The Anterior Cingulate Cortex in Contextual Fear Memory: From Formation, Consolidation, and Reconsolidation, to Mediating Context Generalization

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Abstract

To persist, new memories must undergo a consolidation process, during which they are sensitive to disruption. This process, referred to as cellular consolidation, is posited to be completed within the first hours following learning and involves stabilization of changes in synaptic connectivity. Memories can also consolidate at the level of brain systems. Systems-consolidation is a more prolonged process involving gradual reorganization of brain systems that support memory expression, where memories that initially depend on the hippocampus increasingly come to depend on specific cortical structures. A number of recent studies have suggested that the anterior cingulate cortex (ACC) is one such cortical structure that is not required for the expression of recent memory, but becomes critical for the expression of remote memory. In addition to reorganization of the anatomical substrates of memory, memories are known to change in other ways over time. Contextual memory, initially hippocampus-dependent, can become less specific over time, whereby animals generalize conditioned responding to novel contexts. However, a number of recent studies have described how following retrieval, memories can return to a labile state, a process referred to as cellular reconsolidation. Similarly, retrieval has been found to transiently return a hippocampus-independent remote memory to a hippocampus-dependent state, a process of systems reconsolidation. Moreover, memory retrieval reactivating the hippocampus has been found to renew memory precision with reduced behavioural generalization.

To date, little is known about how retrieval affects the involvement of the ACC, more specifically: (1) whether memory undergoes cellular consolidation and

reconsolidation in the ACC, and if so, if this is the case for recent as well as remote memories; (2) whether ACC-dependent remote memory transiently becomes ACC-independent following retrieval; and (3) whether increased context memory generalization is mediated by the ACC. This thesis aims to answer these questions in the following two manuscripts. In all experiments, rats underwent contextual fear conditioning. The first manuscript addresses the first two questions. To answer the first question, protein-synthesis inhibitor was infused into the ACC immediately following conditioning or memory retrieval. The results indicate that the ACC is involved in cellular consolidation and reconsolidation of recent and remote contextual fear memory. To answer the second question, the ACC was pharmacologically inactivated at different time-points before testing at time-points after a reactivation trial. The results suggest that at 6 hours following memory retrieval, memory expression can be supported by either the ACC or the dorsal hippocampus (DH), whereas at 24 hours after the retrieval, the memory is once more ACC-dependent. The second manuscript examines the third question, examining the effects of memory retrieval on subsequent context generalization. The results indicate that following memory retrieval, context discrimination is renewed at 24 hours, but not at 6 or 48 hours. Furthermore, pharmacological inactivation of the ACC before testing at 6 hours restores context discrimination, suggesting that the ACC mediates context generalization.

In summary, research presented in this thesis suggests that the ACC plays an important role in the brain networks consolidating and reconsolidating contextual fear memory, and becomes more critical for memory retrieval with time as memory expression becomes more generalized.

Résumé

Afin d'être préservés, les nouveaux souvenirs doivent être soumis au processus de la consolidation, pendant lequel ces souvenirs sont sensibles à toute modification. Ce processus nommé consolidation cellulaire, a lieu pendant les premières heures suivant un apprentissage, et implique la stabilisation de certains changements au niveau des connections synaptiques. Les souvenirs peuvent également être consolidés au niveau des réseaux neuronaux, lors de la consolidation systémique. La consolidation systémique est un processus prolongé qui implique une réorganisation graduelle des réseaux neuronaux qui sous-tendent l'expression de la mémoire. Ainsi, un souvenir dépend initialement de l'hippocampe mais devient progressivement dépendant de structures corticales spécifiques. Plusieurs études récentes ont suggéré que le cortex cingulaire antérieur (CCA) est l'une de ces structures qui n'est pas requise pour l'expression de souvenirs récents, mais devient nécessaire pour l'expression de souvenirs anciens. En plus de la réorganisation des substrats anatomiques d'un souvenir, les souvenirs peuvent êtres modifiés autrement avec le temps. Les souvenirs contextuels, initialement dépendants de l'hippocampe, peuvent devenir moins spécifiques avec le temps, et les animaux vont généraliser leur réponse conditionnée à de nouveaux contextes. Cependant, selon plusieurs études récentes, suite au rappel, les souvenirs peuvent revenir à un état instable par un processus appelé la reconsolidation cellulaire. Il a également été montré qu' un souvenir ancien, et donc indépendant de l'hippocampe, peut de nouveau dépendre de cette structure suite au rappel , par un processus de reconsolidation systémique. De plus, il a été démontré que la réactivation de l'hippocampe suite au rappel d'un souvenir ancien

peut renouveler la précision des souvenirs, et donc diminuer la généralisation comportementale normalement observée avec les souvenirs anciens.

