 binding can impair memory maintenance or late-LTP (Migues et al., 2014; Yao et al., 2008). While perfusion of PKMζ in vitro can potentiate synapses, inhibition of NSF-GluA2 binding prevents this effect (Yao et al., 2008). Further, blocking this interaction does not alter PKMζ translation or protein levels, suggesting that NSF-GluA2 binding is downstream of PKMζ synthesis (Yao et al., 2008).

PKMζ may also increase the availability of GluA2-AMPARs that can be moved to the PSD through interactions with PICK1. PICK1-GluA2 binding seems to promote intracellular retention and extrasynaptic pooling of GluA2-AMPARs (Lin & Huganir, 2007; Yao et al., 2008). Impairing PICK1-GluA2 binding has a similar effect as perfusing

PKMζ into cells, which suggests that PKMζ might also work to disrupt this binding (Yao et al., 2008). If so, this process is likely to occur through the action of NSF, which is known to disrupt PICK1-GluA2 binding (Hanley et al., 2002). While speculative, this could explain in part why overexpression of PKMζ increases the synaptic expression of GluA2-AMPARs (Shao et al., 2012; Xue et al., 2015). Although it is also possible that this effect of PKMζ overexpression is the result of reduced endocytosis of GluA2-AMPARs.

Notably, one study also discovered a possible role of PKMζ in the nucleus of neurons (Ko et al., 2016). The authors found that PKMζ protein seems to move into the nucleus in an activity-dependent manner. PKMζ can phosphorylate CREB-binding protein (CBP) at Ser436 and possibly other sites. CBP is important for transcription of memory-related genes, and PKMζ regulation of CBP seems to promote histone acetylation. The authors show that ZIP reduces histone acetylation, although this is blocked by a histone deacetylase (HDAC) inhibitor. Perhaps most interestingly, the co-infusion of ZIP and the HDAC inhibitor prevents the amnesic effect of ZIP even though HDAC inhibition did not affect memory alone. Thus, PKMζ seems to play a role in promoting gene expression via histone acetylation. While there is still much to be investigated, PKMζ may play some memory-related role in the nucleus as well.

1.5.3. Challenges to the PKMζ Memory Maintenance Hypothesis

As mentioned above, a significant portion of PKMζ research utilizes ZIP to inhibit this kinase. The reliance on ZIP has been somewhat of a liability for the field, given several identified problems with this peptide. Most notably, PKMζ-null mice show intact learning and memory that, importantly, is vulnerable to ZIP (Lee et al., 2013; Volk,

Bachman, Johnson, Yu, & Huganir, 2013). PKMζ-KO mice show LTP indistinguishable from wild-type mice that decays with application of ZIP (Volk et al., 2013). Other work found that PKMζ-KO mice also have normal performance in auditory fear conditioning, object recognition, object location memory, and cocaine CPP (Lee et al., 2013). The authors showed that CPP memory in PKMζ-KO is also vulnerable to ZIP (Lee et al., 2013). Since publication of these two papers, others have also reported that ZIP effectively impairs memory in PKMζ-KOs in other tasks (Deutschmann, Lenz, McGrath, & Briand, 2019). These results cast doubt on whether PKMζ is necessary for memory maintenance and whether some other ZIP-sensitive mechanism is responsible instead. Another problem with ZIP is a lack of binding specificity. Despite a high affinity for PKMζ, ZIP can bind to several other PKC isoforms like PKCα and PKCι/λ (Bogard & Tavalin, 2015; Lee et al., 2013; Ren et al., 2013). However, the significance of this nonspecific binding is still debated since the relative abundance of these isoforms varies throughout the brain and during memory maintenance (Bogard & Tavalin, 2015; Osten et al., 1996). ZIP also seems capable of binding to p62 and thereby impairing its interaction with AMPAR subunits, which could affect learning and memory as well (Tsai et al., 2015). Finally, others have found that ZIP can disrupt neuronal physiology in ways that may confound previous findings. One study found that ZIP infusions into the hippocampus can cause neural silencing for at least two hours (LeBlancq, McKinney, & Dickson, 2016). However, another report showed that ZIP could increase spontaneous neural activity in vitro (Sadeh, Verbitsky, Dudai, & Segal, 2015). More work is needed to identify the off-target effects of ZIP in order to understand its mechanism of action beyond PKMζ inhibition.

Unsurprisingly, the publication of two studies showing normal memory and ZIP-sensitivity in PKMζ constitutive KO mice cast doubt on past claims about the nature of PKMζ in memory maintenance (Frankland & Josselyn, 2013; Yong, 2013). However, there is good reason to believe that PKMζ plays an essential role in memory maintenance. Firstly, subsequent research provides evidence that PKMζ constitutive KO mice may compensate for the lack of PKMζ using a unique mechanism (Tsokas et al., 2016). This work showed that, in the absence of PKMζ, these KO mice seem to use another atypical PKC isoform to support long-term memory persistence, PKCι/λ. The authors found that in wildtype mice, elevated expression of PKMζ persists for at least three hours post-tetanization whereas PKCι/λ levels return to baseline by this timepoint. However, in PKMζ-null mice, expression of PKCι/λ remains elevated even three hours post-tetanization suggesting that it may become persistently active in the absence of PKMζ (Tsokas et al., 2016). Interestingly, one experiment found that conditional knockout of PKMζ in the NAc is not compensated by an increase in PKCι/λ expression (McGrath, Lenz, & Briand, 2018). Previous work showed that ZIP can also disrupt PKCι/λ (Lee et al., 2013), which suggests that ZIP might impair memory in PKMζ-KO mice by acting on PKCι/λ. In fact, the authors found that LTP maintenance can be impaired in PKMζ-KO, but not wild-type, hippocampal slices using the PKCι/λ inhibitor [4- (5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl] methyl dihydrogen (ICAP; Tsokas et al., 2016). This drug can also impair spatial memory maintenance in PKMζ-KO mice but not wild-type mice. Altogether, this work provides reasonable evidence that PKMζ-KO mice represent, by definition, a unique system where the loss of PKMζ is compensated for by PKCι/λ, and possibly other signalling pathways.

Interestingly, more recent work found that PKCι/λ-KO mice seem to compensate using PKMζ, providing further evidence that these proteins' homology enables one to rescue the function of the other (Sheng et al., 2017).

The use of PKMζ-antisense also provides evidence to support the role of PKMζ in long-term memory. Tsokas and colleagues showed that antisense ODNs specific to PKMζ impair L-LTP in wild-type animals but not PKMζ-KOs. Similarly, infusion of PKMζ-antisense impairs memory in wild-type but not PKMζ-KO mice (Hsieh et al., 2016; Tsokas et al., 2016). These two sets of findings provide further evidence for an important role of PKMζ in long-term memory.

Importantly, the hypothesis that PKMζ maintains long-term memory is not founded solely on ZIP-based experiments. A number of different and more specific tools have been used to target PKMζ, leading to similar conclusions. For example, overexpression of PKMζ has numerous synaptic effects that promote long-term memory: it enhances LTP; it promotes clustering of PSD-95; it increases the size of dendritic spines; it increases the number of mature spines; it increases the number of synaptic GluA2-AMPARs; and it enhances conditioned taste aversion, auditory fear conditioning, and contextual fear memory (Ling et al., 2006; Ron, Dudai, & Segal, 2012; Schuette, Fernandez-Fernandez, Lamla, Rosenbrock, & Hobson, 2016; Shao et al., 2012; Shema et al., 2011; Xue et al., 2015). Importantly, overexpression of PKMζ can enhance the strength of both existing and new memories (Shema et al., 2011). In *Drosophila*, transiently overexpressing a PKMζ homolog (DaPKM) also enhances existing memory (Drier et al., 2002). On the other hand, expression of a dominant-negative PKMζ impairs existing conditioned taste aversion memory in rats (Shema et

al., 2011) and disrupts LTP 20 minutes after stimulation (Ling et al., 2002). Similarly, expressing a dominant-negative of the atypical PKM (PKM Apl III) disrupts intermediate-term and long-term facilitation in *Aplysia* (Bougie et al., 2012; Hu et al., 2017). Expressing PKMζ-shRNA three days after training can impair an existing trace fear conditioning memory in rats (Wang, Sheng, Ren, Tian, & Lu, 2016). In another experiment, expression of shRNA targeting PKMζ impaired inhibitory avoidance memory in rats but infusing $GluA2_{3Y}$ rescued this phenotype (Dong et al., 2015). Additionally, elevated PKMζ protein levels can be observed even one month after training (Hsieh et al., 2016) whereas other PKC isoforms return to baseline shortly after stimulation or training (Sacktor et al., 1993; Sheng et al., 2017). PKMζ correlates with memory strength in rats and monkeys (Hara et al., 2012; Hsieh et al., 2016; Sebastian et al., 2013). Lastly, as previously mentioned, PKMζ-antisense impairs spatial memory and LTP in hippocampal slices (Hsieh et al., 2016; Tsokas et al., 2016). In light of this broad collection of findings using many different methods, each more specific to PKMζ than ZIP, it seems reasonable to conclude that PKMζ maintains memory by limiting GluA2-AMPAR endocytosis.

What remains unclear, however, is what role PKMζ plays during memory destabilization and reconsolidation. It is clear that the movement of GluA2-AMPARs plays an essential role in this process. As mentioned, internalization of these GluA2-AMPARs occurs during memory destabilization and they are re-inserted into the synaptic membrane around the time of reconsolidation (Hong et al., 2013; Rao-Ruiz et al., 2011). Given that PKMζ seems to regulate the internalization and movement of

GluA2-AMPARs (Dong et al., 2015; Migues et al., 2010; Yao et al., 2008; Yu et al., 2017), it seems likely that PKMζ is implicated in this plasticity process as well.

1.5.4. Sex-Differences and PKMζ

An unresolved question concerns the role of sex-differences in PKMζ-mediated memory maintenance. While sex-differences have not been the primary focus of most memory studies in animals—if females are included at all—a peculiar pattern seems to have emerged for PKMζ that may provide insights.

Experiments with PKMζ-KO mice show that knocking out or knocking down expression of this protein differentially affects males and females. Knocking down PKMζ in the nucleus accumbens increases cocaine self-administration in male mice but has no effect in female mice (McGrath et al., 2018). In another report, Nasir and colleagues knocked out the gene encoding both PKCζ and PKMζ to study its effect in pain models (Nasir et al., 2016). The authors found PKC/Mζ-KO male mice developed only temporary allodynia (increased pain sensitivity), lasting less than 2 hours, after intracolonic capsaicin administration (Nasir et al., 2016). On the other hand, allodynia persisted for at least two hours in PKC/Mζ-KO females, wild-type males, and wild-type females. In another experiment, PKC/Mζ-KO males showed significantly less mechanical allodynia compared to WT males. However, in females, WT and KO mice showed similar levels of allodynia (Nasir et al., 2016). These authors also found ZIP to be less effective in females than in males, a finding replicated by George, Laferrière, and Coderre (2019). While these findings are difficult to interpret, it is clear that males and females are differentially sensitive to PKMζ knockout or knockdown.

Another study investigated the expression of PKMζ protein and its relationship to male and female rats' performance in a radial arm maze task. The authors found some sex-differences in performance but, more interestingly, sex-differences in PKMζ expression (Sebastian et al., 2013). While males and females had similar levels of GluA2-AMPARs, males consistently had more synaptic PKMζ than females—in some cases, roughly twice as much. Similarly, synaptic GluA2-AMPAR expression in the hippocampus correlated with memory performance in both males and females. However, synaptic PKMζ levels only correlated with performance in males. These data suggest that while GluA2-AMPARs seem to be central for maintaining memory in both sexes, PKMζ may play a different role in males and females.

Of note, ZIP disrupts memory in both male and female animals. Numerous studies have shown that ZIP impairs memory in male animals (Kwapis et al., 2012; Migues et al., 2010; Serrano et al., 2008; Shema et al., 2009). Studies have also shown that ZIP infusion in the sensorimotor cortex of female rats can impair memory in a reaching task (Gao, Goodman, Sacktor, & Francis, 2018; von Kraus et al., 2010). Notably, ZIP injection into the spinal cord seems to affect males but not females in certain pain models (Nasir et al., 2016). This discrepancy could suggest an additional difference between the brain and spinal cord, although it might simply be a consequence of other known sex-differences in pain (Rosen, Ham, & Mogil, 2017). As mentioned, ZIP has come under scrutiny due to its unspecific binding (Bogard & Tavalin, 2015) and the finding that it impairs memory in PKMζ-KO animals (Deutschmann et al., 2019; Lee et al., 2013; Volk et al., 2013). Given that more specific tools—namely Western blotting, knockout, and knockdown—show a sex-difference in

behaviour and protein expression (McGrath et al., 2018; Nasir et al., 2016; Sebastian et al., 2013), these ZIP findings should be considered cautiously.

One way of resolving this discrepancy is by using a more specific method to target PKMζ to determine if this protein maintains memory in male and female animals. While PKMζ antisense has been used to impair its synthesis, to the best of our knowledge, it has not been used to disrupt memory maintenance. Thus, some alternative interference molecule with higher selectivity for PKMζ might be useful to address these issues. 8-hydroxynaphthalene-1,3,6-trisulfonic acid (ζ-stat) is an inhibitor that has recently been used in cancer research to disrupt PKCζ (Islam, Dey, Patel, Smalley, & Acevedo-Duncan, 2020; Islam, Patel, & Acevedo-Duncan, 2018; Ratnayake et al., 2018). ζ-stat binds to a pocket in the C-terminal catalytic domain that is common to both PKCζ and PKMζ (Ratnayake et al., 2018). Further, ζ-stat shows specificity for the catalytic domain shared by PKMζ and PKCζ compared to the closely related PKCι/λ protein (Ratnayake et al., 2018). Administration of ζ-stat also causes a significant decrease in p-PKCζ and total PKCζ, but not PKCι/λ in cultured cells (Islam et al., 2018). Hence, ζ-stat could be used to more specifically study the role of PKMζ in memory maintenance in male and female animals.

1.6. Fear Conditioning and the Basolateral Amygdala

Evaluating the role of a given mechanism or protein in memory requires a robust and straightforward model system. Rats and mice are the models of choice in the majority of neuroscience research (Beery & Zucker, 2011). They are evolutionarily closer to humans than other models like *Drosophila*, snails, crabs, or *Aplysia*, but cheaper than more closely related models like rhesus monkeys. PKMζ has been

investigated in several different species (Bougie et al., 2012; Drier et al., 2002; Mei et al., 2011; Migues et al., 2010; Serrano et al., 2008; Solntseva et al., 2015) as has memory reconsolidation (Barreiro et al., 2013; Gainutdinova et al., 2005; Holehonnur et al., 2016; Lee et al., 2012; Nader et al., 2000; Soeter & Kindt, 2015). Thus, both PKMζ-dependent memory maintenance and reconsolidation seem to be well-conserved across species.

Similarly, both reconsolidation and PKMζ-dependent memory maintenance have been studied across many different brain regions although, in rodents, many studies have investigated the role of PKMζ in the hippocampus and amygdala (Dong et al., 2015; Hardt et al., 2010; Migues et al., 2010; Schuette et al., 2016; Sebastian et al., 2013; Serrano et al., 2008). Likewise, research in memory destabilization and reconsolidation targeted mostly hippocampus- and amygdala-dependent fear conditioning memory in rodents (Ben Mamou et al., 2006; De Oliveira Alvares et al., 2013; Frankland et al., 2006; Milekic & Alberini, 2002; Nader et al., 2000; Wang et al., 2009).

Peculiarly, while pharmacological manipulations in the dorsal hippocampus impair reconsolidation of contextual fear memory (Debiec et al., 2002; Frankland et al., 2006), hippocampal ZIP infusions do not disrupt contextual fear memories (Serrano et al., 2008). Instead, this type of memory is vulnerable to ZIP in the amygdala (Kwapis et al., 2012). Given this discrepancy in contextual fear memory—reconsolidation vulnerability in the hippocampus but maintenance vulnerability in the amygdala—auditory fear conditioning in the BLA offers a more straightforward alternative.

Auditory fear conditioning is a commonly used and well-characterized paradigm to study memory. In a typical experiment, an animal is placed a conditioning chamber and, after a brief period of acclimation, a tone is played which ends with a footshock (Nader et al., 2000). The next day, the animal is re-exposed to the tone and will show freezing behaviour if it has learned that the tone predicts the footshock. The amount of freezing behaviour—canonically defined as the cessation of all movement except breathing—is then used as a measure of memory strength.

The underlying circuitry of this fear memory has been well-defined. Auditory information reaches the amygdala through thalamic and cortical input, and output to the periaqueductal grey participates in controlling the specific fear response (Doyère et al., 2007; Kim & Jung, 2011; Romanski & LeDoux, 1992). Synapses in the BLA seem to be crucial for learning, storage, and expression of this memory. Auditory fear conditioning strengthens auditory-evoked field potentials at thalamo-amygdala connections and reconsolidation blockade depotentiates these synapses (Doyère et al., 2007). This depotentiation is specific to the reactivated tone, not unrelated ones (Doyère et al., 2007). As mentioned before, auditory fear memories are vulnerable both to ZIP (Migues et al., 2010; Serrano et al., 2008) and reconsolidation blockade in the BLA (Ben Mamou et al., 2006; Nader et al., 2000; Wang et al., 2009). Thus, auditory fear conditioning offers a more straightforward paradigm since both reconsolidation and memory maintenance have been shown to occur in the same brain structure, unlike in contextual fear conditioning. Taken together, auditory fear conditioning in the amygdala is a well-suited paradigm to study the role of PKMζ in memory destabilization and reconsolidation.

1.7. Summary and Rationale

After retrieval, long-term memories can become transiently labile, requiring reconsolidation within a few hours in order to persist. Several mechanisms trigger destabilization, including activation of NMDARs. Following NMDAR activation, protein degradation is an essential component of destabilizing the memory. Within a few hours, new protein synthesis is necessary to reconsolidate the memory. However, which proteins must be synthesized is mostly unknown. One clue comes from another important component of destabilization/reconsolidation, namely the exchange of AMPARs at the PSD. During destabilization, GluA2-AMPARs are internalized and replaced with GluA2-lacking, CP-AMPARs. As the memory reconsolidates, CP-AMPARs are internalized and GluA2-AMPARs return to the synaptic membrane.

During memory storage, GluA2-AMPARs seem to be maintained at the PSD by the activity of PKMζ, a persistently active atypical PKC isoform. Disrupting the activity of this kinase appears to cause internalization of GluA2-AMPARs and memory loss. Such findings have led to the theory that PKMζ maintains long-term synaptic changes involved in long-term memory. However, its role—if any—during memory destabilization and reconsolidation has not been thoroughly investigated. Given the rapid synaptic reorganization during destabilization, changes to the PKMζ maintenance process seem likely. More specifically, if GluA2-AMPARs are internalized during destabilization, and PKMζ serves to prevent endocytosis, presumably destabilization disrupts PKMζ activity somehow. Furthermore, since reconsolidation involves returning GluA2-AMPARs to the synapse, some restoration of the PKMζ maintenance process seems likely to occur as well.

In order to fully understand the role of PKMζ in memory processes, a recurring question about possible-sex differences must also be clarified. Past research shows contradictory results: on the one hand, ZIP disrupts long-term memory with equal efficacy in males and females; on the other hand, sex differences are revealed when PKMζ is knocked out, knocked down, or quantified in Western blots. Therefore, more precise tools are needed to determine if PKMζ has a different role in memory in males and females.

