Behavioural and neural correlates of memory loss: investigating storage impairment in consolidation, reconsolidation, and active decay

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General abstract

Although the loss of memory has long been recognised as fundamental to memory function as initial memory formation, considerably less is known about the neurobiology of forgetting. Therefore, we explore neurobiological correlates of three distinct forgetting phenomena: (1) experimental amnesia induced by impairing memory consolidation, (2) amnesia induced by impairing memory reconsolidation, and (3) active decay of long-term memory.

(1) The nature of experimental amnesia has traditionally been discussed within a framework of analysis based on the distinction of storage versus retrieval impairment, with the view that experimental amnesia arises either from memories being unavailable (impaired storage) or inaccessible (impaired retrieval). Using a second learning protocol that takes advantage of the fact that *N*-methyl-D-aspartate receptors (NMDARs) are required for the initial but not subsequent learning of a task, it is possible to behaviourally dissociate whether loss of a specific memory is caused by a storage or retrieval impairment. In Chapter 2, we describe experiments in which we employed this approach to show that infusions of ZIP, an inhibitor of a protein required for memory maintenance (PKMζ), leads to a memory impairment consistent with the view that experimental amnesia reflects impaired memory storage. Animals first acquired long-term memory for object locations in an open field. The subsequent day, we infused ZIP into the dorsal hippocampus (dHPC), which promoted the loss of these object location memories. Animals then learned another set of object locations. We found that this learning did not require NMDAR activation, which is normally necessary in animals naïve to this task. Western blot quantification of GluA2-subunit containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (GluA2-AMPAR) expression levels in the dHPC revealed an

unexpected correlation, in that decreased memory expression in animals infused with ZIP went along with increased GluA2-AMPARs. This suggests that the correlation between GluA2-AMPAR expression and memory expression might not be as linear as we expected.

(2) In Chapter 3, we used the second learning protocol to explore whether impairing the reconsolidation of an object location memory leads to memory loss consistent with a storage or retrieval impairment view of amnesia. To this end, we infused the transcription inhibitor sulfasalazine (SSZ) into the dHPC after reactivating long-term object location memories. We then trained these animals in a different object location task and found that infusing an NMDAR-blocker prior to this second learning did not affect memory acquisition the way it normally would in untrained animals. Thus, similar to the results reported in Chapter 2, these findings suggest that impairing memory reconsolidation leads to a memory loss consistent with the position that the intervention impaired memory storage processes, and not memory retrieval. Western blot assays showed no difference in GluA2-AMPAR expression levels in dHPC neurons, which may indicate that the changes that are likely occurring as a result of reconsolidation blockade are too minimal to be detected with this technique.

(3) In Chapter 4, we explore biochemical correlates of decay of long-term object location memories. We varied the time between memory acquisition and test, and measured the expression of GluA2-AMPARs in the dHPC. We found the hypothesized gradual decrease in memory expression with increasing retention time, leading to apparent full memory loss after seven days. However, in contrast to these behavioural observations, we found that the expression of GluA2-AMPARs in the dHPC increases during the retention interval, unlike the positive correlation between GluA2-AMPAR expression and memory strength expected, based on literature. Taken together with the results from Chapter 2 and 3, the inconsistency of GluA2-

AMPAR expression in our studies suggest that western blots might not be a suitable method of assessment for the types of memories studied in our experiments.

In summary, our data indicate that experimental amnesia observed after impairing memory consolidation and reconsolidation reflects the actual loss of a memory trace, and thus supports the idea that these forms of memory loss are best explained with compromised memory storage, not memory retrieval. Based on the unexpected results from our study on the relationship between memory loss during natural memory decay over time and expression of GluA2-AMPARs, we remain cautious regarding the corresponding biochemical assessments in our consolidation and reconsolidation studies. In chapter 5, we discuss these issues in detail. Indeed, understanding how behavioural phenomena relate to synaptic phenomena and vice versa seems essential in deepening our understanding of memory. The results put forward in this dissertation illustrates the importance of this goal for progress in this field, and provide a set of solid behavioural paradigms that will promote future research efforts.

Résumé

Bien que la perte de mémoire ait longtemps été reconnue comme une fonction fondamentale de la formation initiale de la mémoire, la neurobiologie de l'oubli est considérablement moins connue. Nous explorons donc les marqueurs neurobiologiques de trois phénomènes de l'oubli : (1) l'amnésie expérimentale induite par l'altération de la consolidation de la mémoire (2) l'amnésie induite par l'altération de la reconsolidation de la mémoire, et (3) le déclin actif de la mémoire à long terme.

(1) Traditionnellement, la nature de l'amnésie expérimentale a été discuté dans un cadre d'analyse basé sur la distinction entre un problème de stockage ou de rappel, en tenant compte que l'amnésie expérimentale résulte soit de souvenirs non-disponibles (problème de stockage) ou inaccessibles (problème de rappel). En utilisant un protocole de deuxième apprentissage, qui tire avantage du fait que les récepteurs *N*-methyl-D-aspartate (NMDARs) sont nécessaires pour le premier mais non le deuxième apprentissage d'une tâche, il est possible de dissocier de manière comportementale si la perte d'un souvenir spécifique est causée par un problème de stockage ou de rappel. Au chapitre 2, nous décrivons des expériences qui emploient cette approche afin de démontrer que des infusions de ZIP, un inhibiteur d'une protéine nécessaire au maintien de la mémoire (PKMζ), mène à une diminution de la mémoire en accord avec la vision que l'amnésie expérimentale reflète un problème de stockage. Les animaux ont d'abord acquis un souvenir à long terme pour la localisation d'objets situés dans une arène. Le jour suivant, nous avons infusé ZIP dans l'hippocampe dorsal (dHPC), ce qui a stimulé la perte de ces souvenirs de localisation d'objets. Les animaux ont ensuite appris la localisation d'une autre paire d'objets. Nous avons constaté que cet apprentissage ne nécessitait pas l'activation des NMDARs, qui sont

normalement essentiels chez les animaux naïfs à cette tâche. La quantification par *Western blot* des niveaux d'expression des récepteurs de l'acide propionique alpha-amino-3-hydroxy-5-methyl-4-isoxazole contenant la sous-unité GluA2 (GluA2-AMPARs) dans le dHPC a révélé une corrélation inattendue, c'est-à-dire qu'une diminution de l'expression de la mémoire chez les animaux infusés avec ZIP correspondait avec une augmentation des GluA2-AMPARs. Ceci suggère que la corrélation entre l'expression des GluA2-AMPARs et l'expression de la mémoire ne sont pas aussi linéaires que nous l'avions anticipé.

(2) Au chapitre 3, nous avons utilisé le protocole de deuxième apprentissage pour explorer si altérer la reconsolidation d'un souvenir de localisation d'objets amène à une perte de mémoire cohérente avec la vision selon laquelle l'amnésie reflète un problème de stockage ou de rappel. Pour ce faire, nous avons infusé l'inhibiteur de transcription sulfasalazine (SSZ) dans le dHPC après avoir réactivé la mémoire à long terme liée à la localisation d'objets. Nous avons ensuite entraîné ces animaux dans une tâche différente de localisation d'objets. Nous avons constaté que l'infusion de l'inhibiteur des NMDARs avant ce second apprentissage n'a pas affecté l'acquisition de ce souvenir de la façon normalement observée chez des animaux non-entraînés. Ainsi, comme les résultats rapportés au chapitre 2, ces données suggèrent que de bloquer la reconsolidation amène à une perte de mémoire cohérente avec le concept que l'intervention a modifié les processus de stockage de la mémoire, et non pas ceux du rappel. Les analyses par *Western blot* n'ont montré aucune différence sur les niveaux d'expression des GluA2-AMPARs dans les neurones du dHPC, ce qui peut indiquer que les changements probables en conséquence de l'inhibition de la reconsolidation qui se produisent sont trop minimes pour être détectés par cette technique.

(3) Au chapitre 4, nous explorons les marqueurs biochimiques du déclin des souvenirs à long terme liés à la localisation d'objets. Nous avons varié la durée entre l'acquisition du souvenir et le test, puis mesurons l'expression de GluA2-AMPARs dans le dHPC. Nous avons constaté que l'hypothétique diminution graduelle de l'expression de la mémoire au fur et à mesure que la durée de rétention augmente mène à une apparente perte de mémoire complète après sept jours. Toutefois, contrairement aux observations comportementales, nous avons découvert que l'expression des GluA2-AMPARs dans le dHPC augmente dans l'intervalle de rétention, à l'inverse de la corrélation positive attendue entre l'expression des GluA2-AMPARs et l'intensité de la mémoire montrée dans la littérature. En tenant compte de ces résultats et de ceux des chapitres 2 et 3, l'incohérence de l'expression des GluA2-AMPARs dans nos études suggère que la quantification par *Western blot* n'est peut-être pas une technique adéquate d'analyse pour le type de souvenirs étudiés dans nos expériences.

En conclusion, nos données indiquent que l'amnésie expérimentale observée après l'altération de la consolidation et de la reconsolidation révèle la perte réelle de l'origine de la mémoire et ainsi supporte l'idée que ces formes de pertes de mémoire sont expliquées davantage par un problème de stockage, et non pas par un problème de rappel. En s'appuyant sur les résultats inattendus de notre étude sur la relation entre la perte de mémoire lors du déclin naturel au fil du temps et l'expression des GluA2-AMPARs, nous demeurons prudents à l'égard des analyses biochimiques correspondantes dans nos études en consolidation et reconsolidation. Au chapitre 5, nous discutons de cette problématique en détails. En effet, comprendre comment les phénomènes comportementaux sont reliés aux phénomènes synaptiques et vice versa paraît essentiel à l'approfondissement de notre compréhension de la mémoire. Les résultats présentés dans cette dissertation démontrent l'importance de ce but pour le progrès dans ce domaine, et

fourni un ensemble de paradigmes comportementaux fiables qui assurera de future efforts en recherche.

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Contribution of original work

The work presented in this dissertation focuses on the storage impairment of experimental amnesia, the possible storage impairment of natural forgetting, and examines how this impairment may in part be due to endocytosis of GluA2-containing AMPA receptors (GluA2-AMPARs) from the postsynaptic membrane. It comprises of three unpublished manuscripts.

In Chapters 2 and 3, we show that training in object location learning leads subsequent learning of the same task in a different context to be NMDAR-independent. NMDAR-independent learning has been shown in contextual learning and water maze learning, but not yet a non-aversive, non-Pavlovian spatial task like object location learning. Moreover, we took advantage of this NMDAR-independent quality in a second learning protocol, which uniquely allows a positive prediction of whether memory loss is caused by a storage or retrieval impairment of the memory representation. To our knowledge, this protocol is the only kind that can behaviourally parse these two sources of amnesia. Using this protocol, we showed that memory impairment caused by ZIP infusions as well as reconsolidation blockade caused by sulfasalazine infusions lead to a storage loss of the memory.

Additionally, the reconsolidation blockade in Chapter 2 is the first instance in which reconsolidation mechanisms have been observed in object location learning. Historically, very few experiments have examined reconsolidation of non-Pavlovian learning; our work is one of a small handful that have shown the effect, and the first one that has shown it in object location learning. Lastly, the effects of the nuclear factor κB (NF-κB) pathway is a relatively new but growing research interest within the field of learning and memory. Our data contributes to the

emerging literature about how inhibiting this pathway can block transcription needed for reconsolidation.

Mechanisms of GluA2-AMPAR maintenance in the postsynaptic membrane are beginning to be understood: it has been shown that removal of GluA2-AMPARs cause decay of the long-term potentiation (LTP) signal (Dong et al., 2015) and disrupts long-term memory (LTM). Importantly, there is a correlation between surface GluA2-AMPAR expression and memory strength (Migues et al., 2010), and pharmacologically extending maintenance of GluA2-AMPAR in the postsynaptic membrane seems to attenuate forgetting (Migues et al., 2016). However, the exact relationship between the rate and amount of GluA2-AMPARs internalized, and memory loss, has not yet been shown. The sum of the biochemical data gathered across these three projects show that GluA2-AMPAR expression does not seem to correlate with behavioural changes related to memory loss. As this is overwhelmingly contrary to the literature, the data overall suggests that western blots are not sensitive enough to detect these putative molecular changes. As experiments directly measuring variations of GluA2-AMPAR expression with respect to object location memory loss has not yet been shown, our data highlights many of the issues to be considered in future research.

Contributions of authors

The work presented in Chapter 2 was completed by me and undergraduate student Sophia Papp. Dr Oliver Hardt helped design the experiments and helped with analysis for Experiments 1 and 3 (part 2.5.1. and 2.5.3). I performed all the surgeries, most of the training, all of the testing, and all of the scoring. I mixed the drugs used for infusions. Sophie helped with handling, training and infusions. Sacrifice, histology, and biochemistry was done by me. Analysis and writing was done by me under the supervision of Dr Karim Nader.

The work presented in Chapter 3 was completed by me and undergraduate Amaryllis Mirlycourtois. I designed the experiments and did all surgeries. Running the experiment (handling, training, infusions, testing) was done by Amaryllis and I. Drug mix was done by me. Sacrifice and histology was done by me; biochemistry was performed by Amaryllis and I. Scoring and analysis was also done by me and Amaryllis. Writing was done by me under the supervision of Dr Karim Nader.

The work presented in Chapter 4 was completed by me and undergraduate Yun Lu. I designed the experiments and performed all surgeries. Handling and training was done by Yun, with testing done by Yun and I. Sacrifice and histology was done by me; biochemistry was done by Yun and I. Yun did the scoring, and both Yun and I contributed to the analysis. Writing was done by me under the supervision of Dr Karim Nader.

Commonly used abbreviations

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
GluA1-AMPAR	AMPAR containing GluA1 subunits
GluA2-AMPAR	AMPAR containing GluA2 subunits
CP-AMPAR	AMPAR that are impermeable to calcium (i.e. not GluA2)
D-AP5	NMDAR antagonist
dHPC	dorsal hippocampus
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
NMDAR	*N*-methyl-D-aspartate receptor
NSF	*N*-ethylmaleimide-sensitive factor
PKMζ	Protein kinase M zeta
SSZ	sulfasalazine, inhibitor of nuclear factor κB transcription factors
TAT-GluA2$_{3Y}$	GluA2-AMPAR endocytosis inhibitor
Veh	vehicle
ZIP	Zeta inhibitory peptide, PKMζ inhibitor
Scr-ZIP	Scrambled ZIP, inactive form of ZIP

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CHAPTER TWO: ZIP erasure of an object location memory leads to NMDAR-dependency of later object location learning

CHAPTER THREE: Reconsolidation blockade leads to experience-driven changes in NMDAR-dependency in later spatial learning

CHAPTER ONE

General introduction

1.1. *How memories are lost*

As humans, our life experience is shaped by our memories. This involves not only memories that are kept, but also memories that are lost. Since Hermann Ebbinghaus' seminal work in 1885 (Murre & Dros, 2015) characterizing a forgetting curve that illustrated how his own retention of nonsense syllables decreased over time, there has been a drive to understand how memory works, and how it fails.

The vast majority of past and current memory research explored how we encode, store, and recall memories. Mechanisms of acquisition, consolidation, maintenance, and retrieval have been extensively studied through both behaviour and biochemistry. In comparison, our knowledge of how memories are lost is lacking. Over the years, there have been various debates over how memories are lost—for instance, does forgetting occur passively or actively (R. L. Davis & Zhong, 2017), are memories lost through decay or interference (Hardt, Nader, & Nadel, 2013), is amnesia caused by a storage or retrieval impairment (L R Squire, 1982)? Memories can be lost pathologically or naturally, through diverse causes, in permanent or temporary ways; and because there are so many nuances to memory loss, there are many labels and hypothesized mechanisms.

In an extensive review, Davis and Zhong (2017) summarize a list of ways memory loss can occur. For example, forgetting can occur as neural correlates of memory (i.e. components of the memory trace, also sometimes called the engram) naturally decay. Evidence that supports this mechanism includes observations that long-term potentiation (LTP), a sustained increased of synaptic transmission strength that models how synaptic plasticity might underpin learning and memory (Bliss & Collingridge, 1993; Malenka, Nicoll, & A., 1999; Teyler & DiScenna, 1987), can decay over time (W. C. Abraham et al., 1993; Otani, Marshall, Tate, Goddard, & Abraham,

1989; Villarreal, Do, Haddad, & Derrick, 2002). Some have hypothesized that LTP in the hippocampus occurs due to a passive decrease of LTP maintenance processes (R. G. Morris, Davis, & Butcher, 1991; Villarreal et al., 2002). In this case, the memory is not being altered by extrinsic forces, but by a natural degradation of upkeep mechanisms. Alternatively, memory loss also can occur by means that counter LTP maintenance, such as reversing LTP (depotentiation) (Doyère, Srebro, & Laroche, 1997; Fujii, Saito, Miyakawa, Ito, & Kato, 1991; Martin, 1998) or by inducing long-term depression (LTD), the long-lasting reduction of synaptic transmission strength (W. Abraham & Goddard, 1983; Bear & Abraham, 1996; Xiao, Niu, & Wigström, 1996). At the molecular level, decay processes seem to occur as a function of time (Barnes, 1979; Wittenberg & Tsien, 2002), while processes like interference occur when other mechanisms impede the storage or retrieval of the target memory (Jarrard, 1975; Winocur, 1988) (Winocur, 1988; Jarrad & Leonard, 1975), essentially blocking the memory of interest from being accessed. While interference mechanisms are thought of as "active" because they involve brain activity occurring before or after the learning event, or from top-down cognitive control, such as with mental suppression, decay processes are also considered by some as an active process due to their well-regulated nature and the theory that our biological systems can dynamically remove neural correlates of memory based on the memory's relevance or recency (Hardt et al., 2013). Overall, many different mechanisms exist that can lead to loss of integrity of the memory trace, or to impair successful retrieval, both of which lead to forgetting at the behavioural level (R. L. Davis & Zhong, 2017).

Due to the emphasis on synaptic strengthening and maintenance in neurobiology literature, there already exists abundant research on one possible molecular mechanism of synaptic strength weakening, which may underlie certain types of memory loss—specifically,

removal of a subtype of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) from the postsynaptic membrane of excitatory neurons (Carroll, Beattie, von Zastrow, & Malenka, 2001; Collingridge, Isaac, & Wang, 2004; Lüscher et al., 1999). AMPARs, a type of glutamate receptor that have extensively been shown to modulate synaptic plasticity (for example Lee et al., 2000; but see Malinow & Malenka, 2002, and Huganir & Nicoll, 2013, for in-depth reviews), seem to play a crucially important role on memory retention due to the positive correlation of AMPAR in the postsynaptic density with synaptic strength (for example Hayashi et al., 2000; but see Kessels & Malinow, 2009, for a review). Much of the extant data focuses on how trafficking of AMPARs from the postsynaptic plasma membrane can lead to disrupted LTP or induction of LTD; in contrast, relatively little emphasis is placed on how AMPAR endocytosis is involved in long-term memory loss. This thesis therefore aims to show how postsynaptic AMPAR expression levels change as a memory is lost, and endeavours to demonstrate that these changes are representative of a storage impairment of the memory trace. Our work here shows how different types of memory loss—that is, experimentally-induced amnesia of consolidation (Chapters 2) and reconsolidation (Chapter 3)—are caused by a storage impairment of the memory, and how both these processes, along with active decay processes of forgetting (Chapter 4), are affected by GluA2-AMPAR internalization. This evidence for AMPAR endocytosis as a molecular correlate of memory loss can further our understanding of both natural and pathological forms of forgetting.

1.2. *The nature of amnesia*

1.2.1. *History of recovery from amnesia experiments*

The study of the mechanisms of memory loss has been difficult prior to the onset of molecular techniques, as researchers could only rely on behavioural output to make inferences on how the memory loss occurred. This understandably led to conflicting theories, as the end point of a forgetting experiment is always a performance deficit, regardless of where or how that deficit originated. One long-held debate about the nature of experimentally-induced amnesia therefore argued whether a trained memory is lost through a storage impairment, where the memory trace is no longer available, or a retrieval impairment, where the trace is rendered inaccessible (Gold & King, 1974; Hardt, Wang, & Nader, 2009; Nader & Hardt, 2009; Larry R. Squire, 1980).

As early as 1966, Endel Tulving suggested that forgetting, the loss of ability to express memory, can occur through either the unavailability (the memory representation is gone) or the inaccessibility of the trace (the memory representation is blocked from expression). In other words, a problem can occur with the retention (storage) or the recall (retrieval) of the memory (Tulving & Pearlstone, 1966). For many years, retrograde amnesia was thought to be necessarily caused by a storage impairment because the memory could not be recalled at later times, thus suggesting a permanent amnesia that damaged physical substrates of the memory (Larry R. Squire & Alvarez, 1995). In 1973, Miller and Springer developed an alternate explanation based on experiments that administered reminder cues after an amnesic treatment. Experiments had already shown that amnesic animals given a reminder cue in the form of a non-contingent treatment (e.g. in passive avoidance experiments, this could be a footshock in a different context), later seemingly recover the originally trained memory at a subsequent retention test

(Koppenaal, Jagoda, & Cruce, 1967; Lewis, Misanin, & Miller, 1968; Quartermain, McEwen, & Azmitia, 1970). Because the reminder cue prompted the previously lost memory to return, these "recovery from amnesia" studies were taken as evidence that even with an amnesic treatment, the memory remained latent, and was merely inaccessible until a reminder was given. Miller and Springer therefore posited that these cases of experimental amnesia must be caused by a retrieval failure of the memory (Miller & Springer, 1973, 1974).

At the same time, Jim McGaugh's group showed that both storage and retrieval failure theories were possible explanations for experimental amnesia (Gold, Haycock, Macri, & McGaugh, 1973; Gold & King, 1974). As expected, their experiments revealed that animals trained in a passive avoidance task, later rendered amnesic, could then recover their memory following presentation of a non-contingent footshock (reminder cue). Naïve animals given the reminder unsurprisingly did not show any change in performance at a later test. However, Gold et al. (1973) separated the amnesic animals into two groups: those that performed at naïve levels, and those that performed at a level higher than naïve animals—at the same levels as animals that had been given very weak footshock training. They found that the fully amnesic animals were not affected by the reminder cue. But amnesic animals that showed weak memory at an initial retention test were capable of recovering their memory after being given a reminder cue. This led them to believe that the non-contingent footshock was not acting purely as a reminder, but possibly also as new learning. This learning could enhance a memory greatly weakened through amnesic treatments and bring performance back up to levels of control animals that had experienced strong training. Crucially, animals that were initially weakly trained, and had poor performance, also improved their performance level through the non-contingent footshock

reminder cue, in a manner similar to the animals that had partially forgotten the training (Gold et al., 1973).

Gold and King (1974) therefore theorized that the amnesic affect could not only be explained by a retrieval impairment, but also by a storage loss of the memory trace. The trained memory was impaired by an amnesic treatment to the extent that the animals could not perform well at an initial retention test, but because the memory was partially retained, it was able to be boosted to expressible levels after a reminder cue acting ostensibly as a weak training session. The authors state that this does not disprove all cases where a reminder cue helped overcome a retrieval deficit, but does provide additional interpretations of data. However, this showed that in studies of experimental amnesia using a recovery from amnesia paradigm, it is impossible to distinguish whether the amnesia was caused by a storage or retrieval failure, as both interpretations are possible (also see Nader, 2009; Hardt et al., 2009). Both storage and retrieval impairments lead to a similar performance loss at a retention test, resulting in ambiguous interpretations of how the trained memory is lost when only behavioural data available. As well, these experiments rely on negative data, that is, the absence of performance; thus it was not possible to make positive predictions about the cause of observed amnesic behaviour. This revealed not only a need to be cognizant of how amnesia could be produced, but a need for a behavioural paradigm that could positively differentiate between situations of storage and retrieval impairment.

1.2.2. *The second learning phenomenon*

Ideally, both behavioural and molecular evidence should be used to decipher the nature of amnesia. However, as stated, behavioural tests cannot easily distinguish between cases of storage impairment (leading to memory unavailability) and retrieval impairment (leading to memory

inaccessibility) because a lack of memory universally leads to decreased performance. To this end, Hardt et al. (2009) developed a second learning paradigm to positively dissociate the source of experimental amnesia.

The second learning paradigm takes advantage of the fact that there is a differential requirement for *N*-methyl-D-aspartate receptor (NMDAR) activation depending on whether a hippocampal-dependent memory is learned for the first or second time. This effect was first noted by Bannerman and colleagues (1995) in rats trained in two different Morris water mazes, thus employing two different contexts to provide separate learning experiences of principally the same task. Rats infused with AP5, an NMDAR-antagonist, into the dorsal hippocampus (dHPC) prior to the first training experience were not able to successfully learn the task. This was expected, as NMDAR activation has been shown to be necessary for new spatial learning in the hippocampus (S. Davis, Butcher, & Morris, 1992; R. G. M. Morris, Anderson, Lynch, & Baudry, 1986; Nakazawa, McHugh, Wilson, & Tonegawa, 2004). But the researchers further showed that rats then trained in a second water maze setup were not affected by pre-training AP5 infusions. These rats were able to learn the task like control rats, showing that subsequent learning of a familiar task does not require NMDAR activation. Since then, NMDAR-independency of later learning has been observed in other experiments: in water maze learning (Saucier and Cain, 1995), contextual fear learning (Hardt et al., 2009; Sanders & Fanselow, 2003; Tayler et al., 2011; Wiltgen, Wood, & Levy, 2011), re-extinction of auditory fear (Langton & Richardson, 2010), and even relearning of auditory fear conditioning after forgetting (Chan, Baker, & Richardson, 2015; Li & Richardson, 2013).

Such findings suggest that giving prior exposure to a context or experience, whether through habituation or training, initiates plasticity mechanisms that facilitate future learning.

Because of this, NMDAR-independent learning may be a form of metaplasticity (Ryan G. Parsons, 2017), a phenomenon describing the plasticity of synaptic plasticity (Wickliffe C Abraham, 2008; Wickliffe C Abraham & Bear, 1996). The neural mechanisms involved in NMDAR-independent learning are not fully clear, although there seems to be a need to involve the hippocampus, as the exemplary protocol to induce NMDAR-independent learning has so far only been shown in hippocampus-dependent spatial memories (Tayler et al., 2011; Parsons, 2017), although similar effects have been seen with other memory phenomenon using auditory fear conditioning (extinction and relearning: Langton & Richardson, 2010; Li & Richardson, 2013, respectively). That the subsequent learning must build upon the same training experience as the initial learning (Wiltgen et al., 2011) perhaps indicates a shared or overlapping neural ensemble between the two experiences which allows the second learning to avoid NMDAR activation due to reliance on the first learning ensemble in some capacity. Evidence has indeed shown that different sets of training in the same context, given in close time proximity, do involve shared neural ensembles (Cai et al., 2016). Although the purpose of this thesis is not to explore the mechanisms of NMDAR-independent learning, this unique property of second learning acquisition is significant, as it allowed Hardt et al., (2009) to build a protocol that behaviourally dissociated between experimental amnesia caused by a storage deficit or a retrieval deficit.

In their study, rats were taught a contextual fear memory in one context, then given an amnesic treatment (infusions of anisomycin, a protein synthesis inhibitor) after training to block consolidation of this memory. Rats later experienced another set of fear conditioning sessions in a markedly different context. Infusions of AP5 were administered in the hippocampus prior to each of these “second learning” training sessions. Results showed that by impairing the initially-

learned memory with post-training anisomycin infusions, second learning acquisition became NMDAR-dependent. All other experimental groups showed adequate performance at the test for the second learning experience. Since NMDAR-dependent acquisition is a property of naïve learning, Hardt et al. concluded that the second learning experience had been acquired as if it was naïve learning. This suggested that anisomycin impairment of the first-learned memory erased the memory to a degree that the brain interpreted a familiar experience as new—inferring that the storage of the first learning trace had been lost to a large degree. Had the post-training amnesic treatment only impaired retrieval of the first-learned memory, the hypothesis would predict that subsequent learning would remain NMDAR-independent due to the physical persistence of the neural ensemble representing the first learning memory. Thus, the sensitivity of second learning to NMDAR impairment revealed whether the first learning memory was lost through a storage or retrieval impairment.

This protocol is a clear way to elucidate the nature of amnesia or forgetting through behavioural means. We were interested in finding out if erasing a consolidated memory, or blocking reconsolidation of a memory, could be characterized as a storage or retrieval impairment. This would allow clear disambiguation of the nature of these two forms of induced amnesia. By using the second learning protocol on a memory task, we could impair first learning memory (i.e. by erasing consolidation or blocking reconsolidation), and then evaluate the dependency of second learning acquisition on NMDARs, thus inferring how the intervention caused the memory loss.

1.2.3. *Types of memory and memory loss*

Memory can be lost in myriad ways, and three methods will be focused upon here. Not only erasing long-term memory (Chapter 2), and reconsolidation blockade (Chapter 3), but also

forgetting that occurs naturally, without intervention (Chapter 4). If all three situations cause a storage loss of the memory trace, and moreover, involve the loss of the same neural correlates, then this would imply some level of similarity in their neurobiological mechanisms. Because natural forgetting can be caused either by decay (neural correlates gradually being disintegrated), or interference (memories affecting recall or content of other memories) (Hardt et al., 2013), comparing analogous memory loss processes would allow indirect study of how forgetting occurs. Thus, experimental amnesia, which can be more strictly manipulated, can act as a proxy to study forgetting. Our first project, then, was to characterize the nature of amnesia of impairing a consolidated memory, which we believe is an appropriate parallel to forgetting mechanisms.

Secondly, we thought it was crucial to study amnesia caused by reconsolidate blockade. Reconsolidation describes the phenomenon of a consolidated, stable memory becoming temporarily labile after recall (Debiec, LeDoux, & Nader, 2002; Milekic & Alberini, 2002; Nader, Schafe, & Le Doux, 2000; Przybyslawski & Sara, 1997; Susan J. Sara, 2000). During the period of lability, the memory can be manipulated, for example to block a memory (Nader et al., 2000), block an emotional component of a memory (Schiller et al., 2009), or even strengthen a memory (Tronson, Wiseman, Olausson, & Taylor, 2006). But reconsolidation does not occur ubiquitously; some have theorized that memories are most likely to reconsolidate when a prediction error is experienced, i.e. the experience that reactivates the memory involves new learning (Exton-McGuinness, Lee, & Reichelt, 2015; Sevenster, Beckers, & Kindt, 2013, 2014). The incorporation of new data has been thought of as a form of memory updating needed to keep memory relevant (J. L. C. Lee, 2009), thus reconsolidation may be an adaptive memory process (J. L. C. Lee, Nader, & Schiller, 2017). Therefore, reconsolidation blockade—of spatial

memories especially—is a needed area of study in the greater framework of memory loss mechanisms.

If these instances of memory loss are caused by a retrieval impairment, then neural correlates of the memory are likely to be retained despite being temporarily obstructed from being expressed. But if they are caused by a storage impairment, then the neural correlates of the memory are likely to be lost, removed, or degraded by the intervention method or as a factor of time. Indeed, the accumulated literature on long-term memory impairment, reconsolidation blockade, and forgetting, suggest that the latter is what occurs. Specifically, all three situations have been observed to involve internalization of a subtype of glutamate receptor, GluA2-subunit containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (GluA2-AMPARs), from the postsynaptic membrane. Under mechanisms of Hebbian plasticity, it may be that GluA2-AMPAR endocytosis is a correlate of storage loss of the memory trace, and any method that induces AMPAR endocytosis must be causing a storage impairment. However, this seemingly evident concept has never been shown empirically. Additionally, it is unclear what degree of GluA2-AMPAR endocytosis is required to cause an inability to behaviourally express the memory (i.e. a performance loss). Therefore, to investigate this, we explored changes in postsynaptic GluA2-AMPAR expression in different types of experimental amnesia and forgetting. This would allow linkage of behavioural and molecular evidence to show how changes in this neural correlate can be classified as an impairment of storage or retrieval of the memory trace, and how these changes may correlate to memory loss and changes to memory strength.