À l'heure actuelle, la façon dont le rappel affecte l'implication du CCA est assez méconnue. Plus spécifiquement, plusieurs questions se posent: (1) le souvenir est-il sujet à la consolidation et à la reconsolidation cellulaire au niveau du CCA, et si oui, est-ce le cas pour les souvenirs récents et anciens; (2) les souvenirs anciens dépendants du CCA deviennent-ils momentanément indépendants de cette structure après le rappel; et (3) la généralisation accrue des souvenirs contextuels est-elle gérée par le CCA? Cette thèse a pour but de répondre à ces questions dans les deux manuscrits suivants. Pour toutes les expériences, les rats ont été entrainés dans une tache de conditionnement de peur au contexte. Le premier manuscrit porte sur les deux premières questions. Pour répondre à la première question, un inhibiteur de synthèse protéique a été infusé dans le CCA immédiatement après le conditionnement ou le rappel. Les résultats montrent que le CCA est impliqué dans la consolidation et reconsolidation cellulaire des souvenirs de peur contextuels à la fois récents et anciens. Pour répondre à la deuxième question, le CCA a été pharmacologiquement inactivé à différents temps avant le test et après le rappel. Les résultats suggèrent que l'expression de la mémoire peut être sous-tendue par le CCA ou l'hippocampe dorsal 6 heures après le rappel. Néanmoins, le souvenir est de nouveau dépendant du CCA 24 heures après le rappel. Le second manuscrit examine la troisième question : étudier les effets du rappel sur la généralisation de contextes. Les résultats indiquent que suite au rappel d'un souvenir ancien, la discrimination des contextes est observée à 24 heures, mais pas à 6 ou 48 heures. De plus, l'inactivation pharmacologique

du CCA avant le test à 6 heures rétablit la discrimination des contextes. Ceci suggère que le CCA gère la généralisation des contextes.

En résumé, la recherche présentée dans cette thèse suggère que le CCA joue un rôle important dans les circuits neuronaux consolidant et reconsolidant les souvenirs de peur contextuels. De plus, le rôle du CCA semble prendre de l'importance avec le temps alors les souvenirs se généralisent.

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Statement of Original Contribution

The research presented in the current dissertation constitutes an original contribution to the field of neuroscience of memory, in particular the role of the ACC. To date, few studies have examined the evolving role of the ACC to contextual memory; from cellular consolidation to reconsolidation at different ages of the memory; its involvement in supporting memory retrieval and how it interacts with hippocampal contribution, and the nature of the ACC contribution.

Research on the involvement of the ACC in contextual memory has focused on structural changes (e.g. imaging changes in spine density) in the ACC following memory acquisition, and the role of the ACC in supporting memory retrieval of recent and remote memory. Studies on consolidation and reconsolidation of contextual memory have mainly focused on the involvement of the dorsal hippocampus. At the same time, research on context memory transformation over time, as reflected by increased contextual fear generalization, has examined on the role of the dorsal hippocampus in mediating detailed memory expression.

The research presented in chapter 2 examines the involvement of the ACC in consolidation and reconsolidation, both on a cellular and systems level. The study is the first to examine the effects of remote memory retrieval on systems dynamics between the ACC and dorsal hippocampus in supporting subsequent memory expression. Following up on findings presented in chapter 2 suggesting that following a reminder session, memory expression can be supported by either the ACC or dorsal hippocampus, research presented in chapter 3 tests the effects of a reminder on contextual generalization on a

subsequent test, and whether the ACC contributes specifically to the expression of generalized memory expression. The study is the first to examine the qualitative nature of the ACC involvement in contextual memory expression, made possible by the unique experimental conditions of parallel ACC and dorsal hippocampus support for memory expression described in chapter 2.

Contribution of Authors

This dissertation is primarily based on two manuscripts. Chapter 2 is based on the first study, “Involvement of the Anterior Cingulate Cortex in the Acquisition, Consolidation and Reconsolidation of Contextual Fear Memory”, which was co-authored by myself and Dr. Karim Nader, and has been prepared for submission to Current Biology. Chapter 3 is based on the second study, “The Anterior Cingulate Cortex Mediates the Expression of Generalized Contextual Fear Memory”, which was co-authored by myself, Jennifer Pors and Karim Nader, and has been prepared for submission to Journal of Neuroscience. Both studies were developed through discussions with Dr. Nader. I designed and ran the experiments, performed the data analysis, and wrote the manuscripts under the supervision of Dr. Nader. In the study presented in Chapter 3, my co-author, Jennifer Pors, assisted with behavioural experiments, animal surgery, and histology work.