This thesis, therefore, investigates (a) whether memory destabilization and reconsolidation dynamically regulate expression of synaptic PKMζ, and (b) whether PKMζ contributes to long-term memory differently in males and females.

In Chapter 2, we investigate the role of PKMζ in memory destabilization. During destabilization, GluA2-AMPARs are internalized. Given that PKMζ seems to prevent this internalization, destabilization likely disrupts PKMζ expression as well. To this end, we tested three hypotheses: (1) retrieval reduces synaptic expression of PKMζ, (2) this reduction of PKMζ requires NMDAR-dependent memory destabilization, not simply retrieval, (3) activation of the proteasome is necessary to reduce PKMζ expression following destabilization.

In Chapter 3, we investigate whether reconsolidation restores PKMζ to the synaptic membrane. If destabilization reduces PKMζ, we expect that additional PKMζ is required to restabilize the memory. In this chapter, we report experiments testing four hypotheses concerning the role of PKMζ in reconsolidation: (1) synaptic levels of PKMζ increase following reconsolidation, (2) reconsolidation requires synthesis of PKMζ, (3) ongoing memory maintenance does not require uninterrupted synthesis of PKMζ, (4)

without memory destabilization, memories are not vulnerable to acute disruptions in PKMζ synthesis.

Finally, in Chapter 4, we investigate whether PKMζ is equally important for memory maintenance in males and females. The experiments reported in this chapter use a recently published PKCζ inhibitor, ζ-stat, which appears to be specific to the ζ catalytic domain shared by PKCζ and PKMζ. Using this inhibitor, we tested two hypotheses concerning the specificity of this drug and the role of PKMζ: (1) ζ-stat is specific to PKMζ and disrupts memory in wild-type mice but not PKMζ-KO animals, (2) ζ-stat does not impair memory in female mice, but a different, unspecified ZIP-sensitive process does.

Chapter 2: Memory destabilization reduces synaptic PKMζ within one hour after retrieval

2.1. Preface

Maintaining GluA2-AMPARs at the PSD seems to be crucial for long-term memory storage (Dong et al., 2015; Migues et al., 2010, 2016). This maintenance relies in part on the binding of NSF to the GluA2 tail and the persistent activity of PKMζ (Migues et al., 2014, 2010). However, during memory destabilization, synaptic GluA2-AMPARs are internalized and NSF is degraded (Hong et al., 2013; Ren et al., 2013). Thus, destabilization seems to represent a unique case in which memory storage mechanisms are suspended as the synapse is transiently reorganized (Hong et al., 2013).

As mentioned in Chapter 1, PKMζ seems to be a central component of memory maintenance (Ling et al., 2002), although its role in memory destabilization/ reconsolidation has not been well-examined. The predominant hypothesis is that PKMζ, in connection with NSF, maintains long-term memory by preventing endocytosis of GluA2-AMPARs (Migues et al., 2010; Sacktor, 2011). Given that destabilization alters GluA2-AMPAR and NSF regulation, it seems likely that it will also affect PKMζ. Therefore, we investigated here how memory destabilization impacts the expression of synaptic PKMζ.

To this end, we tested three hypotheses related to PKMζ in memory destabilization. (1) Retrieval of a consolidated, long-term auditory fear memory decreases the expression of synaptic PKMζ in the BLA. (2) This reduction in PKMζ

expression depends on destabilization. That is, NMDAR activation is necessary to decrease the levels of PKMζ at synapses. (3) Proteasome activation is necessary to decrease PKMζ following retrieval. In this chapter, we describe how memory destabilization leads to the downregulation of PKMζ.

2.2. Abstract

Retrieval can initiate a destabilization process that renders a retrieved long-term memory transiently labile. While this process requires re-exposure to a stimulus to reactivate the memory, behavioural expression is not necessary to trigger memory destabilization. Instead, destabilization seems to require other processes, such as the activation of NMDARs and the ubiquitin-proteasome system. Internalization of GluA2-AMPARs from the postsynaptic membrane and their replacement with GluA2-lacking, CP-AMPARs are essential components of destabilization. What is not clear is how these GluA2-AMPARs are removed, given that the protein PKMζ seems to prevent their endocytosis. Here we investigated the fate of PKMζ during post-retrieval memory destabilization. We found that within 1 hour after retrieval of an auditory fear memory, there was a significant reduction in PKMζ at BLA synapses. This reduction was destabilization-dependent because blocking NMDARs prevented it. Furthermore, protein degradation is necessary for reducing PKMζ because impairing proteasome activity also prevented a reduction in PKMζ. These results suggest that memory destabilization causes a dynamic downregulation of PKMζ.

2.3. Introduction

Following retrieval, memories can become transiently plastic through a process of synaptic destabilization. For about 6 hours, the reactivated memory becomes plastic. Once destabilized, the memory can be modified (De Oliveira Alvares et al., 2013), enhanced (Debiec et al., 2011), or weakened (Ben Mamou et al., 2006; Jarome et al., 2016; Nader et al., 2000). Without destabilization, memories do not undergo reconsolidation and are not sensitive to treatments that block reconsolidation (Ben Mamou et al., 2006; Jarome et al., 2016; Lee & Flavell, 2014). Importantly, destabilization seems to be specific to the retrieved or “reactivated” memory, leaving other ones intact (Debiec, Doyère, Nader, & Ledoux, 2006).

Initiation of destabilization after retrieval requires activation of many different neurotransmitter receptors including GluN2B-containing NMDARs (Ben Mamou et al., 2006; Ferrer Monti et al., 2016; Lopez et al., 2015; Milton et al., 2013), cannabinoid receptor 1 (CB1 receptors; Lee & Flavell, 2014; Suzuki, Mukawa, Tsukagoshi, Frankland, & Kida, 2008), dopamine receptors (Merlo et al., 2015; Reichelt et al., 2013), and L-type voltage-gated calcium channels (LVGCCs; De Oliveira Alvares et al., 2013; Lee & Flavell, 2014; Suzuki et al., 2008). An important consequence of NMDAR activation is the activation of CaMKII (Fukunaga, Soderling, & Miyamoto, 1992). This second messenger has several targets including the ubiquitin-proteasome system (UPS), which is crucial to destabilizing retrieved memories (Jarome, Werner, Kwapis, & Helmstetter, 2011; A. S. Lee et al., 2008).

Additionally, destabilization initiates a transient movement of postsynaptic AMPARs (Clem & Huganir, 2010; Hong et al., 2013; Rao-Ruiz et al., 2011). Almost

immediately after retrieval, GluA2-AMPARs are internalized from the postsynaptic membrane and replaced with GluA2-lacking, calcium-permeable AMPARs (CP-AMPARs; Hong et al., 2013). As in acquisition, these CP-AMPARs are important for new learning following retrieval (Clem & Huganir, 2010; Diering & Huganir, 2018; Hong et al., 2013; Malinow & Malenka, 2002; Rumpel et al., 2005). Importantly, the removal of GluA2-AMPARs seems to be a prerequisite for the subsequent insertion of CP-AMPARs (Hong et al., 2013). Thus, endocytosis of GluA2-AMPARs is a crucial component of post-reactivation plasticity (Rao-Ruiz et al., 2011).

While GluA1-containing AMPARs are inserted to the synapse in an activity-dependent manner, GluA2-AMPARs undergo both independent cycling and activity-dependent trafficking (Hayashi et al., 2000; Malinow & Malenka, 2002; Rumpel et al., 2005; Takahashi, Svoboda, & Malinow, 2003). These GluA2-AMPARs are cycled from intracellular stores, inserted to extrasynaptic sites on the plasma membrane, and then laterally diffuse to the post-synaptic density (PSD; Diering & Huganir, 2018; Malinow & Malenka, 2002). From the PSD, GluA2-AMPARs first move to extrasynaptic sites and are then endocytosed through clathrin-coated pits (Diering & Huganir, 2018; Malinow & Malenka, 2002). Endocytosis of GluA2-AMPARs is highly regulated, involving many proteins binding to the AMPAR and other sites (Awasthi et al., 2019; Diering & Huganir, 2018; Hayashi et al., 2000; Malinow & Malenka, 2002; Takahashi et al., 2003; Yao et al., 2008).

PKMζ, an atypical PKC isoform, is one protein that seems essential to the regulation of GluA2-AMPAR endocytosis. Previous work has shown that the administration of ZIP, which inhibits PKMζ activity (Laudanna et al., 1998; Yao et al.,

2013), leads to GluA2-AMPAR endocytosis (Migues et al., 2010). This finding suggests that PKMζ may be in part responsible for the maintenance of these AMPARs at the PSD. Additional research shows that overexpression of PKMζ increases GluA2-AMPAR content at the PSD (Shao et al., 2012) and limits their movement (Yu et al., 2017). These findings provide additional support for the hypothesis that PKMζ helps regulate endocytosis of GluA2-AMPARs.

If GluA2-AMPAR internalization is integral to destabilization and if PKMζ works to prevent this endocytosis from occurring, it seems likely that PKMζ is implicated in memory destabilization. Here we investigated the fate of PKMζ during post-retrieval memory destabilization. We show that following retrieval, there is an NMDAR-dependent reduction in PKMζ protein. We also show that this reduction relies on UPS activation.

2.4. Materials and Methods

2.4.1. Animals

Male Sprague-Dawley rats (275-300 g) were obtained from Charles River, Saint-Constant, Quebec. Rats were housed in pairs and maintained on a 12 h light/dark cycle (lights on at 07:00, lights off at 19:00). Experiments began at 09:00 each morning. Rats received food and water *ad libitum*. All methods and procedures were approved by McGill University's Animal Care Committee and conformed to Canadian Council on Animal Care's guidelines.

2.4.2. Surgery

Rats received an intraperitoneal injection of anesthetic cocktail (1 mL/kg) containing ketamine (50 mg/mL), xylazine (3 mg/mL), and dexdomitor (0.175 mg/mL).

Prior to surgery rats also received carprofen analgesic (5 mg/mL; 1 mL/kg) subcutaneously. Rats were bilaterally implanted with 22-gauge guide cannulas (Plastics One, Roanoke, VA) targeting the basolateral amygdala (from bregma: AP -3.0 mm; ML +5.3 mm; DV -8.0 mm). BLA coordinates were determined using a rat brain atlas (Paxinos & Watson, 2004). Cannulas were secured to the skull with dental cement and three jeweller's screws. To ensure the interior of the cannula remained clear of debris, metal dummies were inserted and remained in place except during infusions. Following surgery, rats received an intraperitoneal injection of 0.5 mg/mL of antisedan to reverse anesthesia. Following surgery, rats were monitored and individually handled for at least seven days before the start of behavioural experiments.

2.4.3. Infusions

DL-2-Amino-5-phosphonopentanoic acid (APV, Sigma A5282) was dissolved in saline to reach a final concentration of 5 µg/µL. Clasto-Lactacystin beta-lactone (β-lac, Abcam ab141412) was first dissolved in 2% DMSO-HCl and brought to a final concentration of 32 ng/µL in saline.

All infusions were performed bilaterally into the basolateral amygdala at a rate of 0.2 µL/min with a total volume of 0.5 µL/side. Intracranial infusions utilized 23-gauge injectors (Plastics One) connected to 20-gauge polyethylene tubing (Braintree Scientific, Inc.) which were connected to 26-gauge Hamilton syringes (Model 1701N). Injectors extended 1.5 mm beyond the guide cannula and remained in place for an additional 1 minute following the infusion to ensure proper drug diffusion. Rats were handled by the experimenter during infusions and returned to their home cage following each infusion. For experiments where infusions occurred prior to retrieval, each rat was given a "sham

infusion" before each day of habituation to familiarize rats with the infusion experience. Sham infusions followed the same procedure, but no solutions were injected.

2.4.4. Fear conditioning

Each day, rats were transported to a nearby holding room 30 min before the start of the experiment. Experiments utilized two different Coulbourn Habitest (model I-I10-24A) conditioning chambers referred to here as Context A and Context B. Context A had white, curved, plastic walls, and a plastic, white floor. Context B had square, checkered walls, stainless-steel grid floors, and a vanilla scent was sprayed in the chamber before each rat entered. Additionally, conditioning boxes for Context A were housed in a room of bright ambient lighting whereas Context B was in a different room with very low lighting.

In each experiment, rats were habituated and trained as follows. On Days 1 and 2, rats were placed in Context A for 20 minutes in order to habituate them to the context. On Day 3, rats were placed in Context B for training. During training, rats were allowed to habituate to the context for 2 minutes. Then, a 30-second tone (4 kHz, 75 dB) was played, which co-terminated with a 1 second, 1.0 mA footshock. Rats remained in the context for an additional 30 seconds before being removed. After this procedure, the three experiments continued as follows:

Experiment 2.1: On Day 4, one group of rats ("1h Post-Retrieval" group) was placed back in Context A for a retrieval test. After 2 minutes in the chamber, these rats were exposed to one unpaired tone (30 seconds, 4 kHz, 75 dB) and remained in the context for an additional 30 seconds. Rats were returned to their home cage following this reactivation session and sacrificed one hour later. The other group ("No Retrieval"

group) did not undergo reactivation and remained in their home cage during this period. These non-reactivated rats were sacrificed at the same time as the 1h Post-Retrieval group.

Experiment 2.2: On Day 4, rats received an intracranial infusion of APV (5 μg/μL; 0.5 μL per side) or vehicle to the BLA. After 30 min, rats underwent a retrieval test (as in Experiment 2.1). Rats were returned to their home cage following the retrieval test and sacrificed one hour later.

Experiment 2.3: On Day 4, rats underwent a retrieval test (as in Experiment 2.1). Immediately after retrieval, rats were infused intracranially with β-lac (32 ng/μL; 0.5 μL per side) or vehicle in the BLA. Rats were returned to their home cage following the infusion and sacrificed one hour after retrieval.

After each animal was taken out of a conditioning box (Context A or B) on each day of the experiment, the floor and walls were wiped clean using a damp paper towel with 2% Versa-Clean (Fisher, 18200700) in dH_2O before the next animal was placed inside.

2.4.5. Sacrificing

Rats' brains were quickly collected and flash frozen. First, rats were placed in an induction chamber containing isoflurane (Baxter, 02225875). Once deep anaesthesia had been induced, the rat was removed and quickly decapitated using a guillotine. Its brain was quickly retrieved. The brain was immediately submerged in a beaker containing 2-methylbutane (Fisher, O3551-4) which was within a container of dry ice. Once brains were frozen, they were wrapped in aluminum foil and submerged in the dry ice before final storage at -80°C.

2.4.6. Subcellular fractionation

Subcellular fractionation followed a previously established protocol to obtain synaptosome fractions (Bai & Witzmann, 2007). Frozen brains were mounted on a cryostat and basolateral amygdala tissue was collected using a tissue puncher (Fine Science Tools). The tissue was homogenized using a Pellet Pestle (Fisher, #12141361) in 200 µL of homogenization buffer containing 20 mM HEPES, 1 mM EDTA, 2 mM EGTA, 320 mM sucrose, along with one protease inhibitor tablet (Roche, 05892791001) and one phosphatase inhibitor tablet (Roche, 04906837001). Homogenized tissue was centrifuged at 1000 g for 10 min. The supernatant was collected and centrifuged at 17,000 g for 15 min. The pellet was resuspended in 50 µL of homogenization buffer and layered on a sucrose gradient containing 100 µL of 0.8 M sucrose (1 mM EDTA, 2 mM EGTA) and 100 µL of 1.2 M sucrose (1 mM EDTA, 2 mM EGTA). This mixture was centrifuged at 54,000 g for 90 min. The layer between the 0.8 M and 1.2 M sucrose, containing the synaptosomal fraction (Bai & Witzmann, 2007), was collected and used for Western blotting following protein quantification with a BCA protein assay kit (Pierce).

2.4.7. Western Blotting

After protein quantification, 15 µg of protein was loaded in 8% SDS-PAGE gels. Proteins were transferred overnight (at 4 °C) onto nitrocellulose membranes. Following transfer, Ponceau solution (Sigma, P7170) was applied to membranes to reveal protein bands. Along with the molecular weight marker (Thermo Scientific, 26634), this enabled the cutting of membranes at 70 kDa and 40 kDa to produce two membranes. One membrane contained PKMζ protein (55 kDa) and the other contained GAPDH (37 kDa).

Membranes were washed in 0.1% Tween 20, Tris-buffered saline (TBS-Tween). Blocking of membranes was done for 1 h at room temperature in TBS-Tween containing 5% bovine serum albumin (BSA). Membranes were then incubated overnight with antibodies in 5% BSA TBS-Tween. To target PKMζ, we used a PKCζ polyclonal antibody (1:1000, Abcam, ab59364) previously used to quantify PKMζ in Western blots (Dong et al., 2015). Given that PKCζ and PKMζ share the same C-terminal catalytic domain, this antibody is capable of identifying both proteins as well as the closely-related PKCι/λ. To target the loading control, GAPDH, we used a monoclonal antibody (1:10,000, Abcam, ab8245). Following incubation overnight, membranes were washed three times with TBS-Tween. Membranes then underwent incubation with secondary antibody (Anti-rabbit IgG HRP-conjugated from Amersham, NA934V; Anti-mouse IgG HRP-conjugated from Amersham, NA931V) for one hour at room temperature. After secondary antibody incubation, membranes were washed four times for 10 min in TBS-Tween. Membranes were revealed using Pierce ECL 2 Western Blotting Substrate and scanned using a Storm Scanner (Molecular Dynamics). Scanned images were quantified using Image Lab (Bio-Rad). PKMζ protein values were compared to the GAPDH loading control for each sample. Values were then standardized as a percent of one of the two groups (2.1. No retrieval group; 2.2. APV group; 2.3. β-lac group).

2.4.8. Statistical analyses

Data were analyzed using Jamovi (Version 1.0.7.0.). Data were normally distributed and showed homogeneity of variance. PKMζ protein from Western blots was analyzed using independent samples t-tests. The null hypothesis was rejected where $p < 0.05$. Figures present data as means ±1 SEM.

2.5. Results

2.5.1. Memory retrieval reduces synaptic PKMζ

We expected that rats sacrificed one hour after retrieval would show lower synaptic PKMζ expression in the BLA compared to rats sacrificed without prior retrieval. Indeed, rats sacrificed 1 hour after the reactivation session (n=5) showed less PKMζ than animals that remained in their home cage prior to sacrificing (n=6; independent samples t-test, t_9=2.755, p=0.022, Figure 2.1). Thus, memory retrieval seems to induce a decrease in synaptic PKMζ in the basolateral amygdala.

2.5.2. Reduction in PKMζ is destabilization-dependent

Retrieval does not necessarily lead to memory destabilization. While memory retrieval can occur in the absence of NMDAR activation, destabilization does require their activation (Ben Mamou et al., 2006; Garcia-delaTorre et al., 2014; Pineyro, Ferrer Monti, Alfei, Bueno, & Urcelay, 2013). Therefore, we reasoned that NMDAR activation is likely necessary for reducing PKMζ one hour after retrieval. Indeed, rats infused with APV before retrieval (n=10) showed higher levels of PKMζ compared to vehicle-infused animals (n=10; independent samples t-test, t_{18}=2.358, p=0.030, Figure 2.2). This suggests that PKMζ expression decreases in response to NMDAR-dependent memory destabilization.