All three chapters in this thesis are based on object location learning, a type of novelty preference task. The representation of object location memories critically requires the dorsal

hippocampus (Assini, Duzzioni, & Takahashi, 2009; Abdelkader Ennaceur, Neave, & Aggleton, 1997), and is a non-Pavlovian learning task. As mentioned above, most studies that involve NMDAR-independent learning use contextual fear conditioning. However, non-Pavlovian, hippocampus-based learning also plays an important role in everyday memory function. The hippocampus in particular has been implicated in not only spatial memory representation, but in episodic memories (Tulving & Markowitsch, 1998). Although episodic memories and spatial memories have shown to be independently coded in the hippocampus (Leutgeb et al., 2005), studying object location learning can increase understanding of how non-associative, hippocampus-dependent memories are lost. More importantly, to study forgetting (Project 3: Chapter 4), we required a memory task that would show a reliably steady degradation of memory strength over time, which makes object location memory an ideal behavioural task to use.

We expected that behavioural results would confirm that these treatments cause a storage impairment of the memory trace. And by correlating behavioural results with molecular data, we aimed to show how GluA2-AMPAR endocytosis is related with these three types of memory loss.

1.3. *Spotlight on a specific type of AMPA receptor*

To explore how AMPARs contribute to memory loss, it is important to understand their role in synaptic plasticity. Fast excitatory synaptic transmission in the mature mammalian central nervous system is mainly mediated by AMPARs (Traynelis et al., 2010). They are heterotetramers made up of subunits GluA1 to GluA4 (Hollmann & Heinemann, 1994; Wisden & Seeburg, 1993); trafficking and phosphorylation of GluA1 and GluA2 subtypes especially play significant, but different, roles in processes of synaptic plasticity (Huganir & Nicoll, 2013;

Newpher & Ehlers, 2009; Shepherd & Huganir, 2007). A full review of these functions are beyond the scope of this thesis, but in short, GluA1-containing AMPARs (GluA1-AMPARs) are inserted into the postsynaptic membrane after LTP stimulation, triggering a cascade of events that leads to eventual replacement of GluA1-AMPARs with GluA2-containing AMPARs (GluA2-AMPARs), which seem to stabilize LTP expression in the synapse. In the section below we summarize the function of AMPARs and especially GluA2-AMPARs in learning and memory. If these roles in the maintenance of increased synaptic strength also applies to maintenance of long-term memory, investigating GluA2-AMPAR removal from the postsynaptic membrane will reveal how these processes are disrupted and eventually lost—leading to memory impairment.

1.3.1. *The role of GluA2-AMPARs in synaptic plasticity*

In rat hippocampal pyramidal neurons, the majority of AMPAR are arranged into heteromers of GluA1/GluA2, with GluA2/GluA3 heteromers being the second most plentiful type of AMPAR (W. Lu et al., 2009; Wenthold, Petralia, Blahos J, & Niedzielski, 1996). GluA2 subunits have unique properties that differentiate them from other AMPAR subunits. Most noticeably, AMPAR that contain GluA2 are not permeable to calcium. This is due to a posttranscriptional modification of the Q/R editing site located in the ion channel pore region of the AMPAR (Araki, Lin, & Huganir, 2010; Seeburg, Higuchi, & Sprengel, 1998). Here, the usual amino acid glutamine (Q) is replaced with arginine (R), flipping the site from having no charge to having a positive charge, which prevents calcium from entering the ion pore (Burnashev, Villarroel, & Sakmann, 1996). This structural change can regulate trafficking of GluA2-AMPARs into the plasma membrane by allowing binding to proteins that promote GluA2-AMPAR insertion (Araki et al., 2010).

In addition to the changed codon at the Q/R site, AMPAR subunits notably differ at their intracellular cytoplasmic tail, though otherwise have largely similar composition (Malinow & Malenka, 2002). GluA1 and GluA4 subunits have longer tails, and GluA2 and GluA3 subunits have shorter tails (Malinow & Malenka, 2002). Trafficking of AMPARs to membranes is dependent on the interaction of these tails with other intracellular proteins, as well as posttranslational modifications at the cytoplasmic carboxyl-terminus through processes such as phosphorylation and ubiquitination (Huganir & Nicoll, 2013; Shepherd & Huganir, 2007; Anggono & Huganir, 2012). For example, phosphorylation of the amino acid serine 880 in GluA2 subunits by protein kinase C leads to a chain of events that ultimately removes GluA2-AMPARs from the postsynaptic membrane (Chung, Steinberg, Huganir, & Linden, 2003; Matsuda, Launey, Mikawa, & Hirai, 2000; Santos, Carvalho, Caldeira, & Duarte, 2009), which is thought to be a possible mechanism of hippocampal and cerebellar LTD (Chung et al., 2003; C.-H. Kim & Lisman, 2001; Matsuda et al., 2000; Perez et al., 2001).

In terms of function, the long tail of the GluA1 subunit restricts delivery of GluA1-AMPAR to the synapse when there is a lack of activity (Sheng & Lee, 2001). Thus activity-dependent insertion of long-tailed AMPARs is hypothesized to contribute to synaptic strengthening during LTP. On the other hand, the short tails of GluA2 subunits mediate constitutive delivery to synapses (Sheng & Lee, 2001), which can occur in the absence of activity (Kessels & Malinow, 2009; Shi, Hayashi, Esteban, & Malinow, 2001). The accumulated evidence suggests that LTP induction triggers insertion of GluA1-AMPARs into the plasma membrane, which are then replaced with GluA2-AMPARs over time (Plant et al., 2006; Sheng & Lee, 2001; Shi et al., 2001). Plant et al. (2006) showed that activity-dependent upregulation of calcium-permeable AMPARs (CP-AMPARs—that is, AMPAR that do not contain GluA2

subunits) into the synaptic surface are gradually exchanged with GluA2-AMPARs within 25 minutes following LTP induction, without resulting in loss of synaptic strength. This activity-independent delivery is one reason why GluA2-AMPARs may provide stabilization of the LTP or LTD signal (Meng, Zhang, & Jia, 2003), despite regular molecular turnover (Kessels & Malinow, 2009). Indeed, this stabilization via exchange of CP-AMPARs with GluA2-AMPARs may be a molecular model of memory consolidation (Kessels & Malinow, 2009). In general, calcium-permeable AMPARs (GluA1-AMPARs) are associated with LTP induction and strengthening of the synaptic signal, whereas GluA2-AMPARs are associated with maintenance of this signal (Man, 2011).

1.3.2. *The role of GluA2-AMPARs in long-term memory maintenance*

A well-established theory of how synaptic GluA2-AMPARs are trafficked to and maintained in the postsynaptic membrane has emerged over the years. The carboxyl-terminus tail of GluA2-AMPARs can bind to *N*-ethylmaleimide sensitive factor (NSF), a chaperone protein that is heavily involved in trafficking mechanisms and membrane fusion events (Malinow & Malenka, 2002; Sheng & Lee, 2001; Whiteheart, Schraw, & Matveeva, 2001). Studies have shown that disrupting the interaction of GluA2-AMPARs with NSF leads to a rapid decrease of synaptic GluA2-AMPARs (C.-H. Kim & Lisman, 2001; Lüscher et al., 1999; Noel et al., 1999; Shi et al., 2001; I. Song et al., 1998) and therefore weakening of synaptic strength (Nishimune et al., 1998), similar to how LTD is also caused by GluA2-AMPAR endocytosis (Sang Hyoung Lee, Liu, Wang, & Sheng, 2002; Man et al., 2000). This downregulation was not seen in mice with GluA2-AMPARs knocked out (Shi et al., 2001). NSF may accomplish this by impeding interactions between GluA2-AMPARs and the protein PICK1 (protein interacting with C kinase 1), which has been implicated in GluA2-AMPAR removal from the synapse (Hanley, Khatri,

Hanson, & Ziff, 2002), creating an LTD-like decrease in basal synaptic transmission (Sacktor, 2011). By preventing GluA2/PICK1 binding, NSF interactions can maintain GluA2-AMPARs in the postsynaptic membrane (Sacktor, 2011).

Another protein that is crucially involved in the persistence of GluA2-AMPARs at the postsynaptic membrane is atypical protein kinase C isoform M ζ (PKMζ), which is widely hypothesized to be a molecular correlate of long-term memory (Migues et al., 2010; Sacktor, 2011; Serrano et al., 2008). PKMζ has been shown to bind with NSF in order to traffic GluA2-AMPARs to the postsynaptic density (Migues et al., 2010) and is uniquely suited to maintain the NSF-GluA2 complex in the membrane (Sacktor, 2011; Yao et al., 2008). During LTP induction, several substrates act on PKMζ to release the translational block from its regulatory domain. The resulting open catalytic domain of PKMζ becomes constitutively active and autophosphorylates, allowing perpetual synthesis of more PKMζ. This makes PKMζ ideally suited to maintaining changes induced by LTP (Kwapis & Helmstetter, 2014). Together, it is clear that sustaining GluA2-AMPARs in the postsynaptic membrane is necessary for memory maintenance.

1.4. *GluA2-AMPAR endocytosis is part of a neural mechanism of many forms of memory loss*

If pharmacologically-induced amnesia of a consolidated memory is a result of a storage impairment of the memory as some research has shown (Hardt et al., 2009), then we would expect postsynaptic GluA2-AMPAR internalization to be a manifestation of this storage loss. This would hold true when a memory was erased, or was prevented from reconsolidating, or forgotten. In this section, I highlight the role of GluA2-AMPAR endocytosis across various forms of memory deficits, and what questions about these processes are yet to be answered.

1.4.1. *Endocytosis of GluA2-AMPARs disrupts long-term memory*

Following the discovery of PKMζ, it became increasingly possible to study impairment of long-term memory (LTM) through pharmacological interventions with drugs like the pseudosubtrate, zeta inhibitory peptide, that regulates the activity of PKMζ (ZIP), which acts as a protein kinase C inhibitor. Blocking PKMζ activity with application of ZIP functionally erases LTM, an effect that has been seen across different types of memory and in different species (Kwapis & Helmstetter, 2014). In the hippocampus, the increased amounts of synaptic AMPAR resulting from LTP return to baseline levels after infusions of ZIP (Ling et al., 2002; Pastalkova et al., 2006; Serrano, Yao, & Sacktor, 2005). This strongly suggests that the decay of LTP and loss of LTM are associated with endocytosis of GluA2-AMPARs from the postsynaptic density (PSD). Other studies have corroborated this theory by showing that ZIP-mediated deficits of LTP maintenance are prevented if there is a prior application of TAT-GluA2$_{3Y}$, a synthetic protein that mimics the GluA2 subunit C-terminal tail (Migues et al., 2010). GluA2$_{3Y}$ has been shown to prevent AMPAR endocytosis induced with insulin stimulation and LTD in hippocampal slices (Ahmadian et al., 2004); it is thought to inhibit the endocytic pathway that usually would remove GluA2-AMPARs by blocking binding to BRAG2, a protein that is involved in trafficking GluA2-AMPARs out of the membrane (Sacktor, 2011). At the behavioural level, GluA2$_{3Y}$ can prevent ZIP-induced amnesia of an amygdala-dependent fear conditioning task, as well as a hippocampus-dependent object location task (Migues et al., 2010).

Migues et al. (2010) showed the rescue effects of blocking GluA2-AMPAR endocytosis in vitro and in vivo in the basal lateral amygdala (BLA). Bath application of ZIP to BLA slices was able to reverse an induced LTP signal, but if GluA2$_{3Y}$ was perfused intracellularly, the LTP reversal was prevented. In addition, infusing GluA2$_{3Y}$ into the basolateral amygdala one hour

prior to post-training ZIP infusions can attenuate the behavioural impairment in conditioned fear memory usually induced by ZIP. Western blot quantification of GluA2-AMPARs in subcellular fractions of BLA neurons revealed that the amount of postsynaptic GluA2-AMPARs was linearly correlated with the strength of memory (Migues et al., 2010). This suggested that GluA2-AMPAR endocytosis from the PSD is directly related to a decrease of fear expression (Migues et al., 2010; Hardt et al., 2013). Interestingly, the data points for this correlation also showed a relatively large decrease of GluA2-AMPARs correlated to freezing no longer being expressed. This prompts the question: what proportion of surface GluA2-AMPARs are internalized to lead to erasure of memory expression?

To answer this question, we impaired a dHPC-dependent long-term spatial memory—object location memory—with infusions of ZIP, and correlated performance at test with expression of GluA2-AMPARs in the PSD (Project 1: Chapter 2). The object location task has a clear index of measurement to define when the animal shows memory or not. Thus, we expected to see that a memory erased with ZIP infusions will show a lower amount of GluA2-AMPAR expression in the PSD of dHPC CA1 neurons compared to when the memory is intact. However, as stated previously, also of interest is determining the relative percentage of GluA2-AMPAR endocytosis that leads to experimental amnesia, and relating this value with how much endocytosis is observed in other forms of amnesia. To this end, we also explored endocytosis of GluA2-AMPAR after reconsolidation blockade of an object location memory.

1.4.2. *Post-retrieval AMPAR endocytosis causes long-term memory destabilization*

It is now accepted that many types of memories, upon retrieval, destabilize and then need to restabilize over subsequent hours (S.-H. Wang, de Oliveira Alvares, & Nader, 2009; Winters, Tucci, & DaCosta-Furtado, 2009). This restabilization process—known as reconsolidation—

requires protein synthesis (Debiec et al., 2002; Inda, Delgado-García, & Carrión., 2005; Nader et al., 2000; see Dudai & Eisenberg, 2004, or Alberini, 2005, for a review) . During a period of time after retrieval, memories are labile and susceptible to manipulation (Nader et al., 2000), whether impairment (for example, Lee, Di Ciano, Thomas, & Everitt, 2005), enhancement (for example, Fukushima et al., 2014), or inserting new information (for example, Jarome, Ferrara, Kwapis, & Helmstette, 2015). Infusions of protein synthesis inhibitors, such as anisomycin, can block reconsolidation and cause amnesia (Nader, 2003; Nader et al., 2000). This suggests that protein synthesis is needed to sufficiently reform the memory, otherwise the entire process is arrested.

Many studies have shown evidence of GluA2-AMPAR endocytosis after retrieval of a long-term memory (Bhattacharya et al., 2017; Cazakoff & Howland, 2011; Hong et al., 2013; Rao-Ruiz et al., 2011). This GluA2-AMPAR internalization is likely to be the mechanism of retrieval-induced memory destabilization. Rao-Ruiz and colleagues quantified GluA2-AMPAR expression in synaptic membrane fractions in dHPC CA1 cells to assess post-retrieval mechanisms. They showed that 1 hour after retrieval, GluA2-AMPAR expression levels in the synaptic membrane fraction decreased, but then increased again 7 hours post-retrieval. Notably, within this period, memory expression was not affected, likely due to the replacement of GluA2-AMPARs with GluA1-AMPARs, allowing for consistent synaptic strength. Indeed, this timeframe approximately matched the approximate 6-hour time window purportedly needed for reconsolidation of a fear memory in rats (Nader et al., 2000). Additionally, they showed that blocking retrieval-induced endocytosis with infusions of $GluA2_{3Y}$ 1 hour before retrieval led to an enhancement of fear expression at test, lending support to the theory that GluA2-AMPAR expression in the PSD is linked to LTM. However, they do not explicitly link behavioural results

with molecular data, and thus the amount of GluA2-AMPAR internalized in relation to fear expression destabilization (i.e. reconsolidation blockade) remains unknown.

To examine this, and to directly compare any observed changes of GluA2-AMPAR expression with what was observed with LTM impairment, we used an object location task and probed neurons in the dHPC (Project 2: Chapter 3). We blocked reconsolidation with infusions of sulfasalazine, an inhibitor of nuclear factor kappa B transcription factors (NF-κB) (Wahl, Liptay, Adler, & Schmid, 1998), which has previously been shown to selectively impair reconsolidation but not consolidation of contextual fear memory (J. L. C. Lee & Hynds, 2013). Because retrieval leads to a downregulation of GluA2-AMPARs, a memory that is prevented from reconsolidating was expected to show less surface GluA2-AMPAR expression in dHPC CA1 neurons compared to control animals with unimpaired memory. Additionally, we were interested in comparing the percentage of GluA2-AMPARs internalized to the amount internalized due to ZIP-induced LTM blockade. If the performance output for these two situations of experimental amnesia are comparable, and if GluA2-AMPAR expression is linearly correlated to strength of memory expression (i.e. performance), then it is plausible to hypothesize that they both would involve similar amounts of GluA2-AMPAR internalization.

Even if we detect similar changes in GluA2-AMPAR expression through both types of experimental amnesia, these results require context to frame how much GluA2-AMPAR endocytosis is needed for a memory to be functionally lost. For example, does experimental amnesia, either through LTM erasure, reconsolidation blockade, or both, cause GluA2-AMPAR expression levels to return to pre-training levels seen in naïve rats? If not, what is the threshold of GluA2-AMPAR expression levels needed for a memory to no longer be expressible? Or are all changes in postsynaptic GluA2-AMPAR levels directly translatable to behavioural output? To

answer these questions, we thought a third experiment was required, where GluA2-AMPAR endocytosis is measured as an object location memory is forgotten.

1.4.3. *Endocytosis of GluA2-AMPARs contributes to forgetting*

There is much support for the role of GluA2-AMPARs in LTP and LTM maintenance, as we have already outlined. However, many types of memories, such as object location memories, do not seem to last forever. Object location memories have been observed to last up to 32 days after a strong training protocol (Hardt et al., 2010). Without any interventions, expression of object location memory gradually weakens as time passes (i.e. is forgotten), likely through an innate decay process involving removal of synaptic GluA2-AMPARs (Migues et al., 2016). As with LTM impairment and reconsolidation blockade, this would correspond to forgetting being caused by a storage loss of the memory. Indeed, a strong line of evidence for the necessary role of GluA2-AMPARs in forgetting was found in experiments where memories are extended for longer than their typical lifespan. Migues et al. (2016) taught rats an object location task over 7 days, then tested their memory 3, 7, 10, 12 or 14 days after training. Without intervention, a gradual loss of the object location memory was observed: the rats that experienced the fewest days of retention time (3 days) had the strongest memory, as indicated by their performance at the test session. Rats that experienced a midrange retention time (7 days) had worse (but still statistically significant) performance in comparison. Performance of rats after 10, 12, and 14 days of retention was not significantly different from rats with no memory. Plotting this behaviour over time displayed a negative exponential slope that eventually reaches a behavioural asymptote, echoing the pattern seen in Ebbinghaus' famous forgetting curve (Murre & Dros, 2015), where a lot of training was forgotten soon after training completed, but as time increased, less and less information was additionally forgotten. Daily infusions of $GluA2_{3Y}$ after training

proved to prevent this forgetting, allowing memory expression to be seen even 14 days after training. The $GluA2_{3Y}$ treatment thus lead the memory to persist longer than it would normally last without intervention (Migues et al., 2016).

The literature therefore strongly supports GluA2-AMPAR endocytosis as a possible mechanism of forgetting. Taken into consideration with previous data showing GluA2-AMPAR expression in the PSD is linearly correlated to memory strength (Migues et al., 2010), it should be possible to observe a gradual decrease in GluA2-AMPAR over time that corresponds to LTM weakening/forgetting. It is unclear whether this endogenous GluA2-AMPAR endocytosis occurs at the same rate as experimental amnesia—in other words, does forgetting involve a similar correlation between decreasing GluA2-AMPAR expression and memory strength as seen with cases of experimental amnesia? This might indicate a rate of GluA2-AMPAR endocytosis that is consistent across multiple forms of memory loss.

To determine the rate of GluA2-AMPAR endocytosis as a memory is forgotten, we taught separate groups of rats the same object location memory, then tested them after varying days of retention (Project 3: Chapter 4), expecting memory to weaken as retention time increases, as seen in Migues et al., 2016. We also probed for levels of GluA2-AMPAR in the PSD found at each of these time points. If the decrease of memory strength as a function of time is paralleled by decreasing GluA2-AMPARs in the PSD, we would expect to see a positive correlation between test performance and GluA2-AMPAR expression levels. This would further support the relationship between GluA2-AMPAR expression and memory strength. This data may also be able to interpret changes in GluA2-AMPAR expression seen in the previous experiments on LTM impairment and reconsolidation blockade. For example, at what time point does the performance deficit from LTM impairment or reconsolidation blockade match up to the

performance deficit of a forgotten memory? At this point, are GluA2-AMPAR expression levels similar across these three situations? If not, this would reveal if the amount of GluA2-AMPAR endocytosed via experimental amnesia is more or less than when the memory has just been forgotten. Furthermore, if groups show similar behaviour but different levels of GluA2-AMPAR, this would possibly indicate that the degree of correlation between GluA2-AMPARs and memory differs depending on how memory loss is induced (i.e. pharmacologically versus endogenously).

1.4.4. *GluA2-AMPAR expression levels may increase due to additional learning*

Lastly, we wish to note a potentially interesting area of analysis of GluA2-AMPAR expression while using the second learning paradigm. Current research suggests that NMDAR-independent learning occurs only when recently activated hippocampal neurons are recruited for second learning (Tayler et al., 2011). Thus, there exists a degree of overlap in the neurons of a first- and second-learned spatial memory, perhaps due to the similarity of the contexts of the two learning experiences. There indeed have been studies that indicate similar contexts will activate overlapping neuronal populations, while very distinct contexts will instead activate different populations (Vazdarjanova & Guzowski, 2004). Similarly, shared memory traces have been observed when pre-exposure to a context is given close in time to a later training session in that context (Cai et al., 2016). It is unknown how the degree of overlapping neural ensembles translates into GluA2-AMPAR expression change, but the second learning paradigm allows a unique method to investigate this.

Because two different training experiences are given, the protocol produces four experiment groups based on the amnesic treatment given after first learning (ZIP/scrambled-ZIP,

or sulfasalazine/vehicle) and whether an NMDAR-antagonist given prior to second learning (AP5/vehicle): drug-AP5, drug-veh, veh-AP5, and veh-veh. Thus, these combinations might allow comparisons of GluA2-AMPAR expression associated with neuronal representation of two similar memories to when there is a single memory, or indeed, no memory. If the amnesic treatment impairs the storage of the memory, then we can expect less GluA2-AMPAR expression in the drug-AP5 group compared to the veh-veh group, in which animals will retain memories both first and second learning. Additionally, these levels of GluA2-AMPAR expression across conditions can then be compared to when the same object location memory is forgotten naturally (Project 3: Chapter 4). This can provide new insights to how the amount of GluA2-AMPAR endocytosed from the PSD of dHPC neurons is correlated to not only how memory is lost, but how many memories are lost.

1.5. *Summary: GluA2-AMPAR endocytosis as a biological correlate of experimental amnesia and active decay*

In conclusion, amnesia can be caused by a storage or retrieval impairment of the memory trace. Maintenance of GluA2-AMPARs in the PSD, through interactions with molecules like PKMζ, has been shown to provide stability to LTP and LTM, whereas internalization of GluA2-AMPARs from the synaptic surface has been correlated destabilization of these processes, leading to forgetting. Some studies have suggested that there is a positive relationship between GluA2-AMPAR expression and LTM strength (Migues et al., 2010), thus, a decrease of GluA2-AMPARs from the synapse must correlate to weakening and eventual loss of the memory. However, it remains unknown what percentage of internalized GluA2-AMPARs are necessary to lead a quantifiable behavioural deficit. Additionally, molecular data about GluA2-AMPAR

endocytosis and behavioural data showing the nature of amnesia have not been properly reconciled.

The research described in this thesis therefore aimed to find the relative percentage of surface GluA2-AMPAR endocytosed when a memory was lost through various means. We taught object location memory to rats and induced LTM erasure with infusions of ZIP (Chapter 2) or blocked reconsolidation with infusions of sulfasalazine (Chapter 3), or allowed forgetting to naturally occur (Chapter 4), then evaluated GluA2-AMPAR expression in the dHPC CA1 with western blot assays in each of these cases. We aimed to use the second learning paradigm to verify that amnesia involving GluA2-AMPAR endocytosis can be thought of as causing a storage impairment of the memory trace, and compare these changes in GluA2-AMPAR expression with the rate seen as a memory is forgotten. If similarities exist in the correlation of GluA2-AMPAR levels and memory expression across all three projects, this would perhaps provide evidence of a generalized pattern of how much GluA2-AMPAR internalization can be expected when memory is lost, regardless of the cause. This would increase our understanding of how GluA2-AMPAR expression levels change across different situations of memory deficits, with possible application to pathological memory impairment (e.g. Alzheimer's disease). Thus, on the whole, we aimed to build a holistic profile of GluA2-AMPARs' role in memory loss using both molecular and behavioural data.

In the next three chapters, we outline the results from our experiments. We indeed show that blocking LTM or reconsolidation impairs the storage of an object location memory trace (Chapters 2 and 3, respectively), and that an object location memory is gradually forgotten over time (Chapter 4). However, how GluA2-AMPAR expression levels change with these

impairments is less clear, making it difficult to make comparisons across the three projects. The possible reasons for these conflicting results are extensively discussed in Chapter 5.

CHAPTER TWO

ZIP erasure of an object location memory leads to NMDAR-dependency of later object location learning

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2.1. *Preface*

To study experimental amnesia of a consolidated memory, the first project for this dissertation examined the effects of PKMζ, a kinase that in the recent decades has been shown to be necessary for LTM maintenance (Sacktor, 2011). PKMζ can be inhibited with infusions of zeta-inhibitory peptide (ZIP). PKMζ has been shown to maintain GluA2-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (GluA2-AMPAR) at the postsynaptic membrane, which allows persistence of the memory (Migues et al., 2010; Sacktor, 2011; Serrano et al., 2008). Thus, blocking PKMζ is hypothesized to result in GluA2-AMPAR endocytosis from the postsynaptic membrane. Thus, we hypothesized that ZIP infusions would impair storage of a memory. We had two main research questions: 1) Does ZIP-induced amnesia cause a storage or retrieval impairment of the memory? 2) If there is indeed a storage loss of the memory, would it correlate with endocytosis of GluA2-AMPAR, and if so, by how much?

To answer the first question, we employed a second learning protocol where we taught rats two different experiences of an object location task, first demonstrated by Hardt et al., 2009. We impaired the first-learned memory with bilateral infusions of ZIP into the dorsal hippocampus (dHPC), then evaluated the dependence of acquisition in a second learning experience on *N*-methyl-D-aspartate receptors (NMDARs). Second learning of certain hippocampus-dependent spatial tasks has been shown to not require NMDAR activation (e.g. Bannerman et al., 1995; Hardt et al., 2009; Tayler et al., 2011; Wiltgen et al., 2011), thus if ZIP infusions erase storage of a first-learned LTM, then any subsequent learning would require NMDAR activation as if it was being learned naïvely. Indeed, this is what we observed. These data also provide evidence that the object location task can produce NMDAR-independent learning.

To see how postsynaptic GluA2-AMPAR expression correlated with this putative storage loss of the first learned memory, we performed western blot assays on subcellular fractions of the postsynaptic density (PSD) of CA1 area neurons in the dHPC, the brain region where object location memory is thought to be represented (Assini et al., 2009). If GluA2-AMPAR endocytosis correlates with storage impairment, then rats infused bilaterally with ZIP in the dorsal hippocampus (dHPC) would have lower levels of GluA2-AMPAR expression compared to other treatment groups. If second learning is not successfully acquired after this impairment, these levels should remain low in comparison to groups that did acquire the additional learning.

However, GluA2-AMPAR expression in the PSD did not correlate with the memory loss seen in behavioural results as we had hypothesized. Interestingly, we observed a negative linear correlation between memory expression at second learning test and relative GluA2-AMPAR expression, such that increased GluA2-AMPARs were seen with lower preference for the novel object location. Furthermore, this pattern was only seen in ZIP-infused rats. Thus, the molecular data, while not able to confirm that GluA2-AMPAR endocytosis correlated with ZIP-induced storage impairment of a memory, did show an intriguing nuance of the role GluA2-AMPAR plays in storage impairment. These data suggest that the relationship between GluA2-AMPAR internalization from the postsynaptic membrane and memory loss is more complex than initially hypothesized.

2.2. *Abstract*

Blocking PKMζ, a kinase critical for maintaining long-term memory, with infusions of the peptide ZIP has been shown to impair long-term memory. Behavioural studies historically have had difficulty in differentiating if such experimentally-induced amnesia is caused by a storage impairment (the memory is no longer available due to loss of neural correlates) or a retrieval impairment (the memory is available, but not accessible). To test this, we employed a protocol that takes advantage of the indifference of object location learning to *N*-methyl-D-aspartate receptor (NMDAR) activation after prior experience with the task. We showed that a ZIP-induced impairment of a memory leads a subsequently learned task to require NMDARs for acquisition, thus suggesting that the initial memory trace experienced damage to its storage. Western blot analysis revealed that this storage loss of the memory trace did not produce a correlated decrease of postsynaptic GluA2-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (GluA2-AMPARs). Rather, ZIP-infused animals were observed to have less surface GluA2-AMPAR expression after they showed strong memory at test. This indicates a more nuanced relationship between GluA2-AMPARs and memory loss than has previously been reported.

2.3. *Introduction*

Mechanisms of memory maintenance are beginning to be understood. In contrast, there is less knowledge on how memories are lost. Disrupting the mechanisms and molecular substrates that are implicated in long-term memory (LTM) storage can result in forgetting, but the rates at which these substrates change in relation to memory loss, and if these patterns of change are similar across different situations of forgetting are unknown. There are many types of forgetting (Davis & Zhong, 2017), and although functionally, the end result is the same, the causes of forgetting might vary. One way to characterize how molecules necessary for memory maintenance are affected by different conditions of memory loss is to examine cases of experimental amnesia.

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are glutamate receptors that are thought to mediate fast synaptic transmission in the neuron. They have been shown to have an integral role in learning and memory mechanisms and long-term potentiation (LTP). In particular, GluA2-containing AMPARs (GluA2-AMPARs) seem to be a key player in memory maintenance. Compared to the other AMPAR subunits (GluA1-GluA4), GluA2-AMPARs are calcium-impermeable, and display unique properties compared to calcium-permeable AMPARs (CP-AMPARs) (Hollmann, Hartley, & Heinemann, 1991; Malinow & Malenka, 2002). Unlike activity-dependent GluA1-containing AMPARs insertion into the postsynaptic density, GluA2/GluA3 AMPARs can be inserted independent of activity (Y. Hayashi et al., 2000; Isaac, Ashby, & McBain, 2007; Shi et al., 2001), and loss of GluA2-AMPARs from the synaptic fraction leads to destabilization of a memory (Hong et al., 2013). Indeed, many studies have shown that endocytosis of GluA2-AMPARs from the synaptic surface

underlies LTP disruption (Dong et al., 2015) and induction of long-term depression (LTD) (C.-H. Kim & Lisman, 2001; Luthi et al., 1999; Steinberg, Huganir, & Linden, 2004).