General Introduction

One of the main tenets in the study of memory is that our memories are not formed instantly, but progress from an initial labile state to a more fixed one. This idea of gradual stabilization was first introduced by Müller and Pilzecker (1900) first used the term "Konsolidierung" (consolidation) to describe a memory stabilization interval over minutes in their studies on retroactive interference of new learning of nonsense syllables. A few years earlier, the French psychologist Theodule-Armand Ribot (1882) had been the first to suggest that new memories go through qualitatively distinct phases. Ribot noted in his observations that amnesic patients seemed most prone to lose memories of their recent past, while more remote events could still be remembered. Ribot concluded that over time, a biological process unfolds that progressively strengthens memories from a vulnerable new state to a fixed permanent state.

These fundamental notions inspired two of the most successful research programs studying the biological basis of memory. Two memory models emerged out of the broad empirical fundament laid down by these efforts - cellular and systems consolidation (Dudai, 1996)[1]. The former, mostly derived from animal-research models, is thought to occur over a similar time-frame as that described by Müller and Pilzecker (1900), describes the molecular mechanisms unfolding after learning that stabilize changes in synaptic efficacy that constitute memory on a neuronal level. Cellular consolidation

[1] It should be noted that the terminology initially referred to the methods used in studying the two gradients, where, for example, subcellular processes were manipulated in the study of the shorter gradient (cellular consolidation), whereas lesioning or imaging of whole brain systems was used in the study of the longer gradient (systems consolidation).

posits that as the process advances, any disruptions of these processes will lead to weaker memory impairments. Once these processes have run their courses and the same manipulations cease to be effective, the memory is considered consolidated.

Systems consolidation, on the other hand, is a hypothesis that refers more to a memory transfer or reorganization process than a memory stabilization process, describing a time-dependent shift in brain systems that support performance. Research that gave rise to this model began with Scoville and Milner's (1957) case study of the late patient HM, who lost most of this medial temporal lobe in a surgery to relieve him from intractable epilepsy. The operation left him with a profound impairment in forming new memories (anterograde amnesia), and an extensive memory loss, extending back years into the past (retrograde amnesia). His and similar cases, as well as animal models, suggested that such memories initially depend on the hippocampus, but come to rely on extra-hippocampal, cortical areas over time. Similar to the cellular consolidation model, systems consolidation entails a temporally graded sensitivity of memory to amnesic treatments: the less complete systems consolidation, the more memory will be impaired following hippocampal damage. Temporally retrograde amnesia following localized disruptions of the hippocampus has since been modelled in a number of non-human animal paradigms further supporting the systems consolidation hypothesis.

In the past two decades a number of animal studies have focused on the cortical structures hypothesized to be loci of systems consolidated memories. These studies have used pharmacological and lesioning approaches targeting specific structures, as well as imaging and mouse genetic approaches in identifying the network of cortical areas involved. One structure in particular, the anterior cingulate cortex, has been suggested to

play an increasingly larger role in the retrieval of remote memory as the hippocampus becomes less critical.

Both standard models of cellular and systems consolidation assume that each memory can only undergo consolidation once, after which they remain in a relatively stable state. This assumption was challenged early on by findings that had undergone cellular consolidation could return to a transient labile state sensitive to the same manipulations that impair consolidation, a state that came to be called reconsolidation. Later studies have suggested that remote memories can also undergo reconsolidation on a systems level (Debiec et al., 2002), where following memory retrieval they once more become transiently sensitive to hippocampal manipulations. However, to date, few studies have focused on the effects of memory retrieval on key cortical areas involved in systems consolidation.

The focus of this thesis is the study the role of the ACC in cellular consolidation and reconsolidation of contextual fear memory, and how its role in mediating increased context generalization of remote memory expression. Before getting to the two manuscripts that describe the findings, a general introduction will familiarize the reader with the history and some of the evidence for cellular consolidation and reconsolidation mostly derived from animal studies. Similarly, the history of systems consolidation will be reviewed where evidence from human and animal studies is discussed. In particular, contextual fear conditioning, the animal model used in the manuscripts, will be discussed in detail with regards to what information is being acquired and how does it change over time and systems consolidation.

Cellular Consolidation

In 1900, Müller and Pilzecker observed in their classic studies on retroactive interference that participants memory of nonsense syllables was impaired if additional lists of syllables to learn were presented in the immediate minutes after the initial learning. In their attempts to explain the phenomenon, Müller and Pilzecker suggested "the tendency to perseverate […] might serve to consolidate the associations among [the syllables]." (p. 68). However, a physiological explanation for Müller and Pilzecker's perseveration-consolidation hypothesis was first provided by Hebb's (1949); see also (Gerard, 1949) dual-trace memory theory. Hebb proposed that new memories are stabilized by recurrent activity of the neuronal network representing the new experience, corresponding to short-term memory (but see McGaugh and Landfield, 1970; Miller and Springer, 1971) [2]. If no interference occurred, morphological alterations of the network's synapses would take place, rendering the connections permanent allowing for later regeneration of the activity pattern. Such structural modifications were proposed to correspond to stabilization of long-term memory.