2.5.3. Proteasome activation is necessary for post-retrieval decrease of PKMζ

One downstream consequence of NMDA receptor activation is the activation of CaMKII (Fukunaga et al., 1992), which leads to an increase in proteasome activity after retrieval (Jarome et al., 2016). Protein degradation seems to be necessary for

destabilization since inhibiting proteasome activity with β-lac prevents reconsolidation blockade (Lee et al., 2008). Thus, we expected that inhibiting proteasome activity with β-lac would prevent a reduction in PKMζ after retrieval. In fact, we found that rats infused with vehicle (n=7) showed less PKMζ one hour after retrieval than rats infused with β-lac (n=7; independent samples t-test, t_{12}=2.377, p=0.035, Figure 2.3). Therefore, it seems that protein degradation after retrieval does lead to a reduction in PKMζ.

2.6. Discussion

Following memory retrieval, there is a significant reduction in PKMζ protein within one hour. This decrease depends on both NMDAR and proteasome activation. These results suggest that memory destabilization involves the downregulation of synaptic PKMζ to labilize the memory.

Given the putative role of PKMζ in maintaining GluA2-AMPARs at the synaptic membrane (Migues et al., 2010), our work closely parallels other findings showing dynamic AMPAR regulation after retrieval. Memory retrieval reduces synaptic GluA2-AMPARs within one hour (Hong et al., 2013; Rao-Ruiz et al., 2011), the same time point at which we found PKMζ reduced. Furthermore, infusion of APV before retrieval prevents the removal of GluA2-AMPARs (Hong et al., 2013) and the reduction of PKMζ expression, as we show here. Thus, the correlated change of expression in GluA2-AMPARs and PKMζ during memory destabilization suggests they may be linked by a shared mechanism.

Downstream of NMDARs, CAMKII promotes proteasome activity following retrieval, which is necessary to destabilize the memory (Jarome et al., 2016). That is, in the absence of protein degradation, protein synthesis inhibitors no longer block

reconsolidation (Fukushima et al., 2014; Lee et al., 2008; Lee et al., 2012). Our data show that inhibiting the UPS with β-lac similarly prevents the decrease in PKMζ after retrieval.

However, it is not clear whether PKMζ itself is degraded or whether its reduction is simply downstream of the degradation pathway. PKMζ can be polyubiquitinated and degraded by the proteasome (Vogt-Eisele et al., 2014). Normally, the interaction of PKMζ and KIBRA prevents PKMζ from being degraded (Vogt-Eisele et al., 2014). Blocking the interaction of these two proteins or knocking down KIBRA using siRNA leads to a rapid reduction in PKMζ (Vogt-Eisele et al., 2014). It is possible that, after retrieval, some process disrupts the binding of KIBRA and PKMζ, leading to ubiquitination and degradation of PKMζ. However, we show that in the absence of proteasome activity PKMζ cannot be degraded and remains enriched at the synapse. Perhaps this process might involve mitogen-activated protein kinase or extracellular signal-related kinase (MAPK/ERK), which is known to bind with KIBRA (Yang et al., 2014). Indeed, NMDAR activity can lead to activation of MAPK/ERK (Kurino, Fukunaga, Ushio, & Miyamoto, 1995; Xia, Dudek, Miranti, & Greenberg, 1996). Further, phosphorylation of MAPK/ERK peaks at roughly 15 minutes post-retrieval (Chen et al., 2005), preceding the decrease in PKMζ we have seen here. Of course, this is purely speculative. Future research may investigate changes to KIBRA following retrieval to establish if KIBRA is also depleted following retrieval or if just its association with PKMζ is disrupted instead.

While degradation of PKMζ following retrieval seems likely, it could presumably be trafficked elsewhere in the dendrite along with the internalized GluA2-AMPARs. One

clue regarding the fate of PKMζ could come from whether the protein is synthesized after destabilization. That is, if reconsolidation involves the synthesis of new PKMζ, it seems likely that the post-retrieval reduction in PKMζ is due to degradation.

2.7. Figures

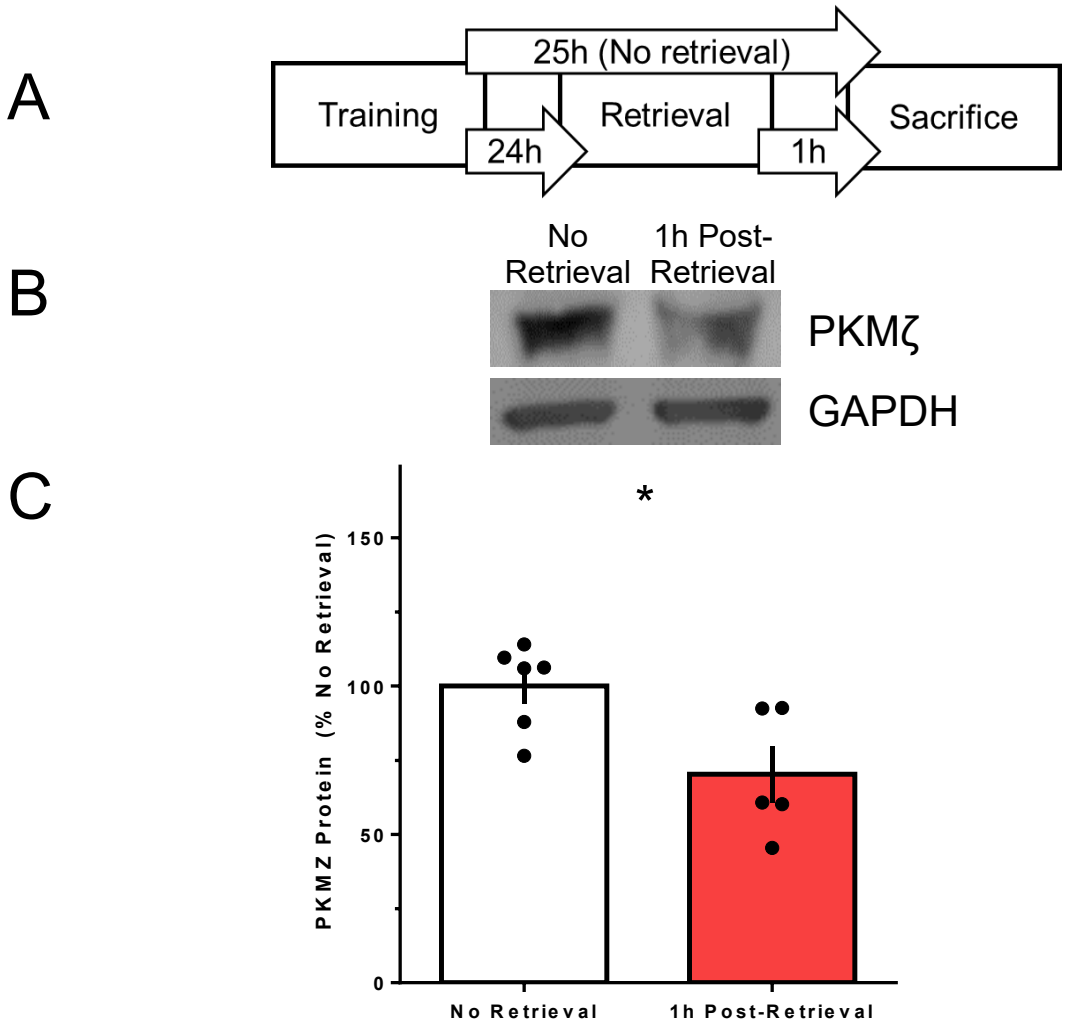

Figure 2.1. Memory retrieval reduces synaptic PKMζ.

A) Following habituation, rats underwent auditory fear conditioning training in which a 30-second tone co-terminated with a 1.0 mA shock. One group of rats experienced a retrieval test 24 hours later and sacrificed one-hour post-retrieval ("1h Post-Retrieval" group, n=5). Another group of rats was sacrificed 25 hours after training ("No Retrieval" group, n=6). B) Representative Western blot. C) PKMζ protein levels from Western blots. Rats sacrificed one-hour post-retrieval showed significantly lower synaptic PKMζ protein in BLA tissue compared to rats that did not undergo retrieval (t_9=2.755, p=0.022).

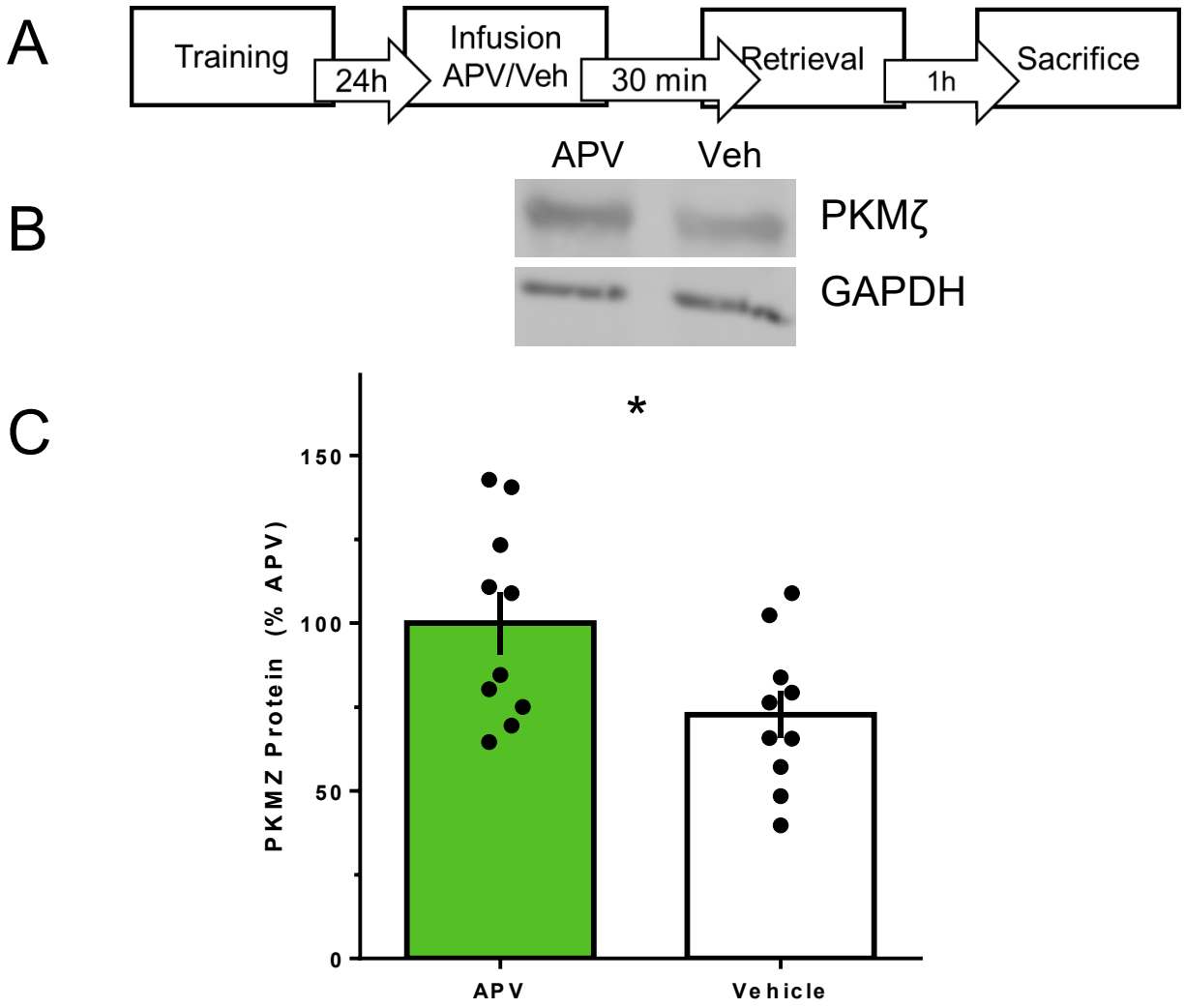

Figure 2.2. APV prevents post-retrieval reduction in PKMζ.

A) Rats underwent habituation and auditory fear conditioning training. Thirty minutes before a retrieval test the next day, rats received an intracranial infusion of APV (5 µg/µL in saline; 0.5 µL/side) or vehicle to the BLA. All rats were sacrificed 24 hours post-retrieval. B) Representative Western blot. C) PKMζ protein levels from Western blots. Rats that received APV infusion prior to retrieval (n=10) showed significantly higher PKMζ protein compared to rats that received vehicle (n=10; t_{18}=2.358, p=0.030).

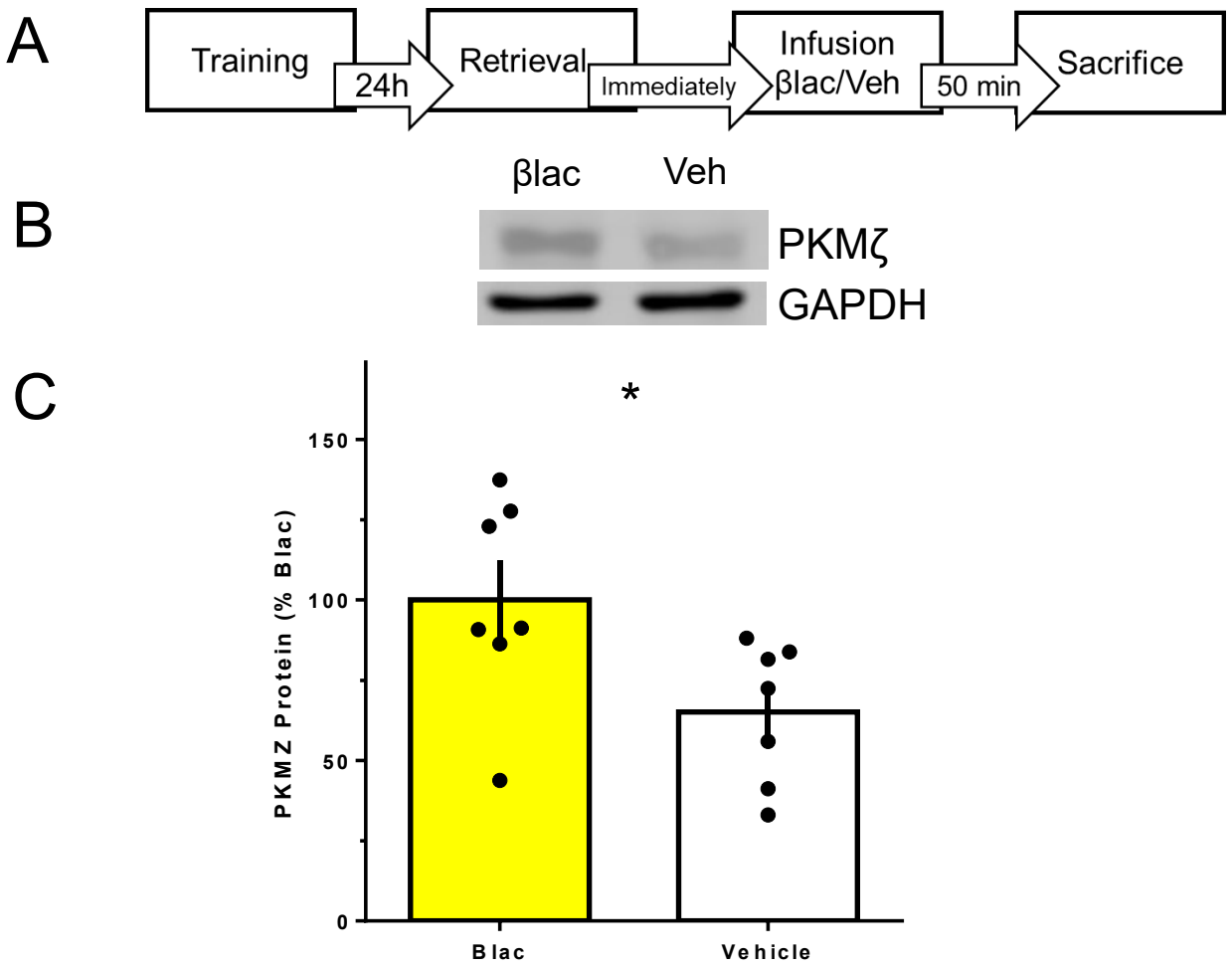

Figure 2.3. β-lac prevents post-retrieval reduction in PKMζ.

A) Rats underwent habituation, training, and a retrieval test. Immediately following retrieval, rats received an intracranial infusion of β-lac (32 ng/μL; 0.5 μL per side) or vehicle in the BLA. All rats were sacrificed one-hour post-retrieval, roughly 50 min post-infusion. B) Representative Western blot. C) PKMζ protein levels from Western blots. Rats that received β-lac infusion after retrieval (n=7) showed significantly higher PKMζ protein compared to rats that received vehicle (n=7; t_{12}=2.377, p=0.035).

Chapter 3: Memory reconsolidation requires de novo synthesis of PKMζ

3.1. Preface

Disrupting activity of PKMζ during memory storage leads to synaptic removal of GluA2-AMPARs (Dong et al., 2015; Migues et al., 2010). This disruption causes long-term amnesia that does not recover once the PKMζ inhibitor is removed. In Chapter 2, we observed a reduction in PKMζ within one hour after retrieval. Our data coincide with previous work showing internalization of GluA2-AMPARs at the same timepoint following memory retrieval (Hong et al., 2013). Nonetheless, memory destabilization does not result in amnesia per se, as the memory is soon reconsolidated. Thus, memory destabilization represents a unique situation in which the transient disruption of PKMζ activity does not lead to long-lasting amnesia. This is likely due to the presence of GluA2-lacking, CP-AMPARs which sustain synaptic transmission during this lability period (Hong et al., 2013). However, these CP-AMPARs are eventually replaced with GluA2-AMPARs during memory reconsolidation. Given that PKMζ seems vital for maintaining synaptic expression of GluA2-AMPARs in consolidated memories, it is likely that PKMζ is necessary for reconsolidated memories as well.

If PKMζ expression returns to pre-retrieval levels during reconsolidation, it could mean that existing PKMζ is translocated back or that new PKMζ is synthesized. Data from Chapter 2 suggest that the reduction in PKMζ requires protein degradation since inhibiting proteasome activity prevents the loss of PKMζ. This is consistent with the hypothesis that destabilization causes degradation of PKMζ itself. If PKMζ is degraded during destabilization and its expression increases during reconsolidation, presumably this additional protein comes from de novo protein synthesis. Indeed, translation of new proteins does seem to be crucial for reconsolidation (Arguello et al., 2013; Jobim et al.,

2012; Lee et al., 2004; Milekic et al., 2007). Thus, PKMζ could be one such protein that must be synthesized in order to restabilize the labile memory.

Here we investigated whether translation of PKMζ is indeed necessary for reconsolidation. We first examined whether reconsolidation leads to an increase in PKMζ expression from its low during destabilization. Next, we used PKMζ-antisense to determine whether blocking translation of PKMζ impairs reconsolidation. Finding that this did impair long-term memory, we then tested if this effect is specific to reconsolidation. That is, does PKMζ-antisense impair memory at any time point or only shortly after retrieval? We infused PKMζ-antisense after reconsolidation, during the maintenance phase of memory, to see if maintenance requires uninterrupted PKMζ synthesis. We also infused PKMζ-antisense in the presence of an NMDAR antagonist to determine whether NMDAR activity is necessary to render reactivated memories vulnerable to PKMζ-antisense. In short, this chapter investigated how reconsolidation responds to the loss of PKMζ that occurs during memory destabilization.