Studies have shown that the chaperone protein *N*-ethylmaleimide-sensitive factor (NSF) binds with and traffics GluA2-AMPARs to the postsynaptic membrane (Nishimune et al., 1998; Noel et al., 1999; I. Song et al., 1998). Disruption of the GluA2-NSF complex allows endocytosis of surface GluA2-AMPARs, which results in LTP decay (Dong et al., 2015) or long-term depression (Luthi et al., 1999; Steinberg et al., 2004). Blocking this disruption with infusions of synthetic peptide $GluA2_{3Y}$ can rescue LTP decay in the amygdala and hippocampus (Dong et al., 2015; Rao-Ruiz et al., 2011; Sacktor et al., 2011). Blocking GluA2-AMPAR endocytosis can also extend the lifetime of an object location memory (Migues et al., 2016). Because of this putative role in memory maintenance and memory loss, it follows that there might exist a quantitative relationship between amount of GluA2-AMPAR expression in the postsynaptic density (PSD) and memory, such that upregulation of GluA2-AMPARs are correlated with a retained memory and downregulation of GluA2-AMPARs are correlated with a lost memory. However, it is unclear how levels of GluA2-AMPARs change when a memory is lost. Therefore, we aim to characterize levels of GluA2-AMPAR expression associated with the loss of an object location memory trace.

One method to experimentally produce LTM erasure is to block PKMζ, a constitutively-active protein kinase that is thought to uniquely mediate LTM maintenance (Migues et al., 2010; Serrano et al., 2008; Shema, Sacktor, & Dudai, 2007). PKMζ interacts with the GluA2-NSF complex to maintain high levels of GluA2-AMPARs in the postsynaptic membrane inserted after LTP induction (Sacktor, 2011). Inhibiting activity of PKMζ with application of zeta-inhibitory peptide (ZIP) has been shown to block LTM across multiple different paradigms and species

(Kwapis & Helmstetter, 2014). Thus, we can induce amnesia through infusions of ZIP and compare how GluA2-AMPAR expression in the PSD of neurons recruited in the memory changes in comparison to GluA2-AMPAR levels of a retained memory.

Observation of postsynaptic GluA2-AMPAR endocytosis also allows us to address a theoretical question about the nature of amnesia. One model to conceptualize memory loss attributes amnesia to either of two general mechanisms: a storage impairment, where a memory trace is rendered unavailable, or a retrieval impairment, when a memory is inaccessible (Nader & Hardt, 2009). Historically it has been difficult to deduce the source of amnesia through only behavioural testing (Gold & King, 1974). With the onset of increasingly advanced quantification and imaging techniques, it is possible to measure changes in neural correlates of a memory trace in order to deduce its cause. However, behavioural and molecular data have not yet been reconciled to show the cause of experimental amnesia, and it remains unclear how GluA2-AMPAR endocytosis fits within that framework.

We are able to behaviourally differentiate experimental amnesia caused by storage or retrieval impairment by taking advantage of the fact that experience with a specific task changes how the brain forms new memories of similar experiences. Bannerman et al. (1995) showed that *N*-methyl-D-aspartate receptors (NMDARs) were required when rats acquired a spatial memory in the Morris water maze task, but not when rats were taught another spatial memory task in a different water maze. Acquisition of this second-learned memory was not affected by NMDAR-blockade, suggesting that unlike naïve learning, second learning acquisition mechanisms were NMDAR-independent. NMDAR-independent learning has also been shown for contextual fear learning (Crestani et al., 2018; Hardt et al., 2011; Tayler et al., 2011; Wiltgen et al., 2011). This opposing reliance on NMDARs allows inference of the nature of amnesia of a first-learned

memory by evaluating if acquisition of the second-learned memory is affected by NMDAR-impairment. If the storage of the first learning memory is impaired, a subsequently-learned memory will be acquired as if it is the first time the animal encountered such training, and thus rely on NMDAR activation. Alternatively, if retrieval of the first learning memory is impaired but storage of the memory trace is largely unaltered, subsequent learning should not be treated as naïve learning, and thus not require NMDAR-mediated acquisition. This paradigm therefore allows formation of a positive prediction about the nature of the amnesic intervention given to the first learning memory.

Additionally, the second learning (L2) protocol allows investigating multiple situations of memory loss within the same experiment. By having first learning (L1) memory retained or erased, then having the animal learn second learning or not, four situations will result in which GluA2-AMPAR expression can be measured: L1-L2, no L1-L2, L1-no L2, and no L1-no L2. This allows, for example, comparison of GluA2-AMPAR expression after erasure of first learning but impaired second learning, with erasure of first learning and successful second learning. In sum, changes in GluA2-AMPAR expression in the PSD will be corroborated with behavioural results to allow us to identify how ZIP-induced amnesia affects the memory on a molecular level.

Our behavioral results showed that ZIP-induced impairment of a first learning memory renders the second learning NMDAR-dependent, strongly suggesting that ZIP causes amnesia through storage impairment. Correlating molecular results with behaviour, however, was inconclusive, as we found different interpretations might apply to the data.

2.4. *Methods*

2.4.1. Animal subjects

Male Long Evans rats (received at the weight of 275g-325g; Charles River) were housed in pairs in rectangular polyethylene cages with a PVC tube for environmental enrichment. Rats had *ad libitum* access to food and water. Lights in the colony were turned on at 7 a.m. and turned off at 7 p.m. daily. Behavioural studies were always performed between 9 a.m. and 5 p.m. All procedures were approved by the McGill University Animal Care and Use Committee, and complied with the Canadian Council on Animal Care guidelines.

2.4.2. Surgeries

Rats were anaesthetized with a solution of Ketamine (50 mg/ml), Xylazine (3 mg/ml), and Dexdomitor (0.175 mg/mL), injected intraperitoneally (i.p.) at a volume of 1 ml/kg. Bilateral cannulations in the dorsal hippocampus were then performed with coordinates based on the rat atlas (Paxinos and Watson). 22-gauge steel cannula were implanted using a Kopf stereotaxic frame (coordinates from bregma: A/P -3.6 mm, M/L +/- 3.1 mm, D/V -2.4 mm, 10° away from midline). Dental cement was adhered to the skull to stabilize the cannula. After surgeries, rats were administered an analgesic (Carprofen 5 mg/ml, subcutaneous at 1 ml/kg) and then anesthesia was reversed with Antisedan (0.5 mg/ml, i.p. at 0.66ml/kg). A week of recovery time was given, with regular handling throughout, before the experiment began.

2.4.3. Apparatus

Two open field contexts were set up in separate rooms.

Open Field A was set up in a windowless room (context room A; area 15.85 m^2, 3.98 m × 3.98 m × 2.65 m) with dim lighting. The floor level of the open field was illuminated with, on average, 13 lux. The open field was a circular area made with clear Plexiglas walls, with a total

area of 3600 cm^2. The height of the walls measured 60 cm. A strip of black paper covered the lower 15 cm of each Plexiglas panel in order to minimize distraction of rats peering through the Plexiglas. One Plexiglas panel was labeled with a black and white striped pattern to act as a disambiguating cue. Three objects were set up at northwest (NW), north (N), and northeast (NE) locations, with north arbitrarily assigned to the corner of the room opposite to the door. NE and NW objects were identical, N objects were of the same height and texture but different colour and shape. Object combinations were counterbalanced between rats. Bedding was made up of a 1:1 dried corn and woodchips mix. Flooring was made of a wooden pegboard into which objects could be screwed in and out and fastened securely. A camera hung 50 cm above the context. Behavioural testing occurred with the door closed, and the experimenter outside of the room.

Open Field B was set up to be in a distinctly different context compared to Open Field A to facilitate second learning as a separate experience. It was placed in a cubicle-sized, windowless room (context room B; 2.13 m^2, 2.13 m × 1 m × 2.65 m), lit brightly. The floor level of the open field was illuminated with, on average, 201 lux. The open field was a square area made with thin plywood walls, with a total area of 3600 cm^2 (60 cm × 60 cm × 60 cm). The bedding was made of woodchips. A black and white striped disambiguating cue was taped at the top of one of the walls. Two identical objects were located at N and S locations within the open field, and were of similar height but different size and shape to the objects from Open Field A. Objects were counterbalanced across rats, such that all object combinations across both open fields were experienced by comparable numbers of rats. A camera hung 80 cm above the context. Behavioural testing occurred with room curtains pulled closed and the experimenter outside of the room.

2.4.4. Drug Infusions

PKMζ inhibitor ZIP and inactive, scrambled ZIP (Eurogentec) were each mixed with TRIS-buffered saline (pH 7.0) at 10 nmol/µl and infused bilaterally into the dHPC (1.0 µl/hemisphere at 0.25 µl/min) with a microinfusion pump. The NMDAR-antagonist D-AP5 (Santa Cruz Biotechnology) was mixed with phosphate buffered saline (PBS, pH 7.4) at 5µg/µl and infused bilaterally into the dHPC (1.0 µl/hemisphere at 0.25 µl/min). Injector tips remained in the cannula for 60 seconds after infusions completed in order to ensure complete diffusion of the drug. Placements were checked at the time of tissue sample collection for western blots.

2.4.5. Behavioural procedure

2.4.5.1. First learning

Rats were brought to the context room (context B in experiment 1; A in all other experiments) and left to rest for 20 minutes before training. As habituation could be construed as a learning experience, no habituation sessions were given; rats proceeded directly to first learning (L1). L1 training sessions consisted of daily 10-minute free exploration trials across seven days in Open Field A or twice daily for 2 days in Open Field B. Rats were lowered into the open field at a different corner (Open Field B) or quadrant (Open Field A) of the open field each day (SE, SW, NW, NE), with their noses pointed away from the centre of the field to allow the rat to self-orient towards the objects.

After each training session, rats were removed from the open field and returned to their home cage. They were allowed to rest for 20 minutes following the conclusion of training of all rats before being returned to the colony. To ensure that rats experienced equal amounts of rest time across experimental days, the order of training was alternated daily (i.e. rats were trained in backwards order every other day). Rats received either bilateral AP5 or vehicle infusions prior to

each training session (Experiment 1), no infusions prior to training (Experiment 2 and 3), or ZIP or scrambled ZIP infusions 24 hours after completion of all training sessions (Experiment 4).

Following the conclusion of first learning training (Experiments 1, 2, 3) or following ZIP/Scr-ZIP infusions (Experiment 4), rats either proceeded with a first learning test 48 hours later (Experiment 1) or 2, 5, or 7 days (Experiment 2) later, or received a second learning protocol (L2) 48 hours (Experiment 3) or 24 hours (Experiment 4) later.

For a test session performed in Open Field A, the middle object in the open field was moved across the floor. The other two objects remained in the same position. Rats were placed in the centre of the open field, facing away from all three objects and allowed to explore for 3 minutes.

For at a test session in Open Field B, one of the objects was moved to a corner of the open field. The other object remained in the same position. Rats were placed in a corner of the open field that was equidistant from both objects, facing the walls and allowed to explore for 3 minutes.

2.4.5.2. Second learning

Second learning training (L2) occurred in experiments 3 and 4 only. Rats were brought to context room B and left to rest for 20 minutes before training. Same as above, rats were lowered into the open field at a different quadrant of the open field each day (SE, SW, NW, NE), with their noses pointed away from the centre of the field to allow the rat to self-orient towards the objects.

L2 consisted of 10-min exploration sessions in Open Field B twice daily for 2 days. Bilateral infusions of AP5 or vehicle were administered immediately prior to each training session. After training, rats were returned to their home cage. Twenty minutes after completion

of training of all rats, rats were brought back to the colony room. Four infusions and training sessions in total occurred across two days, with each morning and afternoon session separated by a minimum of 4 hours during which rats rested in the colony.

Forty-eight hours after the final session, rats were tested for second learning memory in Open Field B, where one of the objects was moved to a corner of the open field. The other object remained in the same position. Rats were placed in a corner of the open field that was equidistant from both objects, facing the walls and allowed to explore for 3 minutes, after which rats were returned to their home cage. After all rats were tested, rats were returned to the colony room.

2.4.6. Sacrifice

Twenty-four hours after testing, rats were sacrificed for western blot analysis. Rats were anaesthetized with isoflurane then decapitated. Brains were removed and flash frozen over dry ice, then preserved in a -80°C freezer.

2.4.7. Behavioural scoring

Behavioral videos were scored manually. Exploratory behaviour was diagnosed when the rat showed directed interest in the object, e.g. smelling from at most 3 cm away from the object, touching, climbing into the object. Rats climbing on top of the object was considered exploratory activity, but while there, usage of the object as a platform to explore the walls of the context was not included in measuring object exploration. Scoring began immediately prior to the first instance of directed interest towards the object, and continued for 30 seconds.

Novel object location preference (novelty ratio) for first learning (in Open Field A) was calculated with the following formula:

$$\frac{time\ exploring\ new\ location)}{time\ exploring\ new\ location + (\frac{time\ spent\ exploring\ old\ location\ 1 + old\ location\ 2}{2})}$$

Novelty ratio for second learning (in Open Field B) was calculated with the following formula:

$$\frac{time\ exploring\ new\ location}{total\ time\ exploring}$$

Open Field B had two objects, therefore time spent exploring each object location had an equal weight of 50% of the total time exploring (equation denominator). However, because Open Field A had 3 objects, the time spent with the object at the new location would be underweighted against the other two objects (33% versus 66%). To account for this, the total time spent exploring the two objects at familiar locations was averaged, thus exploration of the two familiar locations was treated as exploration of one object. In this way, time spent at the new location and the familiar locations would be weighed equally, allowing use of the same ratio scale in equations involving both two or three open field objects.

For both novelty ratio calculations, a value of 0.5 indicates equal preference for both objects or no preference for either object, while a value significantly higher than 0.5 indicates a significant preference for the new object location. Because rats prefer novelty over familiarity (Berlyne & Slater, 1957; A. Ennaceur & Delacour, 1988), this behavior suggests that they took notable interest in the new object location, suggesting that they retained the memory of the previous object locations. Therefore, a high novelty ratio implies a strong memory, and a no preference ratio of 0.5 implies weak or impaired memory. A low novelty ratio indicates preference for the old object location, and thus insufficient performance for the trained memory. To determine significant preference for the object at the new location in Experiments 1 to 3, t-tests were run comparing group novelty ratio means to an estimated mean of 0.5. For Experiment 4, a one-way ANOVA was used to probe for group differences across four treatment groups.

Furthermore, western blot data of Experiment 4 was analyzed with behavioural data using a one-way ANOVA and Pearson's correlation. Analyses were completed with SPSS and JMP software.

2.4.8. Biochemistry

2.4.8.1. Subcellular fractionation for postsynaptic density

The dorsal hippocampus CA1 region was dissected from frozen brains with a 1 mm neuro punch (Fine Science Tools), and homogenized in cold Tris-HCl buffer (30 mM, pH 7.4) containing 4 mM EDTA, 1 mM EGTA and a cocktail of protease inhibitors (Complete, Roche). The hippocampus homogenates were then centrifuged at 4°C at 500 g for 10 min to remove nuclei and other debris. The supernatants were removed and centrifuged at 100,000 g at 4°C for 60 min. Resulting pellets were resuspended in a solution of the homogenization buffer and 0.5% Triton X-100 and incubated on ice for 20 min, then layered over 1 M sucrose. This was centrifuged again at 100,000 g for 60 min, allowing detergent-soluble membrane components and extra-synaptic receptors to remain in the Triton-soluble fraction. The remaining Triton-insoluble materials were pulled down into the sucrose layer, where proteins of the postsynaptic densities also lay. This final pellet was resuspended in homogenization buffer and stored at -80°C. Total protein concentration was determined with the BCA protein assay kit (Pierce).

2.4.8.2. Western blots

Western blots were performed using 8% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes overnight. Membranes were washed with 2% BSA in TBS-Tween for 1 hour at room temperature, then incubated with polyclonal antibodies to GluA2 (1 mg/ml, Millipore; dilution 1/2000) and monoclonal β-actin (2 mg/ml, Sigma; 1/10,000) for 20 hours at 4°C. Membranes were washed with TBS-Tween and incubated in rabbit horseradish antibody (GluA2, NR2B; GE Healthcare UK; 1/10,000) or mouse horseradish

antibody (actin; GE Healthcare UK; 1/10,000) at room temperature for 1 hour. Detection of bands was completed with Pierce ECL2 western blotting substrate (ThermoScientific). Membrane blots were scanned with a Storm Laser Scanner (Molecular Dynamics) and analyzed with ImageQuant software (ABI).

2.5. *Results*

2.5.1. Experiment 1: First learning of an object location task requires NMDARs for acquisition

To confirm the requirement of NMDARs in acquisition of a novel object location memory, rats were infused with either AP5, an NMDAR antagonist, or its vehicle, phosphate buffered saline (Veh), in the dHPC immediately prior to 4 training sessions in Open Field B across 2 days. To ensure that the rats would be naïve during the spatial task and thus experience a first learning of spatial locations, they were not habituated before training (Figure 1A).

Mean group novelty ratio scores were compared against a hypothesized mean of 0.5 (no preference) to determine preference for the new object location, which indicated the strength of memory expression. Because mean novelty ratios often varied from between 0.5 to 1.0, significant group differences groups may not be detected despite groups differing on their relation to the 0.5 ratio, therefore differences between groups were irrelevant for our analyses of whether memory was expressed or not. At the probe test, AP5-infused rats expressed no novelty preference for either object (NR=0.52 ± 0.12, $t<1$; data presented as mean novelty ratio ± s.e.m.), while Veh-infused rats showed significantly more preference to the novel object location (mean NR =0.76 ± 0.09, $t_{(8)}=2.77$, $P=0.024$) (Figure 1B). We conclude that learning of an object location task is sensitive to AP5-impairment, and thus is dependent on NMDAR activation.

2.5.2. Experiment 2: First learning object location memory lasts longer than a week

Before proceeding with a second learning protocol, we wanted to verify that the first learning memory would be retained throughout the duration of an added second learning protocol. Therefore, we tested for novelty preference of learning in Open Field A after varying retention times in different groups of rats (Figure 2A). Separate groups of rats were trained with first learning, and then tested in Open Field A 2, 5, or 7 days following the final training session. The memory was found to persist even 7 days post-training (Figure 2B). This suggests that under regular conditions, second learning in Open Field B in our protocol within a week from the first learning will indeed be acquired on the background of an available and accessible memory for the first-learning of object locations in Open Field A.

2.5.3. Experiment 3: Second learning of an object location task does not require NMDARs for acquisition

To confirm that acquisition of a second learning memory does not require NMDAR activation, rats were given 7 daily sessions of 10-min training in Open Field A, followed the next day with 2 days of training with AP5 or Veh infusions in Open Field B as in Experiment 2. After completion of second learning training in Open Field B, rats were given 2 days of rest before the 3-min probe test in Open Field B (Figure 3A).

At test, both groups showed significant preference for the new object location in open field 2 (Veh-infused rats: NR=0.75 ± 0.10, $t_{(5)}$=2.61, P=0.047; AP5-infused rats: NR=0.77 ± 0.09, $t_{(5)}$=3.22, P=0.023), showing that AP5 affected performance at second learning test. We conclude that NMDAR activation is not needed for acquisition of second learning of object location memory (Figure 3B).

2.5.4. Experiment 4: ZIP impairment of first learning memory renders second learning NMDAR-dependent

To test for the nature of amnesia induced by disruption of the first learning, rats were infused with the PKMζ inhibitor ZIP or the inactive form, scrambled ZIP (Scr), 24 hours after first training in Open Field A. One day later, rats were given a second learning protocol as in Experiment 3, with AP5 or Veh infusions preceding four object location training sessions. 48 hours after second learning training, rats were returned to Open Field B to test their second learning memory (Figure 4A). This created a total of four experimental groups, ZIP-AP5, ZIP-Veh, Scr-AP5, and Scr-Veh, of which the ZIP-AP5 group was predicted to have impaired performance at second learning test (Table 1).

Table 1. Behavioural hypotheses of the four experimental groups based on ZIP or scrambled ZIP (Scr) infusions following first learning training, and AP5 or vehicle (Veh) infusions prior to second learning training.

		Infusions after first learning	
		ZIP	**Scr**
Infusions prior to second learning	**AP5**	Impaired first learning memory; second learning acquisition is AP5-sensitive. Second learning test performance is poor.	Non-impaired first learning memory; second learning acquisition is AP5-insensitive. Second learning test performance is good.
	Veh	Impaired first learning memory; second learning acquisition is unaffected by Veh. Second learning test performance is good.	Non-impaired first learning memory; second learning acquisition is unaffected by Veh. Second learning test performance is good.

As predicted, rats from the ZIP-AP5 group showed no preference for the object at the new location at second learning test (NR=.51 ± .06, t<1), indicating they had no second learning memory (Figure 4B). All other groups did show significant novel location preference (ZIP-Veh NR=.85 ± .14, $t_{(3)}$=2.41, P=.048; Scr-Veh NR=.69 ± .09, $t_{(5)}$=2.09, P=.046; Scr-AP5 NR=.72 ± .09, $t_{(6)}$=2.33, P=.029), indicating rats had acquired and retained the second learning memory. A one-way ANOVA showed no significant difference between group means; however, as stated above, the comparison of interest is the difference from 0.5 ratio, and significant group differences in novelty ratio are not required to determine whether the memory exists or not within separate treatment groups. There was no significant difference in time spent exploring across all four groups, suggesting no treatment effects on motility or motivation to explore.

These results suggest that both Scr-AP5 and Scr-Veh rats had unimpaired first learning and therefore NMDAR-independent second learning, indicated by significant preference for the new object location at the second learning probe test (high novelty ratio). ZIP-Veh animals also showed unimpaired second learning memory through significant object location preference at the second learning probe test. However, ZIP-AP5 rats displayed no object location preference at test. This indicates that ZIP treatment impaired storage of the first learning memory trace in a way that rendered second learning acquisition NMDAR-dependent. Thus, ZIP-AP5 rats acquired the second learning experience as if it was a naïve learning experience.

2.5.5. GluA2-AMPAR expression level is negatively correlated with novelty ratio score following ZIP-induced impairment

Because ZIP-AP5 animals had, on average, weaker memory of the second learning experience than other groups, this might also have been reflected in a lower level of GluA2-AMPAR expression compared to the other groups. To verify this, we probed for GluA2-AMPAR

expression in PSD fractions of dHPC CA1 neurons in rats from Experiment 4 (Figure 5A). We isolated the PSD of neurons in the CA1 hippocampus 24 hours after second learning test and probed for levels of GluA2-AMPARs, standardized to β-actin as loading control. As there was no hypothesized mean from which to compare group means, group means were compared to each other. Levene's test for the homogeneity of variance was significant ($F_{(3,20)}$=3.21, P=.450), therefore we used a non-parametric test to determine differences between groups. A Kruskal-Wallis test found no significant difference in β-actin expression levels across the 4 experimental groups ($\chi^2_{(3)}$=5.25, P=.154). When average GluA2-AMPAR levels of each group was set relative to the Scr-Veh group, we also found no significant difference in GluA2-AMPAR levels across groups ($\chi^2_{(3)}$=4.87, P=.182; Figure 5B).

Due to variation of behaviour within groups, we thought it was possible to see a correlation of GluA2-AMPAR expression in relation to performance (novelty ratio score) at an individual level. Therefore, we correlated the novelty ratio score and relative GluA2-AMPAR expression of all samples (Figure 6). If GluA2-AMPAR expression is associated with memory expression at test, we would anticipate low levels of GluA2-AMPARs to be correlated to low performance at test (i.e. novelty ratio score close to 0.5), and high levels of GluA2-AMPARs to strong memory performance. Contrary to our hypothesis, we found a significant *negative* correlation of GluA2-AMPAR expression to novelty ratio score ($r_{(22)}$=-.62 ± .06, P=.001), indicating that the less GluA2-AMPARs were found in the PSD, the higher the novelty ratio score of the animal. We did not see any significant correlation between GluA2-AMPAR expression and amount of time spent exploring ($r_{(22)}$=-.19 ± 1.33, P=.384).

To further understand this correlation, we separated these results by group. In Scr-AP5 and Scr-Veh rats, the correlation between GluA2-AMPAR expression and novelty ratio was non-

significant. However, it was significant for ZIP-AP5 ($r_{(4)}$=-.90 ± .04, P=.016) and ZIP-Veh rats ($r_{(2)}$=-.97 ± .04, P=.026) (Figure 7). The data suggested that ZIP infusions after first learning memory led to a negative correlation of GluA2-AMPARs and memory expression at second learning test, which contradicted our original hypothesis.

Overall, the data showed that infusing ZIP into the dHPC after animals had acquired object location memory causes a memory storage impairment, leading to second learning being NMDAR-dependent. Furthermore, a correlation between memory expression during second learning test and GluA2-AMPAR expression in the CA1 dHPC was seen in ZIP-infused animals.

2.6. *Discussion*

Much progress has been made to understand how memories are retained, especially since the discovery that the protein kinase PKMζ seems to be uniquely critical for maintaining LTP and long-term memories (Sacktor, 2011). A PKMζ-mediated mechanism is hypothesized to retain GluA2-containing AMPARs (GluA2-AMPARs) at the post-synaptic density (PSD) of neurons recruited during memory formation (Migues et al., 2010; Migues et al., 2014; Migues et al., 2016). Removal of GluA2-AMPARs has been associated with the decay of long-term potentiation (LTP) and long-term memory (LTM) (Rao-Ruiz et al., 2011, Dong et al., 2013). Here, we aimed to quantify the percentage of GluA2-AMPARs expression in the PSD of CA1 neurons is associated with maintenance of an object location memory, compared to how much is internalized when the memory is erased.

Utilizing a second learning paradigm, we showed behavioural evidence that ZIP-induced erasure of a first learning object location memory renders subsequent learning of the location of different objects in a different context dependent on NMDARs. This supports the view that ZIP

impairs the storage of memory representations related to the first learning of object locations. Indeed, recent work shows that retrieving a conditioned taste aversion memory after ZIP infusions interrupts the amnesic effects of ZIP, but effects can once again be induced after the memory has reconsolidated, suggesting that maintenance processes involving ZIP are mutually exclusive from retrieval processes (Levitan et al., 2016). This supports the effects of ZIP in impairing memory maintenance only. Our behavioural data is in line with existing literature on the effects of ZIP impairment, which have extensively shown that ZIP disrupts LTM in the hippocampus (see Sacktor and Fenton, 2011, for a list). The other experimental groups did not show AP5-sensitivity at second learning test (Figure 4B), indicated by their preference for the object in a new location within the open field. These results add to the growing literature of NMDAR-independent learning (Bannerman et al., 1995; Crestani et al., 2018; Hardt et al., 2011; Tayler et al., 2011; Wiltgen et al., 2011). The process of initial learning experience affecting future learning may be an example of metaplasticity, which is the plasticity of synaptic plasticity (Abraham and Bear, 1996). NMDAR-independent object location learning is one example of affecting future synaptic plasticity through behavioural methods (see Parsons, 2017, for a brief review). Metaplasticity may provide a means to interpret the observed molecular data, as contrary to expectations, our findings suggested that the memory impairment induced by ZIP did not correlate with a decrease of postsynaptic GluA2-AMPAR expression.

We originally hypothesized that the biochemical analyses would complement the behavioural results. Therefore, the relative expression of postsynaptic GluA2-AMPARs quantified from the ZIP-AP5 group would be less than levels found in the groups ZIP-Veh, Scr-AP5, and Scr-Veh. We anticipated that the Scr-Veh group, which experienced no impairment for either first or second learning memory, would show the highest relative GluA2-AMPAR

expression, and the levels seen in Scr-AP5 and Scr-Veh groups would lie in between these two points. However, our electrophoretic analysis found no such differences between groups, preventing us from drawing conclusions about the relationship of GluA2-AMPAR expression and memory performance during the test probing for memory of the second learning.

The lack of group differences in average GluA2-AMPAR expression levels may reflect lack of differences in memory expression at test (i.e. novelty ratio), or that the GluA2-AMPAR signalling in the dHPC was too small to be adequately detected with western blots. However, even if no significant group differences were found, we hypothesized that the GluA2-AMPAR expression of the ZIP-AP5 group would be clearly lower than those of the other three treatment groups. Instead, the Scr-AP5 showed the lowest expression of the four groups, suggesting that the relationship between GluA2-AMPAR and novelty ratio score was different than expected. Indeed, when we specifically correlated individual behavioural scores and GluA2-AMPAR expression, surprisingly, we found a negative correlation: the data suggest that larger amounts GluA2-AMPAR expression are associated with low novelty ratio (NR) scores, which reflect a tendency to explore the object at the old, familiar location. This pattern in ZIP-infused rats is counterintuitive, as we would expect low NR (no object location memory) to correlate with lower GluA2-AMPAR levels compared to animals with high NR, or, based on our above data, no difference in GluA2-AMPAR expression between low and high NR animals. One explanation for this data could be that for ZIP-AP5 rats, the test session acted as a new learning trial, which was more salient because this group had previous learning erased by ZIP infusions. It might be that in ZIP-treated animals, a significant amount of memory in the hippocampus was erased, thus lowering background noise, thereby allowing subsequent learning—whether in the form of the

second learning training sessions (ZIP-Veh group) or the second learning test session (ZIP-AP5 group)—to be detected by western blot.

Why impairing the storage of an initially learned object location memory leads to upregulated GluA2-AMPARs when rats preferentially explore an old object location (ZIP-Veh group) remains unclear, but may reflect a lack of specificity in hippocampal place fields. Place fields, specific environmental locations to which place cells in the hippocampus preferentially respond (O'Keefe & Dostrovsky, 1971), underlie object location association learning (Eacott & Norman, 2004; Komorowski, Manns, & Eichenbaum, 2009; Manns & Eichenbaum, 2009). Larger place fields are associated with decreased specificity of spatial representation (Royer, Sirota, Patel, & Buzsáki, 2010) and more hippocampal cells are likely to be recruited for larger place fields (Fenton et al., 2008). There is evidence that infusing ZIP while place fields are being expressed will decrease place field specificity (Barry et al., 2012). Thus, rats that did not show memory expression at test and preferred exploring the old object location might have less specific place fields, which might recruit more neurons and thereby will involve more GluA2-AMPAR expression compared to rats who did prefer the novel object location. However, this would not apply to ZIP-AP5 rats, as NMDAR antagonism in the hippocampus can disrupt place field stability and expansion (Ekstrom, Meltzer, McNaughton, & Barnes, 2001; Kentros et al., 1998).

Alternatively, the lower GluA2-AMPAR levels seen in Scr-Veh and Scr-AP5 rats expressing normal novelty preference may be indicative of a more efficient learning mechanism, employed during the second training experience. This could suggest that the first learning experience primed future learning in a metaplastic-like manner, despite ZIP treatment, in a similar way to how reminder cues can boost weak memories that were incompletely erased.

These post-amnesia cues can enhance weak performance levels to match performance of non-amnesic animals because they essentially act as a new learning session (Gold & King, 1973). In this interpretation, ZIP-Veh rats showing higher GluA2-AMPAR expression after preference for the old object location would reflect coarseness of the memory representation. But it would not explain why high levels GluA2-AMPAR expression in ZIP-AP5 rats correlated with old location preference, as in this group, only the test session could induce a learning experience, and there was no post-test recall experience to examine resultant novelty preference, if any.

Considering multiple but not fully comprehensive explanations for our data, it might be informative in future studies to use techniques outside of western blots to analyse changes in GluA2-AMPAR expression based on object location memory loss. The behavioural results showed that NMDAR-independent second learning resulted from a storage impairment of the first learning memory, and as the literature strongly supports the role of GluA2-AMPAR internalization in LTM impairment, a more sensitive technique may reveal a more accurate characterization of how GluA2-AMPAR expression changes due to the second learning paradigm. Labeling hippocampal cells recruited for first and second learning can shed light on how neural ensembles change and overlap with increasing or decreasing memory expression, as well as if ZIP infusions leave any residual memory substrates from the first learning experience that can affect second learning efficiency.