Since Hebb's (1949) proposal, a large body of empirical evidence has accumulated over the years, demonstrating that for some period after learning, memories are labile and vulnerable to modification; a period termed the consolidation interval. Three lines of evidence have been advanced to support the existence of such a cellular consolidation process: First, amnesia can be induced if treatments such as

[2] Hebb's hypothesis of preservative recurrent neural activity as the STM trace has since been weakened by findings showing electroconvulsive shocking leading to intact memory expression on a STM-test but impaired on a LTM-test (McGaugh et al., 1970; Miller et al., 1971).

electroconvulsive shock (Duncan, 1949) or protein synthesis inhibitors (Flexner et al., 1965) are given shortly after learning. Second, performance can be impaired if new competing learning occurs in short temporal proximity to the initial learning (Gordon and Spear, 1973). Third, retention can be enhanced by administration of various compounds, such as strychnine (McGaugh and Krivanek, 1970). Critically, all three manipulations, impairment by treatment, impairment by new learning, and enhancement by treatment are effective only when given shortly after new learning, not when given after a delay. Accumulating evidence of this nature led to several propositions of a consolidation theory of memory (Gerard, 1949; Hebb, 1949; McGaugh, 1966). These theories propose an initial unstable short-term memory trace that lasts on the order of hours and a more permanent long-term memory trace that consolidates over that period. Although initial formulations of the theory assumed a serial process between the memory stages, (Gerard, 1949; Hebb, 1949) later ones did not (McGaugh, 1966).

The field has enjoyed numerous successes in creating models at different levels of analysis to describe the changes that occur when a memory is converted from labile trace to a fixed one. In addition to studies at the behavioural level, models of consolidation include long-term potentiation (LTP), a long-lasting enhancement in signal transmission between two neurons following high-frequency stimulation of a chemical synapse, typically in a hippocampal slice preparation, thought to be analogous to cellular mechanisms of memory.(Bliss and Lomo, 1973; Martin et al., 2000) Similar to the distinction between short-term and long-term memory, LTP can be divided into an early transient phase (E-LTP), and a more persistent late phase (L-LTP), which similar to long-term memory, requires protein-synthesis in order to stabilize. Lastly, on a molecular

level, specific signal transduction factors such as cAMP-response element binding protein (CREB) have been identified to be critical for the formation of a long-term memory (Dash et al., 1990; Bourtchuladze et al., 1994; Yin et al., 1994).

Cellular Reconsolidation

Since its inception, consolidation theory was challenged by a small number of studies demonstrating that a consolidated memory could return to an unstable state and then re-stabilize within minutes to hours following retrieval. As with consolidation, three lines of evidence were put forth to support the existence of a re-stabilization period. First, performance can be impaired if amnesic treatments such as electroconvulsive shock are given shortly after reactivation (Misanin et al., 1968; Schneider and Sherman, 1968). Second, performance can be impaired if new competing learning occurs in short temporal proximity to the reactivation (Gordon, 1977a). Third, retention can be enhanced by administration of various compounds, such as strychnine after retrieval (Gordon, 1977b). Critically, all three manipulations are effective only when given shortly after memory reactivation but not when given after a delay. These findings, acquired by different investigators, in different tasks and species, fundamentally challenged consolidation theory (Miller and Springer, 1973; Lewis, 1979; Miller and Marlin, 1984; Spear and Mueller, 1984).

The implications of these findings, originally referred to as cue-dependent amnesia, were that long-term memory was not the end of the road in terms of memory lability but simply a momentary pause until the memory was reactivated. Based on these findings, a model of memory as a dynamic process entailing two memory states was proposed (Miller and Springer, 1973; Lewis, 1979; Spear and Mueller, 1984). First, an

active state in which new and reactivated memories were labile and vulnerable to disruption, and second, an inactive state of stabilized memory. The model accounts for both the evidence supporting consolidation theory, as well as findings of cue-dependent amnesia which consolidation theory could not explain. However, for reasons that remain unclear, the dozens of studies that demonstrated reconsolidation across species, tasks and amnesic agents had little impact in the status of consolidation theory in the field of memory research (Sara, 2000).