3.2. Abstract

After destabilization, labile memories must be reconsolidated in order to persist in long-term memory. A central component of this process seems to be the synthesis of new proteins. However, it is mostly unknown which proteins need to be synthesized. PKMζ seems to be necessary for late LTP and maintaining GluA2-AMPARs at the postsynaptic membrane. Additionally, we found that PKMζ decreases during memory destabilization. Therefore, we investigated whether reconsolidation of an auditory fear memory involves restoration of synaptic PKMζ in the BLA, a brain area critical for long-term storage of these memories. We found that reconsolidation leads to an increase in synaptic PKMζ. Blocking translation of new PKMζ using antisense ODNs during reconsolidation impaired memory. We also observed that only labile memories are vulnerable to PKMζ-antisense. That is, transiently disrupting PKMζ synthesis did not impair memories after reconsolidation had ended. PKMζ-antisense also had no effect when animals first received NMDAR inhibition, which prevented memory destabilization after retrieval. These findings suggest that increasing PKMζ through new synthesis is an integral component of reconsolidation. They also show that labile memories are uniquely vulnerable to disruptions in PKMζ translation.

3.3. Introduction

Following post-retrieval destabilization, memories must be reconsolidated in order to persist as long-term memory. Reconsolidation seems to require several different processes including activation of NMDARs (Ben Mamou et al., 2006; R. Kim et al., 2011; Milton et al., 2013), β-adrenergic receptors (Debiec et al., 2011; Debiec & Ledoux, 2004; Przybyslawski et al., 1999; Soeter & Kindt, 2015), CREB (Fukushima et al., 2014; R. Kim et al., 2011), PKA (Tronson et al., 2006), ERK/MAPK (Duvarci et al., 2005; Merlo et al., 2018), gene transcription (Kida et al., 2002; Villain et al., 2016), protein synthesis (Duvarci et al., 2005; Jobim et al., 2012; S. Lee et al., 2012; Milekic & Alberini, 2002; Nader et al., 2000; S.-H. Wang et al., 2009), and ongoing neuronal excitation (Bustos et al., 2010; Espejo et al., 2016; Lux et al., 2015; Reichelt et al., 2013) to name a few.

Gene expression seems to be a central component of reconsolidation. Inhibition of the transcription factor CREB after retrieval disrupts long-term memory (Fukushima et al., 2014; Kida et al., 2002; R. Kim et al., 2011). On the other hand, post-retrieval administration of histone deacetylase (HDAC) inhibitors, which promote transcription, actually enhances long-term memory (Villain et al., 2016). Protein synthesis also appears to be crucial to reconsolidation. Many studies have found that administration of protein synthesis inhibitors after retrieval impairs long-term memory (Jobim et al., 2012; R. Kim et al., 2011; S. Lee et al., 2012; Milekic & Alberini, 2002; Nader et al., 2000; S.-H. Wang et al., 2009). However, which specific proteins must be synthesized remains mostly unknown.

Antisense oligonucleotides are a powerful tool that can help address this question. These oligodeoxynucleotides (ODNs) are sequence-specific complements that bind to the mRNA encoding a protein of interest, preventing its translation (Dias & Stein, 2002). In the context of reconsolidation, this means that antisense ODNs can transiently knockdown a protein of interest to determine if it is necessary for reconsolidation. For instance, infusions of antisense-ODNs specific to zif268 impair reconsolidation of a contextual fear memory but BDNF-antisense infusions do not (Lee, 2010; Lee et al., 2004). That is, zif268, but not BDNF, seems to be necessary for reconsolidation. Antisense ODNs have also been used to show that C/EBPβ (Milekic et al., 2007), C/EBPδ (Arguello et al., 2013), and Arc (Maddox & Schafe, 2011) must be synthesized during reconsolidation as well.

To determine whether a protein of interest or molecular process is necessary for reconsolidation, the manipulation should affect only destabilized, reactivated memories. For instance, anisomycin disrupts long-term memory only after post-retrieval memory destabilization (Ben Mamou et al., 2006; Hong et al., 2013; Nader et al., 2000). Similarly, infusions of zif268- or C/EBPδ-antisense have no effect on long-term memory in the absence of retrieval (Arguello et al., 2013; Lee et al., 2004).

As shown in Chapter 2, there is a destabilization-dependent reduction in postsynaptic PKMζ after retrieval. Importantly, this decrease in PKMζ expression at the synapse occurs at the same time that previous work has shown a reduction in GluA2-AMPARs (Hong et al., 2013; Rao-Ruiz et al., 2011). However, GluA2-AMPARs are later reinserted at a timepoint roughly corresponding to the end of reconsolidation (Hong et al., 2013). Considering the role of PKMζ in regulating these receptors at the PSD, it

seems likely that PKMζ expression also recovers at reactivated synapses during reconsolidation.

Here, we tested whether PKMζ is integral to memory reconsolidation. We found that, following reconsolidation, there is an increase in synaptic PKMζ. Blocking translation of PKMζ after reactivation using antisense-ODNs disrupts reconsolidation and leads to long-term memory impairment. Further, only destabilized memories are vulnerable to the amnesic effect of PKMζ-antisense, suggesting that only labile memories require uninterrupted synthesis of PKMζ.

3.4. Materials and Methods

3.4.1. Animals

Male Sprague-Dawley rats (275-300 g) were obtained from Charles River, Saint-Constant, Quebec. Rats were housed in pairs and maintained on a 12h light/dark cycle (lights on at 07:00, lights off at 19:00). Experiments began at 09:00 each morning. Rats received food and water ad libitum. All methods and procedures were approved by McGill University's Animal Care Committee and conformed to Canadian Council on Animal Care's guidelines.

3.4.2. Surgery

Rats received an intraperitoneal injection of anesthetic cocktail (1 mL/kg) containing ketamine (50 mg/mL), xylazine (3 mg/mL), and dexdomitor (0.175 mg/mL). Prior to surgery rats also received carprofen analgesic (5 mg/mL; 1 mL/kg) subcutaneously. Rats were bilaterally implanted with 22-gauge guide cannulas (Plastics One, Roanoke, VA) aiming at the basolateral amygdala (from bregma: AP -3.0 mm; ML +5.3 mm; DV -8.0 mm). BLA coordinates were

determined using a rat brain atlas (Paxinos & Watson, 2004). Cannulas were secured to the skull with dental cement and three jeweller's screws. To ensure the interior of the cannula remained clear of debris, metal dummies were inserted and remained in place except during infusions. Following surgery, rats received an intraperitoneal injection of anesthetic reversal containing 0.5 mg/mL of antisedan. Following surgery, rats were monitored and individually handled for at least seven days before the start of behavioural experiments.

3.4.3. Infusions

DL-2-Amino-5-phosphonopentanoic acid (APV, Sigma A5282) was dissolved in saline to reach a final concentration of 5 μg/μL.

Antisense oligodeoxynucleotides (ODNs) were obtained from Integrated DNA Technologies at 2 nmol/μL dissolved in TE Buffer-PBS, for both PKMζ-antisense and scrambled controls. The sequence for single-stranded PKMζ-antisense was C*T*C*TTGGGAAGGCAT*G*A*C, and the sequence for the scrambled control was A*A*C*AATGGGTCGTCT*C*G*G where each asterisk represents a phosphorothioate linkage from 5' to 3'. These sequences followed previous work showing selective impairment of PKMζ synthesis following PKMζ-antisense but not the scrambled control ODN (Tsokas et al., 2016). The antisense sequence did not affect synthesis of the closely related isoform PKCι/λ and was shown to disrupt L-LTP in wild-type but not PKMζ-null mice (Hsieh et al., 2016). The PKMζ-antisense is the complementary sequence to the start site of PKMζ mRNA, whereas the scrambled control had no complementarity to a known mRNA sequence (Tsokas et al., 2016).

All infusions were performed bilaterally into the basolateral amygdala at a rate of 0.2 μL/min with a total volume of 0.5 μL/side. Intracranial infusions utilized 23-gauge injectors (Plastics One) connected to 20-gauge polyethylene tubing (Braintree Scientific, Inc.) which were connected to 26-gauge Hamilton syringes (Model 1701N). Injectors extended 1.5 mm beyond the guide cannula and remained in place for an additional one minute following the infusion to ensure proper drug diffusion. Rats were handled by the experimenter during infusions and returned to their home cage following each infusion. For experiments where infusions occurred prior to retrieval, each rat was given a “sham infusion” before each day of habituation to habituate rats to the infusion experience. Sham infusions followed the same procedure, but no solutions were injected.

3.4.4. Fear conditioning

Each day, rats were transported to a nearby holding room 30 min before the start of the experiment. Experiments utilized two different Coulbourn Habitest (model I-I10-24A) conditioning chambers referred to here as Context A and Context B. Context A had white, curved, plastic walls, and a plastic, white floor. Context B had square, checkered walls, stainless-steel grid floors, and a vanilla scent was sprayed in the chamber before each rat entered. Additionally, conditioning boxes for Context A were housed in a room of bright ambient lighting whereas Context B was in a different room with very low lighting.

In each experiment, rats were habituated and trained as follows. On Days 1 and 2, rats were placed in Context A for 20 minutes in order to habituate to the context. On Day 3, rats were placed in Context B for training. During training, rats

were allowed to habituate to the context for 2 minutes followed by a 30 second tone (4 kHz, 75 dB) which co-terminated with a 1 second, 1.0 mA footshock. Rats remained in the context for an additional 30 seconds before being removed. Following habituation and training:

Experiment 3.1: On Day 4, rats were placed back in Context A for a retrieval test. After 2 minutes in the chamber, these rats were exposed to one unpaired tone (30 seconds, 4 kHz, 75 dB) and remained in the context for an additional 30 seconds. After retrieval, rats returned to their home cage and one group was sacrificed 1 hour later while the other group was sacrificed 24 hours later.

Experiment 3.2: On Day 4, rats underwent a reactivation test (as in Experiment 3.1). Immediately after retrieval, rats were infused intracranially with PKMζ-antisense (2 nmol/μL; 0.5 μL per side) or the scrambled control sequence into the BLA and then returned to their home cage. One day later (Day 5, PRLTM test), rats were again placed in Context A and received one unpaired tone as on Day 4. Freezing behaviour for both the reactivation and post-reactivation long-term memory (PRLTM) test days was recorded.

Experiment 3.3: Rats underwent the same procedure as in Experiment 3.2 except that infusions occurred 24 hours post-retrieval. As in Experiment 3.2, the post-infusion test occurred 24 hours after the infusion.

Experiment 3.4: On Day 4, rats received an intracranial infusion of APV (5 μg/μL; 0.5 μL per side) or vehicle to the BLA and then returned to their home cage. After 30 minutes, rats underwent a reactivation test (as in Experiment 3.2). Immediately following retrieval, rats were intracranially infused with PKMζ-antisense (2 nmol/μL; 0.5 μL per

side) or the scrambled control in the BLA and then returned to their home cage. One day later (Day 5, PRLTM test), rats were again placed in Context A and received one unpaired tone as on Day 4. Freezing behaviour for both the reactivation and PRLTM test days was recorded.

After each animal was taken out of a conditioning box (Context A or B) on each day of the experiment, the floor and walls were wiped clean using a damp paper towel with 2% Versa-Clean (Fisher, 18200700) in dH_2O before the next animal was placed inside.

Rats were recorded during training using FreezeFrame software (Actimetrics) and during tests using GeoVision GV-600 System.

3.4.5. Sacrificing

Rats' brains were quickly collected and flash frozen. First, rats were placed in an induction chamber containing isoflurane (Baxter, 02225875). Once deep anaesthesia had been induced, the rat was removed and quickly decapitated using a guillotine. Its brain was quickly retrieved. The brain was immediately submerged in a beaker containing 2-methylbutane (Fisher, O3551-4) which was within a container of dry ice. Once brains were frozen, they were wrapped in aluminum foil and submerged in the dry ice before final storage at -80°C.

3.4.6. Subcellular fractionation

Subcellular fractionation followed a previously established protocol to obtain synaptosome fractions (Bai & Witzmann, 2007). Frozen brains were mounted on a cryostat and basolateral amygdala tissue was collected using a tissue puncher (Fine Science Tools). The tissue was homogenized using a Pellet Pestle (Fisher, #12141361)

in 200 µL of homogenization buffer containing 20 mM HEPES, 1 mM EDTA, 2 mM EGTA, 320 mM sucrose, along with one protease inhibitor tablet (Roche, 05892791001) and one phosphatase inhibitor tablet (Roche, 04906837001). Homogenized tissue was centrifuged at 1000 g for 10 min. The supernatant was collected and centrifuged at 17,000 g for 15 min. The pellet was resuspended in 50 µL of homogenization buffer and layered on a sucrose gradient containing 100 µL of 0.8 M sucrose (1 mM EDTA, 2 mM EGTA) and 100 µL of 1.2 M sucrose (1 mM EDTA, 2 mM EGTA). This mixture was centrifuged at 54,000 g for 90 min. The layer between the 0.8 M and 1.2 M sucrose, containing the synaptosomal fraction (Bai & Witzmann, 2007), was collected and used for Western blotting following protein quantification with a BCA protein assay kit (Pierce).

3.4.7. Western Blotting

After protein quantification, 15 µg of protein was loaded in 8% SDS-PAGE gels. Proteins were transferred overnight (at 4 °C) onto nitrocellulose membranes. Following transfer, Ponceau solution (Sigma, P7170) was applied to membranes to reveal protein bands. Along with the molecular weight marker (Thermo Scientific, 26634), this enabled the cutting of membranes at 70 kDa and 40 kDa to produce two membranes. One membrane contained PKMζ protein (55 kDa) and the other contained GAPDH (37 kDa). Membranes were washed in 0.1% Tween 20, Tris-buffered saline (TBS-Tween). Blocking of membranes was done for 1 h at room temperature in TBS-Tween containing 5% bovine serum albumin (BSA). Membranes were then incubated overnight with antibodies in 5% BSA TBS-Tween: 1:1000 PKCζ (Abcam, ab59364), 1:10,000 GAPDH (Abcam, ab8245). Following overnight incubation, membranes were washed three times

with TBS-Tween. Membranes then underwent incubation with secondary antibody (Anti-rabbit IgG HRP-conjugated from Amersham, NA934V; Anti-mouse IgG HRP-conjugated from Amersham, NA931V) for 1 h at room temperature. After secondary antibody incubation, membranes were washed four times for 10 min in TBS-Tween. Membranes were revealed using Pierce ECL 2 Western Blotting Substrate and scanned using a Storm Scanner (Molecular Dynamics). Scanned images were quantified using Image Lab (Bio-Rad). PKMζ protein values were compared to the GAPDH loading control for each sample. Values were then standardized as a percent of one of the two groups (i.e. 24 hours post-retrieval group).

3.4.8. Statistical analyses

Data were analyzed using Jamovi (Version 1.0.7.0.). Neither homogeneity of variance, normal distribution of data, nor sphericity assumptions were violated where relevant for the following data. PKMζ protein from Western blots was analyzed using independent samples student's t-tests. Freezing data from behavioural experiments were analyzed using one- or two-way repeated-measures ANOVAs. Tukey's test was used for post-hoc analyses for statistically significant effects. For all analyses, the null hypothesis was rejected where $p < 0.05$. Figures present data as means ±1 SEM.

3.5. Results

3.5.1. Reconsolidation increases synaptic PKMζ

As shown in Chapter 2, memory destabilization reduces the availability of PKMζ at the synapse. Previous work has revealed that within hours after destabilization, GluA2-AMPARs are reinserted to the postsynaptic membrane at roughly the same time as reconsolidation occurs (Hong et al., 2013). Given the apparent role of PKMζ in regulating GluA2-AMPARs (Dong et al., 2015; Migues et al., 2010), we wondered whether reconsolidation also coincided with an increase in PKMζ expression. Indeed, we found that PKMζ levels were higher in rats sacrificed 24 hours after retrieval (n=7) compared to those sacrificed 1 hour after retrieval (n=8; independent samples t-test, t_{13}=2.365, p=0.034). This suggests that following destabilization, reconsolidation involves an increase of synaptic PKMζ.

3.5.2. Synthesis of PKMζ is necessary for reconsolidation

One well-supported finding in the literature is that protein synthesis inhibitors disrupt reconsolidation and impair long-term memory (Barnes et al., 2012; Ben Mamou et al., 2006; Debiec et al., 2006; Finnie & Nader, 2012; Huynh et al., 2014; Jobim et al., 2012; Lee et al., 2004; Nader et al., 2000). Given that reconsolidation increases the presence of synaptic PKMζ following destabilization, we investigated whether synthesis of new PKMζ is necessary to restabilize the memory. To selectively inhibit PKMζ translation we used antisense oligodeoxynucleotides (ODNs) specific to PKMζ. We employed a sequence previously shown to disrupt synthesis of PKMζ and impair late-LTP in vitro but has no effect in PKMζ-knockout animals (Tsokas et al., 2016). We found that rats' memory differed depending on whether they were infused with PKMζ-

antisense or the scrambled control (repeated measures ANOVA with Day as the repeated factor and ODN sequence as between-subjects factor, $F_{1,18}$=11.005, p=0.004). Specifically, rats receiving PKMζ-antisense showed impaired memory following infusion (n=10; Tukey's test, p<0.001). However, rats receiving the scrambled control showed no difference in performance (n=10; Tukey's test, p=0.210). These findings suggest that PKMζ-antisense impairs reconsolidation by blocking the synthesis of new PKMζ protein.

3.5.3. Acute inhibition of PKMζ synthesis does not impair stable memory

Disruptions in PKMζ catalytic activity impair memory maintenance (Kwapis et al., 2012; Serrano et al., 2008; Shema et al., 2011). We, therefore, wondered whether disrupting PKMζ synthesis during memory maintenance also impairs memory or if the amnesic effect of PKMζ-antisense is specific to the reconsolidation period. Put simply, is reconsolidation exceptionally vulnerable to acute disruption of PKMζ synthesis compared to memory maintenance? To test this, we infused PKMζ-antisense or the control ODN 24 hours after retrieval. We found that infusion of either PKMζ-antisense (n=9) or the scrambled ODN (n=7) did not affect freezing behaviour (repeated measures ANOVA with Day as the repeated factor and ODN sequence as between-subjects factor, $F_{1,14}$= 0.055, p=0.818). That is, neither group showed a memory impairment. Therefore, acute infusion of PKMζ-antisense after reconsolidation does not disrupt long-term memory. Consequently, it seems that labile, but not stable, memories are vulnerable to disruptions of PKMζ synthesis.

3.5.4. Preventing destabilization protects memory from the amnesic effect of PKMζ-antisense

In Chapter 2 we showed that NMDAR-dependent memory destabilization is necessary to decrease PKMζ. Given that labile memories are sensitive to inhibition of PKMζ synthesis, we next tested whether NMDAR activation prevents the amnesic effect of PKMζ-antisense after retrieval. That is, is it specifically destabilization that renders the memory vulnerable to PKMζ-antisense? Indeed, we found that whether rats received APV or vehicle prior to retrieval determined whether the post-retrieval ODN infusion could impair memory at the final test (repeated measures ANOVA with Day as the repeated factor and pre-retrieval infusion and ODN sequence as between-subjects factors, $F_{1,31}$=6.65, p=0.015). Rats infused with the vehicle prior to retrieval followed by infusion of PKMζ-antisense post-retrieval showed impaired performance (n=11; Tukey's test, p<0.001). However, rats that received APV prior to retrieval followed by PKMζ-antisense post-retrieval did not show impaired memory (n=8; Tukey's test, p=0.999). The scrambled control sequence did not affect memory in any group (vehicle-scrambled group, n=7; APV-scrambled group, n=7). This suggests that, absent NMDAR-dependent destabilization, retrieved memories are not vulnerable to acute inhibition of PKMζ synthesis.