2.7. *Conclusion*

The behavioural findings resulting from the second learning paradigm have allowed a positive prediction of the nature of ZIP-induced long-term memory impairment. Although the literature suggests that this storage loss would be reflected in molecular data of postsynaptic

GluA2-AMPAR endocytosis at the dorsal hippocampus CA1, the data did not support this hypothesis. Making conclusions from the data presented is complex and opens many more avenues of study, but at the same time should be seen with a skeptical eye, as our data seem to indicate inherent experimental limits in quantifying the relationship between object location memory loss and GluA2-AMPAR expression as we have here. Future research will help untangle the exact nature of the correlation. Nonetheless, our behavioral data strongly support the view that PKMζ inhibition leads to storage impairment of a memory trace, and that unimpaired first learning object location memory leads to NMDAR-independency of subsequent learning of the same task in a different context.

2.8. *Figures*

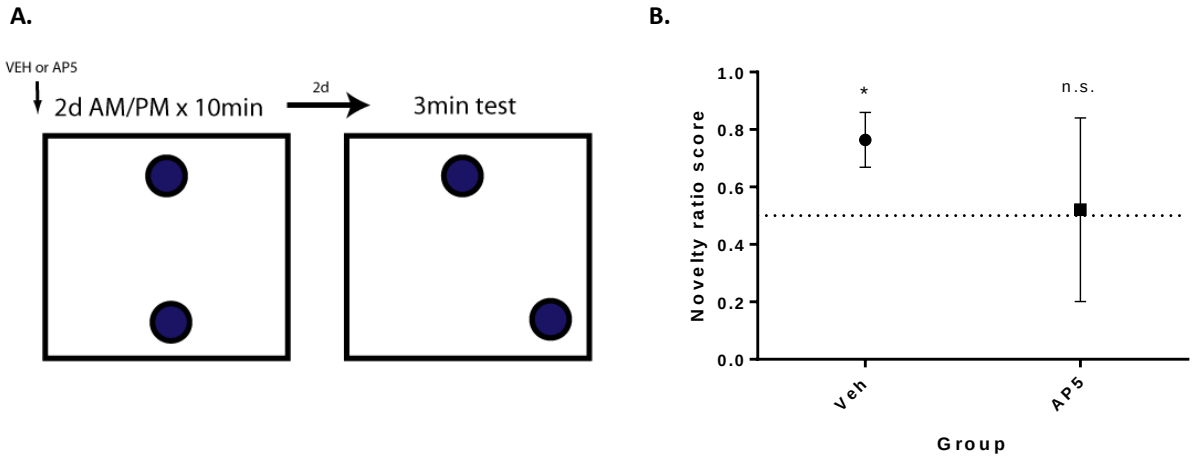

Figure 1. Acquisition of an object location memory for the first time is NMDAR-dependent. *A)* Experimental design. Rats explored an open field for 10 min/day across 2 days, with infusions of AP5 or vehicle (PBS) prior to each session. Forty-eight hours later rats were given a 3-min test session where novel object location recognition was assessed. *B)* Infusions of AP5 blocked first learning. At test, performance of AP5-infused rats was shown to have no location preference compared with vehicle controls, who did show preference for the novel object location. n=9 and 7 for Veh and AP5 groups, respectively. Data are expressed as the mean ± s.e.m. *P<0.05 indicates significance from 0.5, no preference (dotted line).

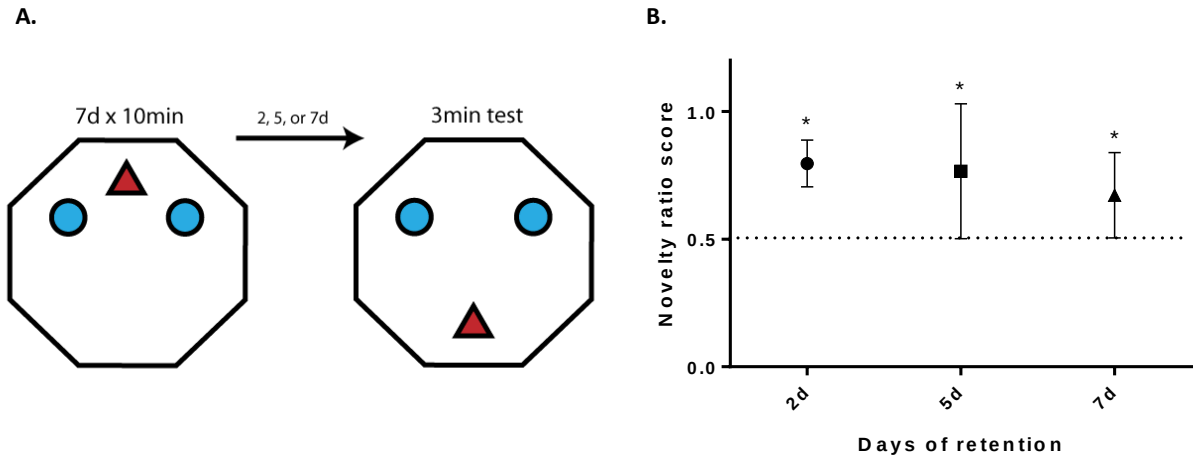

Figure 2. First learning memory of object location lasts up to 7 days post-training. *A)* Experimental design. Rats were tested for 10 min/day across 7 days in Open Field A. Rats then remained in their colony for different durations of time (2, 5, 7 days) before given a 3-minute probe test. B) Rats tested for novel object preference after 2 (NR=0.8 ± 0.09, $t_{(5)}$=3.24, P=0.230), 5 (NR=0.77 ± 0.09, $t_{(5)}$=2.61, P=0.470), or 7 (NR=0.67 ± 0.06, $t_{(8)}$=3.08, P=0.015) days of retention post-training all showed consistent novel object location recognition even as retention time increased. Data are expressed as the mean ± s.e.m. *P<0.05 indicates significance from 0.5, no preference (dotted line).

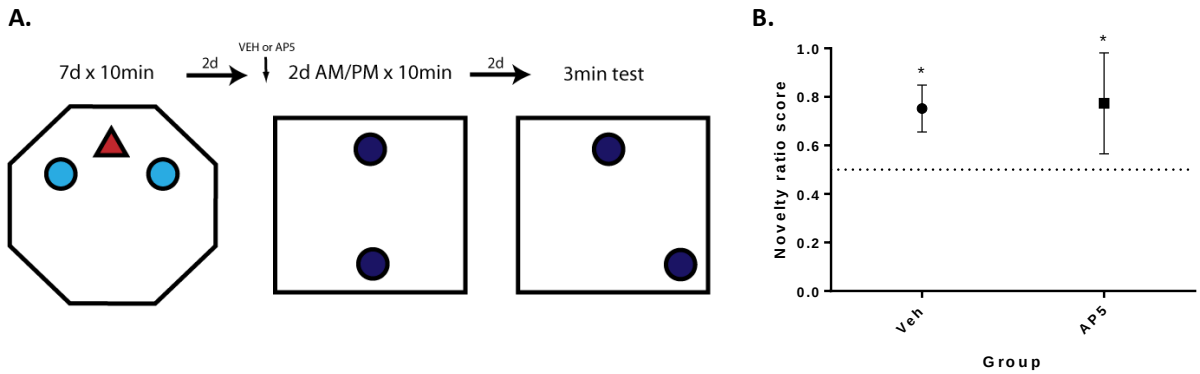

Figure 3. Acquisition of an object location memory after prior experience to the task is NMDAR-independent. *A)* Experimental design. Rats explored Open Field A for 10 min/day across 7 days. One day later, rats explored a different open field for 10 min/day across 2 days, with infusions of AP5 or vehicle (PBS) prior to each session. Forty-eight hours later rats were given a 3-min test session where novel object location recognition was assessed. B) Infusions of AP5 did not affect second learning. At the second learning test, performance of both AP5- and vehicle-infused rats indicated significant preference to the novel object location. Data are expressed as the mean ± s.e.m. n=6 per group. **P*<0.05 indicates significance from 0.5, no preference (dotted line).

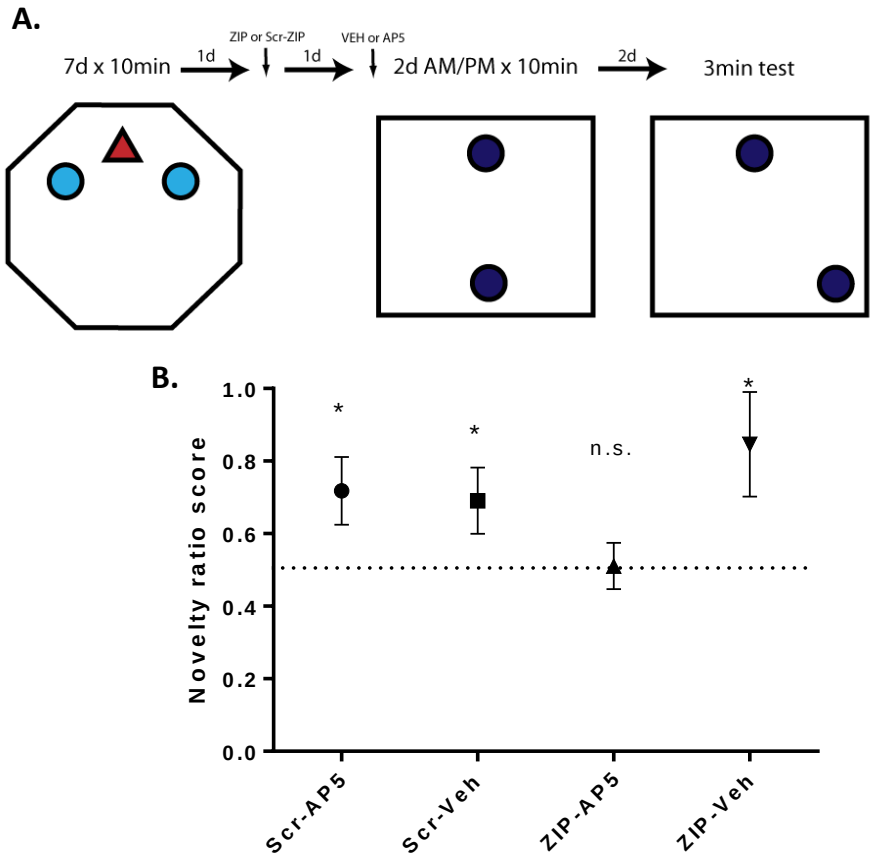

Figure 4. ZIP-induced impairment of a first learning memory leads second learning to be NMDAR-dependent. *A)* Experimental design for second learning of an object location task after first learning memory erasure. Rats explored Open Field A for 10 min/day across 7 days. Twenty-four hours after the last training sessions, rats were infused with AP5 or vehicle (PBS). One day post-infusions, rats were given the second learning protocol, consisting of exploration in Open Field B for 10 min/day across 2 days, with infusions of AP5 or Veh (PBS) prior to each session. Forty-eight hours later rats were given a 3-min test session where novel object location recognition was assessed. *B)* ZIP blocks first learning and renders the second learning AP5-sensitive. Scr-AP5, Scr-Veh, and ZIP-Veh groups showed significant preference for the new object location at second learning test. ZIP-AP5 rats did not show a preference, spending time with new and old locations equally. n=4-8 per group. Data are expressed as the mean ± s.e.m. *P<0.05 indicates significance from 0.5, no preference (dotted line).

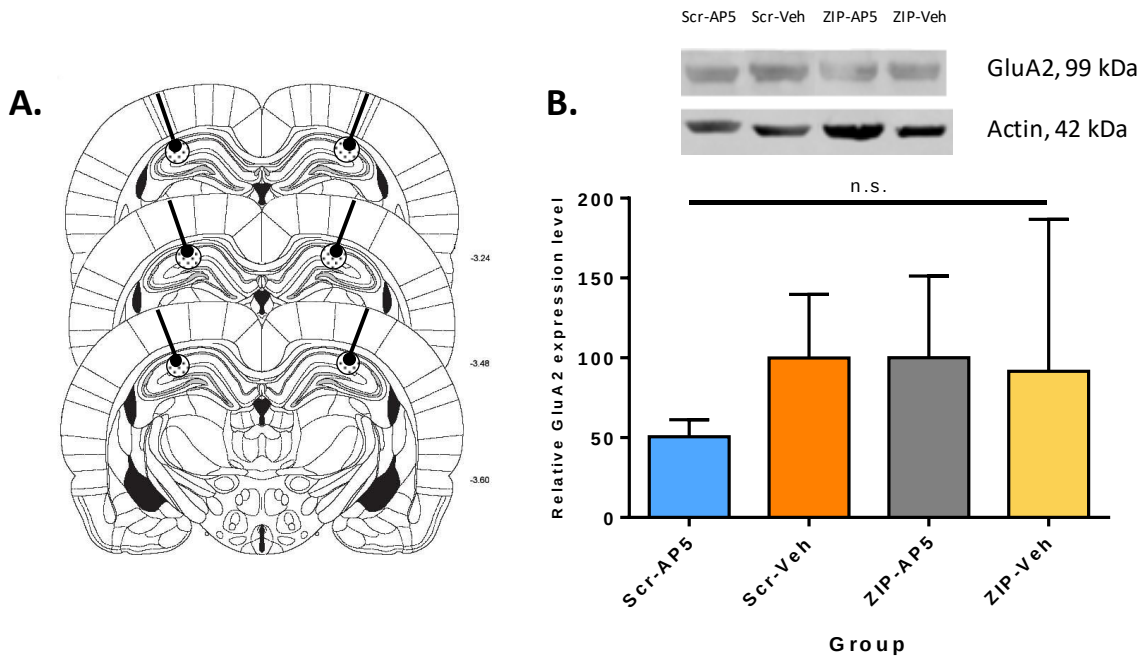

Figure 5. First and second learning impairments do not affect GluA2-AMPAR expressions levels after second learning test. *A)* Schematic representation of locations within the dorsal hippocampus, including injector tracts (thick black line), injection points (black dot) and punched CA1 area (dotted circle) for western blot analysis. Values on right side indicate anterior-posterior position relative to bregma (Paxinos and Watson). *B) Top:* Representative western blot bands of GluA2-AMPAR expression volume of each experimental group. *Bottom*: Relative amount of GluA2 in PSD fractions of CA1 dHPC neurons, standardized to β-actin, in relation to Scr-Veh group (set at 100%). Animals were infused with ZIP or its inactive form, scrambled ZIP (Scr), 24 hours after first learning training, and AP5 or Veh prior to second learning training, resulting in four groups: Scr-AP5 (*n*=8), Scr-Veh (*n*=6), ZIP-AP5 (*n*=6) and ZIP-Veh (*n*=4). No significant difference between groups were observed. Data are expressed as the mean ± s.e.m.

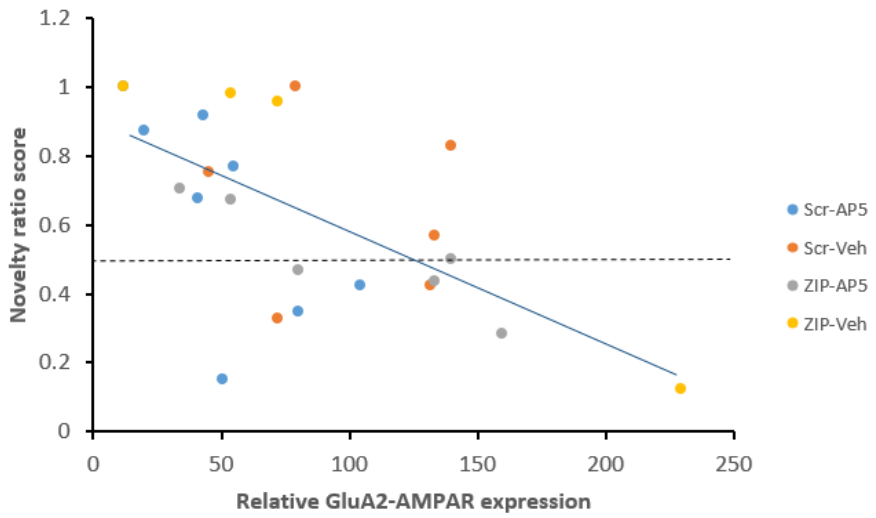

Figure 6. GluA2-AMPAR levels negatively correlates with novelty ratio at second learning probe test. Data shows that increasing GluA2-AMPAR expression is significantly correlated with decreasing memory performance as shown by novelty ratio score (r=-0.62 ± 0.06, N=24, P=0.001). Novelty ratio of 0.5, no preference, is indicated by the dotted line.

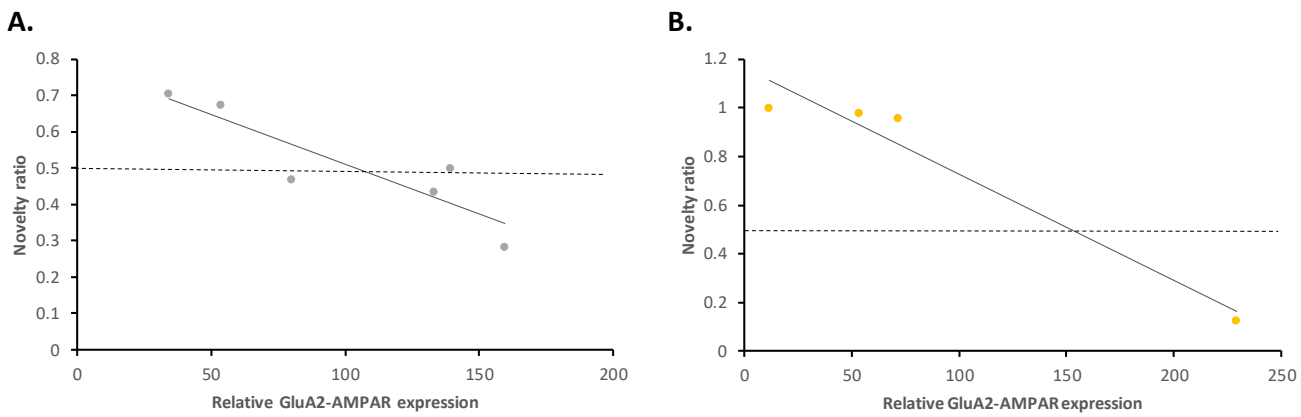

Figure 7. Increasing GluA2-AMPAR expression in PSD of CA1 dHPC neurons is significantly correlated with decreasing novelty ratio score. Data is filtered from Figure 6 to graphically isolate two groups, ZIP-AP5 (*A*) and ZIP-Veh (*B*). Linear regression lines are shown for each group: ZIP-AP5 ($r_{(4)}$=-.89 ± .04, *P*=.016) and ZIP-Veh ($r_{(2)}$=-.97 ± .04, *P*=.026). Novelty ratio of 0.5, no preference, is indicated by the dotted line.

CHAPTER THREE

Reconsolidation blockade leads to experience-driven changes in NMDAR-dependency in later spatial learning

J Jane Zhang, Amaryllis Mirlycourtois, Karim Nader

3.1. *Preface*

After investigating the nature of long-term memory erasure, we examined if storage impairment occurred in a different type of memory loss: reconsolidation impairment. This second example of storage-mediated memory loss may allow more reliable comparisons of changes to neural correlates that are seen in both consolidation and reconsolidation blockade. Like in Project 1 (Chapter 2), we performed both a behavioural test to verify the nature of the amnesia and then western blots of the dorsal hippocampus (dHPC) to probe for changes in postsynaptic GluA2-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (GluA2-AMPAR) expression. We hoped that the observed changes to GluA2-AMPAR expression from this data set would allow stronger interpretation of the molecular data from Project 1. Amnesia caused by reconsolidation blockade provided a good choice for our next avenue of study for multiple reasons: (1) reconsolidation, the process of how a recalled memory slowly restabilizes over time, may be a mechanism through which our memories update and stay relevant (see Lee et al., 2017 for review) and is therefore an important memory phenomenon to study, (2) reconsolidation has not yet been shown to occur in object location memories, and (3) extant literature observed that GluA2-AMPAR endocytosis occurs after retrieval of a memory, and are reinserted as the memory reconsolidates (Rao-Ruiz et al., 2011; Hong et al., 2013). What remains unclear is how the decrease of GluA2-AMPARs correlates to memory destabilization. To examine this, we measured GluA2-AMPAR expression changes in the CA1 dHPC as a correlate of amnesia caused by reconsolidation blockade of an object location memory.

Here, we also used the second learning protocol (Hardt et al., 2009) to allow positive predictions about the nature of the induced amnesia. To block reconsolidation, infusions of sulfasalazine (SSZ), a selective transcription inhibitor which blocks the nuclear factor κB (NF-

κB) transcription pathway (see Shrum & Meffert, 2008, for a review), were infused bilaterally in the rat dHPC. SSZ infusions in the dHPC have been shown to block reconsolidation of hippocampus-dependent contextual fear memories (Lee & Hynds, 2013), and was therefore a good candidate to also block reconsolidation of object location learning. Behavioural results indicated that indeed, like ZIP-induced amnesia, the memory loss caused by blocking reconsolidation with SSZ also can be classified as a storage impairment of the memory. Through this, it was also confirmed that object location memories can reconsolidate, and SSZ impairment is an effective method to block this reconsolidation. Molecular analyses, however, showed no difference between average group expression of GluA2-AMPAR across treatment groups. If GluA2-AMPAR endocytosis did occur after memory retrieval, as the literature suggests, animals infused with SSZ after reactivation and then did not acquire a subsequent learning experience should have shown less GluA2-AMPAR expression relative to the other groups. That we saw no differences between groups led us to believe that western blots were not sensitive enough to detect changes of GluA2-AMPAR expression caused by reconsolidation blockade of an object location memory. Framed against the data from Project 1 (Chapter 2), this suggests that there might exist innate difficulties in reliably quantifying changes in GluA2-AMPAR expression correlated with increasing or decreasing object location memory strength.

3.2. *Abstract*

Reconsolidation processes in non-associative, spatial memories are not well understood. It has not yet been shown that hippocampus-dependent object location memories can undergo reconsolidation. Additionally, although blockade of reconsolidation processes can lead to amnesia of the reactivated memory, it remains unclear whether this memory loss reflects a storage impairment (the memory is not available due to loss of neural correlates) or a retrieval impairment (the memory is available, but inaccessible), and how expression of a correlate of memory destabilization, GluA2-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (GluA2-AMPARs), change as a result of this amnesia. Here, we show that (1) blocking activation of the nuclear factor κB (NF-κB) pathway, which regulates transcription of plasticity-associated genes, after memory reactivation, leads to impaired reconsolidation of object location memory in a novelty recognition task. Furthermore, we demonstrated that (2) this intervention causes a storage impairment of the reactivated memory trace. We used a second learning protocol to predict the nature of amnesia based on whether a familiar learning experience requires *N*-methyl-D-aspartate receptor (NMDAR) activation. Reconsolidation blockade object location memory led subsequent object location training to rely on NMDAR activation for acquisition, which usually is a NMDAR-independent process. This indicated that the initial memory was lost in a way that subsequent learning was acquired as if it is new learning, suggesting a storage loss of the first-learned memory. Changes in postsynaptic expression of a neural correlate of memory destabilization, GluA2-AMPARs, were assayed with western blots to corroborate this storage impairment, but (3) no differences were detected across treatment groups, which may suggest that amnesia-driven changes in GluA2-AMPARs were too

minimal to be detected. Taken together, our data show that blocking NF-κB-regulated gene transcription can impede reconsolidation by impairing storage of the memory, but this impairment did not correlate with changes in postsynaptic GluA2-AMPAR expression.

3.3. *Introduction*

The work of Nader et al. (2000) revived interest in the neuroscientific study of the effects retrieval exerts on memory. In this seminal paper, it was demonstrated that consolidated memories, contrary to predictions of the dominant memory models, do not remain in a stable state permanently. Rather, retrieval prompts memories to become labile and pervious to manipulation for a brief time window until they return again to a stable state. This two-part process (S. H. Lee et al., 2008; S.-H. Wang et al., 2009; Winters et al., 2009) of (1) memory destabilization following retrieval, and (2) subsequent memory restabilization, or reconsolidation, has been observed in many species and research paradigms (Haubrich & Nader, 2018; Kwapis & Helmstetter, 2014).

Yet, few studies have shown reconsolidation of non-Pavlovian hippocampus-dependent tasks, such as location recognition paradigms, or similar paradigms that give rise to episodic-like memories. Because these memories of events in space-time are thought to be require the hippocampus (see Burgess et al., 2002 for a review), understanding how spatial memories reconsolidate is crucial to understand what happens to memories when similar contexts are repeatedly encountered in daily life. Reconsolidation blockade of a hippocampus-dependent spatial memory has been shown in only a small number of studies (R. G. M. Morris et al., 2006; Przybyslawski & Sara, 1997; Suzuki et al., 2004); furthermore, these studies employ maze tasks, motivated either appetitively by food reward (Przybyslawski & Sara, 1997), or aversively by water (Suzuki et al., 2004; Morris et al., 2006). However, many memories in daily life are not strictly associative or motivated by reward or punishment. Thus, it is relevant to study reconsolidation of object location learning, notable in that it is reinforcement-free (Assini et al.,

2009), and is driven by an animal's own exploration and novelty preference (Berlyne & Slater, 1957), providing an excellent model for most of our everyday memories of events.

Another missing aspect of the reconsolidation literature is how to conceptualize the amnesic effect of reconsolidation blockade. Based on behavioural data alone, it is difficult to separate whether the amnesic treatment causes a storage impairment, rendering the memory trace unavailable, or a retrieval impairment, rendering the memory trace inaccessible (Gold et al., 1974; Hardt et al., 2009; Nader, 2009). Although it seems logical to assume that preventing neural correlates of memory from reconsolidating defines a loss of memory storage, this has yet to be confirmed through a paradigm that clearly dissociates it from retrieval impairment. Our lab previously established a protocol that allows a positive prediction for the nature of the experimentally induced amnesia (Hardt et al., 2009). The protocol takes advantage of a second learning phenomenon first observed by Bannerman et al., (1995). They showed that while a naively acquired spatial memory requires *N*-methyl-D-aspartate receptor (NMDAR) activation, a later learning experience of the same task in a different context does not. NMDAR-independent second learning has since been seen in other hippocampus-dependent tasks, although again there is a bias towards studies on contextual fear conditioning (Crestani et al., 2018; Hardt et al., 2009; Sanders & Fanselow, 2003; Tayler et al., 2011; Wiltgen et al., 2011) or water maze (Bannerman et al., 1995; Saucier & Cain, 1995). Hardt et al. (2009) posited that by impairing the storage of the first-learned memory, the sensitivity of a second-learned memory to NMDAR-antagonism can be evaluated as an indicator of the first learning amnesia. If second learning is NMDAR-dependent, this suggests that the initial memory was erased, causing the second learning experienced to be acquired as if it is the first time the animal has been exposed to the task.

Therefore, by employing this protocol after reconsolidation blockade of the first-learned memory, the nature of the reconsolidation impairment can be determined.

If reconsolidation blockade indeed causes a storage impairment of memory, it is likely due to loss of neural correlates of the memory. Internalization of GluA2-subunit containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (GluA2-AMPARs) from the postsynaptic membrane has a critical role in long-term memory (LTM) retrieval and thus reconsolidation. Blocking GluA2-AMPAR endocytosis has been shown to block reconsolidation processes (Rao-Ruiz et al., 2011; Hong et al., 2013; Bhattacharya et al., 2017). For example, Rao-Ruiz et al. (2011) showed that reactivation of a contextual fear memory induces GluA2-AMPAR downregulation in dorsal hippocampus (dHPC) neurons within 1 hour after retrieval, corresponding to a period of memory lability. Seven hours post-retrieval, after the memory reconsolidated, reinserted GluA2-AMPARs were found in the synaptic membrane. Infusions of TAT-GluA2$_{3Y}$, a synthetic protein that blocks GluA2-AMPAR endocytosis, inhibited this initial post-retrieval GluA2-AMPAR downregulation, showing the necessity of these receptors in the destabilization process. This destabilization impairment via blockade of retrieval-induced GluA2-AMPAR endocytosis has also been replicated with fear memory in the lateral amygdala (Hong et al., 2013). However, the proportion of GluA2-AMPAR endocytosed from the synaptic surface after reconsolidation blockade of a memory remains unclear. As blocking reconsolidation, for example with protein synthesis inhibitors, permanently impairs the memory (Nader et al., 2000), GluA2-AMPAR expression levels likely will not return to pre-retrieval levels seen in an unimpaired memory. Evaluating the degree of GluA2-AMPAR endocytosis can thus provide neurobiological evidence for the storage impairment nature of reconsolidation blockade.

The pharmacological interventions of the previously mentioned studies include intraperitoneal injections of NMDAR-antagonist MK-801 (Przybyslawski and Sara, 1997), or general protein synthesis inhibitor anisomycin (Suzuki et al., 2004; Morris et al., 2006). Here, we target inhibition of transcription factors from the nuclear factor κB (NF-κB) family using sulfasalazine (SSZ), which has been shown to block reconsolidation of contextual fear memory (Lee & Hynds, 2013; Lubin & Sweatt, 2007), auditory fear memory (Si et al., 2012), and inhibitory avoidance (Boccia et al., 2007) in rodents. NF-κB transcription factors are commonly known for regulating inflammatory and immune response, however, increasing evidence has linked the NF-κB signalling pathway with long-term potentiation and long-term memory in vertebrates (see Shrum & Meffert, 2008, for a review). NF-κB latently exists in mammalian neurons, bound to its inhibitor (IκB). Activity, for example, with NMDAR activation, can induce phosphorylation of IκB by its kinase complex (IKK) (DiDonato, Hayakawa, Rothwarf, Zandi, & Karin, 1997; Mercurio et al., 1997). This leads to IκB degradation, which frees NF-κB to be translocated in the nucleus, where it regulates expression of plasticity-related genes (Grilli & Memo, 1999; Verma, Stevenson, Schwarz, Van Antwerp, & Miyamoto, 1995; Yeh, Lin, & Gean, 2004). SSZ application inhibits IKK, thus can block the NF-κB activation pathway after stimulation (Wahl et al., 1998). Because this overall process regulates gene transcription activity needed for synaptic changes (Ahn et al., 2008; Ghosh & Karin, 2002; Kaltschmidt et al., 2006), SSZ infusions provides a nuanced way to block reconsolidation.

Therefore, three aims of these experiments are: (1) to show object location memories can reconsolidate, (2) to show that reconsolidation blockade is caused by a storage impairment of the memory, and (3) to assay changes in GluA2-AMPAR expression levels in the dorsal hippocampus CA1 region caused by reconsolidation blockade of the memory, with or without

subsequent learning. Behavioural results confirmed that first learning memory impairment caused by infusions of a transcription inhibitor, SSZ, does indeed reverse the usual NMDAR-independency of second learning acquisition. This suggests that reconsolidation blockade impaired storage of the first learning memory. However, we found no significant changes in GluA2-AMPAR expression levels that correlated with memory loss.

3.4. *Methods*

3.4.1. Animal subjects

Male Long Evans rats (received at weight 275g-325g; Charles River) were housed in pairs in rectangular polyethylene cages with a PVC tube for environmental enrichment. Rats had *ad libitum* access to food and water. Lights in the colony were turned on at 7 a.m. and turned off at 7 p.m. daily. Behavioural studies were always performed between 9 a.m. and 5 p.m. All procedures were approved by the McGill University Animal Care and Use Committee and complied with the Canadian Council on Animal Care guidelines.