Research on the reconsolidation effect was revitalized by its demonstration in auditory fear conditioning in the rat (Nader et al., 2000), a well-defined behavioural paradigm in which the underlying neural circuitry had previously been extensively mapped out (LeDoux, 2000). Targeting directly the basolateral nucleus of the amygdala, known to critically mediate auditory fear conditioning and its consolidation (LeDoux, 2000; Schafe and LeDoux, 2000; Walker and Davis, 2002; Fanselow and Poulos, 2005), and using the commonly used protein synthesis inhibitor anisomycin, Nader and colleagues (2000) showed that reminders could bring well-consolidated fear memories back to a unstable state, in which they could be disrupted by infusing the protein-synthesis inhibitor directly into the basolateral nucleus of the amygdala. As in the original findings of reconsolidation, such impairments were not observed in the absence of reactivation.

Since this study, reconsolidation became an intensive area of investigation in the neurosciences, and has been demonstrated in a range of species (including humans), tasks, and with various amnesic and enhancing agents (for a review of the literature, see Nader & Hardt, 2009). The fact that retrieval can return consolidated memory to an

unstable state from which it must restabilize over time has been established as a fundamental memory process.

Boundary Conditions on Cellular Reconsolidation

However, reconsolidation does not seem to occur after every instance of memory retrieval. This property of reconsolidation was noted early on in the study of the phenomenon, where the induction of reconsolidation was considered dependent on specific parameters in retrieval (Miller and Springer, 1973; Spear, 1973; Lewis, 1979). Several boundary conditions, under which memory that otherwise would undergo reconsolidation no longer does, have been described, most notably age of the memory (Milekic and Alberini, 2002; Frankland et al., 2004b; Suzuki et al., 2004; Wang et al., 2009; Robinson and Franklin, 2010), training intensity (Suzuki et al., 2004; Wang et al., 2009; Robinson and Franklin, 2010), and reactivation parameters (DeVietti and Holliday, 1972; Frankland et al., 2004b; Suzuki et al., 2004). A number of recent studies have confirmed how these conditions can interact in influencing the degree of vulnerability of a reactivated memory. Initially, a number of studies suggested that as memory ages (> ~14 days), it looses its sensitivity to treatments that impair reconsolidation at earlier time points (Milekic and Alberini, 2002; Frankland et al., 2004b; Boccia et al., 2006), leading to the suggestion that only young memories undergo reconsolidation, during a 'lingering consolidation' process after training (Alberini, 2005). However, studies using a more systematic approach have found that with longer reactivation session, older memories again become sensitive to reconsolidation challenges. For example, Suzuki et al (2004) showed that whereas systemic infusions of a protein synthesis inhibitor can disrupt a reactivated 1-21 day old contextual fear memory in mice, a 56 day old memory was

unaffected by the same treatment. However, if the reactivation session was increased from 3 and 5 minutes to 10 minutes, the 56-day-old memory was now disrupted by the treatment. In addition, Suzuki and associated found that stronger fear memory (3 shocks vs. 1 shock) needed longer reactivation to be disrupted one day after training. This kind of parametric manipulation has not been performed for many boundary conditions (e.g. inhibitory avoidance [Milekic & Alberini, 2002; Boccia et al., 2006]). Therefore, it is unclear whether these boundary conditions represent situations in which it is harder to induce reconsolidation, or whether they represent situations in which memory does not undergo reconsolidation. Other studies have not been able to replicate findings of memory age and training intensity as boundary conditions (Debiec et al, 2002; Lee et al., 2005; Bustos et al., 2009). Interestingly, pre-training stress can produce a lingering consolidation gradient for memories that otherwise are sensitive to reconsolidation disruption at early and later time points. In one study, systemic infusions of the benzodiazepine midazolam to rats after reactivation of a contextual fear memory at 1, 7 or 21 days post-training, disrupted reconsolidation, but if subjected to a stress session pre-training, the same treatment disrupted reconsolidation at 1, but not at 7 or 21 days after training (Bustos et al, 2009).

Stronger training can also lead to the opposite gradient of susceptibility to reconsolidation-disruption than that suggested by lingering consolidation, that is a gradient where strong memories are insensitive to reconsolidation challenges until they reach a certain age (~30 days post-training) (Wang et al., 2009; Robinson and Franklin, 2010). In a systematic study of these boundary conditions, Wang et al (2009) delineated the mechanisms mediating this effect on a molecular and brain systems level. Auditory

fear memory which was sensitive to post-retrieval anisomycin infusions into the basolateral nucleus of the amygdala two days after training if trained with one shock, whereas if trained with 10 shocks, the memory was insensitive to the same treatment at 2 and 7 days post-training, but became vulnerable following retrieval at 30 or 60 days. The authors further described a possible molecular mechanism mediating the boundary condition where NMDA-NR2B subunit levels in the basolateral nucleus of the amygdala correlated with sensitivity to the anisomycin infusions, with strong training leading to down-regulation at 2 and 7 days after training, but not at 30 and 60 days. Finally, the authors found that the boundary condition, both at a behavioural and molecular level, was mediated by hippocampus involvement, as lesions of the dorsal hippocampus before a 10 shock training precluded the down-regulation of NDMA-NR2B receptors in the basolateral nucleus of the amygdala and renewed sensitivity to anisomycin infusions 2 days after training. Thus, an apparent boundary condition of training intensity was found to be transient, and mediated by the down-regulation of a molecular mechanism imposed by another memory system.