Of note, rats showed roughly 20% lower freezing during the first retrieval test in this experiment than in Experiments 3.5.2 and 3.5.3. This disparity likely reflects a difference in the procedures used between these experiments. In both Experiments 3.5.2 and 3.5.3, rats received an infusion only *after* retrieval, whereas rats received both a pre-retrieval infusion *and* a post-retrieval infusion here. This unique and extended

handling experience just prior to retrieval may have rendered rats more active during the subsequent session, resulting in lower freezing expressed during the retrieval test.

3.6. Discussion

Here we show that, following destabilization of an auditory fear memory, reconsolidation increased the expression of PKMζ at BLA synapses. We also show that the synthesis of new PKMζ is necessary for reconsolidation because infusing PKMζ-antisense after retrieval impaired long-term memory. Importantly, reconsolidated memories were not vulnerable to disruption by PKMζ-antisense. Similarly, without NMDAR-dependent memory destabilization, PKMζ-antisense did not affect long-term memory.

Our results may help to explain how GluA2-AMPARs stabilize at the PSD during reconsolidation. As previous work has shown, GluA2-AMPARs are inserted into the PSD at roughly the same time when reconsolidation is believed to occur (Hong et al., 2013). Our findings show that reconsolidation seems to recruit protein synthesis to increase postsynaptic expression of PKMζ. Once available at the PSD, newly synthesized PKMζ presumably works to maintain these GluA2-AMPARs at the membrane, as has been suggested during memory maintenance (Dong et al., 2015; Migues et al., 2010; Yu et al., 2017). Other work indicates that PKMζ could act with NSF to reduce PICK1 binding to GluA2-AMPARs (Yao et al., 2008). In this way, PKMζ might also promote the insertion of these AMPARs during reconsolidation in addition to maintaining those already at the PSD. Taken together, it seems that the synthesis of new PKMζ is

necessary to re-stabilize the incoming GluA2-AMPARs during reconsolidation. In the presence of PKMζ-antisense, newly inserted GluA2-AMPARs are liable to be quickly endocytosed, leading to long-term memory impairment.

Our findings add to the previous literature utilizing antisense ODNs to identify proteins necessary for reconsolidation including zif268, C/EBPβ, C/EBPδ, and Arc (Arguello et al., 2013; Lee et al., 2004; Maddox et al., 2011; Maddox & Schafe, 2011; Milekic et al., 2007). Recent work has also shown that infusion of PKMζ-antisense in the dorsal hippocampus impairs reconsolidation of an object recognition memory (Rossato et al., 2019). Interestingly, maintaining this type of long-term memory outside of reconsolidation does not require PKMζ in the dorsal hippocampus (Hardt et al., 2010). This discrepancy may suggest that different structures and/or processes support memory during different phases, such as encoding, storage, and reconsolidation. Similar dissociations have been found for the consolidation of inhibitory avoidance memory, which requires C/EBPβ translation in the hippocampus but not amygdala (Taubenfeld et al., 2001). On the other hand, reconsolidation of this memory requires C/EBPβ translation in the amygdala but not the hippocampus (Milekic et al., 2007).

Importantly, our results suggest that a transient disruption in PKMζ synthesis is not sufficient to impair stable long-term memories. Others have observed that acute administration of PKMζ-antisense does not significantly alter basal levels of PKMζ (Hsieh et al., 2016; Tsokas et al., 2016). Here, we show that brief PKMζ knockdown does not disrupt stable memories either. These findings suggest that the half-life of PKMζ could be quite long, such that existing PKMζ can maintain the memory in the absence of further translation. These data could also imply an excess of PKMζ at the

spine during memory maintenance so that the loss of some protein through turnover does not jeopardize the stability of the trace.

While acutely blocking PKMζ translation does not impair memory, we predict that chronic administration of PKMζ-antisense would eventually lead to amnesia. In previous experiments, chronic disruption of PKMζ activity impaired long-term memory (Dong et al., 2015; Shema et al., 2011; Vogt-Eisele et al., 2014). We expect that reducing PKMζ with repeated administration of antisense ODNs could decrease protein levels sufficient to disrupt memory that would not return. Taken together, we see that during destabilization there is a precariously low concentration of PKMζ that must necessarily be increased through protein synthesis to reconsolidate the memory.

Our empirical findings support a recent paper describing a computational model of PKMζ activity throughout various manipulations (Helfer & Shultz, 2018). First, our data support the model's prediction that reactivation coupled with protein synthesis inhibition (in our case, PKMζ-antisense) is sufficient to cause a long-term memory impairment. Second, our data and the model agree that briefly blocking PKMζ synthesis is insufficient to disrupt memory because the system can recover. While these results conform to the model's predictions, our data suggest a longer timeline than the model describes. This model suggests that, following retrieval, PKMζ levels are reduced almost immediately and return to baseline within 40 minutes. However, our data show that PKMζ protein is still significantly reduced at one hour after retrieval. This observation is in line with other work showing that NSF, another important protein for maintaining GluA2-

AMPARs at the PSD, only begins to decrease at about one hour after retrieval (Ren et al., 2013).

Finally, these results show that memory reconsolidation, in particular, is sensitive to acute disruption of PKMζ synthesis. Inhibiting PKMζ translation many hours after reconsolidation has ended does not impair memory. Similarly, without NMDAR-dependent memory destabilization, PKMζ-antisense will not disrupt reconsolidation. Activation of NMDARs is necessary for inducing memory lability (Ben Mamou et al., 2006; Ferrer Monti et al., 2016; Milton et al., 2013), leading to the removal of synaptic GluA2-AMPARs (Hong et al., 2013). Since NMDAR inhibition prevents the post-retrieval reduction in PKMζ (as shown in Chapter 2), there is no synaptic paucity of PKMζ that must be replaced through de novo synthesis. Thus, the memory can presumably be maintained normally, without additional protein synthesis to maintain the existing GluA2-AMPARs.

In summary, our results show that the synthesis of PKMζ is an essential component of memory reconsolidation.

3.7. Figures

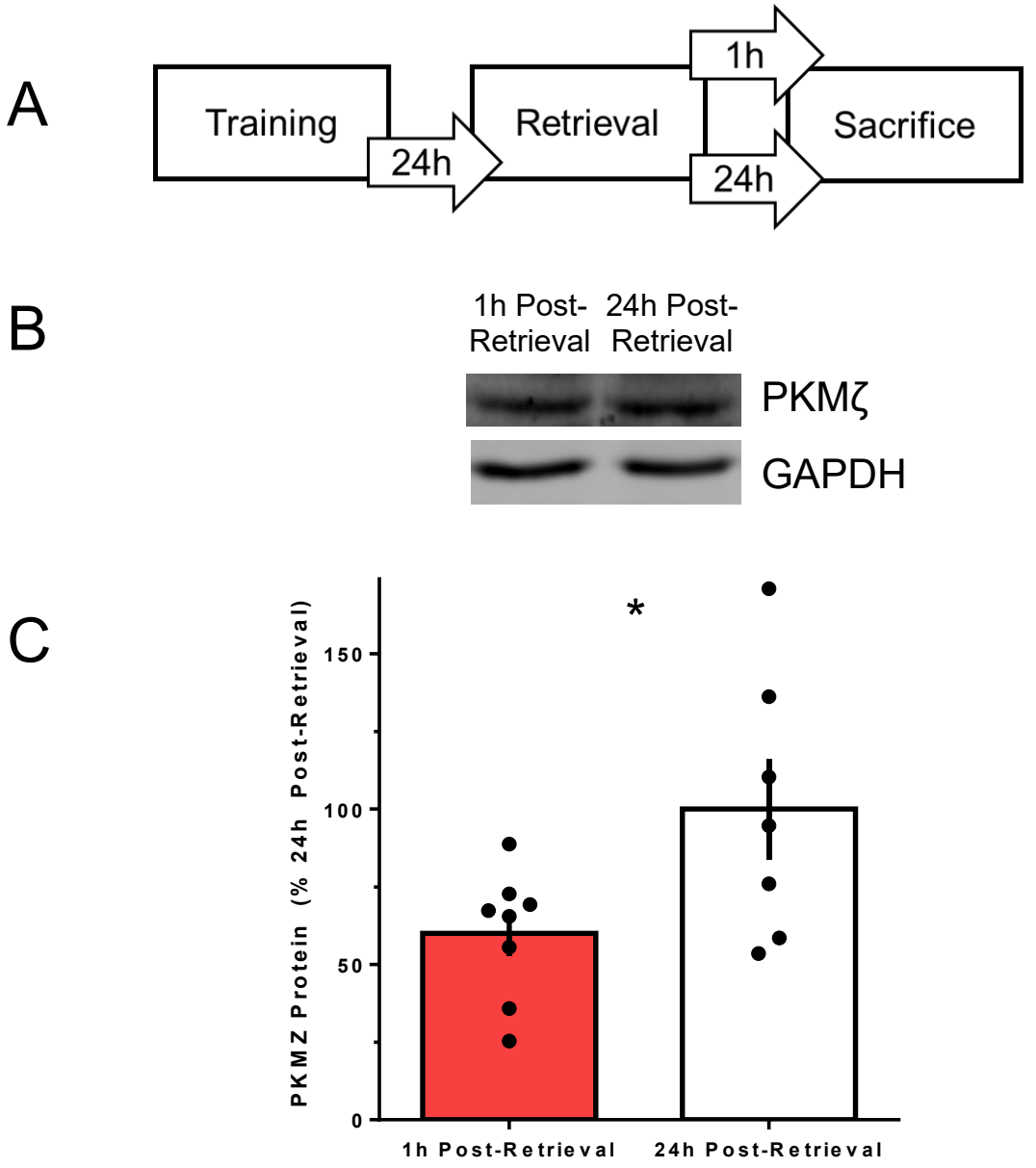

Figure 3.1. Reconsolidation increases synaptic PKMζ.

A) Rats underwent habituation, training, and a retrieval test and were sacrificed either 1- or 24-hours post-retrieval. B) Representative Western blot. C) PKMζ protein levels from Western blots. Rats sacrificed 1-hour post-retrieval (n=8) showed significantly lower PKMζ protein in BLA synaptosomes compared to rats sacrificed 24 hours post-retrieval (n=7; t_{13}=2.365, p=0.034).

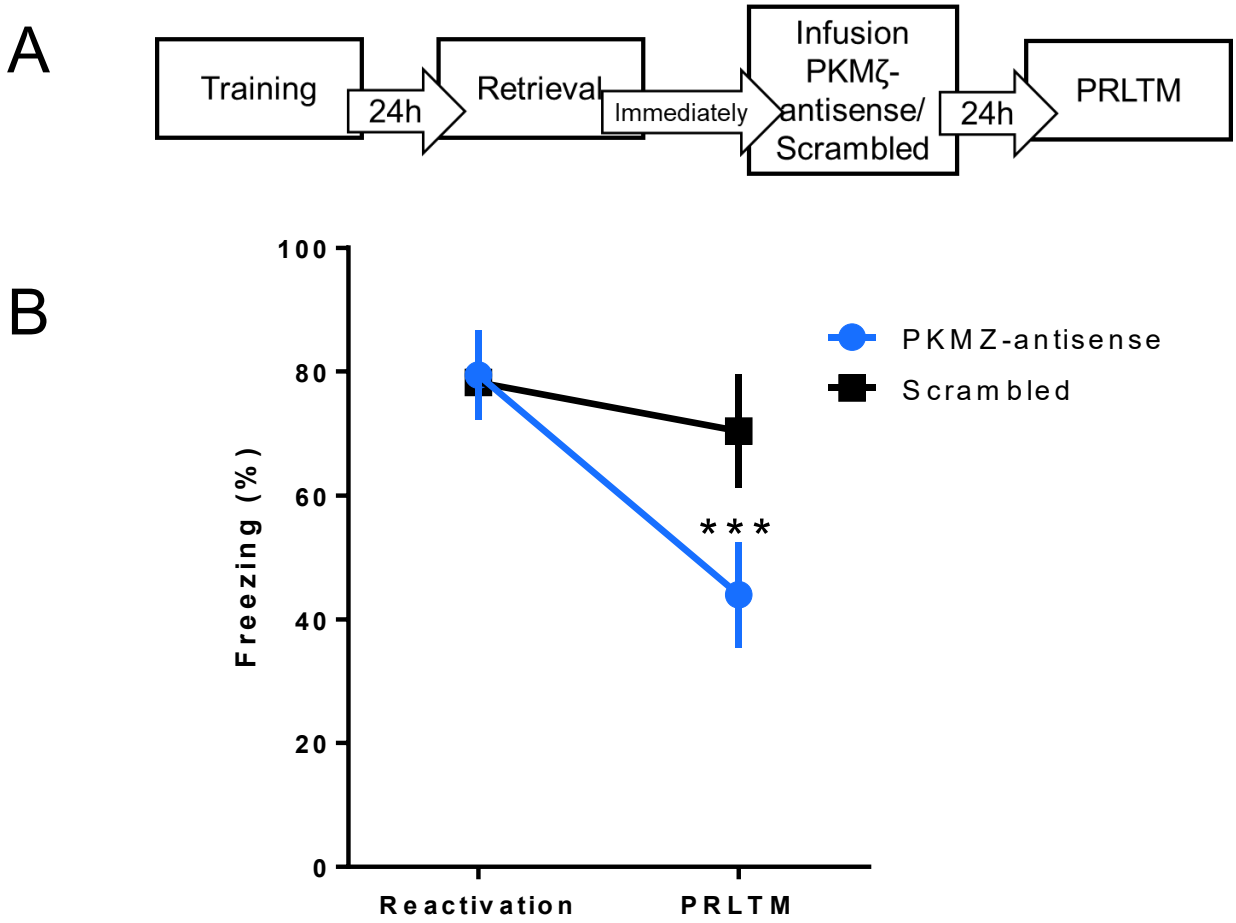

Figure 3.2. Post-retrieval infusion of PKMζ-antisense impairs long-term memory. A) Rats underwent habituation and training. Immediately following retrieval the next day, rats were infused with either PKMζ-antisense or scrambled ODN (2 nmol/µL; 0.5 µL/side). Rats were tested again 24 hours post-infusion. B) Freezing is reported as the percentage of time rats spent freezing during the 30 second tone presentation. Behaviour data showed a significant effect of the infusion on performance ($F_{1,18}$=11.005, p=0.004). Rats that received PKMζ-antisense (n=10) showed a significant impairment in performance when tested 24 hours post-infusion (PRLTM, p<0.001) whereas rats that received the scrambled sequence (n=10) showed no impairment (p=0.210).

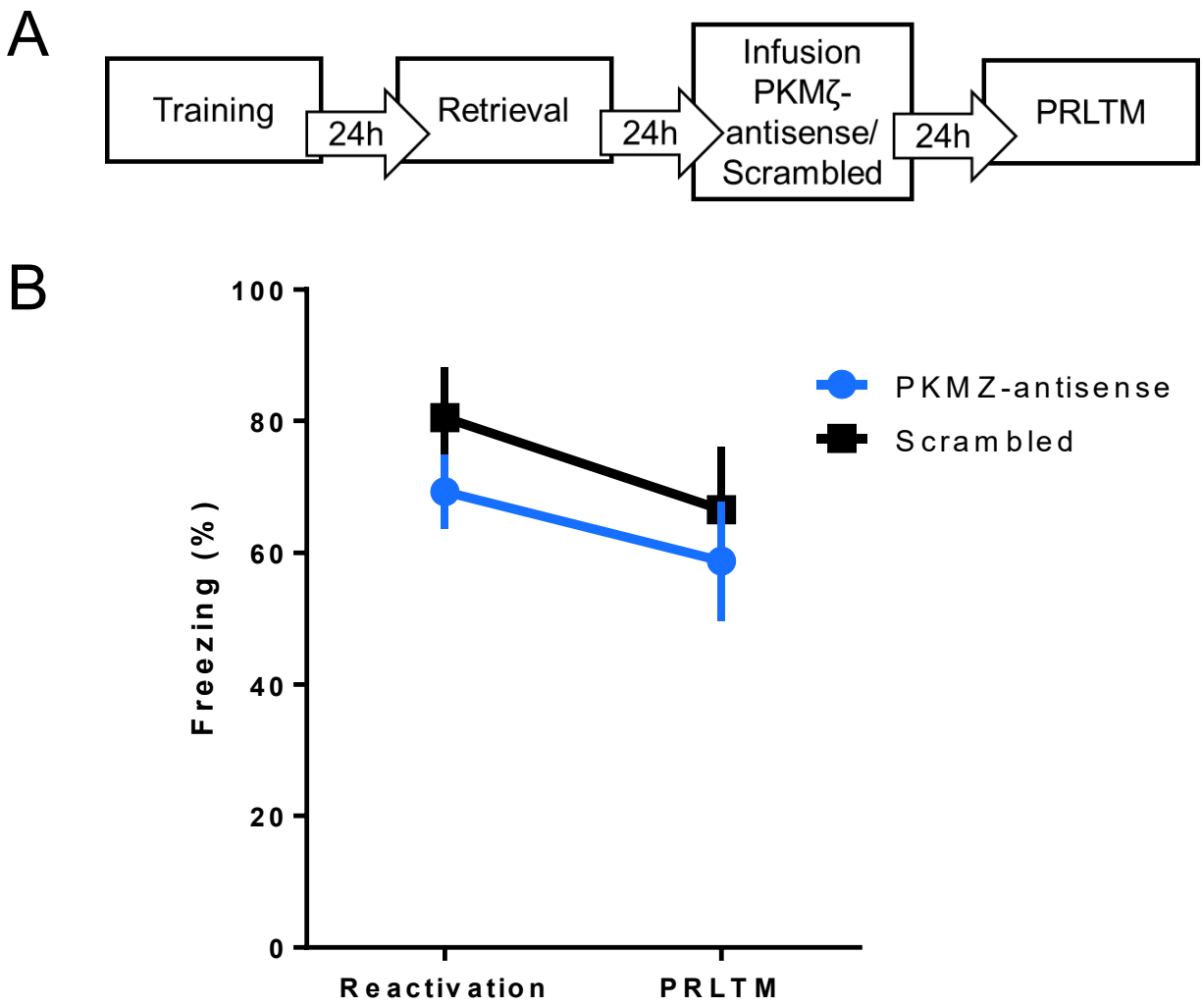

Figure 3.3. Infusion of PKMζ-antisense 24h post-retrieval does not impair memory. A) Rats underwent habituation, training, and a retrieval test. 24 hours following retrieval, rats were infused with either PKMζ-antisense or scrambled ODN (2 nmol/µL; 0.5 µL/side). Rats were tested again 24 hours post-infusion. B) Freezing is reported as the percentage of time rats spent freezing during the 30 second tone presentation. Behaviour data showed no effect of infusion on performance (PKMζ-antisense group, n=9; scrambled group, n=7; $F_{1,14}$= 0.055, p=0.818).