3.4.2. Surgeries

Rats were anaesthetized with a solution of Ketamine (50 mg/ml), Xylazine (3 mg/ml), and Dexdomitor (0.175 mg/mL), injected intraperitoneally (i.p.) at a volume of 1 ml/kg. Bilateral cannulations in the dorsal hippocampus were then performed with coordinates based on the rat atlas (Paxinos and Watson). 22-gauge steel cannula were implanted using a Kopf stereotaxic frame (coordinates from bregma: A/P -3.6 mm, M/L +/- 3.1 mm, D/V -2.4 mm, 10° away from midline). Dental cement was adhered to the skull to stabilize the cannula. After surgeries, rats were administered an analgesic (Carprofen 5 mg/ml, subcutaneous at 1 ml/kg) and then

anesthesia was reversed with Antisedan (0.5 mg/ml, i.p. at 0.66ml/kg). A week of recovery time was given, with regular handling throughout, before the experiment began.

3.4.3. Apparatus

Two open field contexts were set up in separate rooms.

Open Field A was set up in context room A (area 15.85 m^2, 3.98 m × 3.98 m × 2.65 m), a windowless room with dim lighting. The floor level of the open field was illuminated with, on average, 13 lux. The open field was a circular area made with clear Plexiglas walls, with a total area 3600 cm^2, with walls of height 60 cm. A strip of black paper covered the lower 15 cm of each Plexiglas panel in order to minimize distraction of rats peering through the Plexiglas. One Plexiglas panel was labeled with a black and white striped pattern to act as a disambiguating cue. Three objects were set up at northwest (NW), north (N), and northeast (NE) locations, with north arbitrarily assigned at the corner of the room opposite to the door. NE and NW objects were identical, and N objects were of the same height and texture but different colour and shape. The combination of objects used were counterbalanced between rats. Bedding was made up of a 1:1 dried corn and woodchips mix. Flooring was made of a wooden pegboard into which objects could be screwed in, out, and fastened. A camera hung 50 cm above the context. Behavioural testing occurred with the door closed and the experimenter outside of the room.

Open Field B was set up in a distinctly different context compared to Open Field A in order to facilitate second learning as a separate experience. It was placed in context room B (2.13 m^2, 2.13 m × 1 m × 2.65 m), a windowless room lit brightly. The floor level of the open field was illuminated with, on average, 201 lux. The open field was a square area made with thin plywood walls, with a total area of 3600 cm^2 (60 cm × 60 cm × 60 cm). The bedding was made of woodchips. A disambiguating black and white striped cue was taped at the top of one of the

walls. Two identical objects were located at N and S locations within the open field, and were of similar height but differing colour and shape to the objects from Open Field A. A camera hung 80 cm above the context. Behavioural testing occurred with room curtains pulled closed and the experimenter outside of the room.

3.4.4. Drug Infusions

Transcription inhibitor sulfasalazine (Sigma) was mixed in saline with 10 mM HEPES buffer (pH 7.6) plus 20% DMSO at 2 µg/µl, and infused bilaterally into the dHPC (1.0 µl/hemisphere at 0.25 µl/min). The NMDAR-antagonist D-AP5 (Santa Cruz Biotechnology) was mixed with phosphate buffered saline (PBS, pH 7.4) at 5µg/µl and infused bilaterally into the dHPC (1.0 µl/hemisphere at 0.25 µl/min). Injector tips remained in the cannula for 60 seconds after infusions completed in order to ensure complete diffusion of the drug. Placements were checked at the time of tissue sample collection for western blots.

3.4.5. Behavioural procedure

3.4.5.1. First learning in Open Field A

Rats were brought to context room A to rest for 20 minutes before training. First training sessions consisted of daily 10-minute free exploration trials across seven days. As habituation could be construed as a learning experience, no habituation sessions were given prior to training. Rats were lowered into the open field at a different corner of the open field each day (SE, SW, NW, NE), with their noses pointed away from the centre of the field, allowing the rat to orient themselves towards the objects and remove bias for any one object. After each session of training, rats were removed from the open field and returned to their home cage. They were allowed to rest for 20 minutes following the conclusion of training of all rats before being returned to the colony.

3.4.5.2. Reactivation in Open Field A

Twenty-four hours after the last first training session, rats were returned to context room A for a reactivation session. All objects were removed from the open field, but other qualities of the context were unchanged. Rats were placed individually in the middle of the open field, facing away from the previous positions of the objects, and given 10 min of exploration time. Immediately after reactivation, rats were removed from the open field and in a different room given bilateral dorsal hippocampal infusions of sulfasalazine (SSZ), an inhibitor of nuclear factor kappa B transcription factor (NF-κB), or vehicle (Veh; saline/HEPES/DMSO). Rats were returned to their home cage and allowed to rest at least 20 min before being brought back to the colony room.

Next, rats were tested in either Open Field A 24 hours after reactivation (Experiment 1), or underwent subsequent training in Open Field B (Experiment 2). For a test session performed in Open Field A, objects were returned to the floor, with the middle object in the open field moved across the floor from its original position. The other two objects remained in the same position. Rats were placed in the centre of the open field, facing away from all three objects and allowed to explore for 3 minutes. After completion of the test, rats were returned to their cages, then returned to the colony room after all rats finished testing.

3.4.5.3. Second learning in Open Field B

The day following reactivation (Experiment 2), rats were brought to the hallway outside context room B to rest for 20 minutes before training began. Second learning training comprised of bilateral infusions of AP5, an NMDAR-antagonist, or vehicle (Veh; PBS) into the dorsal hippocampus, followed immediately by a 10-min exploration session in Open Field B. For each

session, rats were delivered into the open field while facing the corner of the rectangular field. The origin corner alternated each training session (SE, SW, NW, NE). After completion, rats were returned to their home cage. Following completion of all rats' training, animals were given 20 minutes of rest before being returned to their colony room. Four infusion and training sessions in total occurred across two days, with each morning and afternoon session separated by a minimum of 4 hours during which rats rested in the colony.

Forty-height hours after the final second learning session, rats were returned to context room B for a probe test. One of the objects was moved to a corner of the open field. The other object remained in the same position. Rats were placed in a corner of the open field that was equidistant from both objects, facing the walls and allowed to explore for 3 minutes. Rats were then returned to their cages, left to rest, then returned to the colony room 20 minutes after completion of test sessions for all rats.

3.4.6. Sacrifice

Twenty-four hours after testing, rats were sacrificed. Rats were anaesthetized with isoflurane then decapitated. Brains were removed and flash frozen over dry ice, then preserved at -80°C.

3.4.7. Behavioural scoring

Behavioral videos were scored manually. Exploratory behaviour was diagnosed when the rat showed directed interest in the object, e.g. smelling from at most 3 cm away from the object, touching, climbing into the object. Rats climbing on top of the object was considered exploratory activity, but while there, usage of the object as a platform to explore the walls of the context was not included as object exploration. Scoring began immediately prior to the first instance of directed interest towards the object, and continued for 30 seconds.

Novel object location preference (novelty ratio) for first learning (in Open Field A) was calculated with the following formula:

$$\frac{time\ exploring\ new\ location}{time\ exploring\ new\ location + (\frac{time\ spent\ exploring\ old\ location\ 1 + old\ location\ 2}{2})}$$

Novelty ratio for second learning (in Open Field B) was calculated with the following formula:

$$\frac{time\ exploring\ new\ location}{total\ time\ exploring}$$

Open Field B had two objects, therefore time spent exploring each object location had an equal weight of 50% of the total time exploring (equation denominator). However, because Open Field A had 3 objects, the time spent with the object at the new location would be underweighted against the other two objects (33% versus 66%). To account for this, the total time spent exploring the two objects at familiar locations was averaged, thus exploration of the two familiar locations was treated as exploration of one object. In this way, time spent at the new location and the familiar locations would be weighed equally, allowing use of the same ratio scale in equations involving two or three open field objects.

For both novelty ratio calculations, a value of 0.5 indicates equal preference for both objects or no preference for either object, while a value significantly higher than 0.5 indicates a significant preference for the new object location. Because rats prefer novelty over familiarity (Berlyne & Slater, 1957; Ennaceur & Delacour, 1988), this behavior suggests that they took notable interest in the new object location, suggesting that they retained the memory of the previous object locations. Therefore, a high novelty ratio implies a strong memory, and a no preference ratio of 0.5 implies weak or impaired memory. A low novelty ratio indicates preference for the old object location, and thus insufficient performance for the trained memory. In Experiment 1, a t-test was used to determine significant preference for the object at the new

location, comparing group novelty ratio means to an estimated mean of 0.5. In Experiment 2, a one-way ANOVA was used to probe for group differences across four treatment groups. In Experiment 3, western blot and behavioural data was analyzed with a one-way ANOVA and Pearson's correlation. Analyses were completed with SPSS and JMP software.

3.4.8. Biochemistry

3.4.8.1. Subcellular fractionation for postsynaptic density

The dorsal hippocampus CA1 region was dissected from frozen brains with a 1 mm neuro punch (Fine Science Tools), and homogenized in cold Tris-HCl buffer (30 mM, pH 7.4) containing 4 mM EDTA, 1 mM EGTA and a cocktail of protease inhibitors (Complete, Roche). The hippocampus homogenates were then centrifuged at 4°C at 500 g for 10 min to remove nuclei and other debris. The supernatants were removed and centrifuged at 100,000 g at 4°C for 60 min. Resulting pellets were resuspended in a solution of the homogenization buffer and 0.5% Triton X-100 and incubated on ice for 20 min, then layered over 1 M sucrose. This was centrifuged again at 100,000 g for 60 min, allowing detergent-soluble membrane components and extra-synaptic receptors to remain in the Triton-soluble fraction. The remaining Triton-insoluble materials were pulled down into the sucrose layer, where proteins of the postsynaptic densities also lay. This final pellet was resuspended in homogenization buffer and stored at -80°C. Total protein concentration was determined with the BCA protein assay kit (Pierce).

3.4.8.2. Western blots

Western blots were performed using 8% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes overnight. Membranes were washed with 2% BSA in TBS-Tween for 1 hour at room temperature, then incubated with polyclonal antibodies to GluA2 (1 mg/ml, Millipore; dilution 1/2000) and monoclonal β-actin (2 mg/ml,

Sigma; 1/10,000) for 20 hours at 4°C. Membranes were washed with TBS-Tween and incubated in rabbit horseradish antibody (GluA2, NR2B; GE Healthcare UK; 1/10,000) or mouse horseradish antibody (actin; GE Healthcare UK; 1/10,000) at room temperature for 1 hour. Detection of bands was completed with Pierce ECL2 western blotting substrate (ThermoScientific). Membrane blots were scanned with a Storm Laser Scanner (Molecular Dynamics) and analyzed with ImageQuant software (ABI).

3.5. *Results*

3.5.1. Experiment 1: Post-reactivation infusion of sulfasalazine blocks reconsolidation of object location memory

We first tested if blocking transcription via sulfasalazine infusions impaired reconsolidation of an object location memory. Rats were given 7 days of first learning training in Open Field A with no previous habituation. Twenty-four hours after first learning training, rats were given a reactivation session immediately followed by bilateral infusions of sulfasalazine (SSZ) or vehicle (Veh) into the dHPC. After one day of rest, rats were returned to the open field for a 3-minute probe test (Figure 1A).

Animals that received the post-reactivation sulfasalazine treatment showed no significant preference for the new location at the probe test (NR=.47 ± .97, $t<1$; data presented as mean novelty ratio ± s.e.m.), whereas control rats did (NR=.64 ± .057, $t_{(7)}=2.37$, $P=.05$) (Figure 1B). Because rats are naturally attracted to novelty, significant preference for the new object location at the test session would indicate training memory of the original object locations is intact. A novelty score of 0.5 indicates that the animal is spending equal amounts of time exploring the new and old object locations during test, suggesting the training memory of the old object

locations can no longer be expressed. This memory impairment did not occur when rats did not receive a reactivation session, or when they received no SSZ infusions (Figure 1C). Therefore, SSZ-infused rats showed impaired memory at test, while Veh-infused rats did not, showing that sulfasalazine blocked reconsolidation of the object location memory. The overall time spent exploring objects was not significantly different between groups, indicating no treatment effects on motility or motivation to explore.

3.5.2. Experiment 2: Reconsolidation blockade of first learning memory leads to subsequent learning to become NMDAR-dependent

We wanted explore how blocking reconsolidation of a first-learned object location memory would affect the acquisition of a subsequently learned object location memory. Research has shown that unlike first learning acquisition processes, second learning acquisition of hippocampal-based tasks is independent of NMDARs (Bannerman, Sanders, Tayler, Hardt, Wiltgen). Thus, the sensitivity of a second learning acquisition to an NMDAR-antagonist can act as an indicator of the state of the first learning memory. Interventions that lead to a storage impairment would erase the first learning memory and subsequent learning would be acquired as if it were naïve learning (i.e. in an NMDAR-dependent manner). Retrieval impairment of the first learning memory, because the memory trace is not erased, will not affect the NMDAR-independent nature of second learning memory acquisition.

Rats were given first learning training in Open Field A, followed 24 hours later by a reactivation session and infusions of SSZ or Veh into the dHPC (as in 3.1). Twenty-four hours after infusions, rats were given two days of second learning training with infusions of AP5, an

NMDAR antagonist, or vehicle (Veh; PBS) in Open Field B (Figure 2A). Forty-eight hours after second learning training, rats were given a probe test in Open Field B for 3 minutes.

At the second learning test, all groups showed a significant preference to the novel object location (i.e. novelty ratio significantly greater than 0.5), except the SSZ-AP5 group (NR=.65 ± .082, t<1) (Figure 2B). We reasoned that the Veh-Veh group (NR=.73 ± .089, t=2.56, *P*=.043) had normal acquisition of both first and second learning, as vehicle infusions did not impede learning. Similarly, the Veh-AP5 group (NR=.7 ± .066, t=3.02, *P*=.014) was expected to have intact first learning, and thus would be unaffected by AP5 infusions at second learning. In the SSZ-Veh group (NR=.74 ± .083, t=2.95, *P*=.018), only second learning should have been acquired adequately, as first learning was impaired by sulfasalazine. For SSZ-AP5 rats, blocking reconsolidation of a first learning memory with sulfasalazine resulted in second learning acquisition sensitivity to AP5, i.e. susceptibility to NMDAR-blockade. Thus, it seemed that second learning required NMDAR activation for acquisition in a first-learning-like manner. We concluded that this could occur only if reconsolidation blockade of first learning erased the memory. Therefore, the behavioural evidence shows that SSZ-induced reconsolidation blockade indeed causes a storage impairment of the memory.

We observed no significant differences on average novelty ratio between all groups ($F_{(3,28)}$=0.208, *P*=.89), but this does not affect individual group comparisons with a 0.5 novelty ratio. As memory expression is solely evaluated with individual group mean difference from 0.5, significant group differences in novelty ratio scores are not necessary to determine whether the memory exists or not, and would only supplement the existing behavioural t-test data. There was no difference in total exploration time between all groups ($F_{(3,28)}$=0.573, *P*=.638), again suggesting no treatment effects on motility or exploratory motivation. However, it is worth

noting that these groups have a low N, and patterns may be more evident with increased numbers per group.

3.5.3. Experiment 3: GluA2-AMPAR levels remain unchanged by sulfasalazine-induced reconsolidation blockade of first learning memory, with or without NMDAR blockade of second learning memory acquisition

As described in section 3.2 above, rats were given first learning training followed 24 hours later by a reactivation session and infusions of SSZ or Veh into the dHPC. A day later, rats were given two days of second learning training with infusions of AP5, an NMDAR antagonist, or vehicle (Veh; PBS). Forty-eight hours after second learning training, rats were given a probe test to assess second learning. Twenty-four hours after testing, rats were sacrificed and their brains frozen. CA1 dHPC near the cannulation area was dissected for postsynaptic density (PSD) fractions for western blot analysis (Figure 3A). β-actin was used as a loading control and we found no significant difference in β-actin levels between groups ($F_{(3,28)}$=1.155, P=.344, data not shown).

As there was no hypothesized value from which to compare, we examined group differences in protein expression. Probing for GluA2-AMPAR expression in the PSD of CA1 neurons showed that there was no difference in GluA2-AMPAR levels across group. Although the SSZ-AP5 group numerically had the lowest amount of GluA2, there was no significant difference from the other experimental groups ($F_{(3,28)}$=0.195, P=.899, Figure 3B). To further examine if there was a relationship between GluA2-AMPAR levels and performance at test, we correlated individual novelty ratio score with level of GluA2-AMPAR expression, but found no significant correlation between performance at probe test and amount of GluA2-AMPAR

expression ($F_{(1,30)}$=0.53, P=.471, Figure 4). Additionally, no correlation was seen between GluA2-AMPAR levels and time spent exploring ($F_{(1,30)}$=3.16, P=.086, data not shown).

3.6. *Discussion*

We aimed to show reconsolidation blockade of an object location memory, and describe the nature of the resulting amnesia. Using a second learning protocol, we predicted that blocking the reconsolidation of the memory for the first learning would disrupt its storage. As a consequence, second learning would require NMDAR activation because its acquisition would follow the mechanisms of a new learning experience, which requires NMDARs. We also aimed to detect changes in the expression of GluA2-AMPARs in the PSD of CA1 hippocampal neurons correlated with first and second learning conditions, and link a hypothesized loss of GluA2-AMPARs to the storage impairment of the memory trace.

We showed that object location memories can undergo reconsolidation, like many other types of memories (Haubrich & Nader, 2016, Kwapis & Helmstetter, 2013). Moreover, we confirmed that object location learning can induce NMDAR-independency in future acquisition sessions. Behavioural data showed that reconsolidation of object location memory can be impaired with post-reactivation infusions of NF-κB transcription factor inhibitor sulfasalazine (SSZ). This confirms previous literature about the inhibitory effect of SSZ infusions on transcription (Lee & Hynds, 2012; Boccia et al., 2007; Merlo, Freudenthal, Maldonado, & Romano, 2005; Si et al., 2012), which propose that the family of NF-κB transcription factors are activated by retrieval and are required for memory reconsolidation (Boersma & Meffert, 2008). Additionally, we showed that blocking reconsolidation with infusions of SSZ led to second learning sensitivity to NMDAR-impairment (SSZ-AP5 group). NMDAR-dependency is a

property of naïve learning of hippocampus-based spatial learning, but not of subsequent learning (Bannerman et al., 1995). As only the group that was infused with SSZ after first learning reactivation, and AP5 prior to second learning sessions, showed impaired memory expression at the second learning test, this suggested that reconsolidation blockade erased the storage of first-learned memory and induced second learning acquisition as if it was naïve learning. This result was consistent with the storage impairment hypothesis of experimental amnesia.

We initially hypothesized that reactivation of the memory would induce endocytosis of surface GluA2-AMPARs in dHPC neurons, and thus rats with first learning memory impaired via reconsolidation blockade would have less postsynaptic GluA2-AMPARs relative to rats with retained memory. If SSZ indeed impaired memory storage, SSZ-infused rats subsequently infused with NMDAR-antagonist AP5 prior to second learning (SSZ-AP5 group) sessions should not acquire the second learning training, and therefore we anticipated they would show lower levels of relative surface GluA2-AMPAR expression compared to all other groups (SSZ-Veh, Veh-Veh, Veh-AP5). Additionally, the second learning protocol gave the opportunity to decipher how GluA2-AMPAR expression levels compared between the Veh-Veh group and the Veh-AP5 group: do two learning experiences of the same task increase GluA2-AMPAR expression compared to one learning experience? Would GluA2-AMPAR levels in Veh-AP5 and SSZ-Veh groups lie in between levels seen in SSZ-AP5 (no memories) and Veh-Veh (both memories retained) groups? Through data from these four experimental groups, we anticipated a correlation between memory expression at second learning test and a decrease in surface GluA2-AMPAR expression.

Other studies have shown a correlation between GluA2-AMPARs and performance (Hara et al., 2012; Migues et al., 2010). Migues et al. (2010) showed a positive correlation between

GluA2-AMPAR expression in the PSD with strength of fear memory output in the amygdala. Western blots probing for GluA2-AMPAR levels in a PSD fraction did not reveal any significant differences between groups, despite differences in memory performance between the SSZ-AP5 group and other treatment groups. However, that the SSZ-AP5 group showed lower GluA2-AMPAR expression compared to the other treatment groups is notable, as it follows the hypothesized molecular pattern across groups. The lack of group differences may reflect the same non-significant group differences in novelty ratio—that is, the changes in behaviour between groups may not reach significance, but nevertheless conveys useful information about memory presence. Similarly, lower GluA2-AMPAR expression in the SSZ-AP5 group may in fact indicate a correlation between less GluA2 and lower memory expression. However, further biochemical analysis showed no correlation of GluA2-AMPAR expression to memory performance, so this conclusion could not be drawn. Rather, this non-significance could indicate three alternative interpretations: either the nature of the amnesic effect is not a storage impairment (i.e. the memory trace does not undergo any constitutive changes across experimental conditions), or GluA2-AMPARs are not a neural correlate of object location memory loss under these experimental conditions, or, third, any changes to GluA2-AMPAR expression were not detectable through western blotting.

Framing reconsolidation blockade as a retrieval impairment contradicts our behavioural data. Concluding that surface GluA2-AMPAR levels do not lower after reconsolidation blockade conflicts with previous work that has so far strongly supported the role of GluA2-AMPAR internalization in destabilization of LTP (Yao et al., 2008; Hardt et al., 2014) and LTM (Migues et al., 2010; Hardt et al., 2010). Previous work has also indicated that GluA2-AMPARs are needed for retaining object location memories (Migues et al., 2016). Yet, our data show a slight

trend of decreasing GluA2-AMPAR expression across conditions (see Figure 3B), but group differences were non-significant. It is possible that the test session itself acted as a new learning session, thus possibly promoting the upregulating GluA2-AMPAR expression in all groups—although this posits that a single test session increases GluA2-AMPARs in the SSZ-AP5 group to levels of the Veh-Veh group, which may be unlikely.

Alternatively, perhaps the amount of GluA2-AMPARs internalized was negligible compared to other background trafficking of GluA2-AMPARs in CA1 neurons and thus the dHPC signal induced from the task was too small to be detected. Research has shown that in hippocampal pyramidal neurons, nearly all AMPARs in the synaptic surface contain GluA2, largely in the form of GluA1/A2 heteromers (W. Lu et al., 2009; Wenthold et al., 1996). Exploration of a novel environment has been shown to induce expression of immediate early gene *Arc* in approximately 32% of neurons in the CA1 (Vazdarjanova et al., 2006), thus an exploratory memory trace may be encoded by even fewer cells. Indeed, the amount of neurons activated in mice CA1 hippocampus after a 10-minute exploration of a novel context has been shown to be less than 15% of total neurons—although later exposure to a second context recruits around 50% (Cai et al., 2016). Therefore, the amount of internalized GluA2-AMPARs associated with amnesia of an object location learning memory might be minimal compared to GluA2-AMPARs in non-recruited cells, making the ratio of signal to noise too small to detect. Indeed, other studies have also shown difficulty in detecting activity-mediated GluA2-AMPAR endocytosis at the PSD (Ehlers, 2000; D.-T. Lin & Huganir, 2007; Tao-Cheng et al., 2011).

To best reconcile both our data and the literature, we conclude that any internalization of surface GluA2-AMPARs is likely unable to be detected with western blot assay. Our data would thus encourage amplifying the signal caused by the object location learning, for example via

increasing noradrenergic activity, which has been shown to improve signal and decrease noise in hippocampal cells through the locus ceoruleus (Susan J. Sara, 1985; Segal & Bloom, 1976). With a more robust hippocampal signal, detection of changes in GluA2-AMPAR expression in the CA1 may indeed reveal the changes involved in differences in observed memory expression. However, because these methods ostensibly moderate memory strength, this might affect the probability the memory will reconsolidate. Thus, more research is needed to characterize the neural changes involved in the reconsolidation of object location memories.

3.7. *Conclusion*

Using a second learning protocol, we were able to form a positive prediction about the nature of amnesia caused by reconsolidation blockade. Impairing transcription after reactivation of an object location memory impaired the storage of a memory trace. This prompted a subsequently learned object location memory task to be acquired in an NMDAR-dependent manner (SSZ-AP5 group). Because NMDAR-dependency is a feature of naïve learning acquisition, we conclude that blocking reconsolidation through sulfasalazine infusions erased the first learning memory. The data also showed NMDAR-independent object location learning (Veh-Veh group), and supported existing literature on the effect of SSZ in blocking transcription factors. Further research is needed to conclude the percentage of GluA2-AMPAR internalization that occurs within these situations.

Inhibiting reconsolidation is one of many methods through which to experimentally impair memory. Relating the neurobiological mechanisms of different situations of memory loss, as well as learning about memory phenomenon like NMDAR-independent learning, can allow future application to mechanisms of natural forgetting or dysfunctional forgetting. This

knowledge can contribute to better clinical treatment for memory-based anxiety disorders like Post-Traumatic Stress Disorder.

3.8. *Figures*

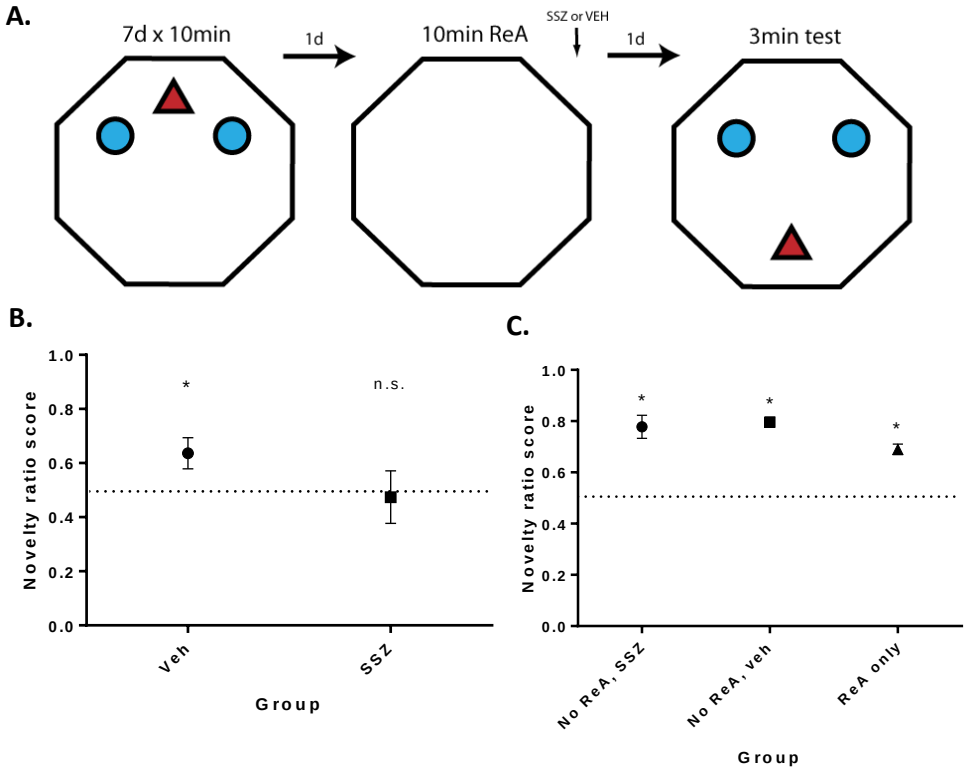

Figure 1. Post-reactivation infusions of sulfasalazine block reconsolidation of object location memory. *A)* First learning reconsolidation blockade experimental design. Rats explored Open Field A for 10 min/day across 7 days, then 24 hours after the last session experienced a 10-min reactivation session in a modified version of Open Field A with post-reactivation infusions of SSZ or Veh. A 3-min test session occurred 24 hours following infusion where object location memory was assessed by moving one object to a novel location in Open field A. *B)* Sulfasalazine infusions post-reactivation blocks reconsolidation of object location memory. Vehicle-infused group (n=9) showed significantly higher average novelty ratio than SSZ group (n=8), which showed no novel location preference at test. *C)* Reconsolidation blockade of object location memory by SSZ is reactivation-dependent. Rats either received sulfasalazine infusion (NR=.78 ± .04, $t_{(3)}$=6.22, P=.008), or vehicle infusion (NR=.8 ± .019, $t_{(2)}$=15.5, P=.004) without prior reactivation session, or rats received no infusions at all (NR=.69 ± .02, $t_{(3)}$=9.33, P=.003) but were subjected to a reactivation session. All groups showed significant preference for novel location at test, indicating no memory impairment. Data are expressed as the mean ± s.e.m. *P<0.05 indicates significance from 0.5, no preference ratio (dotted line).

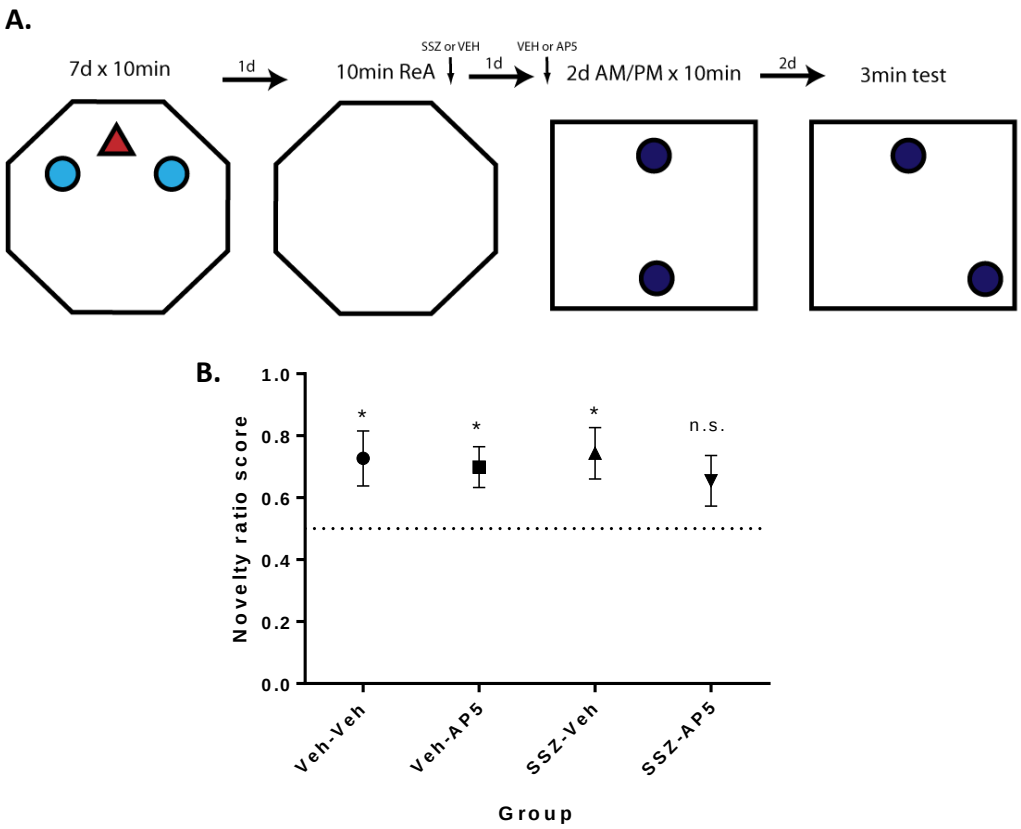

Figure 2. Reconsolidation blockade caused by post-reactivation infusions of sulfasalazine leads subsequent object location learning to be NMDAR-dependent. *A)* Second learning reconsolidation blockade experimental design. Rats explored Open Field A for 10 min/day across 7 days, then 24 hours later experienced a 10-min reactivation session with post-reactivation infusions of SSZ or Veh in a modified version of Open Field A. One day later, rats explored Open Field B for 4 sessions of 10-min across 2 days, with pre-training infusions of AP5 or Veh. Four groups (SSZ-AP5, SSZ-Veh, Veh-AP5, Veh-Veh) were tested on object location second learning memory 48 hours following training in Open Field B where one object was moved to a novel location. *B)* Reconsolidation blockade of first learning memory leads to subsequent learning to become NMDAR-dependent. Rats were infused with either SSZ or Veh after first learning reactivation, and AP5 or Veh prior to second learning training (n=6, 9, 10, 7 for SSZ-AP5, SSZ-Veh, Veh-AP5, Veh-Veh groups respectively). At the second learning probe test, all groups except SSZ-AP5 showed significant preference to the new object location. Data are expressed as the mean ± s.e.m. *P<0.05 indicates significance from 0.5, no preference ratio (dotted line).