In summary, the data suggests that the induction of reconsolidation depends on the parameters of memory acquisition and retrieval. Factors such as previous experience of the animal, training intensity, retrieval parameters and age of the memory interact in influencing the induction of reconsolidation and the vulnerability of a retrieved memory.

Systems Consolidation

Scoville and Milner's (1957) report describing HM's memory impairments marked the beginning of research aimed at identifying specific brain systems and mechanisms underpinning memory. Memory impairments similar to HM have since been found in a

number of patients with medial-temporal lobe (MTL) damage. The extent of the temporal gradient of retrograde amnesia, however, has been found to vary substantially between patients, from several months to decades (e.g. Reed and Squire, 1998), and in some patients, comparable amnesia for both recent and remote memories has been documented, i.e. a flat gradient (Kopelman and Kapur, 2001). These discrepancies have been suggested to be linked to the size and location of the MTL lesion, where patients with damage limited to the hippocampus have sharper gradients, typically covering a decade or less, while individuals with more extensive MTL damage show even more far-reaching retrograde memory loss (Reed and Squire, 1998; Bayley et al., 2005).

It has been suggested that the length of the gradient might differ with the type of memory tested, that is, different gradients have been observed for semantic memory (memories of facts and general knowledge), episodic memory (memories of discrete events or autobiographical episodes), and allocentric spatial memory (memories of spatial relationships between objects within an environment independent of one's viewpoint) (Nadel and Moscovitch, 1997). In support of this view, studies have found that if damage is limited to the hippocampus, patients can present either with a lack of a retrograde gradient for semantic memories or semantic memory loss that extends back no more than about 10 years (Rempel-Clower et al., 1996; Kapur and Brooks, 1999; Manns et al., 2003), whereas a more extensive damage to the MTL is associated with amnesic gradients spanning two or three decades (Bayley et al., 2006; Bright et al., 2006). Similarly, for episodic memories, temporal retrograde gradients of a few years length were detected if damage was limited to the hippocampus (Bayley et al., 2003). When damage extended to adjacent MTL regions, a few studies did either not find a gradient or

have reported retrograde amnesia extending back for decades (Cipolotti et al., 2001; Hirano et al., 2002), whereas others have not (Bayley et al., 2003; Bayley et al., 2005).

While the hippocampus has been implicated in the acquisition of allocentric memories (Maguire et al., 1996), patients with large hippocampus lesions can retain spatial memories for highly familiar environments (Teng and Squire, 1999; Rosenbaum et al., 2005; Maguire et al., 2006). The relatively few studies that have investigated remote spatial memory following hippocampal damage suggest that spatial memory acquired throughout childhood can remain intact (Teng and Squire, 1999), whereas spatial memories acquired later in adulthood may lose some of their detail and are not as flexible for generating new routes (Rosenbaum et al., 2005; Maguire et al., 2006). These later findings suggest qualitative differences between remote hippocampus-dependent and hippocampus-independent spatial memories.

Taken together, studies on amnesic patients suggest, first, that the length of the retrograde amnesia is associated with the size of the brain damage, with shorter gradient if the damage is limited to the hippocampus and longer with more extensive MTL damage. Second, the length of the gradient can differ depending on the type of memory, with episodic and spatial memory being more sensitive to hippocampal and MTL damage than semantic memory.

Neuroimaging Studies in Humans

Studies of brain lesions in patients have given important insights into how memory depends on particular brain regions. Lesions studies, however, have limited value in the study of how an intact brain processes memory. To complement lesion studies, functional neuroimaging of healthy volunteers has been extensively used to map out the activity of

different brain areas in response to memory tasks. However, neuroimaging studies have had their own limitations, one being the lack of standardization between studies as each study has been virtually unique with regards to test material and design. A second limitation is the varying definition of "recent" and "remote" memory, where, for example, recent can vary from 1 day old (Takashima et al., 2006) memory to 2 years (Ryan et al., 2001). A third limitation is the possible confounding effect of using pre-scanning interviews to identify memories for recall in the scanner, conducted, for example, immediately (Ryan et al., 2001) or 2 days before the scanning session (Addis et al., 2004; Soderlund et al., 2011). Here, memory retrieval during the pre-scan interview can lead to hippocampal activation through either the acquisition of a new memory later retrieved in the scanner, or the reconsolidation of the original memory that can re-engage the hippocampal network (see below discussion on systems reconsolidation).