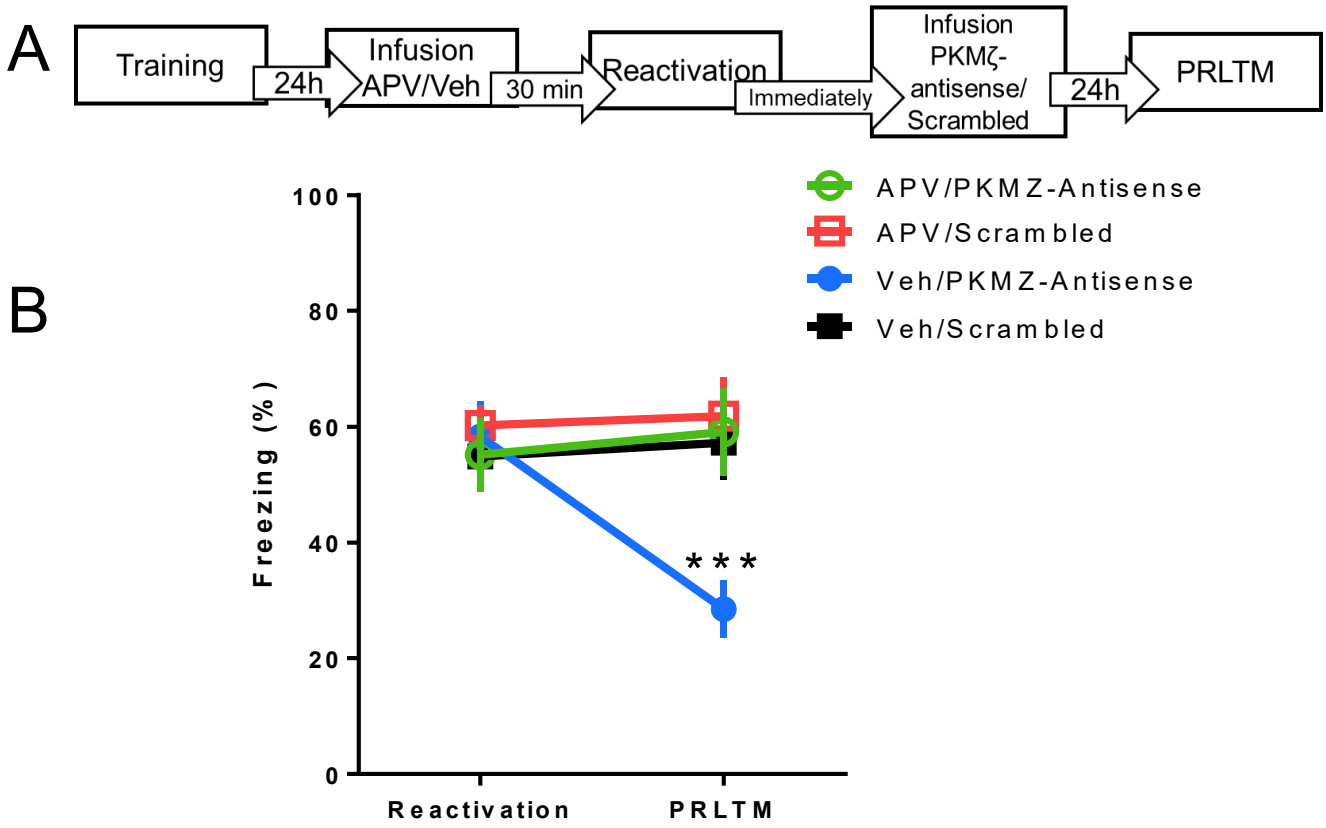

Figure 3.4. Pre-retrieval APV prevents amnesic effect of PKMζ-antisense.

A) Rats underwent habituation and auditory fear conditioning training. One day after training, rats received an intracranial infusion of either APV or vehicle to the BLA 30 min prior to a retrieval test. Immediately following retrieval, rats received an infusion of either PKMζ-antisense or scrambled ODN in the BLA. Rats were tested again 24 hours post-infusion. B) Freezing is reported as the percentage of time rats spent freezing during the 30 second tone presentation. Behaviour data revealed an interaction of pre-retrieval drug and post-retrieval ODN on performance ($F_{1,31}$=6.65, p=0.015). That is, PKMζ-antisense had an amnesic effect in vehicle-pre-treated rats (n=11; p<0.001) but not in APV-pre-treated ones (n=8; p=0.999). Rats receiving the scrambled ODN sequence showed no impairment in performance (vehicle-scrambled group, n=7; APV-scrambled group, n=7).

Chapter 4: ζ-stat is a PKMζ-specific inhibitor that disrupts auditory fear memory in male, but not female, mice

4.1. Preface

In Chapters 2 and 3, we investigated the dynamic regulation of PKMζ during memory destabilization and reconsolidation. This work was done exclusively with male animals, a common practice in neuroscience research (Beery & Zucker, 2011). The inclusion of only one biological sex limits our results as we do not know whether they also apply to female animals.

Sex-differences related to PKMζ are rarely investigated, and only a handful of studies have reported sex-dependent effects (Lee et al., 2013; McGrath et al., 2018; Nasir et al., 2016; Sebastian et al., 2013). This might reflect that sex-differences do not exist for PKMζ, as some work suggests (Deutschmann et al., 2019; Levitan et al., 2016; Volk et al., 2013). This conclusion relies on studies showing that the inhibitory peptide, ZIP, can impair memory maintenance in both males and females (Gao et al., 2018; Serrano et al., 2008; Shema et al., 2009; von Kraus et al., 2010). However, ZIP can inhibit multiple kinases in addition to PKMζ (Bogard & Tavalin, 2015; Lee et al., 2013), making it difficult to conclude that its effect is driven exclusively by PKMζ inhibition. Therefore, more specific tools are required to definitively conclude that PKMζ has a similar function in both males and females.

One well-replicated procedure for studying the role of PKMζ in memory maintenance involves infusing ZIP in the BLA at least 24 hours after auditory fear conditioning and then testing memory retention later on (Kwapis et al., 2009; Migues et al., 2010; Serrano et al., 2008). Given the potential complications of using ZIP outlined above, we examined the role of PKMζ by replicating this experimental design using a more specific inhibitor of PKMζ, ζ-stat, in male and female animals.

To demonstrate that ζ-stat targets PKMζ specifically, we infused it in both wild-type and PKMζ-KO male mice. After demonstrating its specificity, we then tested whether ζ-stat can impair memory in female animals as well. In this case, we expected that ζ-stat would have no effect and therefore included a ZIP-infused group as a positive control. If ZIP can still impair memory in females, as has been shown before (Gao et al., 2018; von Kraus et al., 2010), then the sex-difference we observe is due solely to the drug we infused rather than some other physiological effect.

It should be noted that these experiments utilized mice, whereas Chapters 2 and 3 evaluated the role of PKMζ in rats. The purpose of using mice here was to test the specificity of ζ-stat in PKMζ-KO animals, which would not have been possible in rats.

4.2. Abstract

Considerable research suggests that PKMζ maintains long-term memory in a number of different species. However, most of this work has not investigated whether differences exist between male and female animals. Several studies have found that memory in male rodents is especially vulnerable to knockout or knockdown of PKMζ expression. On the other hand, experiments using ZIP have shown that this peptide impairs memory in both males and females. Importantly ZIP can also inhibit other memory-related proteins. Together these findings raise the possibility that ZIP may be targeting different processes in males and females. Here we sought to clarify this discrepancy using the more specific inhibitor, ζ-stat. We found that ζ-stat impaired maintenance of auditory fear conditioning memory in wild-type males but not PKMζ-KO male mice. However, ζ-stat did not impair memory in female wild-type mice, while ZIP did. Our results thus show that ζ-stat does impair memory by disrupting a PKMζ-specific process. Furthermore, they suggest that female mice do not maintain memory using PKMζ but rely on some other ZIP-sensitive process.

4.3. Introduction

The majority of neuroscience research has focused primarily on males with little attention paid to sex-differences within a species (Beery & Zucker, 2011). This disparity also exists in the neuroscience of learning and memory, despite established differences between male and female animals (Andreano & Cahill, 2009; Cahill, 2006; Choleris, Galea, Sohrabji, & Frick, 2018; Jonasson, 2005). Addressing this issue within the topic of this thesis, we investigated whether the role of PKMζ in memory maintenance differs between males and females.

Sex-differences regarding PKMζ have received little attention in the literature (Nasir et al., 2016; Sebastian et al., 2013). This may be due to some seemingly contradictory findings. One study using a radial arm maze task found that, while synaptic GluA2-AMPARs correlated with performance in both sexes, expression of PKMζ only correlated with performance in males (Sebastian et al., 2013). Other work demonstrates that knocking out (Lee et al., 2013; Nasir et al., 2016) or knocking down (McGrath et al., 2018) PKMζ differentially affects males and females. In one study, PKMζ-KO males had less mechanical allodynia than WT males, but no such difference existed between PKMζ-KO and WT females (Nasir et al., 2016). That is, knocking out PKMζ revealed an effect in males but not females.

These data have been difficult to interpret in light of other experiments utilizing ζ inhibitory peptide (ZIP), the most commonly used inhibitor of PKMζ. ZIP impairs memory in both males and females (Deutschmann et al., 2019; Migues et al., 2010; Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2009; von Kraus et al., 2010). However, recent studies demonstrate that this inhibitor is not specific to PKMζ

(Bogard & Tavalin, 2015; Lee et al., 2013). ZIP can inhibit other PKC isoforms, like PKCι/λ (Lee et al., 2013), which may explain why it disrupts memory in PKMζ-KO animals (Volk et al., 2013). Other work suggests that ZIP may have yet more off-target impacts on neuronal physiology which may confound experimental results. One study found that ZIP promotes neural silencing (LeBlancq et al., 2016) while another showed that ZIP can lead to excitotoxicity in vitro (Sadeh et al., 2015). Thus, ZIP does not provide enough specificity to test sex-differences with PKMζ.

Numerous studies have sought to examine the role of PKMζ in memory maintenance by inhibiting this protein after consolidation in several different memory tasks (Li et al., 2011; Shema et al., 2009; Shema, Sacktor, & Dudai, 2007). For instance, several authors have found that infusing ZIP in the BLA 24 hours after auditory fear conditioning impairs memory performance on a subsequent test (Kwapis et al., 2009; Migues et al., 2010; Serrano et al., 2008). Given the non-specific effects of ZIP outlined above, a more specific method of impairing PKMζ activity is required for more unambiguous conclusions.

PKMζ-antisense provides one option to target PKMζ expression. However, as we have shown in Chapter 3, one acute infusion of PKMζ-antisense is insufficient to disrupt memory maintenance. At present, it is not known how much and how often PKMζ-antisense should be infused to impair memory maintenance. Furthermore, PKMζ-antisense disrupts the translation of new PKMζ rather than disrupting existing PKMζ protein activity. Therefore, some other compound that can disrupt PKMζ activity might be better suited to impair memory maintenance.

8-hydroxy-1,3,6-naphthalenetrisulfonic acid (ζ-stat) is a PKCζ inhibitor which has previously been used to disrupt that kinase in cell cultures (Islam et al., 2020, 2018; Ratnayake et al., 2018). To the best of our knowledge, ζ-stat has not been used to disrupt PKMζ. It is expected to disrupt PKMζ as it does with PKCζ since its binding site appears to be in the C-terminal domain shared by both proteins (Hernandez et al., 2003; Sacktor et al., 1993). If PKMζ does indeed maintain long-term memory and if ζ-stat is specific to that protein, then administration of ζ-stat should impair long-term memory like ZIP.

Here we examined whether ζ-stat can impair long-term memory maintenance in male and female mice by disrupting PKMζ. To test its specificity, we infused ζ-stat in both wild-type males and PKMζ-null males. We found that ζ-stat did indeed impair memory in wild-type males but not PKMζ-null males. Further, we found that ζ-stat did not disrupt memory in wild-type females, suggesting that PKMζ may not maintain memory in female mice.

4.4. Materials and Methods

4.4.1. Animals

C57/B6 PKMζ-null mice were provided by Wayne Sossin from an existing mouse line developed and described by Lee et al. (2013). These mice were bred in-house alongside WT C56/B6 mice from Jackson Laboratories. Female C57/B6 mice used in experiments were also obtained from Jackson Laboratories. Mice were 8-10 weeks at the time of cannulation and 9-11 weeks at the beginning of behavioural experiments. Mice were housed in groups of five males or five females in plastic cages and provided with food and water ad libitum. Mice were maintained on a 12 h light/dark cycle (lights

on at 07:00, off at 19:00) and behavioural experiments began at 09:00. All procedures were approved by McGill's Animal Care Committee and complied with the Canadian Council on Animal Care guidelines.

4.4.2. Surgery

Mice were injected intraperitoneally with an anesthetic cocktail containing ketamine (10 mg/mL) and xylazine (20 mg/mL). Mice were provided with analgesic treatment prior to surgery (carprofen; 5 mg/mL). Guide cannulas (Plastics One, Roanoke, VA) were implanted bilaterally aiming at the basolateral amygdala (from bregma: AP -1.7 mm; L +/- 3.0 mm; DV -4.4mm) and secured to the skull with three jeweller's screws and dental cement. BLA coordinates were determined using a mouse brain atlas (Paxinos & Franklin, 2012). Antisedan (0.5 mg/mL) was given via IP injection after surgery to reverse the anesthesia.

4.4.3. Auditory fear conditioning

For 7 days following surgery, mice were handled by letting them freely explore the experimenter's palm for 2-5 minutes. Mice were habituated, trained, and tested in the same conditioning box (Coulbourn Habitest, Coulbourn Instruments) with differing floors and walls to produce two different contexts (Context A and Context B). For each day of the behaviour experiment, mice were brought to the experiment room at 09:00 and allowed to acclimatize for 30 minutes. Mice were then habituated to the testing context (Context A with a smooth floor and flat, blank walls) for 20 min each day for two consecutive days. The next day, mice were trained in a second context (Context B, with a grid floor, patterned walls, and a curved wall). Training consisted of 2 min of exploration of Context B followed by a tone (2800 Hz, 85 dB, 30s) co-terminating with a

footshock (0.7 mA, 1s). Mice received two tone-shock pairings separated by one minute and remained in Context B for an additional 1 min before returning to their home cage. Mice were tested 24 hours after training in Context A (Test 1). During testing, mice were placed in the conditioning box and, after 2 minutes, were exposed to a 30 second tone (2800 Hz, 85 dB). The next day, mice received bilateral infusions and were tested a second time, 24 hours post-infusion in Context A (Test 2) following the same procedure as Test 1. After each animal was taken out of a conditioning box (Context A or B) on each day of the experiment, the floor and walls were wiped clean using a damp paper towel with 2% Versa-Clean (Fisher, 18200700) in dH_2O before the next animal was placed inside. Freezing behaviour on Test 1 and Test 2 was scored and reported as the percent of time spent immobile during the tone.

4.4.4. Drug Infusions

Mice were bilaterally infused with ζ-stat (20 mM dissolved in PBS, obtained from Drug Synthesis and Chemistry Branch, National Cancer Institute), ζ-inhibitory peptide (ZIP, 10 nmol/μL in 0.1 M Tris-saline, Anaspec AS-63361), or vehicle (PBS). Mice were infused with 0.3 μL at a rate of 0.2 μL/min into each amygdala. Drugs were infused with 28 gauge microinjectors (Plastics One) connected to Hamilton syringes (26 gauge, Model 1701N) by way of polyethylene tubing (Braintree Scientific, Inc). After infusion, injectors remained in place for one minute to ensure the drug sufficiently diffused away from the injector tip.

4.4.5. Statistical analysis

Data were analyzed using Jamovi (Version 1.0.7.0.). Neither homogeneity of variance nor sphericity assumptions were violated. For experiment 4.1, data were

analyzed using a two-way repeated measures (Genotype X Drug X Day) ANOVA. For experiment 4.2, data were analyzed using a one-way repeated measures (Drug X Day) ANOVA. For both experiments, Tukey's test was used for post-hoc analyses for statistically significant effects. The null hypothesis was rejected where $p<0.05$. Figures present data as means with SEM.

4.5. Results

4.5.1. ζ-stat impairs memory in wild-type but not PKMζ-null males

We first tested whether ζ-stat impairs memory similar to ZIP and, if so, whether this amnesic effect is due specifically to PKMζ disruption. To do so, we used both wild-type males and PKMζ-null male mice. We found that drug infusions differentially affected WT and PKMζ-null animals ($F_{1,39}$=10.76, p=0.002, Figure 4.1). Infusions of ζ-stat impaired memory in WT mice (n=10; t_{39}= 5.3837, $p<0.001$) but not PKMζ-null animals (n=9; t_{39}= 0.3651, $p>0.05$). Vehicle infusion did not affect memory in either WT (n=11; t_{39}=-0.5667, $p>0.05$) or PKMζ-null mice (n=13; t_{39}=0.9703, $p>0.05$) and both groups showed no impairment in memory. Therefore, ζ-stat can impair memory maintenance, and this effect is specific to PKMζ.

4.5.2. ζ-stat does not disrupt memory maintenance in female mice, but ZIP does

We next tested if PKMζ maintains long-term memory in wild-type female mice as it does in males. We found that long-term memory was impaired in certain groups after infusion ($F_{2,28}$= 3.90, p=0.032, Figure 4.2). Specifically, while ZIP did impair memory (n=8; t_{28}=3.324, p=0.027), mice infused with ζ-stat showed no impairment in memory at Test 2 (n=13; t_{28}=0.4885, p=0.996). Similarly, vehicle-treated female mice showed no

impairment in memory (n=10; t_{28}=-0.2195, p>0.05). These data suggest that PKMζ does not maintain long-term memory in female mice but a ZIP-sensitive mechanism does.

4.6. Discussion

Here we show that ζ-stat can impair maintenance of an auditory fear memory in male wild-type mice. We also show that ζ-stat does not affect memory in PKMζ-null mice, suggesting that its effect is specific to PKMζ and not some other mechanism. Further, we demonstrate that ζ-stat does not impair the maintenance of auditory fear memory in female mice. However, ZIP does impair this kind of memory. This suggests that auditory fear memory is not maintained by PKMζ in female mice but instead by some other ZIP-sensitive mechanism.

These results provide strong support for the hypothesis that—at least in males—long-term memories are maintained by PKMζ. This hypothesis has been debated, especially since the demonstration that PKMζ-null mice have normal learning and memory, which is impaired by ZIP (Lee et al., 2013; Volk et al., 2013). However, more recent work suggests that in these PKMζ-knockout mice, the closely related atypical PKC isoform, PKCι/λ, may be compensating for the lack of PKMζ (Tsokas et al., 2016). PKCι/λ is also vulnerable to disruption by ZIP, which may explain why that inhibitor also disrupts memory in PKMζ-KO mice (Lee et al., 2013). Importantly, numerous experiments that manipulate PKMζ without using ZIP also reinforce the importance of this kinase in memory maintenance. For instance, using a virus to overexpress PKMζ can enhance contextual fear memory (Schuette et al., 2016), auditory fear memory (Xue et al., 2015), and conditioned taste aversion (Shema et al., 2011). In *Drosophila*, expression of mouse PKMζ or the *Drosophila* homolog, DaPKM, enhances olfactory

memory (Drier et al., 2002). Furthermore, PKMζ-antisense can impair long-term memory in vivo (Hsieh et al., 2016) and LTP in vitro (Tsokas et al., 2016). Our work shows that ζ-stat offers another method to examine the role of PKMζ in long-term memory, one that is specific to PKMζ.