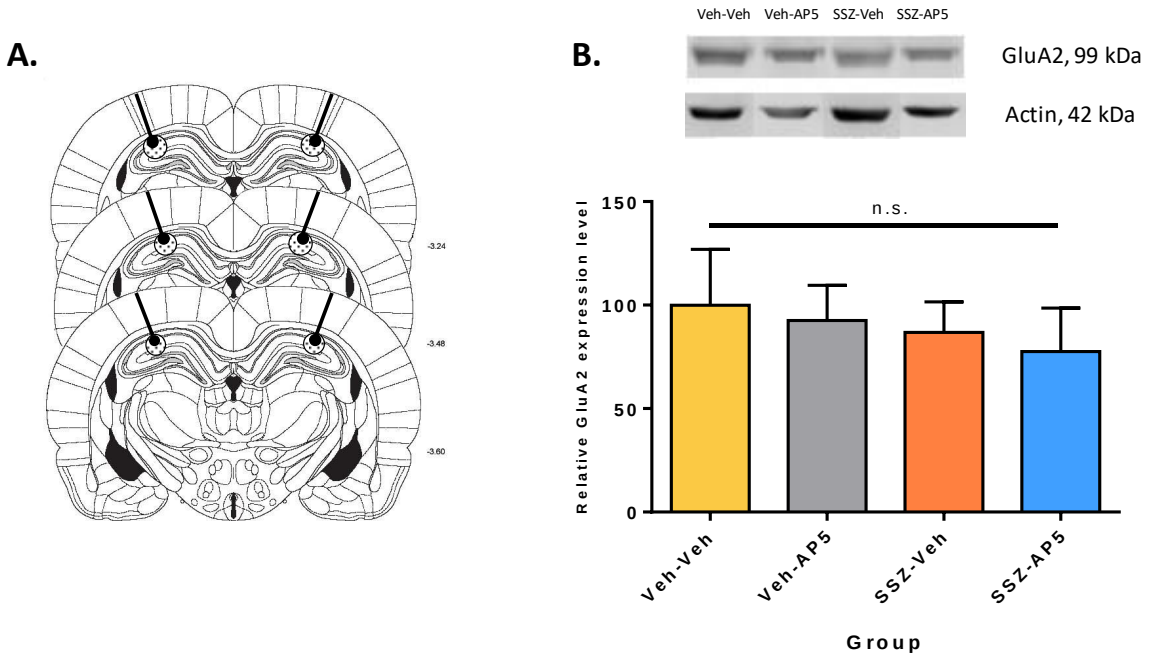

Figure 3. GluA2-AMPAR expression does not correlate with memory expression at second learning test. *A)* Schematic representation of locations within the dorsal hippocampus, including injector tracts (thick black line), injection points (black dot) and punched CA1 area (dotted circle) for western blot analysis. Values on right side indicate anterior-posterior position relative to bregma (Paxinos and Watson). *B) Top:* Representative western blot bands of GluA2-AMPAR expression volume of each experimental group. *Bottom*: Relative amount of GluA2-AMPAR expression in PSD fractions of CA1 dHPC neurons remain unchanged by SSZ-induced reconsolidation blockade with or without NMDAR blockade of the second learning memory. Animals were infused with SSZ or Veh immediately post first learning reactivation, and AP5 or Veh prior to second learning training, resulting in four groups: SSZ-AP5 (*n*=6), SSZ-Veh (*n*=9), Veh-AP5 (*n*=10) and Veh-Veh (*n*=7). No significant difference between groups were observed. GluA2 levels are standardized to β-actin and expressed as relative percentage values of the Veh-Veh group ± s.e.m.

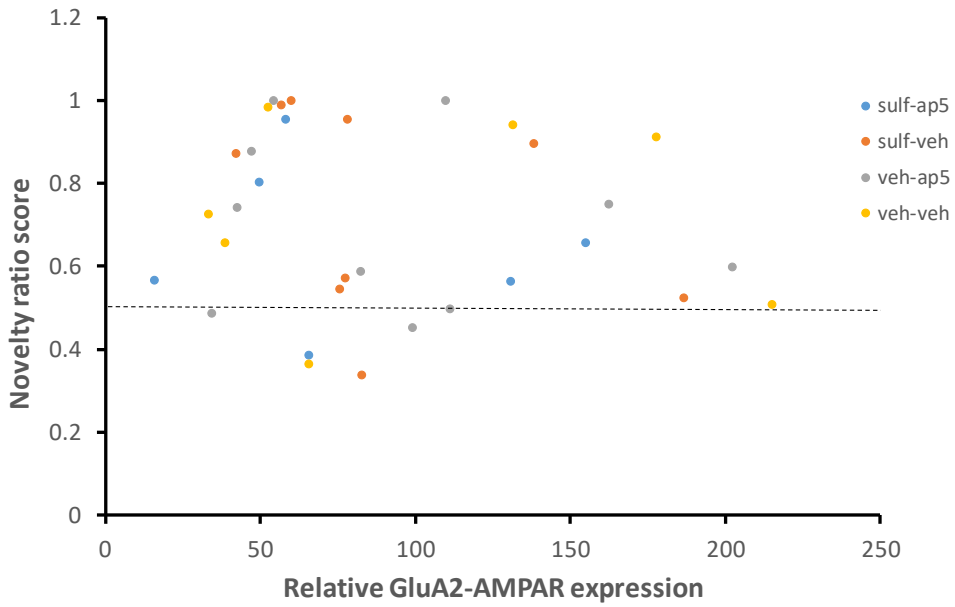

Figure 4. GluA2-AMPAR expression levels do not correlate with novelty ratio scores. No significant correlation found between relative GluA2 expression values to novelty ratio score, across all four experimental groups ($r_{(30)}$=-1.13). Novelty ratio of 0.5, no preference, is denoted by the dotted line.

CHAPTER FOUR

GluA2-containing AMPA receptor expression in CA1 hippocampus does not correlate with decreasing object location memory

J Jane Zhang, Yun Lu, Karim Nader

4.1. *Preface*

After two studies on experimentally-induced amnesia, we stepped away from the second learning protocol to investigate the nature of forgetting, in order to give perspective to data from the previous two projects to memory loss that occurs naturally, without experimental intervention. Our aim was to characterize the rate of forgetting as retention time increased through evaluation at an object location novelty preference test, and obtain snapshots of postsynaptic GluA2-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (GluA2-AMPAR) expression representative of specific time points as the object location memory is forgotten. Previous literature has shown that an object location memory can be gradually lost over time, but this forgetting can be prevented by blocking endocytosis of GluA2-AMPAR. This suggests that presence of surface GluA2-AMPAR is correlated with memory persistence, and their internalization is correlated with decay of a long-term object location memory.

Currently, the rate of GluA2-AMPAR endocytosis as forgetting occurs is not yet known. We therefore aimed to characterize changes to behavioural and molecular correlates of forgetting. Do GluA2-AMPAR expression levels continue to decrease as retention time increases, even after memory is no longer behaviourally expressed? Do levels eventually return to the levels seen in naïve animals (i.e. before learning took place)? Answers to these questions can add context to observations from the previous two projects on experimental amnesia. For instance, does the percentage of GluA2-AMPAR expression level change caused by ZIP- or sulfasalazine-induced impairment also match the changes seen after a memory is forgotten? This would suggest that changes from ZIP or sulfasalazine imitate the effects of a certain number of days of forgetting.

Rats were given the same object location learning training seen in Projects 1 and 2. After training, they remained in the colony for varying retention times before being tested on novel location preference. As retention time increased, performance decreased, showing that the memory was forgotten around 5-7 days post-training. Afterwards, memory expression stayed constant as retention time increased up to 30 days, showing that the expression had reached a floor in terms of behavioural output.

If postsynaptic GluA2-AMPAR expression in the dHPC is a correlate of object location memory, then we would expect to see a decrease of GluA2-AMPAR over time, to match the decrease in performance. However, western blot analyses of dHPC neurons did not show a positive correlation between average group novelty preference score and relative GluA2-AMPAR expression. We observed a non-linear relationship between GluA2-AMPAR expression and behaviour at test. GluA2-AMPAR expression was seen to increase steadily up to 7 days after training, which opposes the weakening of memory expression. Analyses of the loading control β-actin indicated that there were changes of actin expression across conditions, suggesting that there are innate issues with using actin as a loading control to standardize GluA2-AMPAR expression changes from this task. This implied that western blots may be problematic when examining neural changes induced by learning and forgetting, which parsimoniously explains why the expected internalization of GluA2-AMPAR could not be consistently detected in previous projects. The non-linear pattern of GluA2-AMPAR expression observed made it difficult to answer many of our original questions about GluA2-AMPAR endocytosis patterns. In Chapter 5, the Discussion, we go over several interpretations of these data.

4.2. *Abstract*

Rats forget hippocampus-dependent long-term object location memory over time, reflected in reduced preference for location novelty during test. Whether neural correlates of memory reflect this forgetting has not yet been examined. Endocytosis of GluA2-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (GluA2-AMPARs) has been shown to correlate with destabilization of memory and synaptic strength, and maintaining these receptors in the postsynaptic membrane can extend the duration of object location memory beyond its natural retention. Using western blots, we examined how post-synaptic expression of GluA2-AMPAR changes in the dorsal hippocampus CA1 region as a result of increasing time between training and test. Behavioural results indicated that memory is forgotten gradually over 5 days after training, but this decline was not accompanied by a parallel decrease in expressed GluA2-AMPARs. We also found evidence that β-actin may not provide a suitable loading control for these experimental paradigms of learning and memory loss.

4.3. *Introduction*

Although in recent years forgetting processes have attracted increased attention, mechanisms of forgetting remain relatively poorly understood. However, one possible mechanism of forgetting is emerging, involving the endocytosis of GluA2-subunit containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (GluA2-AMPARs) from the postsynaptic density (PSD) over time.

AMPARs mediate fast excitatory synaptic transmission in the mammalian central nervous system, and the trafficking of AMPARs to and from the synapse contributes critically to synaptic plasticity and modulation of synaptic strength (Malinow & Malenka, 2001). Short-term high-frequency stimulation leads to a prolonged increase in the synaptic potentiation (LTP), which has been shown to involve insertion of AMPARs into the postsynaptic density (PSD) (Y. Hayashi et al., 2000; W.-Y. Lu et al., 2001; Park, Penick, Edwards, Kauer, & Ehlers, 2004; Insuk Song & Huganir, 2002). Conversely, repetitive low-frequency stimulation can lead to a reduction of synaptic strength (long-term depression, LTD), which has been associated with the removal of GluA2-AMPARs from the PSD in the hippocampus and the cerebellum (Luthi et al., 1999; Steinberg et al., 2004). As LTP is widely accepted to be a possible cellular model underlying learning and memory processes (for example, in the amygdala, Maren, 1999; or the hippocampus, Bliss & Collingridge, 1993), regulation of AMPAR trafficking therefore must underlie changes in learning and memory (Santos et al., 2009). If maintaining synaptic strength underpins LTP and memory, forgetting mechanisms may likely involve counteracting processes, such as the synaptic weakening occurring in LTD (Tsumoto, 1993).

AMPARs are heterotetramers composed of four different subunit types, GluA1 to GluA4, and, although rare, homotetramers have also been observed (Lu et al., 2009, Wenthold et al.,

1996). Evidence of the role of GluA2-AMPARs in long-term memory has been provided by studies on the role PKMζ, a kinase that has been shown to be involved in long-term memory (LTM) maintenance (Migues et al., 2010; Serrano et al., 2008; Shema et al., 2007). Studies have shown that PKMζ is likely to maintain LTM by stabilizing GluA2-AMPARs in the PSD (Migues et al., 2014; Sacktor, 2011). Blocking PKMζ with pseudosubstrate zeta inhibitory peptide (ZIP) results in removal of GluA2-AMPARs from the postsynaptic membrane of neurons, which has been shown to induce amnesia in amygdala (Serrano et al., 2008), insular cortex (Shema et al., 2007), and hippocampus-dependent memories (Serrano et al., 2008; Migues et al., 2014; Hales, Ocampo, Broadbent, & Clark, 2016), among others. Furthermore, studies have found that blocking the activity-dependent endocytosis of GluA2-AMPARs with the peptide TAT-GluA2$_{3Y}$ prevents the amnesic effects of ZIP (Migues et al., 2010). These findings suggest that PKMζ maintains long-term memories by promoting GluA2-AMPAR expression at the PSD.

Further supporting the critical role of GluA2-AMPARs in memory persistence, daily infusions of GluA2$_{3Y}$ into the dorsal hippocampus (dHPC) have been shown to extend the longevity of object location memory (Migues et al., 2016). Migues and colleagues showed in rats that long-term memory, assessed in an object location novelty preference task, gradually and progressively declined between 7 and 10 days after training, similar to the pattern of forgetting first quantified by Ebbinghaus in 1885 (Murre & Dros, 2015). Migues et al.'s data show that memory strength from an object location recognition task decreases over time until memory is no longer expressed, and suggest that this forgetting reflects active decay of the memory, rather than interference mechanisms. Additionally, postsynaptic GluA2-AMPARs has been shown to have a positive correlation with memory strength—increased memory expression of a contextual fear conditioning task was associated with higher GluA2-AMPAR expression (Migues et al., 2010).

Thus, the available findings strongly suggest that the presence of GluA2-AMPARs at the synapse is positively correlated with memory duration and strength, but the rate of PSD GluA2-AMPAR expression change correlated with forgetting has not yet been determined.

We therefore aimed to quantify levels of GluA2-AMPAR expression as an object location memory is lost over time. To date, the exact nature of how much GluA2-AMPARs must be internalized from the postsynaptic membrane in order to induce memory loss at the behavioural level is unclear. By quantifying the rate of GluA2-AMPAR expression change during natural forgetting, we can observe, for example, if there is a gradual decrease of GluA2-AMPARs correlated with forgetting, if there are sharp drops of GluA2-AMPAR levels at certain time points, and at what point, if at all, expression drops to levels comparable to those seen in naïve animals. The change in GluA2-AMPAR expression can be examined even after object location memory is no longer expressed. For instance, if memory is no longer expressed, but receptor expression levels are higher than those of naïve animals, indicating some lingering substrates of the memory, this could suggest that a threshold of GluA2-AMPARs needs to be reached for a memory can be behaviourally expressed. Thus, to show a clear correlation between loss of GluA2-AMPARs and a decrease in memory strength, we measured declining performance of an object location task in relation to increasing retention time between training and test, and quantified GluA2-AMPAR expression levels in dorsal hippocampus CA1 neurons at these cumulative time points. If endocytosis of GluA2-AMPARs from the synaptic surface is indeed associated with memory loss, we expect to see a decrease of postsynaptic GluA2-AMPARs over time that correlates to forgetting of the object location memory. As this decrease would reflect memory decline that may be caused by a decay-like mechanism of forgetting

(Migues et al., 2016), amounts of GluA2-AMPAR internalization over time may also follow a decay pattern.

Our behavioural results replicate the forgetting curve previously described by Migues et al., (2016), but the biochemical data suggest that the relationship between surface GluA2-AMPAR expression and memory may not resemble a positive linear correlation.

4.4. *Methods*

4.4.1. Animal subjects

Male Long Evans rats (received at weight 275g-325g; Charles River) were housed in pairs in rectangular polyethylene cages with a PVC tube for environmental enrichment. Rats had *ad libitum* access to food and water. Lights in the colony were turned on at 7 a.m. and turned off at 7 p.m. daily. Behavioural studies were always performed between 9 a.m. and 5 p.m. All procedures were approved by the McGill University Animal Care and Use Committee, and complied with the Canadian Council on Animal Care guidelines.

4.4.2. Apparatus

An open field was set up in a windowless room (context room A; area 15.85 m^2, 3.98 m × 3.98 m × 2.65 m) with dim lighting. The floor level of the open field was illuminated with, on average, 13 lux. The open field was a circular area made with clear Plexiglas walls, with a total area of 3600 cm^2, and walls measuring 60 cm in height. A strip of black paper covered the lower 15 cm of each Plexiglas panel in order to minimize distraction of rats peering through the Plexiglas. One Plexiglas panel was labeled with a black and white striped pattern to act as a cue. Three objects were set up at northwest (NW), north (N), and northeast (NE) locations (see Figure 1A), with north arbitrarily assigned at the corner of the room opposite to the door. NE and NW

objects were identical, N objects were of the same height and texture but different colour and shape. Combination of objects were counterbalanced between rats. Bedding was made up of a 1:1 dried corn and woodchips mix. Flooring was a wooden pegboard into which objects can be screwed in and out. A camera hung 50 cm above the context. Behavioural testing occurred with the door closed with the experimenter outside of the room.

At the test session, the middle object in the open field was moved across the floor (see Figure 1A). The other two objects remained in the same position. Rats were placed in the open field facing the corner of the open field that was equidistant from all three objects to allow for rats to self-orient towards the objects.

4.4.3. Behavioural procedure

Rats were brought to a quiet hallway outside the context room to rest for 20 minutes before training. Training sessions consisted of daily 10-minute free exploration trials across seven days. Rats were lowered into the open field at a different corner of the open field each day (SE, SW, NW, NE), with their noses pointed away from the centre of the field. After each training session, rats were removed from the open field and returned to their home cage. Twenty-minutes following completion of training for all rats, animals were returned to the colony room. After various durations of retention, rats were brought back to the context room for a test session. At test, the middle object was moved across the open field, with the other two objects remaining in the same position. Rats were placed in the centre of the open field, facing away from all three objects, and given 3 minutes of exploration time. After completion, rats were returned to their home cage. Following completion of testing of all rats, animals were returned to the colony. One group of rats (NT) were not given training but were kept in the experiment for the same amount of time as the rats given training and one day of retention time.

4.4.4. Sacrifice

For all groups, 24 hours after testing, rats were sacrificed for western blot analysis. Rats were anaesthetized with isoflurane then decapitated. Brains were removed and flash frozen over dry ice, then preserved in a -80°C freezer.

4.4.5. Behavioural scoring

Behavioral videos were scored manually. Exploratory behaviour was counted as the rat showing directed interest in the object, e.g. smelling from at most 3 cm away from the object, touching, climbing into the object. Rats climbing on top of the object was counted, but while there, usage of the object as a platform to explore the walls of the context was not counted. Scoring began immediately prior to the first instance of directed interest towards the object, and continued for 30 seconds.

Novel object location preference (novelty ratio) was calculated with the formula:

$$\frac{time\ exploring\ new\ location\ (s)}{total\ time\ exploring\ (s)}$$

With three objects in the open field, this equation was defined as:

$$\frac{time\ exploring\ new\ location}{time\ exploring\ new\ location + (\frac{time\ spent\ exploring\ old\ location\ 1 + old\ location\ 2}{2})}$$

The typical novelty ratio formula takes consideration of two objects, and therefore time spent exploring each object location has an equal weight of 50% of the total time exploring (equation denominator). However, here, the open field had 3 objects, and the time spent with the object at the new location would be underweighted against the other two objects (33% versus 66%). To account for this, the total time spent exploring the two objects at familiar locations was averaged, thus exploration of the two familiar locations was treated as exploration of one object. In this way, time spent at the new location and the familiar locations would be weighed equally.

A score of 0.5 represents equal preference for both object locations or no preference for either object. Due to the natural preference of rats for novelty over familiarity (Berlyne & Slater, 1957; Ennaceur & Delacour, 1988), a novelty ratio significantly higher than 0.5 indicates their attention to a change in the open field at the test session compared to the training sessions, which infers a retained training memory. A novelty ratio of 0.5 indicates animals show no preference for objects at the new location, and therefore have no memory from training. A low novelty ratio indicates preference for the old object location, and thus insufficient performance for the trained memory.

Behavioural data was analysed with t-tests to compare group mean novelty ratio to an estimated ratio of 0.5, no preference. Means were also compared across groups with a one-way ANOVA. Differences in GluA2-AMPAR expression across groups were also analyzed with one-way ANOVA. GluA2-AMPAR level and novelty ratio correlation was analyzed with Pearson's correlation.

4.4.6. Biochemistry

4.4.6.1. Subcellular fractionation for postsynaptic density

The dorsal hippocampus CA1 region was dissected from frozen brains with a 1 mm neuro punch (Fine Science Tools), and homogenized in cold Tris-HCl buffer (30 mM, pH 7.4) containing 4 mM EDTA, 1 mM EGTA and a cocktail of protease inhibitors (Complete, Roche). The hippocampus homogenates were then centrifuged at 4°C at 500 g for 10 min to remove nuclei and other debris. The supernatants were removed and centrifuged at 100,000 g at 4°C for 60 min. Resulting pellets were resuspended in a solution of the homogenization buffer and 0.5% Triton X-100 and incubated on ice for 20 min, then layered over 1 M sucrose. This was centrifuged again at 100,000 g for 60 min, allowing detergent-soluble membrane components

and extra-synaptic receptors to remain in the Triton-soluble fraction. The remaining Triton-insoluble materials were pulled down into the sucrose layer, where proteins of the postsynaptic densities also lay. This final pellet was resuspended in homogenization buffer and stored at -80°C. Total protein concentration was determined with the BCA protein assay kit (Pierce)

4.4.6.2. Western blots

Western blots were performed using 8% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes overnight. Membranes were washed with 2% BSA in TBS-Tween for 1 hour at room temperature, then incubated with polyclonal antibodies to GluA2 (1 mg/ml, Millipore; dilution 1/2000) and monoclonal β-actin (2 mg/ml, Sigma; 1/10,000) for 20 hours at 4°C. Membranes were washed with TBS-Tween and incubated in rabbit horseradish antibody (GluA2, NR2B; GE Healthcare UK; 1/10,000) or mouse horseradish antibody (actin; GE Healthcare UK; 1/10,000) at room temperature for 1 hour. Detection of bands was completed with Pierce ECL2 western blotting substrate (ThermoScientific). Membrane blots were scanned with a Storm Laser Scanner (Molecular Dynamics) and analyzed with ImageQuant software (ABI).

4.5. *Results*

4.5.1. Object location memory declines over time

To assess when a noticeable decrease in memory expression would be observed, as well as when the animals will have forgotten the memory, we trained different groups of rats on an object location memory task (Figure 1A) followed by varying retention delays. Each group of rats was given differing lengths of retention time between the end of training and test session (R=1d, 3.5d, 5d, 7d, 14d, 30d). Analysis of memory expression at test revealed that groups given

1 day of retention time (NR=.69 ± .069, $t_{(12)}$=2.68, P=.02; data presented as mean novelty ratio ± s.e.m.) or 3.5 days of retention time (NR=.7 ± .078, $t_{(6)}$=2.54, P=.044) had an average novelty ratio that was significantly higher than 0.5, a no-preference value (Figure 1B). Animals that experienced retention of 5 days or longer showed no significant preference for the new or old object location (mean novelty ratio for 5d group=.58 ± .084; 7d=.47 ± .055; 14d=.55 ± .067; 30d=.59 ± .067; t>1 for all). No groups showed a significant preference for the old location.

A one-way ANOVA showed no significant differences between the 5 groups. However, a downwards trend can be seen across increasing retention time (Figure 1B). Across groups R=1d to R=7d, novelty ratio decreased progressively. Groups R=7d, 14d, and 30d, all had novelty ratios around 0.5, which suggests that the trained location memory had been forgotten. Additionally, we saw no differences of total time spent exploring between groups, indicating no group differences in motility or motivated exploratory activity directed towards the objects.

4.5.2. GluA2-AMPAR expression at CA1 PSD does not decrease uniformly over time

To assess if an increase in GluA2-AMPAR expression in the CA1 PSD correlated with acquisition of an object location memory, we quantified the amount of surface GluA2-AMPAR expression in PSD fractions of rats that experienced spatial training and compared them to yoked rats that were not trained. The trained rats were given 1 day of retention time after object location training before returning to the open field for a 3-minute probe test to verify they had acquired the object location memory. Twenty-four hours after test, all rats were sacrificed and their brains frozen over dry ice. The CA1 HC (Figure 2) was sampled then analyzed for GluA2-AMPAR and β-actin expression.

We hypothesized that the expression of GluA2-AMPAR would correlate with performance and expected rats showing learning, indicated by significant novelty ratio scores,

would have higher levels of relative GluA2-AMPARs compared to rats that had no training. However, this was not what was observed. GluA2-AMPAR levels in the dHPC CA1 did not significantly change following object location learning in trained rats (R=1d) compared to untrained rats (no-training, NT; $t_{(19)}<1$; Figure 3A). Therefore, despite the R=1d rats showing significant preference for the novel object location at test (see Figure 1), CA1 neurons did not show higher GluA2-AMPAR expression. Although these results were unexpected, we were still interested in determining the effect of increasing retention time on GluA2 expression. We therefore also probed for GluA2-AMPAR expression across the different groups of rats with varying retention delays (R=3.5, 5d, 7d, 14d, 30d) (Figure 3B). A one-way ANOVA revealed there is a significant difference between groups ($F_{(6, 50)}=5.76$, $P=.0001$). Post-hoc Bonferroni analyses showed that the R=7d group factored in all the group pairs that showed significant differences in GluA2-AMPAR expression: NT and 7d ($P=0.002$), 1d and 7d ($P<.001$), 5d and 7d ($P<.001$), and 30d and 7d ($P=.011$). Nevertheless, we were more concerned about the trend of GluA2-AMPAR expression change in relation to increasing retention time, and how these changes correlated with memory performance (i.e. novelty ratio score). Though behavioural performance of trained rats decreased as retention time lengthened, there appeared to be a trend of GluA2-AMPAR expression increasing as retention time increased. However, this pattern was disturbed by the R=5d and R=30d groups, which showed the lowest and highest relative levels of GluA2-AMPAR expression, respectively. Additionally, a cumulative increase was not observed between consecutive groups (i.e. R=1d group GluA2-AMPAR expression was not less than that of R=3.5d, R=3.5d was not less than from R=5d, and so on), which would have indicated increasing retention time as a main contributor to changes of GluA2-AMPAR expression.

Therefore, on the whole, a direct correlation between retention time and expression of GluA2 in the PSD of dHPC CA1 neurons could not be drawn.

Focusing only on the groups given training, a one-way ANOVA nevertheless showed that there was a significant effect of retention time ($F_{(5, 44)}$=5.873, P<.001). By removing the no-training group from the analyses, the differences lie between the 1d and 7d groups (P=.001), 5d and 7d (P<.001), and 30d and 7d (P=.014). Again it is the group given 7d of retention that contributed to all the pairwise group differences observed. Thus, it is possible that western blot analyses for this group skewed the results of all group analyses. Indeed, after removing the data of the R=7d group from the analysis, an ANOVA showed no significant difference between groups ($F_{(5,43)}$=2.41, P=.520).

4.5.3. GluA2-AMPAR expression at PSD does not correlate with object location memory

To better evaluate the contribution of individual differences, we correlated the relative GluA2-AMPAR levels of samples to the respective novelty ratio. Behavioural results showed no linear increase or decrease of GluA2-AMPAR expression based on retention time. Focusing only on the groups that showed significant novelty preference at test, R=1d and 3d, it seemed that GluA2 increased as novelty preference decreased. However, this pattern was not continued with R=5d. We were interested in seeing if there was a correlation between the GluA2-AMPAR levels and the corresponding average novelty ratio score and found no significant correlation between GluA2-AMPARs and novelty ratio (Figure 4). When looking at individual groups, there was no correlation between GluA2 and novelty ratio for most groups, except R=3.5d ($r_{(48)}$=0.16 ± 0.01, P=0.267).

4.5.4. Actin expression increases with new object location learning

We considered the possibility that the seemingly random pattern of GluA2-AMPAR expression across groups reflected confounds with our biochemical methods. In particular, we wanted to verify that β-actin was indeed appropriate as a loading control for western blots in these learning-based experiments. Although the structural protein β-actin is commonly used as loading control in western blots probing for GluA2-AMPAR expression, this experiment was unique in that it measured changes due only to learning and forgetting. A few studies have purported that actin is not suitable as a loading control for western analysis (Dittmer & Dittmer, 2006). Ideally, the protein chosen as loading control stays at consistent levels across experimental conditions. But actin growth has been correlated with new learning (Fukazawa et al., 2003; Lamprecht, 2014; B. Lin et al., 2005), and thus actin levels might significantly increase after training, disqualifying its appropriateness as a baseline reference for standardization of the protein of interest. However, remaining cognizant of the levels of actin in each assay should nevertheless allow for valid analyses, as long as conclusions are appended with this additional corollary.

The data from the yoked no-training (NT) and trained rats given one day of retention before testing (R=1d) did indeed show that actin levels in the CA1 dHPC increased due to the object location training (Figure 5A). Trained rats had 57% more actin in the PSD of hippocampal CA1 (dHPC CA1) compared to rats with no training. An independent samples t-test indicated that the difference was not significant ($t_{(19)}$=1.903, P=.074). However, when comparing levels across all groups, actin expression increased up to 5d days after training finished, before decreasing at R=7d. Post-hoc Bonferroni analyses showed significant differences between the R=5d group and all other trained groups (P=.001), save R=14d; and R=14d and all other trained

groups (*P*=.001), save R=5d (Figure 5B). Thus, it was possible that the inconsistency of β-actin levels across experimental conditions has biased our analysis.

To investigate this further, we reanalyzed GluA2-AMPAR expression levels that were not previously standardized to actin (i.e. using the direct volume output values of the western bands). A pattern similar to previously standardized results (Figure 3B) was observed (Figure 6). However, compared to the standardized analyses, the R=5d group had much higher levels of GluA2-AMPAR expression. Without the previous anomaly of a lowered R=5d group average, these data showed more strongly that GluA2-AMPAR expression increased despite decreased memory expression, up to R=7d. The overall trend suggested that GluA2-AMPAR expression increased up to R=7d, after which GluA2-AMPAR expression dropped. Post-hoc Bonferroni analyses revealed significant group differences between the no-training group and R=3.5d, 5d, 7d, and 14d. R=1d GluA2-AMPAR expression was also significantly different from R=3.5, 5, 7 and 14d averages. Additionally, a significant difference was seen between R=5d and 30d. Lastly, there did not seem to be a positive linear correlation between GluA2-AMPAR and actin levels, which contributed to the difficulty in making a firm conclusion about GluA2-AMPAR expression changes as a result of increasing retention time.

4.6. *Discussion*

We explored how dHPC-based object location memory performance diminishes as retention time after training increases, and how levels of GluA2-AMPARs in the PSD of dHPC CA1 neurons change as time after training increases. We expected that increased retention time would lead to increased forgetting, which could be observed by measuring novel object preference at test, as well as the relative amount of postsynaptic GluA2-AMPARs endocytosed.