Although some studies have found the hippocampus more active during the retrieval of recent than remote episodic memories (Piefke et al., 2003; Takashima et al., 2006), others have found that the structure is similarly highly active for both recent and remote memories retrieval, both with a pre-scan interview (Ryan et al., 2001; Addis et al., 2004; Soderlund et al., 2011), and without one (Gilboa et al., 2004; Steinvorth et al., 2006; Viard et al., 2007). One study that did not use a pre-scan interview found more hippocampal activity following the retrieval of remote memories than recent ones (Rekkas and Constable, 2005).

This discrepancy between studies may be due to the amount of details recalled, rather than the age of the memory. Evidence suggests that the hippocampus is more engaged in the retrieval of detailed episodic memories (Trinkler et al., 2009), and recent

memories appear more detailed than remote ones (Piefke et al., 2003), leading to stronger hippocampal activity for recent than remote memories. In one study that statistically controlled for the level of detail (or emotionality) of memories, the hippocampus was found to show similar activity when retrieving recent and remote memories (Addis et al., 2004). Further evidence supporting the idea that the hippocampus plays a specific role in supporting rich, detailed episodic memories comes from studies showing that hippocampal activation declines over time as detailed memory recollection fades away (Eldridge et al., 2000; Yonelinas et al., 2005; Viskontas et al., 2009).

A recent study examining hippocampal-neocortical co-activation with retrieval of episodic autobiographical memory at different ages (1 week - 10 years) found, in addition to the hippocampus being highly active regardless of memory age, co-activation with the anterior cingulate cortex only for 10 year old memories (Soderlund et al., 2011). Interestingly, this positive co-activation as delayed within the retrieval session, possibly reflecting that the anterior cingulate is engaged before the hippocampus for such remote memories.

A number of neuroimaging studies on semantic memory have found equal hippocampal activation for recent and remote memories (Kapur et al., 1995; Maguire, 2001; Bernard et al., 2004), while others have found retrograde temporal gradient for activation in the right parahippocampal cortex (Douville et al., 2005) and right entorhinal cortex (Haist et al., 2001). A recent study (Smith and Squire, 2009) found reduced activity following the retrieval of remote semantic memories than recent ones in the medial temporal lobe (including the hippocampus), and increased activity in the frontal lobe, temporal lobe and parietal lobe.

In summary, despite some widespread methodological shortcomings in neuroimaging studies of memory (e.g. lack of standardization between studies), some clear trends have started to emerge from the data. Most notably, evidence from numerous studies indicates that that the hippocampus plays a specific role in supporting rich, detailed episodic memories, a characteristic more typical of recent than remote memories. Moreover, it may not be the age of the memory *per se* that dictates hippocampal involvement, but rather the richness and detail of the memory, which normally declines with time through forgetting.

Animal Models of Systems Consolidation

Studying systems consolidation in animal models has two major advantages: First, memories of different ages can be studied prospectively by performing experimental manipulations at specific time points after memory acquisition. Second, animal models allow targeting specific areas and/or specific molecular processes. One important difference between gradients in human amnesic patients and those found in animal models is the timescale of the temporal gradient, which is vastly shorter in animal models, ranging from days to months in contrast to years and decades in humans.

Temporally Graded Retrograde Amnesia and the Hippocampus

The first two studies to model temporally graded retrograde amnesia used two different paradigms, the socially transmitted food preference task (Winocur, 1990), and contextual fear conditioning (Kim and Fanselow, 1992). In both studies, electrolytic lesions of the dorsal hippocampus of rats were made at different time points after training. Memory of socially transmitted food preference was impaired if lesions were made 1-2 days after training, but not when applied after five days (Winocur, 1990). Contextual fear memory,

however, was severely impaired if lesions were made 1-14 days after training, but intact when applied 28 days after training (Kim and Fanselow, 1992). Since then temporally graded retrograde amnesia following partial or whole hippocampal disruption has been found in studies using rabbits (Kim et al., 1995), mice (Takehara et al., 2002), and monkeys (Zola-Morgan and Squire, 1990), in a number of different paradigms, including inhibitory avoidance (Quillfeldt et al., 1996), auditory trace fear conditioning (Quinn et al., 2008), trace eye-blink conditioning (Kim et al., 1995) and numerous spatial tasks (Ramos, 2000; Maviel et al., 2004) (Table 1). Most recently, temporally graded retrograde amnesia has been demonstrated with pharmacological inactivation of the dorsal hippocampus where infusions of the AMPA receptor antagonist CNQX impaired contextual fear memory 1 day after training, but not at 4 weeks (Wiltgen et al., 2010), and either CNQX or the sodium channel blocker TTX impaired expression of socially transmitted food preference memory at 1 day, but not 4 weeks, after training (Lesburgueres et al., 2011). The length of the temporal gradient described in these studies varies from days to months and most likely depends on parameters such as method and extent of lesioning, type and training parameters of task, and species and age of animals.