Using this more precise tool, we have uncovered a sex-difference regarding PKMζ in memory maintenance. Previous work has indicated that PKMζ may play different roles in males and females. PKMζ correlates with spatial memory performance in males but not females (Sebastian et al., 2013). Knocking down PKMζ in the NAc increases cocaine self-administration in males but not females (McGrath et al., 2018). Finally, knocking out PKMζ differentially affects pain sensitivity in males and females (Nasir et al., 2016). Up to now, it has been difficult to unambiguously test whether disrupting PKMζ impairs memory maintenance since ZIP inhibits other kinases as well (Lee et al., 2013; Volk et al., 2013). Given the specificity of ζ-stat that we have demonstrated here, we believe that our results show PKMζ does not maintain memory in female mice as it does in males.

What molecule or mechanism is maintaining memory in female mice is unclear, but we believe our data offer a clue. Although other work has shown that PKMζ does not correlate with memory performance in females, synaptic GluA2-AMPARs do (Sebastian et al., 2013). This finding suggests that whatever the maintenance process is, it should be capable of maintaining GluA2-AMPARs at the PSD. That ZIP can still impair memory in female animals suggests that this female-specific maintenance mechanism must be ZIP-sensitive as well. The most obvious candidate to maintain memory in female mice is PKCι/λ, which can be inhibited by ZIP (Lee et al., 2013) and

can maintain long-term memory in the absence of PKMζ (Tsokas et al., 2016). Thus, future work should investigate whether female mice rely on PKCι/λ to maintain long-term memory.

While our data show that ζ-stat is a more specific inhibitor than ZIP, our study is limited by the use of only this drug in female animals. In order to fully conclude that a species-specific sex-difference exists, future research should utilize other tools to target PKMζ in these animals. This could include overexpression of PKMζ or perhaps PKMζ-antisense, which we show in Chapter 3 can disrupt reconsolidation in male rats. Further, electrophysiological experiments applying ζ-stat in female brain slices could provide further support to our behavioural data. Presumably if ζ-stat disrupts memory in female mice, then it should similarly disrupt LTP in brain slices.

More research is also needed to understand whether this sex-difference is the result of activational or organizational effects of hormones. Briefly, organizational effects are set in development and remain relatively static in adulthood, whereas activational effects occur from hormone activity even in adulthood (Arnold & Breedlove, 1985). Little to nothing is known about how sex hormones affect PKMζ. However, one report found that estradiol could increase phosphorylation of PKCζ at Thr410, a site shared with PKMζ (Castoria et al., 2004). What this means for memory maintenance in females is not clear, but it suggests an activational effect by estradiol could be possible. One way to test this hypothesis is to use ovariectomized females (Arnold & Breedlove, 1985). That is, if an effect differs in mice ovariectomized as adults, it would suggest that the effect relies on activational effects of hormones (Cooke, Hegstrom, Villeneuve, & Breedlove, 1998). In this context, if ζ-stat can impair memory in adult-ovariectomized

females, it would suggest that this sex-difference results from activational effects. While this experiment was not performed here, it will be important to understand how this difference comes about.

Here we showed that ζ-stat can disrupt long-term memory through a PKMζ-specific pathway. Further, we showed that vulnerability to ζ-stat is sex-dependent—male mice are sensitive, but female mice are not. These data support the importance of PKMζ in long-term memory maintenance with the important caveat that female mice may use an alternative process.

4.7. Figures

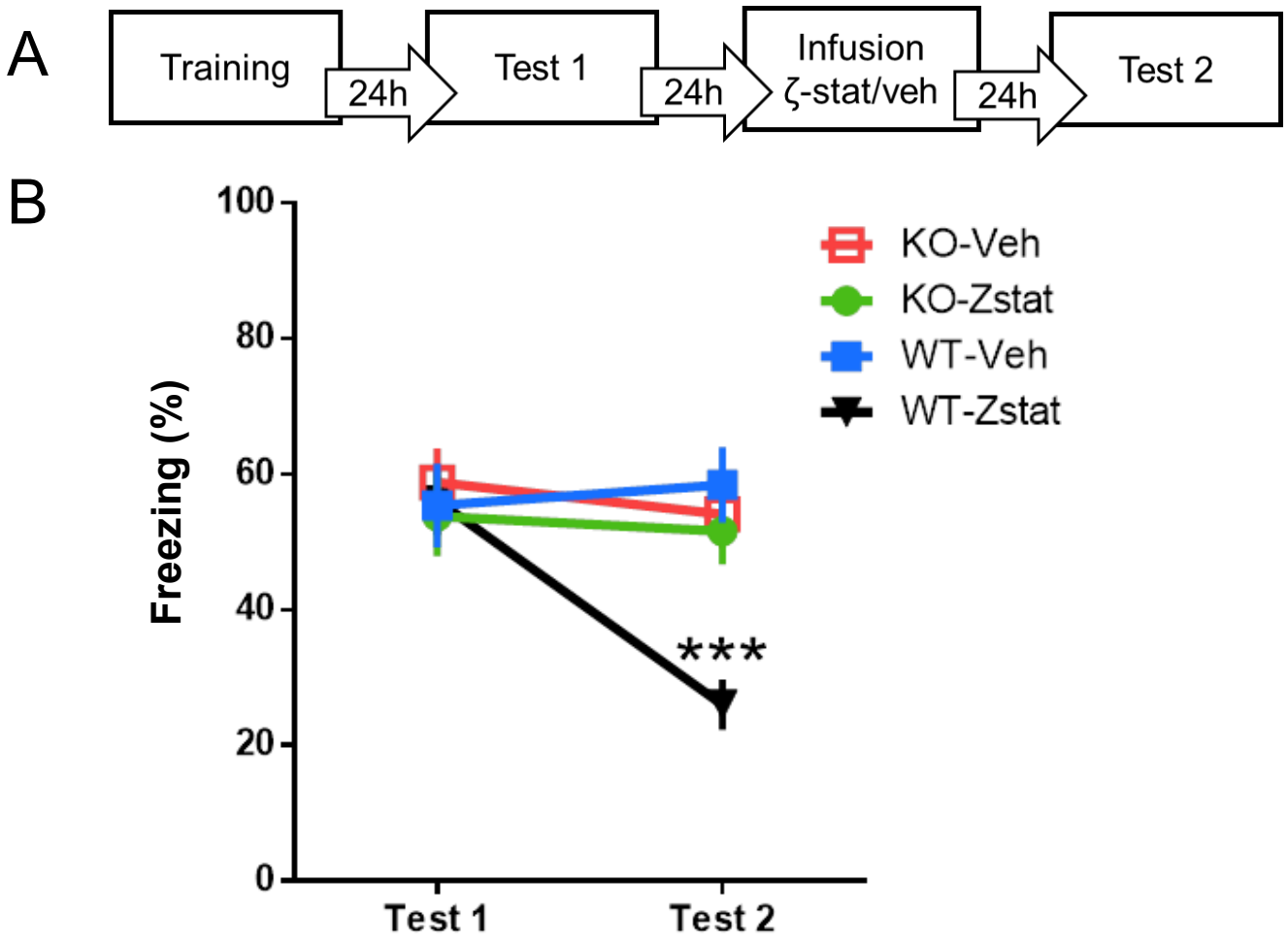

Figure 4.1. ζ-stat impairs memory in wild-type but not PKMζ-null male mice.

A) Wild-type and PKMζ-null mice underwent habituation and auditory fear conditioning. Mice were tested 24 hours post-training (Test 1) with one unpaired tone and infused 24 hours later with ζ-stat (20 mM, 0.3 µL/side) or PBS vehicle in the BLA. Mice were tested again 24 hours post-infusion (Test 2). B) Freezing is reported as the percentage of time mice spent freezing during the 30 second tone presentation. Freezing behaviour revealed that drug effects depended on genotype ($F_{1,39}$=10.76, p=0.002). ζ-stat impaired memory in WT mice (n=10; t_{39}= 5.3837, p<0.001) but not PKMζ-null animals (n=9; t_{39}= 0.3651, p>0.05). PBS had no effect in either WT (n=11) or PKMζ-null animals (n=13).

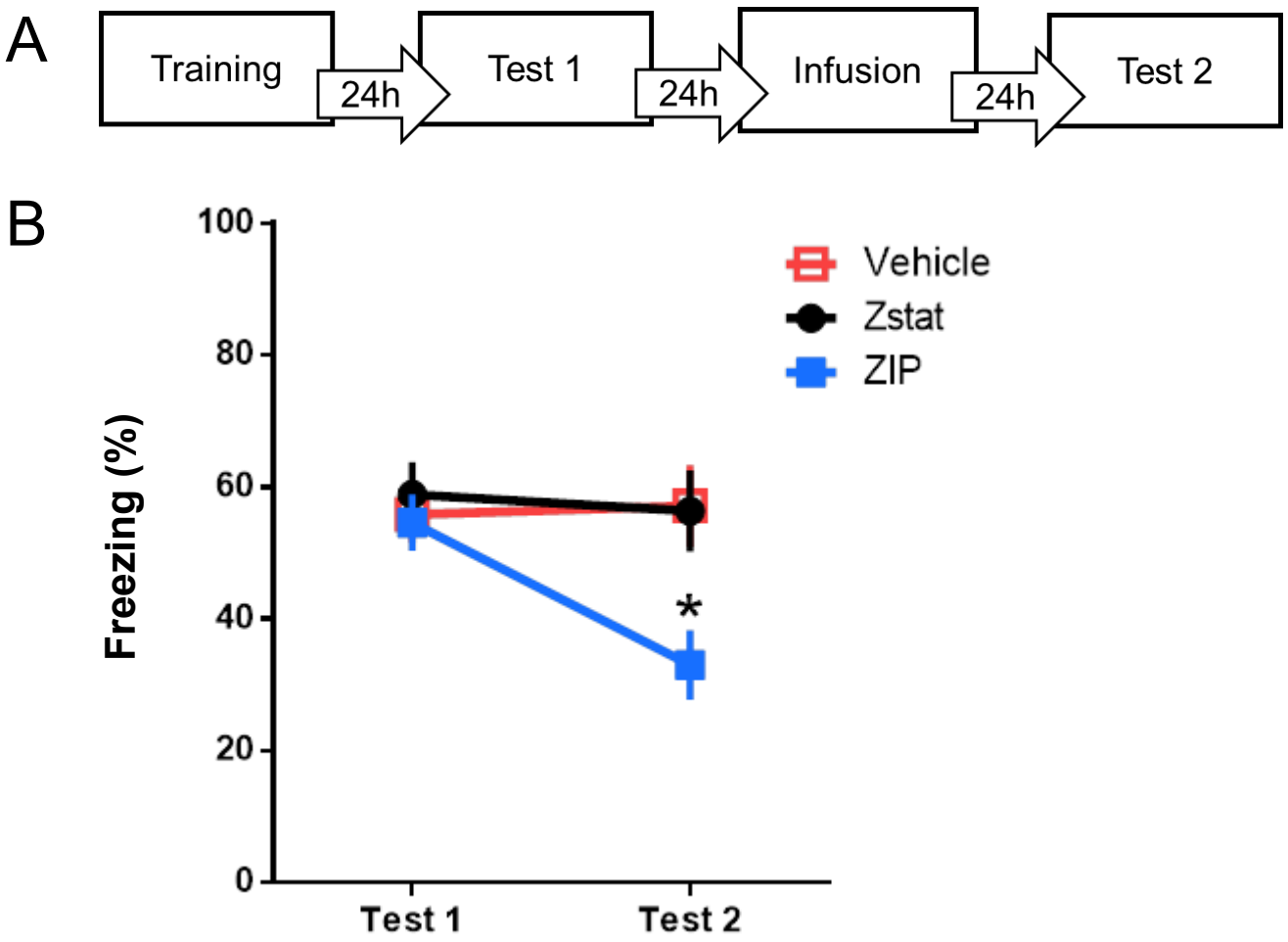

Figure 4.2. ZIP, but not ζ-stat, disrupts memory maintenance in female mice.

A) Wild-type female mice underwent habituation and auditory fear conditioning. Mice were tested 24 hours post-training (Test 1) with one unpaired tone and infused 24 hours later with ζ-stat (20 mM, 0.3 µL/side), ZIP (10 mM), or PBS vehicle in the BLA. Mice were tested again 24 hours post-infusion (Test 2). B) Freezing is reported as the percentage of time mice spent freezing during the 30 second tone presentation. Drug infusions differentially affected long-term memory ($F_{2,28}$= 3.90, p=0.032). While ZIP did impair memory (n=8; t_{28}=3.324, p=0.027), mice infused with ζ-stat showed no impairment in memory at Test 2 (n=13; t_{28}=0.4885, p=0.996). PBS had no effect on memory (n=10; t_{28}=-0.2195, p>0.05)

Chapter 5: Discussion

5.1. Summary

Here we investigated the role of PKMζ in memory destabilization and reconsolidation and whether this protein maintains memory in both males and females. In Chapter 2, we showed that after retrieval of an auditory fear conditioning memory, there is a significant loss of synaptic PKMζ in the BLA. NMDAR activation precedes this reduction since APV prevented the loss in PKMζ post-retrieval. In addition to NMDARs, activation of the ubiquitin-proteasome system (UPS) is also necessary to decrease PKMζ after retrieval. In Chapter 3, we showed that this loss of PKMζ protein is only transient. That is, PKMζ expression at the synapse recovers after destabilization is complete through the process of reconsolidation. In fact, reconsolidation requires synthesis of new PKMζ since blocking its translation leads to a long-term memory impairment. However, labile memories seem to be especially vulnerable to acute disruptions in PKMζ synthesis. We found that acute infusion of PKMζ-antisense after reconsolidation had no impact on memory. Similarly, without prior NMDAR activation, PKMζ-antisense could not disrupt retrieved memories. Altogether, these findings demonstrate a dynamic downregulation of PKMζ during destabilization which is then remedied by synthesis of new PKMζ during reconsolidation.

In Chapter 4, we reported an important sex-difference in the role of PKMζ in memory maintenance. We tested the specificity of the inhibitor ζ-stat in wild-type and PKMζ-KO male mice and found that it only impaired memory in wild-type animals. Finding that this drug specifically disrupted PKMζ activity, we then tested it in female wild-type mice. Strikingly, we found that ζ-stat did not disrupt memory in female mice as it did in males. We also confirmed previous work indicating that ZIP can impair memory

in female mice (Gao et al., 2018; von Kraus et al., 2010). This work supports the role of PKMζ in maintaining long-term memory in males, but it also suggests that PKMζ does not maintain memory in female mice.

Taken together, these findings reveal the dynamic regulation of PKMζ during memory destabilization and reconsolidation. However, they also raise questions about the generalizability of these results to females.

5.2. Implications

5.2.1. Dynamic Regulation of PKMζ

Here we established that PKMζ is an integral protein in memory destabilization and reconsolidation. Previous research identified other proteins that must be synthesized during reconsolidation: zif268 (Barnes et al., 2012; Lee, 2010; Lee et al., 2004; Maddox et al., 2011), C/EBPβ (Milekic et al., 2007), C/EBPδ (Arguello et al., 2013), and Arc (Maddox & Schafe, 2011). Our work provides two additional and unique insights.

First, these previously identified proteins are all components of gene expression whereas PKMζ seems to be particularly important for memory maintenance. That reconsolidation requires gene expression, both transcription and translation, is generally accepted (Kida et al., 2002; Nader et al., 2000; Tronson & Taylor, 2007; Villain et al., 2016). Thus, it should not be surprising that immediate early genes and transcription factors are necessary for reconsolidation to occur. On the other hand, it is less obvious that PKMζ must necessarily be synthesized in this process. Just as existing GluA2-AMPARs are routinely internalized and reinserted to the synapse (Lin & Huganir, 2007), conceivably reconsolidation could involve the recycling of existing PKMζ rather than

synthesis of new protein. Yet the finding that PKMζ-antisense impairs reconsolidation suggests that new PKMζ is necessary to stabilize the memory. Hence, our work identifies PKMζ as a protein that must be synthesized to serve a specific synaptic function in reconsolidation. One important caveat is that PKMζ also functions in the nucleus (Ko et al., 2016). Thus, the amnesic effect of PKMζ-antisense could occur primarily by reducing nuclear PKMζ. Little is known about its role in the nucleus but it promotes transcription via phosphorylation of CREB binding protein (CBP; Ko et al., 2016). In this way, PKMζ could have a more general effect on gene expression in addition to its presumed role in maintaining synaptic AMPARs. This could mean that infusion of PKMζ-antisense impairs reconsolidation in a similar way to C/EBPβ- or C/EBPδ-antisense. Determining whether the amnesic effect of PKMζ-antisense results from loss of synaptic or nuclear PKMζ (or both) will require selective inhibition of PKMζ synthesis in either the dendritic spine or the soma.

Second, our findings demonstrate the dynamic regulation of a synaptic protein during both destabilization and reconsolidation. As mentioned, the other proteins found to be relevant for reconsolidation are primarily required for gene expression. Thus, they are likely to be transiently upregulated during plasticity and then return to baseline. For instance, trained and untrained animals show similar baseline levels of Arc and zif268, but they are upregulated within 2 hours after retrieval (Maddox et al., 2011; Maddox & Schafe, 2011). On the other hand, PKMζ protein is persistently elevated in trained animals (Hsieh et al., 2016) and transiently reduced during destabilization. Thus, our findings imply the existence of two crucial processes: one to reduce PKMζ during destabilization and one to increase PKMζ during reconsolidation.

These data are in line with other work showing a similar transient reduction in NSF in the NAc during destabilization of a CPP memory. In one study, NSF expression was reduced at the synapse as early as one hour after retrieval, remained low for at least another hour, and returned to baseline by 24 hours post-retrieval (Ren et al., 2013). Our data follow a similar time scale, with a reduction in PKMζ by 1-hour post-retrieval and a return of PKMζ within 24 hours. Given that both PKMζ and NSF maintain synaptic GluA2-AMPARs (Migues et al., 2014, 2010; Yao et al., 2008), it seems likely that the reductions in these proteins involve some interrelated process. What this process could entail and how it could lead to a reduction of NSF and/or PKMζ remains unknown.

5.2.2. KIBRA

Kidney/Brain protein (KIBRA) may be part of this broader process that alters PKMζ availability during destabilization and reconsolidation. Infusion of the protein synthesis inhibitor, cycloheximide, can lead to a proteasome-dependent decrease in PKMζ expression. However, overexpression of KIBRA can prevent the loss of PKMζ in the presence of cycloheximide, suggesting that KIBRA prevents PKMζ from degradation (Vogt-Eisele et al., 2014). In Chapter 2, we found that proteasome activity is necessary to reduce synaptic PKMζ suggesting that this protein might be degraded during destabilization. While not examined here, it could be that destabilization causes some change to KIBRA that leaves PKMζ vulnerable to degradation by the UPS.

How KIBRA could be altered to expose PKMζ is not clear. KIBRA can be phosphorylated by several memory-related proteins like ERK/MAPK and PKMζ (Büther et al., 2004; Merlo et al., 2018; Yang et al., 2014; Zhang et al., 2014) and

dephosphorylated by phosphatases like protein phosphatase 1 (PP1; Xiao et al., 2011). Interestingly, memory destabilization requires activation of PP1 (Yu, Huang, Chang, & Gean, 2016), although it is not known if its role in destabilization involves changes to KIBRA.

If memory destabilization disrupts KIBRA activity and thus leads to PKMζ degradation, then reconsolidation, which we have shown increases PKMζ expression, likely involves a reversal of this change to KIBRA. For instance, deactivation of KIBRA could occur during destabilization, dissociating it from PKMζ, and rendering PKMζ vulnerable to degradation. During reconsolidation, KIBRA might then be activated, preventing the newly synthesized PKMζ from being degraded and allowing the memory to be maintained. This process is hypothetical, but future research should investigate changes to KIBRA after retrieval in order to understand the process by which PKMζ is reduced during destabilization.