Our behavioural results showed that unsurprisingly, rats from the R=1d retention group showed the highest novelty preference at test compared to other groups. As retention time increased, average novelty ratio per group decreased, up to 7 days post-training. Both R=1d and 3.5d had novelty ratio scores significantly different from 0.5. The scores from the R=5d group was lower, and after 7 days of retention time (R=7d), scores were not significantly different from 0.5, indicating no novelty preference, suggesting that at this point the memory is functionally lost. This held for 14d and 30d as well, as the behaviour reached a floor. We therefore concluded that the object location memory lasted up to 5d. This behaviour is consistent with previous data (Migues et al., 2016; Hardt et al., 2010). The data confirmed that object location memory will gradually weaken over time if there is no intervention in between training and test. Whether this memory loss is caused by a decay of the memory trace, or interference due to new learning cannot be determined from behavioural data. Yet, based on the work by Migues et al. (2016), who showed that in this task it is unlikely that memory interference contributes to forgetting, our data suggest that this memory loss may likely be driven by an active decay process (Migues et al., 2016; Hardt et al., 2013). However, patterns observed in our molecular data did not lend themselves easily to interpretation, nor did they match previous literature about GluA2-AMPAR. Based on GluA2-AMPAR expression data that was not standardized to loading control, surface GluA2-AMPAR levels across consecutive groups seemed to rise to a peak at R=5d, and then decrease again afterwards (Figure 6). As novelty preference is an indicator of memory strength, the pattern of GluA2-AMPAR expression increasing as memory is forgotten goes against the existing understanding of GluA2-AMPAR endocytosis. As research has shown a positive, linear correlation exists between amount of postsynaptic GluA2-AMPAR in amygdala neurons and conditioned fear memory strength (Migues et al., 2010), our observations were unexpected.

Another surprising observation was that no change in GluA2-AMPAR expression levels was noticed between the R=1d trained group and the no-training group. If GluA2-AMPARs are a neural correlate of LTM maintenance and memory strength, then we would expect to see an increase of GluA2-AMPARs after new learning. One interpretation of this data would suggest that GluA2-AMPARs do not begin to accumulate in the postsynaptic membrane until after 24 hours post-training, at a time point later than when we sacrificed the animal. Typically, after LTP induction, GluA1-AMPARs inserted into the PSD are gradually exchanged with GluA2-AMPARs over a timeline of minutes to hours without causing a change in synaptic strength (Shi et al., 2001; Plant et al., 2006; McCormack et al., 2006). Thus, perhaps for 24 hours post-training, memory of the object location training is mediated primarily by GluA1-containing receptors. However, this contradicts current literature. The insertion of GluA1-AMPARs and their gradual replacement with GluA2-AMPARs is thought to help stabilize LTP at the synapse, and thus is part of the process of memory consolidation (Hong et al., 2013; Joels & Lamprecht, 2010; Shi et al., 2001). Because cellular consolidation of spatial memories has been shown to usually occur over a few hours (see McGaugh, 2000, for review), it therefore seemed unlikely this process could stretch out past one day. Additionally, the data showed no significant increase of GluA2-AMPAR expression even with 3.5 days of retention.

Rather, the results may reflect the test session acting as an additional training session, which would have induced a new learning experience. This new learning may have upregulated GluA2-AMPARs after the test completed and that memory was consolidated. However, all rats experienced the same test session, so any upregulation as a result of new learning is likely to be consistent across all groups. Alternatively, if there are different amounts of GluA2-AMPAR upregulated depending on retention time given, this would suggest that the amount of retention

time affects mechanisms of future learning (i.e. the test session), such that less GluA2-AMPAR increase is seen when the animals are tested closer to training, and more GluA2-AMPARs are seen when tested as retention time increases up to 5 to 7 days. But this interpretation makes it difficult to reconcile the downregulated GluA2-AMPARs observed in R=14d and 30d groups. On the whole, our findings are conflicting and no linear correlation could be detected between postsynaptic GluA2-AMPAR expression in the CA1 and memory expression as indexed by novelty ratio scores. This does not mean that a correlation does not exist, but rather, may indicate that western blots are not a sensitive enough method by which to quantify real GluA2-AMPAR changes in object location memory. The changes detected here might therefore be artifacts compounded from confounding variables, but not specifically due to changes in memory strength.

Immunoblots have been previously used to assess glutamate receptor expression in the rat hippocampus CA1 (for example, Rao-Ruiz et al., 2011; Heynen, Quinlan, Bae, & Bear, 2000), but these studies induce a strong, acute behavioural or electrophysiological response (contextual freezing or high frequency stimulation, respectively). A possible issue with a non-aversive, non-associative task like object location learning is that the hippocampal signal of the memory representation could be too small to be accurately detected against the background noise of all hippocampus function. In other words, the number of neurons recruited in an object location memory might be too small, thus rendering it impossible to detect any significant changes of their signal. GluA2-AMPAR are present in all excitatory synapses in the hippocampus (Lu et al., 2009), and the task likely only recruits a small fraction of dHPC neurons. Although the percentage of neurons recruited to a non-goal oriented exploratory task like object location learning is not well-known, studies have shown that increasing the activity of cAMP response

element binding protein (CREB) in CA1 neurons before a contextual fear memory, a process which may underlie hippocampal memory formation, results in recruitment of around 25% of neurons (Restivo, Tafi, Ammassari-Teule, & Marie, 2009; Rogerson et al., 2014). Presumably, a less emotionally salient memory like object location would result in even fewer neurons and/or synapses being recruited. In comparison, the amount of lateral amygdala neurons that can encode an extremely salient tone-shock presentation exceeds 70%—although some evidence shows that only 10-30% may be needed for a single memory to be expressed (Han et al., 2007; Rumpel, LeDoux, Zador, & Malinow, 2005). Thus, even after acquisition of a spatial memory, the number of recruited neurons may be small compared to all available and active CA1 neurons encoding for daily spatial information, thereby making detection of changes in GluA2-AMPAR expression extremely difficult.

Finally, our interpretations may not be reliable due to the inconsistency of our loading control, actin, across experimental groups. Taking into consideration that the pattern of the biochemical data neither matches the pattern of behavioural data, opposing the literature on GluA2-AMPARs, the most parsimonious interpretation seems to be that the methods used to detect biochemical changes relating to this type of memory were not sensitive enough for our intended analysis. Therefore, the questions of the rate of GluA2-AMPAR expression changes, and what levels are needed for memory expression and/or memory loss, at present cannot be definitively answered. Other techniques might allow more tuned quantification of GluA2-AMPAR in the PSD, such as labelling endocytosed GluA2-AMPARs using a biotinylation assay (Rao-Ruiz et al., 2011). However, this would not solve the innate signal-noise resolution issue of object location memories in the dHPC. If GluA2-AMPAR endocytosis is to be properly studied in these types of memory, one would first need to find a way to increase signal or decrease noise.

Future research should involve either amplifying the neuronal signal representing the object location memory, or reducing noise of hippocampal neurons irrelevant to the target behaviour. For example, noradrenaline application to hippocampal cells (i.e., as a result of connections from the locus ceoruleus, or the hippocampal-ventral tegmental area loop) may be capable of accomplishing this (Otmakhova, Duzel, Deutch, & Lisman, 2013; Susan J. Sara, 1985).

4.7. *Conclusion*

In this study we showed behavioural evidence for the rate of forgetting an object location memory over time. However, we were not able to substantiate the behavioural data with biochemical data, and therefore cannot make firm conclusions about whether the forgetting occurred due to decay of the memory trace through loss of GluA2-AMPARs from the PSD. Over the month of retention time, there seemed to be very little change in GluA2-AMPAR expression in the PSD of hippocampal CA1 neurons compared to control animals, with the exception of rats that were tested 7 days after training. Based on our understanding of how plentiful GluA2-AMPARs are in the hippocampus, the possible low signal in object location memories, and the sensitivity of our biochemical assay, we concluded that western blots may not provide the ideal tool to quantify CA1 GluA2-AMPAR expression levels.

Understanding the regulation and function of GluA2-AMPARs in synapses is increasingly imperative as research interest builds in forgetting mechanisms and memory dysfunction. Downregulation of GluA2-AMPARs in human hippocampus neurons has been implicated with increased glutamate-mediated vulnerability to Alzheimer's pathology (Carter et al., 2004), and cortical neurons in Alzheimer's patients revealed up to 40% less GluA2-AMPAR in the PSD compared to controls (Gong, Lippa, Zhu, Lin, & Rosso, 2009). Upregulation of

GluA2-AMPAR has also been proposed to offset mild cognitive impairment that results from patients with subcortical ischemic vascular dementia (Mohamed et al., 2011). Therefore, characterizing how GluA2-AMPAR levels are related to memory retention and associated memory strength may help creating possible therapies to prevent memory dysfunctions. In addition to preventing endocytosis, work can also be done to pharmacologically increase GluA2-AMPAR levels, which may be used in clinical treatments to boost memory longevity.

4.8. *Figures*

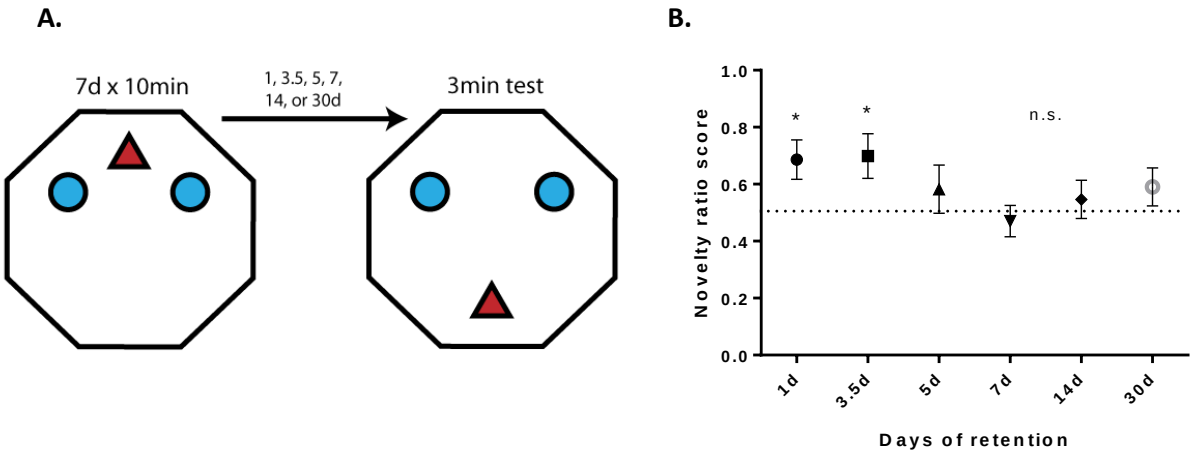

Figure 1. Object location memory as indicated by novelty preference decreases as retention time increases. A) Experimental design. Rats explored Open Field A for 10 min/day across 7 days, then were left undisturbed in their colony room for 1, 3.5, 5, 7, 14, and 30 days. A 3-min test session occurred after retention where object location memory was assessed by moving one object to a novel location in Open field A. B) Object location memory as indicated by novelty preference ratio decreased with increasing retention time. n=8 per group, except R=1d group (n=7). *P<0.05 indicates significance from 0.5, no preference, denoted by the dotted line. Data presented as means ± s.e.m.

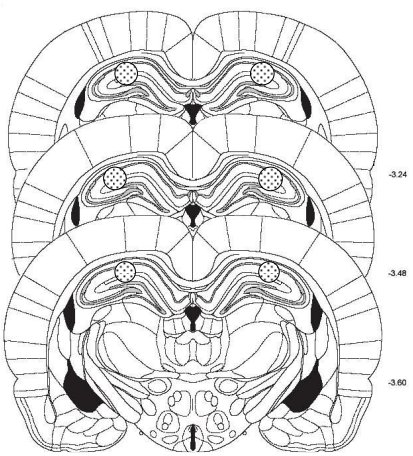

Figure 2. Schematic representation of sampled locations within the dorsal hippocampus and punched CA1 area (dotted circle) for western blot analysis. Values on right side indicate anterior-posterior position relative to bregma (Paxinos and Watson, 2013).

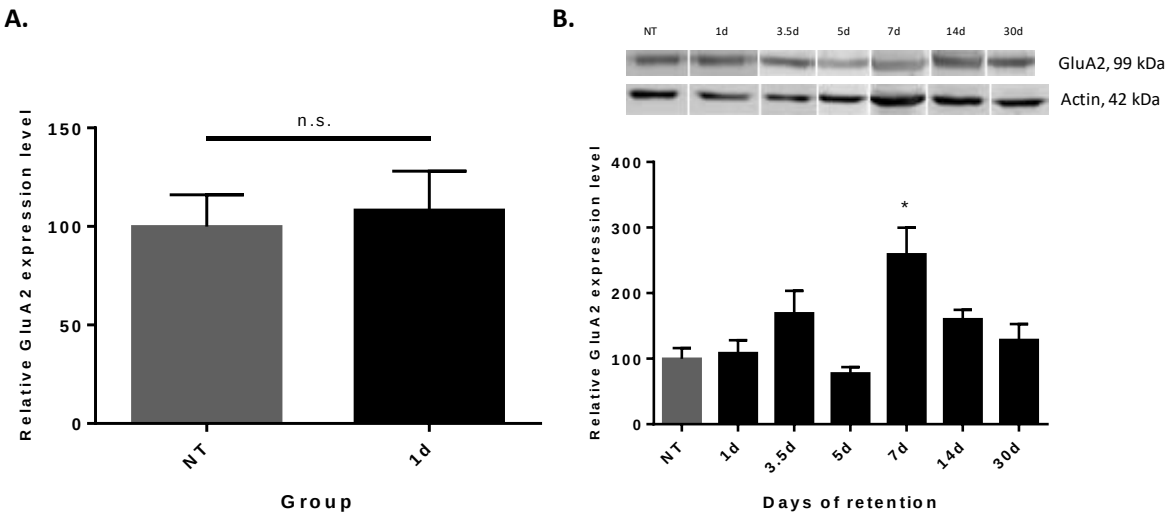

Figure 3. GluA2-AMPAR expression levels in CA1 PSD do not change linearly as a function of training or retention time. *A)* No significant difference was observed between surface GluA2-AMPAR expression of the no-training group and R=1d group. GluA2-AMPAR levels are expressed as relative percentages values of the no-training group (set at 100%). Raw GluA2-AMPAR expression values were standardized to group actin expression. *n*=7 for no-training group, *n*=14 for retention=1d. *B) Top:* Representative western blot bands of GluA2-AMPAR expression volume of each experimental group. *Bottom:* GluA2-AMPAR expression levels in CA1 PSD do not decrease with time. A significant difference between groups was found for R=7d and no-training (NT), 1d, 3,5d, 5d, and 30d. All values are presented as percentages of average GluA2-AMPAR expression in the R=1d group compared with the no-training group (set at 100%). Output GluA2-AMPAR expression values were standardized to β-actin as loading control. *n*=6-8 rats per group. Two outliers in the R=14d group were removed due to darkness of the bands. **P*<.05 for all groups besides R=3.5d and R=14d. Data are expressed as the mean ± s.e.m.

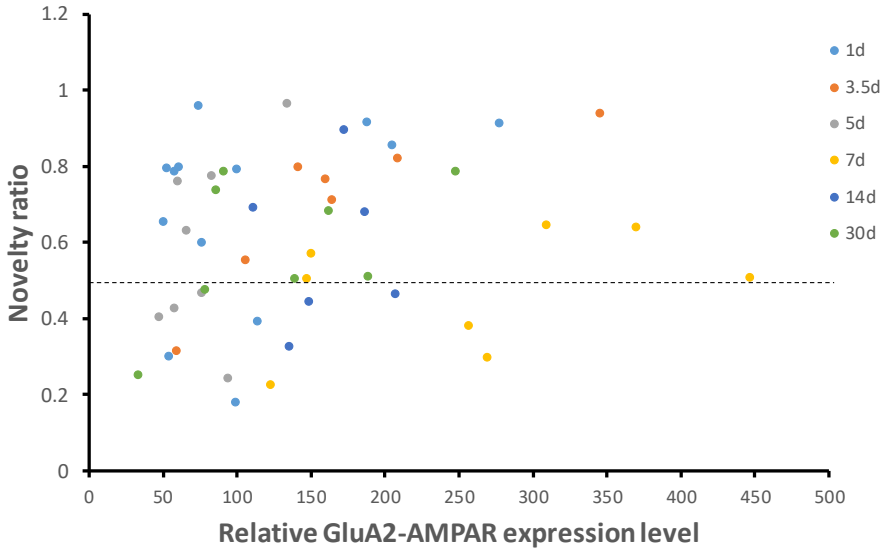

Figure 4. No correlation observed between individual novelty ratio versus GluA2-AMPAR levels in trained rats ($r_{(48)}$=0.16). All GluA2-AMPAR percentage levels are standardized to the no-training (NT) group, as in Figure 4. NT rats are excluded. *n*=6-8 rats per group, except R=1d, *n*=13. Novelty ratio of 0.5, no preference, is denoted by the dotted line.

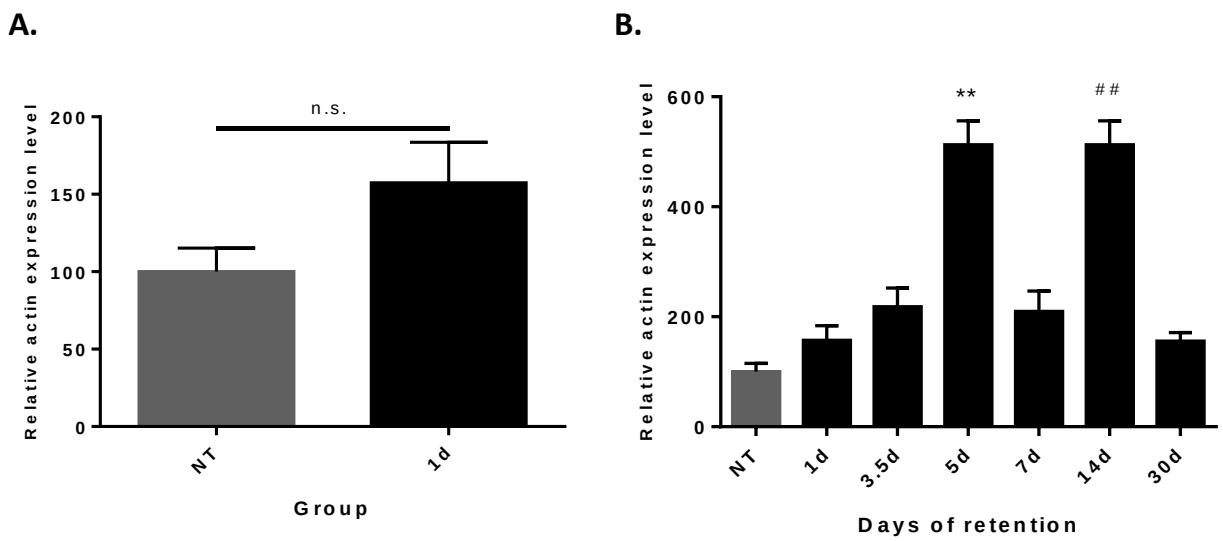

Figure 5. Actin expression levels change as retention time increases. *A)* Actin levels in CA1 PSD increases after object location training. Average amount of actin are shown as a percentage of no-training controls (set as 100%). *n*=7 for no-training (NT), *n*=13 for retention=1d. *B)* Actin levels in CA1 PSD increases after object location training up to 5d. Average amount of actin is shown as a percentage of no-training controls (set as 100%). *n*=7-8 rats per group except R=1d. ***P*<.001 for all groups except R=14d. ##*P*<.001 for all groups except R=5d. Data are expressed as the mean ± s.e.m.

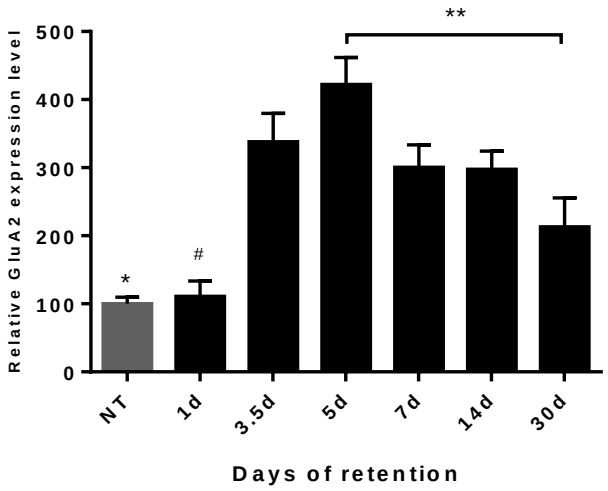

Figure 6. GluA2-AMPAR expression levels not standardized to loading control (actin) in CA1 PSD do not change linearly as a function of training or retention time. Average amount of GluA2-AMPAR expression is shown as a percentage of no-training controls (set as 100%). *significance from R=3d and R=5d: $P<0.001$; from R=7d: $P=0.002$; from R=14d: $P=0.006$. #significance from R=3d and R=5d: $P<0.001$; from R=7d: $P=0.001$; and from R=14d: $P=0.003$. **$P=0.001$ between R=5d and R=30d. Data are expressed as the mean ± s.e.m.

CHAPTER FIVE

General discussion

5.1. Rationale of studies

In general, there are two ways to frame how experimental amnesia of a consolidated memory or a reactivated memory can occur. The memory is lost either through a storage impairment, where the memory representation is rendered unavailable, or a retrieval impairment, where the memory representation is rendered inaccessible. We were interested in showing how erasure of a long-term object location memory (LTM) through infusions of PKMζ inhibitor ZIP, or blocking reconsolidation through post-reactivation infusions of transcription inhibitor sulfasalazine (SSZ), caused either a storage or retrieval impairment of the memory. In addition, we were interested in discovering the relationship of the emergent behavioural impairments with the loss of a neural correlate of memory (GluA2-AMPARs), in not only cases of experimentally-induced amnesia, but also in forgetting, which likely occurs through a decay-like mechanism. We wanted to show both behavioural and molecular evidence for how storage impairment of a memory can lead to memory deficits. Therefore, overall, we hoped to increase our understanding of how mechanisms of memory loss involving GluA2-containing AMPARs occurs.

The exact nature of the relationship between α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and memory loss is still being established. The cellular models of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD), involve insertion and removal of AMPARs, respectively, from the plasma membrane of the postsynaptic density (PSD), a mechanism that seems to modulate postsynaptic expression of synaptic strength (Kessels & Malinow, 2009). This ability marks AMPARs as prominent neural correlates of many types of memory, including object location memories represented in the dorsal hippocampus (Assini et al., 2009; Gilbert & Kesner, 2004; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002). Current research has built a wealth of knowledge about how

AMPAR trafficking in and out of the excitatory synapses leads to learning and memory, including findings about how insertion of AMPARs containing the GluA2 subunit (GluA2-AMPARs) in the PSD are required for sustained LTP, memory strengthening, and memory maintenance, and how removal of these receptors from the synapse result in LTD and memory weakening (see Kessels & Malinow, 2009, and Huganir & Nicoll, 2013, for a review). There is compelling evidence that removal of GluA2-AMPARs from the synaptic surface leads to decay of LTP (Dong et al., 2015), and loss of LTM (Migues et al., 2010; Migues et al., 2014), and blocking GluA2-AMPAR endocytosis has been shown to prevent these decay effects (Migues et al., 2016). In support of this, a proposed mechanism of memory maintenance involving constitutive activity of PKMζ is hypothesized to preserve GluA2-AMPARs in the PSD (Migues et al., 2010; Sacktor, 2011; Yao et al., 2008). Data on the whole suggest that GluA2-AMPARs confer stability to the putative memory trace (Man, 2011), and removal of GluA2-AMPARs will disrupt the memory (Hardt, Nader, & Wang, 2014).

This research led us to the question of how the rate or amount of GluA2-AMPAR endocytosis affects memory loss. Studies have linked increasing surface GluA2-AMPAR presence to increasing memory strength (Migues et al., 2010), but the opposite relationship, of decreasing amounts of GluA2-AMPARs to decreasing memory strength, leading to memory loss, had not yet been explicitly shown. Furthermore, the relative percentage of GluA2-AMPARs that must be internalized from the postsynaptic membrane to manifest behavioural impairment was not known. We intended to examine if observed levels of GluA2-AMPAR endocytosis in neurons recruited in the memory representation directly correlate to a decrease in memory expression, as measured by an object location preference task. Thus, overall, our research goal was to relate how removal of surface GluA2-AMPARs in dorsal hippocampus neurons leads to a

storage loss of the memory trace, leading to experimental amnesia (Project 1, blocking long-term memory maintenance, and Project 2, blocking reconsolidation), and forgetting over time (Project 3). Comparing results from the three projects would allow us to determine whether changes to GluA2-AMPAR expression as a result of experimentally-induced amnesia caused similar changes to those associated with natural forgetting.

5.2. Summary of findings

5.2.1. Experimental amnesia causes storage loss of spatial learning memory

For Projects 1 and 2, we employed a second learning paradigm originally developed in our lab (Hardt et al., 2009), to decipher the nature of experimental amnesia. This protocol makes a prediction about whether an amnesic intervention (i.e. here, either post-consolidation ZIP or post-reactivation sulfasalazine infusions) leads to a storage or retrieval impairment of a first learning memory based on the efficacy of AP5 infusions (i.e. NMDAR impairment) on second learning acquisition. While NMDAR activation is required for the first time a task is learned, second learning has been shown not to require NMDAR activation (for example, Bannerman et al., 1995; Hardt et al., 2009; Tayler et al., 2011; Wiltgen et al., 2011). We therefore hypothesized that if the storage of a first-learned memory was impaired, then a subsequently learned object location memory would be acquired in an NMDAR-dependent manner, as if the learning was naïve. Had the amnesia been a product of retrieval impairment, acquisition of a second learning experience would have remained NMDAR-independent. This procedure thus forms a positive prediction about the cause of the amnesia, rather than relying on negative results (i.e. a lack of memory) that historically led to confounding interpretations of behavioural data (see Gold & King, 1974; Nader, 2009). With this procedure, we were able to confirm that ZIP-induced

impairment of LTM and sulfasalazine-induced reconsolidation blockade—and any associated GluA2-AMPAR changes—could be categorized as a storage impairment of the memory trace.

Our hypotheses were confirmed for the behavioural components of both Projects 1 and 2. Erasing long-term memory through infusions of PKMζ-inhibitor ZIP or blocking reconsolidation of a memory with post-reactivation infusions of transcription blocker sulfasalazine lead to a second learning memory to be NMDAR-dependent, suggesting that these treatments lead to a storage impairment of object location memory (Chapter 2, Figure 4; Chapter 3, Figure 2, respectively). Building off these results, if experimental amnesia was indeed caused by a storage impairment of the memory trace, then it is possible to measure molecular changes in the PSD that are the root of the storage of loss. Therefore, the behavioural results lend support to our hypothesis that GluA2-AMPAR expression levels would decrease after inducing amnesia. Blocking PKMζ with ZIP will disrupt the maintenance of GluA2-AMPARs in the PSD (Migues et al., 2010). Reactivation of a memory has also been shown to involve surface GluA2-AMPAR internalization, causing destabilization (Hong et al., 2013; Rao-Ruiz et al., 2011); blocking the subsequent restabilization process known as reconsolidation, must therefore prevent reinsertion of GluA2-AMPARs. Thus, in both situations, we anticipated that GluA2-AMPAR levels in the PSD would decrease after inducing experimental amnesia of first learning memory.

However, our biochemical analyses did not confirm our hypothesis. Neither Project 1 nor 2 found a significant difference in surface GluA2-AMPAR expression in PSD fractions of CA1 neurons between the fully amnesic group (drug-AP5) and other treatment groups (Chapter 2, Figure 4; Chapter 3, Figure 2). This might be reflective of the pattern seen in the behavioural results, as neither project showed a significant difference between their treatment groups in terms of novelty ratio score (Chapter 2, Figure 5; Chapter 3, Figure 3). In Project 1, interestingly, we

found an upregulation of GluA2-AMPARs associated with decreasing novel location preference, but only in animals infused with ZIP (Chapter 2, Figure 7). But we did not see any similar changes in Project 2 (Chapter 3, Figure 3). The amount of GluA2-AMPARs internalized in these conditions was inconsistent with what we expected in both projects. In Project 1, some groups showed increased levels of GluA2-AMPAR expression after impairing LTM; in Project 2, we saw no changes in GluA2-AMPAR expression in rats given reconsolidation blockade, compared to those with non-impaired memories. Thus, because of these inconsistencies, the data overall suggest that western blotting may not be a sensitive technique enough to detect changes in surface GluA2-AMPAR expression caused by our experimental method. These interpretations will be discussed more in depth in Section 4.

5.2.2. Forgetting of the object location task does not correlate with changes in surface GluA2-AMPAR expression in CA1

We wanted to compare the changes seen in GluA2-AMPAR expression after experimental amnesia with changes that occurred as memory was forgotten. If similarities between GluA2-AMPAR expression patterns in situations of experimental amnesia and regular forgetting were found, this would allow formation of broader conclusions about the role of GluA2-AMPAR internalization in memory loss. Thus in Project 3, different groups of rats received object location learning and then were tested after varying retention intervals. Behaviourally, we expected to see a gradual decline of novel object location preference (i.e. decreasing novelty ratio) as retention time increased, until the performance plateaued at no preference or equal preference ratio (0.5 novelty ratio score). Based on the literature, we then hypothesized that we would see a corresponding decline in GluA2-AMPAR expression in CA1 that would match the behavioural pattern.

Being able to show GluA2-AMPAR expression changes as forgetting progresses would provide information about the percentage of GluA2-AMPARs internalized in order for the animal to behaviourally show no memory expression. Many behavioural tasks such as the object location protocol have a performance floor—a point where the animal has functionally forgotten the memory and cannot show "worse" performance. In this task, this point is the no preference ratio of 0.5. If GluA2-AMPAR levels continued to change (i.e. decline) even after the animal has reached this limit of memory expression (i.e. the memory has been forgotten), this would suggest that GluA2-AMPAR changes only correlate with memory expression up to a point. In other words, this might indicate a threshold of GluA2-AMPAR expression needed in order for the neural representation of memory to be expressible as behaviour. Thus, levels of GluA2-AMPAR expression in groups that had forgotten the memory were of unique interest for our study. Equally important at the outset of the project was the percentage of GluA2-AMPAR change between good and inadequate performance at test, and if these changes were relatively similar to those that would be observed when the memory is experimentally erased (Projects 1 and 2). For example, would the relative percentage of GluA2-AMPARs internalized after ZIP infusions correspond to the GluA2-AMPAR levels in rats that experienced retention time of 5 to 7 days, when the memory has recently been forgotten, or at 30 days, when the memory may have been forgotten for weeks? Compiling the data from all three projects would allow us to build a profile of how much GluA2-AMPAR internalization leads to functional memory loss. This would show the ratio of GluA2-AMPARs that could be lost through experimental means versus endogenous means. Unfortunately, inconsistent molecular data did not allow for these comparisons.

Like Projects 1 and 2, the behavioural results of Project 3 matched our predictions. Corroborating previous work (Migues et al., 2016), we saw a gradual decline in novelty ratio

score as retention time increased. As retention time increased past a week, average novelty ratio scores remained around 0.5. This showed that object location memory weakened over time, and was fully forgotten between 5 to 7 days after training. The memory may have been lost through decay or interference processes, but decay is more likely for three reasons: because animals were not exposed to any new learning experiences during the retention time, because these hippocampus-dependent LTM have been shown to be relatively immune to interference (Migues et al., 2016), and because it would fit in with the framework of losing postsynaptic GluA2-AMPARs over time.

However, western blot analysis did not show results matching our hypothesis. We observed overall a large spike of GluA2-AMPAR expression after 7 days of retention time; it was significantly higher than the expression seen in many other groups. This uptick followed a dip in GluA2-AMPAR expression after 5 days of retention, which showed the lowest GluA2-AMPAR expression relative to all groups (Chapter 4, Figure 3). When we correlated individual novelty ratio score with GluA2-AMPAR expression of the sample, only the group that was given three and a half days of retention (R=3.5d) displayed a significant correlation, in which increasing GluA2-AMPAR expression correlated with increasing novelty ratio score. This in itself seemed to oppose the negative correlation that was observed in ZIP-infused animals in Project 1. The lack of consistent results across groups and across experiments makes interpretation difficult. However, as stated in Chapter 4, drawing firm conclusions here is problematic since, due to issues with the loading control, we could not be certain that GluA2-AMPAR levels seen in the no-training group accurately represented true GluA2-AMPAR levels, and thus standardizing other experimental groups to the data of the no-training group may have skewed actual GluA2-AMPAR levels across all groups.

Despite these reservations, we would like to note a potentially interesting finding when looking and Projects 1 and 3. The possibility of an upregulation of GluA2-AMPAR expression at R=7d (Chapter 3, Figure 3) or even R=5d (unstandardized GluA2-AMPAR expression; Chapter 3, Figure 6) is surprising because at that point, the memory is no longer expressed behaviourally (i.e. it has been forgotten), so we would expect less GluA2-AMPARs in the PSD fractions. It was also surprising to find more GluA2-AMPAR expression in ZIP-infused rats that preferred to explore the old object location at a second learning test (Chapter 2, Figure 7). These situations both are snapshots in time when the object location memory is forgotten, as indicated by behavioural results. Could there be a subsequent increase in GluA2-AMPARs that occurs after a first-learned object location memory is lost? If so, what is the function of this upregulation? One plausible explanation is that new GluA2-AMPARs are being inserted as a result of a new learning experience. For example, the probe test could have induced a new learning experience that translated to upregulated GluA2-AMPARs seen 24 hours later. However, this would not explain why some treatment groups showed greater upregulation than others, since all rats received the same test session. In particular, we did not observe the same GluA2-AMPAR upregulation for the R=14d and 30d group in Project 3, which would be expected if forgetting was a prerequisite to GluA2-AMPAR upregulation after testing. Thus, even if the nature of GluA2-AMPAR involvement is more nuanced than we originally predicted, the current data does not lend themselves to clear conclusions.

Taking into consideration the difficulties in detecting GluA2-AMPAR changes across groups throughout all three projects, the most likely conclusion may be that western blotting did not accurately quantify GluA2-AMPAR expression across all three experiments. It may even be that the observed molecular changes were due to artifacts in the procedure, and thus the

fluctuations were at least somewhat random. Ultimately, the ambiguity of our molecular results prevent us from making conclusions about GluA2-AMPARs' correlation with memory loss, comparing the patterns across the three projects, and from inferring how GluA2-AMPAR levels change as a result of the various conditions of the second learning paradigm (i.e. no memory, one retained memory, two retained memories; see section 1.4.4. in the Introduction). However, if our results do accurately reflect true GluA2-AMPAR expression levels, at least to some degree, it is nevertheless important to consider what might lead to these results.

5.3. Theoretical implications of the data

Overall, many parts of the biochemical data do not align what the literature would suggest. Below we speculate about possible theories that may account for some of our findings, and whether these interpretations are plausible.

5.3.1. Post-retrieval GluA2-AMPAR exchange with CP-AMPARs

In Project 1, we observed that following erasure of a first learning memory, adequate second learning memory was correlated with relatively low levels of GluA2-AMPARs. Previous research has shown that retrieval of a memory leads to a temporary destabilization period of the memory trace, in which GluA2-AMPAR are exchanged with GluA1-AMPARs (Rao-Ruiz et al., 2011). GluA2-AMPARs are rapidly removed from the PSD to be replaced with GluA1-AMPARs, which are then gradually exchanged again for GluA2-AMPARs as the memory reconsolidates and thus restabilizes (Rao-Ruiz et al., 2011; Hong et al., 2013). We therefore considered the possibility that memory retrieval induced by the probe test caused GluA2-AMPAR exchange with GluA1-AMPARs, which were not exchanged back at the time of sacrifice. In Rao-Ruiz and colleagues' study using contextual fear memory, GluA2-AMPAR

reinsertion during reconsolidation had occurred by 7 hours post-retrieval. Perhaps the timeline of GluA2-AMPAR reinsertion post-retrieval is different for object location memories?

Morris et al. (2006) showed reconsolidation of a water maze memory, but to our knowledge there is no other study that shows reconsolidation in an object location memory. If our data reflects actual changes in GluA2-AMPARs, they suggest that object location memories take significantly longer to reconsolidate than contextual fear memories, leading to a longer duration where GluA2-AMPARs are downregulated after retrieval. This may also explain the peculiar behaviour observed in the ZIP-infused rats from Project 1 showing high levels of GluA2-AMPAR expression and preference to the old object location at test. ZIP-Veh rats in this subset may not have correctly retrieved the second learning memory, thus there was no internalization of GluA2-AMPARs prompted by the test session. ZIP-AP5 rats in the subset, on the other hand, had not acquired any of the training, and thus higher GluA2-AMPAR levels may be indicative of the probe test acting as new learning. However, if new learning occurred at test, all individuals in the ZIP-AP5 group should show similarly high levels of GluA2-AMPAR expression, which was not observed. Therefore, we would then have to infer that the observed GluA2-AMPAR expression in these rats directly correlates to an individual's learning experience, and thus not all rats learned at the test session. Although individual variation in learning and retention is expected, this theory opens an additional layer of complexity regarding the efficacy of the object learning task. This would further confound any behavioural interpretation.

Indeed, if data from Project 2 was interpreted with the above framework, we would reason that the lack of difference in GluA2-AMPAR expression levels between groups indicates that none of the memories retrieved at test had reconsolidated at the time of sacrifice. But as

these results do not show any treatment group differences, and there was no correlation between GluA2-AMPARs and novelty ratio, the data does not allow any further speculation. With Project 3 data, a reconsolidation period lasting over 24 hours would infer that post-retrieval GluA2-AMPAR internalization should lower GluA2-AMPAR expression levels to be similar to baseline levels of the no-training group. This could explain the results of the R=1d group. However, the data would imply that this putative reversal of GluA2-AMPAR expression continued for 5 days after testing, which is extremely unlikely (Chapter 3, Figure 3). Thus, on the whole, there is no parsimonious interpretation involving post-retrieval GluA2-AMPAR internalization that accounts for all observations.

5.3.2. Place field size correlates to neuronal recruitment

Place cells in the hippocampus are neurons that respond to an animal's current location (O'Keefe & Dostrovsky, 1971), as well as past locations (O'Keefe and Speakman, 1987) and upcoming locations (Ferbinteanu & Shapiro, 2003), by firing when animals are in their respective place fields. In an open field less than 1 m^2, approximately 20 to 50% of dorsal CA1 pyramidal neurons will become place cells (Karlsson & Frank, 2008; Thompson & Best, 1989; Wilson & McNaughton, 1993). Research has shown that over time, place cells do not solely reflect location information, but also information about events that took place in that space (Leutgeb et al., 2005; Moser, Rowland, & Moser, 2015). As the object location task would imply, hippocampal neurons have been shown to respond to object-in-space information (Komorowski et al., 2009; Manns & Eichenbaum, 2009). Additionally, neurons in the distal CA1 express more place fields when objects are in the open field (Burke et al., 2011). Therefore, as our task deals with object location, a second way to interpret our GluA2-AMPAR findings is to relate them to place field expression.

An increase in GluA2-AMPAR expression seen at second learning test after ZIP-induced erasure of first learning memory (Project 1) may reflect an increase in cells being recruited for a larger place field (Fenton et al., 2008). Studies of the ventral hippocampus have suggested that larger place fields of individual neurons are related to a decline in precision of spatial representation (Royer et al., 2010). This could suggest that the relatively high GluA2-AMPAR levels after second learning test reflects greater neuronal recruitment, which may be related to less precise place fields. Thus, the preference to the old object location may be an artifact of imprecise place fields. Indeed, Barry et al. (2012) have shown that infusing ZIP into the hippocampus while place fields are being expressed makes them less precise. Thus, if the ZIP infusions resulted in larger place fields, the preference to the old location is not necessarily an indication of preference for familiarity, but rather may reflect neurons firing at more locations, or perhaps that more hippocampal neurons were activated near the old object location, thus producing a cruder memory. Following this logic, less GluA2-AMPAR expression implies smaller, more precise place fields, which may be a measure of optimized performance (i.e., better novelty preference).

However, while this interpretation may hold for Project 1, it does not clearly explain the GluA2-AMPAR results from Project 3. Following this explanation, the increase of GluA2-AMPARs seen after 7 days of retention time may indicate less precise place fields, which may be a consequence of forgetting, but we would also expect molecular data from R=14d and R=30d groups to display similarly, or even more imprecise place fields. This would output as similarly high or even higher levels of GluA2-AMPAR expression compared to R=7d rats, which is opposite to the declining levels seen in our data. Additionally, it is impossible to conclude whether imprecision in place fields also occurs after reconsolidation blockade via sulfasalazine

infusions as seen in Project 2, as no any significant correlations between behaviour and GluA2-AMPAR expression were observed. Thus, this theory does not adequately explain all results.

5.3.3. Metaplasticity and NMDAR-independent learning

The phenomenon of metaplasticity, described as the plasticity of synaptic plasticity, is another means through which we may be able to interpret our data. Situations in which prior experience will influence future learning and memory (Abraham and Bear, 1996; Abraham, 2008) may be a form of metaplasticity; thus the second learning phenomenon first observed by Bannerman et al., (1995) may fall under this description. Other studies have also shown the effect of NMDAR-independent learning (Sanders & Fanselow, 2003; Saucier & Cain, 1995; Tayler et al., 2011; Wiltgen et al., 2011). Wiltgen et al. (2011) put forth evidence that this effect only occurs when the subsequent learning experience occurs using the same behavioural task as the initial experience. Some data has recently emerged showing that if two separate hippocampus-based learning events are related such that one learning event influences the other, this can increase the degree of overlap of each memory's associated neuronal populations (Cai et al., 2016; Nomoto et al., 2016). For example, Cai et al.'s 2016 study showed that exposing mice to two separate contexts within one day shows a higher overlap of neural ensembles in the CA1, compared to when events are separated by a week. Furthermore, the shared neurons link together both memories, allowing the fear associated to the first context to be activated when animals were put in the second context, and increased excitability of the neurons representing the first contextual fear memory increase the strength of the later memory. Thus, it may be possible that the second learning effect reflects recruitment of overlapping neurons across two learning experiences, and the existence of a first-learned memory provides support for the subsequent

memory to be acquired without NMDAR activation. In this sense, the first experience with the object location task can be viewed as a type of priming for later learning.

Interestingly, some research has shown that disrupting the synaptic mechanisms of memory can result in a later rebound of the behaviour (Chen et al., 2014; R. G. Parsons & Davis, 2011; Ryan, Roy, Pignatelli, Arons, & Tonegawa, 2015). The observed recovery suggested that initial learning may leave some form of priming that facilitates future learning, despite memory disruption of the initial learning (Parsons, 2017). Using *Aplysia*, in which sensitization of the siphon withdrawal reflex can occur by inducing long-term facilitation of the neurons involved in that circuit (Montarolo et al., 1986), the Glanzman group have shown that LTM can still be formed with a partial training protocol when protein synthesis was blocked after training, and therefore despite impaired consolidation (Pearce, Cai, Roberts, & Glanzman, 2017)(Pearce et al., 2017). Importantly, the same training would not induce LTM in untrained animals. The authors suggest that the early proteins formed by the original training constitute a "priming signal" that is not affected by protein synthesis inhibition (Pearce et al., 2017). As well, since no intervention is ever one hundred percent effective, any leftover neural correlates may provide a partial foundation or scaffolding that streamlines future learning. Similar results of reinstated memory with partial training were seen after blocking reconsolidation of long-term facilitation with anisomycin treatment to *Aplysia* cell cultures (Chen et al., 2014).

Observations from the ZIP-infused groups in Project 1 may also be evidence of this effect. ZIP infusions erased the first learning memory, but likely in an incomplete way. Any lingering traces of the first learning memory then acted as a priming effect for second learning acquisition (ZIP-Veh group), or for the second learning probe test to act as a learning session (ZIP-AP5 group). In the ZIP-Veh group, second learning memory was facilitated; perhaps

training required recruitment of fewer new GluA2-AMPARs compared to rats that did not properly acquire the training. Thus, when rats correctly explored the new location at test, this would involve less GluA2-AMPAR expression. However, in the ZIP-AP5 group, if the probe test acted as new learning, there was no subsequent session to test that learning, and thus the current GluA2-AMPAR data cannot infer that the "training" was acquired more efficiently. And, as before, this interpretation can neither be confirmed nor rejected in light of the non-significant results from Project 2. The results from Project 3 are not easily applicable for the same reasons: there is no experience given to solely the R=7d group that would imply the upregulation in GluA2-AMPAR expression resulted from facilitated learning. Thus, overall, it is tenuous that our results could be explain by facilitated second learning through a metaplastic mechanism.

Because none of these possible explanations fully satisfy our range of data, it might therefore be more likely that the western blot technique could not reliably quantify GluA2-AMPAR expression across the experimental conditions. In the next section, we discuss reasons why using this molecular technique to probe for GluA2-AMPAR level changes caused by object location learning and forgetting may be problematic.

5.4. Usage of western blotting to probe for GluA2-AMPARs in the hippocampus

The previous sections outline the challenge in building a comprehensive theory that applies to all our biochemical findings and are difficult to reconcile with the behavioural results. Therefore, it is possible that a common methodological issue underpins these outcomes: either GluA2-AMPARs were not recruited in the way we hypothesized, or the western blot technique was not effective in reliably measuring GluA2-AMPAR expression changes caused by the experimental paradigms.

5.4.1. Dorsal hippocampus is necessary for spatial memory representation

One possibility is that we targeted the incorrect brain area in the experiments. Both infusions and brain tissue collection for subcellular fractionation occurred at the dorsal CA1 subregion of the hippocampus (for example see Chapter 2, Figure 5). But the likelihood that spatial memory representation of the object location task is not based in the dorsal hippocampus (dHPC), but perhaps elsewhere, such as in the ventral hippocampus (vHPC), is very low. This is supported by several studies. For example, a seminal study by Moser et al. (1995), showed that the dHPC is required for rats to perform in the Morris water maze task, which is a standard method to assess spatial memory. Acquisition of this task was impaired after lesions to a quarter of the dHPC, but was not affected by lesions to the vHPC (Moser et al., 1995). Additionally, research has shown that the dHPC and vHPC serve, broadly, two different functions, with the dHPC being involved more in spatial information processing and cognition, and the vHPC being involved more involved with emotional reactions (Fanselow & Dong, 2010). Furthermore, density of place fields is higher in the dHPC compared to the vHPC (Jung, Wiener, & McNaughton, 1994), suggesting that the dHPC is more biased towards location-based tasks than the vHPC. And, most importantly, multiple studies have shown that object location tasks depend on the dHPC (Assini et al., 2009; Gilbert & Kesner, 2004; Mumby et al., 2002). Thus, it seems unlikely that we probed the wrong brain area in our experiments.

5.4.2. Long-term retention of spatial memories recruit GluA2-AMPARs

Another possible interpretation of our findings is that long-term spatial memories do not depend on postsynaptic GluA2-AMPARs. However, the available empirical evidence strongly argues against this option. As mentioned above, several studies have directly implicated GluA2-AMPARs in long-term object location memories (Migues et al., 2014; Migues et al., 2016; see

Hardt et al., 2013). Moreover, hippocampus-dependent spatial memory has also extensively been shown to require PKMζ for maintenance (Pastalkova et al., 2006; Serrano et al., 2008; see Sacktor, 2011, or Kwapis & Helmstetter, 2013, for a review), which participates in a memory maintenance mechanism that sustains GluA2-AMPARs in the postsynaptic membrane (Migues et al., 2010; Migues et al., 2016; Yao et al., 2008; Dong et al., 2015). It therefore seems reasonable to conclude that GluA2-AMPARs are involved in object location memory maintenance. That our data suggest otherwise therefore more likely indicates a methodological issue, rather than the discovery of a form of long-term object location memory that is not reliant on GluA2-AMPARs for maintenance.

5.4.3. Actin is not an appropriate loading control for experiments measuring learning-based changes

New learning involves growth and expansion of dendrites (see Rudy, 2015, for a review), which will necessarily increase levels of structural proteins like β-actin (Fukazawa et al., 2003; Lamprecht & LeDoux, 2004; Okamoto, Nagai, Miyawaki, & Hayashi, 2004). In our experiments, we isolated the PSD fraction, which enlarges as a result of LTP (Desmond & Levy, 1986) due in part to insertion of proteins involved in LTP processes, such as Ca^{2+}/calmodulin-dependent protein kinase II (Soderling & Derkach, 2000), or, more pertinently, AMPARs into the plasma membrane. In Project 3, comparing actin expression between the no-training group versus R=1d group, we found that expression of actin increased with new learning. Although it was not a significant increase, further analysis of actin expression levels across the different groups nevertheless showed fluctuations in actin as retention time increased. In western blot protocols, loading control expression levels need to be consistent across treatment groups if they are to act as an internal measure through which target proteins can be standardized. The changes

observed in actin across groups abolishes its effectiveness as a loading control for our experiments. We tested if another commonly used loading control protein, β-tubulin, remained constant between the no-learning group and the 1d retention group, but also found it increases with new learning. Because we aimed to detect changes in protein expression related to learning and memory loss, choosing a valid loading control protein for our study is difficult. In light of loading control unreliability, conclusions about GluA2-AMPAR levels in our data should be taken with some discretion. Indeed, some articles have also highlighted complications in using actin as a loading control in western blot analyses (Dittmer & Dittmer, 2006), despite its ubiquity in immunoblot protocols.

5.4.4. Spatial memories likely are coded sparsely in the dorsal hippocampus

There may be a disadvantageous signal-to-noise ratio complicating detection of changes in GluA2-AMPAR expression in CA1-dependent object location memories. Pyramidal cells in CA1 receive tens of thousands of synaptic inputs, and hundreds of simultaneous synaptic events are required to produce an action potential (Andersen, 1990). Furthermore, GluA2-AMPARs are the most common AMPAR found in CA1 pyramidal cells (Wenthold et al., 1996). This makes it extremely challenging to detect changes related to one new learning experience over the background activity. Indeed, there is a probability that GluA2-AMPAR signalling specific for our manipulations are below the threshold for detection (Stacey & Durand, 2001). Some of our data support this interpretation. In Project 1, we found that we could detect a significant relationship between GluA2-AMPAR expression and individual novelty ratio score within groups for ZIP-AP5 and ZIP-Veh infused rats. If ZIP infusions erased the LTM previously represented in dHPC, this would significantly reduce background noise, which would allow detection of changes in GluA2-AMPAR expression caused by second learning. Rats infused with

scrambled-ZIP after first learning would therefore retain a signal to noise ratio in CA1 unfavorable to our measuring methods.

As well, in Project 2, we did not see any significant correlation between novelty ratio score and GluA2-AMPAR expression, nor were there any differences in GluA2-AMPAR expression across treatment groups. Therefore, this result might be reflecting an inability to detect changes in GluA2-AMPAR levels associated with reconsolidation blockade. In Project 3, because the changes in GluA2-AMPARs seem somewhat random, this data may also support the lack of reliability in using western blot for molecular analyses of object location based learning and forgetting. Indeed, the signal to noise issue may be one reason why quantifying learning-based AMPAR changes in the CA1 proves challenging for a non-Pavlovian, exploratory task, compared to an aversive fear-memory task. Activation of around 15% of neurons might occur after exploration of a novel context, although this recruitment can increase to approximately 50% with the addition of a second learning experience (Cai et al., 2016). Tao-Cheng et al. (2011) showed that NMDAR-mediated endocytosis led to upwards of a 42% increase in GluA2-AMPARs found in clathrin-coated pits near the PSD. Following stimulation, clathrin-coated pits can internalize AMPARs for recycling and thus help regulate surface expression of proteins (Carroll et al., 2001), and have been shown to mediate endocytosis after insulin stimulation, leading to LTD in the dHPC CA1 (Man et al., 2000). However, Tao-Cheng and colleagues did not observe noticeable changes in labeled GluA2-AMPAR levels at the PSD, which they noted was consistent with other studies (Ehlers, 2000; D.-T. Lin & Huganir, 2007). They conclude that despite occurrence of GluA2-AMPAR endocytosis in peri-PSD areas, endocytosis at the PSD is highly regulated by activity. Likely, there needs to be a strong activity to induce significant amounts of endocytosis of PSD GluA2-AMPARs. Therefore, perhaps our protocols involving

experimental amnesia and forgetting were not strong enough to cause a noticeable change in GluA2-AMPAR expression.

Regardless of these issues with our biochemical analyses, our behavioural results are sound. The literature strongly supports the role of GluA2-AMPAR internalization in memory loss. Thus, overall, the most parsimonious conclusion of the biochemical analyses is that induced amnesia or innately-occurring forgetting of an object location learning memory very likely results in internalization of GluA2-AMPARs from the PSD, but western blots are not the ideal method to evaluate these changes. In the last section, we therefore touch on a few techniques or molecular targets that may be viable alternatives.

5.5. Future directions

Considering the methodological challenges outlined above, we here highlight a few alternative directions to approach our question or to further explore our current findings.

5.5.1. Alternative neural correlates associated with memory destabilization and/or memory loss

If probing for PSD GluA2-AMPARs in the CA1 hippocampus is difficult because the signal of GluA2-AMPAR expression involved in the object location memory representation is too small, then one option is to probe for a larger population of GluA2-AMPARs. It may be possible to access a larger pool of GluA2-AMPARs by targeting phosphorylated GluA2-AMPARs in the synaptosome. Phosphorylation of amino acids on the GluA2-AMPAR C-terminus tail—serine 880 (ser-880) by protein kinase C (PKC) and tyrosine (tyr-876) by non-receptor Src family tyrosine kinases (Trks)—can trigger GluA2-AMPAR internalization (J. Q. Wang et al., 2005). Phosphorylation at these locations disrupts GluA2 subunit binding to scaffolding proteins such as GRIP (Trk phosphorylation of tyr-876; Hayashi & Huganir, 2004),

and PICK1 (PKC phosphorylation of ser-880; Chung, Xia, Scannevin, Zhang, & Huganir, 2000; Seidenman, Steinberg, Huganir, & Malinow, 2003), which have both been implicated in regulation of AMPAR trafficking and memory maintenance mechanisms involving PKMζ (Sacktor, 2011). Therefore, levels of GluA2-AMPARs phosphorylated at these amino acids may reflect GluA2-AMPARs internalized via this mechanism. As AMPARs have been found to mostly internalize from peri-PSD zones (within 200 nm from the edge of the PSD) after NMDAR treatment (Tao-Cheng et al., 2011), as well as possibly endocytic zones at the lateral edges of excitatory synapses (Blanpied, Scott, & Ehlers, 2002; Rácz, Blanpied, Ehlers, & Weinberg, 2004), expanding the postsynaptic region of interest to the synaptosome is required.

Similarly, it may be interesting to measure the expression of ubiquitinated GluA2-AMPARs. Ubiquitination is a post-translation attaching of the ubiquitin molecule to a substrate protein, which helps regulate various processes such as trafficking, endocytosis, and degradation (Hershko & Ciechanover, 1998) of these proteins. AMPAR processes have also been shown to be regulated by the ubiquitin-proteasome system (Hou, Gilbert, & Man, 2011; Patrick, Bingol, Weld, & Schuman, 2003; Zhang et al., 2009). Moreover, synaptic activity can induce GluA2-AMPAR ubiquitination following GluA2-AMPAR endocytosis (Lussier, Nasu-Nishimura, & Roche, 2011). The ubiquitin-proteasome system allows GluA2-AMPAR to be targeted for degradation or for recycling them back into the plasma membrane (Lussier et al., 2011; Widagdo et al., 2015; Zhang et al., 2009). An increased amount of polyubiquitinated proteins were found in the CA1 hippocampal PSD following retrieval of a contextual fear memory in rats (Jarome, Werner, Kwapis, & Helmstetter, 2011; S. H. Lee et al., 2008). Additionally, the effects of inhibiting protein synthesis during reconsolidation with anisomycin infusions can be prevented by infusing the proteasome inhibitor lactacystin immediately following retrieval (Lee et al.,

2008; Jarome et al., 2011) suggesting that protein degradation mediated by the ubiquitin-proteasome system may underlie destabilization following fear memory retrieval in the amygdala (Lee et al., 2008) and cocaine reward memory in the nucleus accumbens (Ren et al., 2012). Ren et al. (2012) showed a reversal of retrieval-induced GluA2-AMPAR downregulation with lactacystin. Although it is unknown if all internalized GluA2-AMPARs are tagged with ubiquitin after memory retrieval, nor has the exact relationship between ubiquitinated GluA2-AMPARs and memory loss been clearly characterized, ubiquitinated GluA2-AMPARs may provide another molecular target for our research interests.

We note that if other molecular correlates are targeted to characterize memory loss, the time course of the molecular assay will necessarily change from what is described in our current procedure (i.e. sacrifice 24 hours after test). Indeed, different proteins do not require all the same amount of time to express. For example, to quantify ubiquitinated GluA2-AMPARs, the tissue samples must be procured from brains frozen within 24 hours following retrieval.

5.5.2. Other techniques

A different angle to approach our research questions is to use another method to either increase GluA2-AMPAR signalling in the dHPC, or improve its detection. Assuming there is indeed a signal-to-noise issue that underlies the learning-based changes of the object location task, other molecular quantification or visualization techniques may also lack efficacy because of this issue. Thus, it is necessary either to find techniques to increase signal, decrease noise, or specifically are able to label and quantify only the GluA2-AMPARs that are recruited for the memory representation.

A unique way to increase in vitro hippocampal CA1 signal is using electrode stimulation to create stochastic resonance (Stacey & Durand, 2001; Wiesenfeld & Moss, 1995; Yoshida,

Hayashi, Tateno, & Ishizuka, 2002). In essence, stochastic resonance describes the phenomenon of supplementing the endogenous noise of a system—that is, "white noise"—to enhance a weak input signal. In behaving animals, noise can be created by administering periodic stimulation that by itself is too weak to induce firing. For example, perturbing the water at a specific periodicity can stimulate crayfish mechanoreceptors (Douglass, Wilkens, Pantazelou, & Moss, 1993), or the perturbing air flow can stimulate a cricket mechanosensory system (Levin & Miller, 1996), boosting originally weak signals to detectable levels. Creating an optimal amount of noise resonates and thereby can amplify the weak signal (Gammaitoni, Hänggi, Jung, & Marchesoni, 1998). In vivo, stochastic resonance has been applied to CA1 slices via sets of electrode pulses given at random intervals in response to subthreshold "signal" stimulation at 5 Hz (Stacey & Durand, 2001). Although this technique has not been applied to in vivo rat experiments, computer simulations of CA1 neurons (Stacey & Durand, 2001) and in vitro models of in vivo neocortical activity (Fellous, Rudolph, Destexhe, & Sejnowski, 2003) suggest efficacy.

It may also be possible to increase a signal by increasing extracellular noradrenaline in the hippocampus (Sara, 2009). Stimulation of the locus coeruleus has been shown to increase noradrenergic activity of hippocampus cells, which may improve signal/noise differentials by enhancing the neuronal responses to reinforcing stimuli and inhibiting background spontaneous activity (Susan J. Sara, 1985; Segal & Bloom, 1976). Noradrenaline can also enhance LTP at hippocampal mossy fibres (Hopkins & Johnston, 1988). Behaviourally, noradrenaline increase caused by chronic psychosocial stress has been shown to improve hippocampus-dependent spatial distribution learning in tree shrews (Bartolomucci, Biurrun, Czéh, Kampen, & Fuchs, 2002), and infusing noradrenaline into the basolateral amygdala after water maze training enhances retention of the platform location (Hatfield & McGaugh, 1999). More recently,

optogenetic activation of noradrenergic locus coeruleus neurons have been shown to improve object location memory by increasing dopamine released in the hippocampus (Kempadoo, Mosharov, Choi, Sulzer, & Kandel, 2016). This noradrenergic neuromodulation in essence imitates stimulation that typically would result from behavioural training with aversive or appetitive protocols, such a fear conditioning. Since fear conditioning training has been known to generate robust memories, we would expect that pharmacologically increasing the dHPC signal would enhance object location memory strength to similarly robust levels, thus increasing the signal to noise ratio. However, strong fear memories are known to resist decay, lasting for over a month (J. Kim & Fanselow, 1992) or two months after extensive training (Pickens, Golden, Adams-Deutsch, Nair, & Shaham, 2009), or upwards of over a year (Fanselow & Gale, 2003). It is unclear if modulating object location memory strength with noradrenaline may also lead to a similar resistance to decay. But, as the type of training we use does not specifically target use of the amygdala and the fear memory circuit, object location memories enhanced this way may have a higher chance of decaying than true fear conditioning memories.

In terms of improving detection, the goal would be to identify GluA2-AMPAR expression only in neurons that are allocated to the object location memory. There exist several neurobiological visualization methods that can be useful. For example, collecting tissue samples specific time points and treating brain slices with biotin, which can label surface AMPARs for immunoblot quantification (shown in mice, Rao-Ruiz et al., 2011). For increased temporal resolution, to detect real-time GluA2-AMPAR insertion and removal in vitro, some labs have tagged GluA2-AMPARs with the green fluorescent protein variant pHluorin, which fuses to the N-terminus of GluA2 (Rathje et al., 2013). The dynamics of surface diffusion of hippocampal GluA2-AMPARs in neuronal cultures can then be monitored under total internal reflection

fluorescence microscopy (TIRFM) (Araki et al., 2010). This has been used to visualize NMDAR-mediated AMPAR endocytosis that leads to LTD (Rathje et al., 2013), which might make it appropriate for our needs as well. There is some controversy with this technique (Rathje et al., 2013) though some purport that the technique is sound as long as appropriate caution is exercised (Wilkinson, Ashby, & Henley, 2014). Newly emerging techniques such as Capturing Activated Neuronal Ensembles (CANE) and the Robust Activity Marking (RAM) System take advantage of immediate early genes such as *fos* to specifically label active neuronal ensembles in vivo (Sakurai et al., 2016, and Sørensen et al., 2016, respectively). How these highly specific and temporally precise methods can apply to visualization of AMPAR trafficking has yet to be determined. In particular, despite the fact that these visualization methods are often only tested on transgenic mice, the RAM system is also applicable to rats (Sørensen et al 2016).

5.6. Conclusion

The experiments of this dissertation explored two lines of research, behavioural and biochemical, in order to corroborate how storage loss of a memory trace is related to GluA2-AMPAR endocytosis. Using the second learning paradigm, we showed that experimental amnesia observed after impairing a consolidated memory or reconsolidation of a memory leads to a storage loss of an object location memory trace. This storage impairment was not corroborated with our molecular data, which showed inconsistent expression patterns of postsynaptic GluA2-AMPARs. These studies provide a direction for future research of these phenomena. Characterizing how neural correlates, like GluA2-AMPARs, are related to memory loss can improve our knowledge of how memory mechanisms work, as well as help build clinical

interventions that may improve memory maintenance, or minimize forgetting occurring from memory disorders.

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