5.2.3. Modelling PKMζ Dynamics

Our findings confirm several predictions from a recently published computational model that describes changes to PKMζ expression throughout multiple manipulations like memory reactivation and protein synthesis inhibition (Helfer & Shultz, 2018). This model predicts that shortly after retrieval, destabilization causes a transient reduction in PKMζ, as we have observed here. The model further predicts that the loss of PKMζ occurs almost immediately after retrieval, reaches a trough within 5-10 minutes, and returns to baseline within 40 minutes after retrieval. Our results are generally supportive of these dynamics, albeit suggesting a different time scale, in that synaptic PKMζ will still be depleted significantly one hour after retrieval. The model also predicts about 60%

reduction in PKMζ (Helfer & Shultz, 2018), whereas our results show only a 33% decrease within 1 hour of retrieval. It may be that the timepoint we chose to quantify PKMζ, 1-hour post-retrieval, occurs either before or after the actual trough. That is, we likely observed PKMζ levels before or after their lowest possible point.

Our data also confirm several of the model's predictions regarding the effect of protein synthesis inhibition on PKMζ. In our case, we used PKMζ-antisense to inhibit the translation of PKMζ specifically. The model predicts that inhibition of PKMζ synthesis following retrieval will cause a long-lasting reduction in PKMζ (Helfer & Shultz, 2018). Similarly, our results show that infusing PKMζ-antisense immediately after retrieval disrupts reconsolidation and impairs long-term memory. Our data also confirm the model's prediction that transient disruption in PKMζ synthesis during memory maintenance will not significantly disrupt memory. That is, PKMζ levels may transiently decrease, but they recover shortly thereafter. Altogether, our data support a number of predictions laid out in this model.

5.2.4. Clarifying the Role of PKMζ in Reconsolidation

To date, three publications explored the role of PKMζ in memory reconsolidation (da Silva, Raymundi, Bertoglio, Andreatini, & Stern, 2020; Levitan et al., 2016; Rossato et al., 2019). The results of these papers provide conflicting evidence amongst each other and with the existing literature. Our data may offer some clues about how to reconcile these findings.

One study by Levitan and colleagues found that ZIP infusions in the gustatory cortex did not affect long-term memory when they occurred immediately or 1 hour after retrieval (Levitan et al., 2016). These data conform with our data showing that PKMζ is

significantly decreased in the BLA within 1 hour after retrieval. Presumably, if PKMζ is depleted, then its effect within the spine is diminished, and inhibition of its activity does not have significant consequences. Our data show that new PKMζ must be synthesized during reconsolidation. Therefore, inhibition of existing protein may have little impact since new PKMζ will be made later on. Other work that shows retrieval leads to endocytosis of GluA2-AMPARs (Hong et al., 2013) reinforces this claim that PKMζ-mediated maintenance of GluA2-AMPARs is disrupted during destabilization. One important consideration is that Levitan et al. used exclusively female rats to study the effect of ZIP on reconsolidation (Levitan et al., 2016). Our results show that female mice do not seem to maintain memory using PKMζ but they use some other ZIP-sensitive mechanism instead. Therefore, it may be that our data regarding PKMζ in destabilization/reconsolidation applies only to male animals and not female rats, as used by Levitan and colleagues.

However, another set of findings in male rats shows some overlap with the results of Levitan et al. In this work, ZIP was infused in the prelimbic cortex to disrupt the reconsolidation of a contextual fear memory (Rodrigues et al., 2020). The authors found that ZIP did not affect memory retention when infused immediately after retrieval, similar to the result of Levitan et al. However, ZIP could disrupt reconsolidation when infused 1-hour post-retrieval. This effect seems to be at odds with our data showing loss of PKMζ within 1 hour of retrieval. However, this discrepancy may reflect differences in the time course of destabilization/reconsolidation in different structures.

Finally, work by Rossato and colleagues suggest that PKMζ dynamics may indeed vary by structure. They studied PKMζ in the dorsal hippocampus during

reconsolidation of object recognition memory (Rossato et al., 2019). Just as we demonstrated in Chapter 3, this group also found that post-retrieval infusions of PKMζ-antisense can impair reconsolidation. Yet while we showed that PKMζ decreases following retrieval, they showed that PKMζ increases in the hippocampus during this period. They also found elevated levels of GluA1- and GluA2-AMPARs, peaking at 3- and 6-hours after retrieval, respectively. These findings are in contrast to other work that found a much more rapid exchange of AMPARs after retrieval in the BLA (Hong et al., 2013). Rossato et al. also found that ZIP could disrupt reconsolidation when infused in the hippocampus immediately after retrieval, differing from the work described above.

Other findings from Rossato et al. suggest that their data may not be generalizable to the amygdala. They found that ZIP infusions could impair memory even 6 hours after retrieval (Rossato et al., 2019), which would typically be considered outside the reconsolidation window (Nader et al., 2000). While this might suggest that ZIP is impairing memory maintenance at this time point, ZIP infusions occurring at later time points did not disrupt memory. Thus, ZIP seems to block reconsolidation even 6 hours after retrieval, but it will not impair the storage of object recognition memory in the dorsal hippocampus. Other work also shows that ZIP does not disrupt the maintenance of object recognition memory when infused in the hippocampus one day after training (Hardt et al., 2010). These data suggest that, in the dorsal hippocampus, PKMζ serves a unique role in the reconsolidation, but not maintenance, of object recognition memory. In other words, an early increase in PKMζ may be necessary for reconsolidation, but the maintenance of these memories does not require PKMζ in the hippocampus.

5.2.5. Memory Maintenance in Female Mice

Perhaps most importantly, our data raise important questions about whether PKMζ serves different roles in males and females. Our finding that ζ-stat impairs auditory fear memory in male, but not female, mice suggests that PKMζ may not maintain this type of memory in females. Furthermore, female mice are vulnerable to ZIP, suggesting that a ZIP-sensitive mechanism could maintain their memory. This finding could explain the seemingly contradictory results present in the literature to date. Namely, sex-differences have been demonstrated when PKMζ is targeted or observed using relatively specific methods, like Western blotting, knockout, and knockdown (McGrath et al., 2018; Nasir et al., 2016; Sebastian et al., 2013). Yet ZIP can disrupt memory in both male and female animals (Gao et al., 2018; Migues et al., 2010; Serrano et al., 2008; von Kraus et al., 2010). Thus, the nonspecific binding of ZIP in these experiments seems to have obscured this sex-dependent difference.

If PKMζ does not maintain memory in female mice, it begs the question, what mechanism is responsible? Our data suggest that a ZIP-sensitive process is involved. Given that ZIP contains the inhibitory sequence of both PKMζ and PKCι/λ, the most obvious candidate is PKCι/λ. This kinase is another atypical PKC isoform that seems to be capable of maintaining memory in PKMζ-null mice (Tsokas et al., 2016). PKMζ and PKCι/λ do have different roles in wild-type male animals. PKCι/λ seems to play a role in early LTP and new learning. Perfusing PKCι/λ into cells potentiates synapses (Ren et al., 2013) whereas inhibition of this protein impairs early LTP but not established LTP (Tsokas et al., 2016). PKCι/λ interacts with p62 and activation of PKCι/λ increases phosphorylation of GluA1-AMPARs, an important component of LTP induction (Ren et

al., 2013). However, p-PKCι/λ increases within 30 minutes of stimulation and returns to baseline by 2 hours after LTP induction (Wang et al., 2016). On the other hand, PKMζ appears somewhat later and can remain elevated at synapses for at least one month (Hsieh et al., 2016; Osten et al., 1996; Sacktor et al., 1993). Interestingly, PKMζ and PKCι/λ seem to compensate for each other to maintain relatively normal memory in PKCι/λ- and PKMζ-KO models, respectively (Sheng et al., 2017; Tsokas et al., 2016). Notably, one study found little, if any, PKMι/λ in the hippocampus (Naik et al., 2000). While it is possible that the hippocampus is unique, it seems more likely that this compensatory mechanism relies on full-length PKCι/λ, rather than PKMι/λ.

If female mice maintain memory using PKCι/λ rather than PKMζ, the question remains of how this sex-difference comes about. Traditionally, sex-differences have been broadly considered to be the result of activational or organizational effects (Arnold & Breedlove, 1985). That is, activational effects result from the activity of hormones on existing physiology, whereas organizational effects are shaped by more profound sex-specific changes in development. For instance, activational effects could involve the influence of estradiol on intracellular signalling cascades like PI3K (Choleris et al., 2018). This may be the case for PKMζ and PKCι/λ which are downstream of PI3K (Kelly, Crary, et al., 2007; Ren et al., 2013). Whether estradiol mediates this effect and, if so, how is still unknown. Potential experiments to test this hypothesis are discussed further below.

5.3. Limitations

While the results presented in this thesis advance our understanding of PKMζ, several limitations provide important qualifications.

First, we only measured PKMζ at three points after training: without retrieval, 1 hour after retrieval, or 24 hours after retrieval. These three time points are sufficient to demonstrate a change, but they do not offer much clarity about the rate of change, especially during reconsolidation. It could be that PKMζ disappears from the synapse within minutes. This sudden decrease seems somewhat unlikely given that NSF is not removed until about 1-2 hours after retrieval, and the two proteins seem to interact closely at the PSD. More importantly, we only examined PKMζ levels 1- and 24-hours after retrieval, leaving a significant amount of uncertainty about when PKMζ expression recovers at the synapse. Quantifying PKMζ at additional time points, for instance, 3- and 5-hours post-retrieval, could clarify when the kinase returns to roughly pre-retrieval levels. These data would also provide evidence that could help better delineate the reconsolidation window. For instance, if PKMζ levels remain low at 3 hours but return by 5 hours, it would suggest that the reconsolidation window closes at roughly 4 hours post-retrieval. As mentioned in Chapter 1, however, this reconsolidation window likely varies for different processes (i.e. actin polymerization, synthesis of different proteins) and brain regions.

In Chapter 2, we show that proteasome activation is necessary to deplete PKMζ by 1 hour after retrieval. It is tempting to conclude that PKMζ degrades during destabilization. Our data are consistent with this hypothesis, showing that reconsolidation requires the synthesis of new PKMζ rather than the recycling of existing PKMζ. Nonetheless, our data do not allow us to definitively determine whether PKMζ is degraded or not. It may be that degradation is necessary for some other process that is upstream of PKMζ depletion. As mentioned, GluA2-NSF binding is a prerequisite for

PKMζ-mediated memory maintenance (Migues et al., 2014; Yao et al., 2008). Further, degradation of NSF seems to occur during destabilization (Ren et al., 2013). Therefore, it could be that degradation of NSF leads to the loss of synaptic PKMζ. Determining whether PKMζ is degraded or not will require quantification of ubiquitinated PKMζ during destabilization. If it is indeed ubiquitinated, this would suggest that PKMζ may undergo proteasome-dependent degradation.

While most research has focused on the synaptic function of PKMζ, there is some evidence that PKMζ activity in the nucleus influences memory maintenance as well. PKMζ protein in the neuronal cell body moves into the nucleus where it seems to promote histone acetylation and gene expression in neurons (Ko et al., 2016). Importantly, increasing histone acetylation after retrieval seems to be important for reconsolidation in the BLA (Jarome & Lubin, 2014). Our findings in Chapters 2 and 3 focused on synaptic PKMζ, although some manipulations may have impacted nuclear PKMζ as well. Western blotting experiments tracking PKMζ levels were confined to synaptosome fractions. However, infusions of PKMζ-antisense were liable to disrupt its translation throughout the cell as well. This may have caused a significant reduction in PKMζ available to the nucleus. It could be that depleting nuclear PKMζ limits post-retrieval histone acetylation, thereby disrupting reconsolidation. Thus, we cannot rule out the possibility that the amnesic effect of PKMζ-antisense was due to the depletion of nuclear PKMζ.

Finally, our data from Chapter 4 suggest that PKMζ does not maintain auditory fear memory in the BLA of female mice. This result is somewhat surprising, considering that PKMζ seems to be fundamental to memory maintenance in male animals (Sacktor,

2011, 2012). Our conclusion is based on the finding that ζ-stat does not disrupt memory in female mice. Thus, our data are limited by the use of only one inhibitor, ζ-stat, to target PKMζ. Given that ζ-stat impairs memory in WT males but not PKMζ-null mice, we believe the drug shows reasonable specificity. However, ζ-stat may be targeting some other protein that is also altered in PKMζ-KO mice. In order to more convincingly demonstrate that female mice do not maintain memory using PKMζ, other additional studies will be required. For instance, one could test whether PKMζ-antisense disrupts reconsolidation in female mice. Alternatively, one could determine if repeated administration of PKMζ-antisense leads to memory loss in male but not female mice. Similarly, expression of PKMζ-specific shRNA could be used in female mice to see if it disrupts their memory as it does in males (Dong et al., 2015).

5.4. Future Directions

In light of these limitations, there is still much to be learned about the role of PKMζ in memory plasticity and in memory maintenance in female animals.

First, it will be important to observe changes in PKMζ over multiple time points, in multiple types of memory, and multiple brain regions. Other research indicates that PKMζ increases in the hippocampus following retrieval of object recognition memory (Rossato et al., 2019). Thus, our results may be specific to a limited number of structures and, perhaps, specific tasks as well. As mentioned, future work should also determine whether PKMζ is ubiquitinated following destabilization and whether this process leads to degradation of PKMζ. Other work shows that NSF is ubiquitinated and depleted following destabilization (Ren et al., 2013). Given the close interaction of NSF and PKMζ, degradation of PKMζ likely follows a pattern similar to NSF. Examining post-

retrieval changes in KIBRA should provide insight into whether PKMζ is degraded or not. Evidence suggests that KIBRA protects PKMζ from degradation and levels of the two proteins correlate in neurons (Vogt-Eisele et al., 2014). If destabilization causes a reduction of KIBRA at the spine, it could explain why PKMζ is also depleted—because it is no longer protected from degradation by KIBRA. Likewise, we expect that as PKMζ increases during reconsolidation, KIBRA should as well.

Future work should also investigate whether reducing levels of PKMζ following retrieval is necessary for labilization to occur. Blocking endocytosis of GluA2-AMPARs with $GluA2_{3Y}$ seems to prevent memory destabilization (Hong et al., 2013; Rao-Ruiz et al., 2011; Yu, Chang, & Gean, 2013). GluA2-AMPAR internalization may be the result of disrupted PKMζ activity, which is required to maintain GluA2-AMPARs at the PSD. Importantly, dissociation of NSF-GluA2 is sufficient to internalize GluA2-AMPARs and impair memory even in the presence of PKMζ (Migues et al., 2014; Yao et al., 2008). Thus, destabilization may also involve decoupling of NSF from GluA2 in addition to the loss of PKMζ expression at the PSD we observed. In order to test whether reducing PKMζ alone is necessary for destabilization, one could overexpress KIBRA, which seems to prevent depletion of PKMζ (Vogt-Eisele et al., 2014). This would allow one to test whether overexpression of KIBRA in the BLA prevents memory destabilization and thereby protects the memory from reconsolidation blockade.

Our work opens up a wide field of research to study memory maintenance in female mice compared to male mice. Future work should first replicate our findings in rats as well as in different tasks and brain structures. For instance, will infusing ζ-stat into the insular cortex impair conditioned taste aversion in female rodents in the same

way ZIP infusions impair memory in males? These efforts would benefit from using other PKMζ-specific tools, such as PKMζ-antisense. Thus, future research should investigate whether female rodents show similar PKMζ dynamics as we observed in male rats in Chapters 2 and 3. Likewise, it will be important to determine whether PKMζ-antisense can disrupt reconsolidation in female animals, as we have shown in males.

If future results confirm that PKMζ does not maintain memory in female animals, it will be essential to determine what alternative mechanism may be at work. As mentioned above, a first step would be to determine if PKCι/λ or perhaps PKMι/λ is responsible. To test this, one could infuse the PKCι/λ-specific inhibitor [4- (5-amino-4-carbamoyl-imidazol-1-yl)-2,3-dihydroxycyclopentyl] methyl dihydrogen (ICAP; Pillai et al., 2011; Tsokas et al., 2016). However, it may be possible that PKCι/λ does not actively maintain memory in females, which would mean that ICAP will have no amnesic effect. In this case, the next step might be to test if other conventional or novel PKC isoforms take on this role of memory maintenance. Here, one could utilize bisindolylmaleimide I, which inhibits conventional and novel PKCs (but not atypical PKCs like PKCι/λ, PKCζ, or PKMζ; Toullec et al., 1991; Zhang, Kays, Hodgdon, Sacktor, & Nicol, 2012). Screening for persistently elevated PKC isoforms may also help to narrow down which kinase may be responsible for long-term memory maintenance. While most PKCs show only transient elevation after LTP induction, PKMζ remains elevated for at least 3 hours in vitro and at least one month in vivo (Hsieh et al., 2016; Osten et al., 1996; Tsokas et al., 2016). Therefore, if another PKC maintains memory in females, it might also show elevated expression long after LTP induction or new learning.

It could be that female mice do not maintain long-term memories using any PKC isoform. Identifying the mechanism responsible might be more difficult in this case. However, previous work and our results showing that ZIP can impair auditory fear memory in female mice suggest that some ZIP-sensitive mechanism seems to be responsible (Gao et al., 2018; von Kraus et al., 2010). Notably, synaptic GluA2-AMPARs correlate with memory performance in both males and females (Sebastian et al., 2013). This finding implies that both male and female mice require maintenance of these receptors to keep long-term memory. This narrows the scope of future research to an as-yet-unspecified ZIP-sensitive mechanism for maintaining GluA2-AMPARs. At present, though, the identity of this mechanism remains elusive.

As mentioned above, sex-differences can be mediated by activational or organizational effects of hormones. In this case, it seems that activational effects may play some role. There are some data suggesting that estradiol influences PKCζ phosphorylation and upstream signalling cascades like PI3K (Castoria et al., 2004; Choleris et al., 2018). Thus, future research should test whether ζ-stat impairs memory in ovariectomized females. If the sex-difference we observed is due to activational effects, it might be that ζ-stat indeed impairs memory in these animals. Similarly, inhibiting the still-unknown alternative maintenance mechanism should presumably impair memory in normal females but not ovariectomized ones.

5.5. Conclusion

The findings presented in this thesis answer several questions about synaptic plasticity and memory maintenance. We show that memory destabilization and reconsolidation require suspension and restoration of PKMζ expression. It seems likely

that other proteins and processes critical to memory storage will follow a similar trajectory during periods of plasticity. We also show that female mice seem to rely on other processes entirely. In light of other work, our findings suggest that these processes may differ across the brain and even between males and females.

Understanding how memories are changed and maintained is fundamental to our understanding of the nervous system as a whole. Synaptic connections between neurons must balance between plasticity and long-term stability. These dynamics underpin not only memory but other neural phenomena as well. Intriguing research shows that memory-related proteins and processes, like PKMζ and reconsolidation, exist in pain systems in the spinal cord as well (Bonin & De Koninck, 2014; Laferrière et al., 2011). Thus, chronic pain, at least in some cases, might be considered a disorder of memory-like processes. Recognizing that memory phenomena are fundamental to all neurons provides a necessary framework to understand the nervous system in general. By viewing the brain as essentially a learning organ, and examining memory-related processes across this system, we are sure to uncover new and exciting findings to advance all of neuroscience.

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