In a recent paper, Sutherland and Lehmann (2011) questioned the evidence for systems consolidation, most notably for two reasons: first, most papers describing temporally graded retrograde amnesia supporting systems consolidation, demonstrate memory impairment following lesioning/inactivation of the hippocampus within 100 hours of training, running the risk of confounding memory expression impairment with impairment of cellular consolidation. This is an important point, as there is some support for recurrent rounds of cellular consolidation (e.g. recurrent sensitivity to the protein

synthesis inhibitor anisomycin) lasting up to 12 hours (Bourtchouladze et al., 1998; Bekinschtein et al., 2007), although there is little evidence of cellular consolidation lasting up to 100 hours. Second, in order to convincingly demonstrate temporally graded retrograde amnesia, Sutherland and Lehmann suggest that animals with lesioned/inactivated hippocampus at different time-points should show improved performance with time, the implication being that spared remote memory cannot be inferred from non-significant difference between the groups primarily due to reduced performance of the control group due to forgetting (e.g. Kim & Fanselow, 1992). To pass these two criteria, a study needs to demonstrate retrograde amnesia more than 100 hours after training, and spared memory at a later time where the level of retention performance is both equivalent to that of the corresponding control condition group and higher than the performance indicating amnesia at the more recent time-point. In their review, Sutherland and Lehmann note that only one study meets these standards, Parsons and Otto (2010), leading them to conclude that there is little evidence for a systems consolidation process. However, this conclusion may be somewhat premature for two reasons. First, Sutherland and Lehmann (2011) overlook two studies that also meet the same criteria, that of Ward et al (1999) and Ramos (1998). Second, to date, few studies test for retrograde amnesia at a recent time point later than 100 hours post-training, as studies on examining systems consolidation typically test for retrograde amnesia only at 24 hours and at ~ 30 days post-training. However, of those studies that have tested at a recent time-point >100 hours (Winocur, 1990; Kim and Fanselow, 1992; Ramos, 1998; Ward et al., 1999; Winocur et al., 2001; Parsons and Otto, 2010), half have found

temporally graded retrograde amnesia according to previously mentioned criteria of Sutherland and Lehmann (2011).

Flat Gradients and the Hippocampus

A number of studies have found memory impairments following hippocampal manipulations not to be temporally graded. Findings of such flat gradients can be divided into two classes; first, studies using excitotoxic lesioning, and second, studies using the water maze paradigm (Table 2). First, several studies have found that excitotoxic lesioning of the hippocampus, either partial or whole, leads to ungraded (Gaskin et al., 2003; Winocur et al., 2005a; Winocur et al., 2005b; Lehmann et al., 2007; Sutherland et al., 2008) or an extended temporally graded retrograde amnesia (Maren et al., 1997). It is possible that excitotoxic lesions may not be suitable for localized manipulations as they may be lead to tissue damage in distal sites that the target region projects to (Day et al., 1999; Glenn et al., 2005), or interfere with memory storage in other ways due to the prolonged non-physiological massive excitatory activity emanating from the target area (Anagnostaras et al., 2001). Indeed, some studies (Maren et al., 1997; Sutherland et al., 2008) targeting the hippocampus with excitotoxic techniques have not only found contextual fear memory impaired, but also auditory fear memory, which is not affected by a more restricted electrolytic lesioning of the hippocampus (Kim and Fanselow, 1992; Anagnostaras et al., 1999), suggesting that extrahippocampal structures are also adversely affected. This pattern of findings fits with studies of human patients that have found that the extent of damage in and around the hippocampus is related to the length of retrograde gradient (Rempel-Clower et al., 1996; Reed and Squire, 1998; Bayley et al., 2005), with one study finding that with damage to limited to the CA1 area of the hippocampus,

retrograde amnesia was limited to 1-2 years, whereas more extensive damage to the whole hippocampus and entorhinal cortex led to amnesia for ~15 years (Rempel-Clower et al., 1996). Second, one hippocampus dependent task that has consistently been found to be sensitive to hippocampal manipulations irrespective of memory age is the Morris water maze task. In this task, rodents learn to navigate to a submerged platform in an open featureless environment with only distal cues outside of the maze to go by. Ungraded retrograde amnesia has been found in this task with partial or whole excitotoxic lesions of the hippocampus (Martin et al., 2005), electrolytic lesioning of the hippocampus (Mumby and Glenn, 2000), pharmacological inactivation of the dorsal hippocampus with the sodium channel blocker lidocaine (Broadbent et al., 2006; Teixeira et al., 2006), with extensive training early in life (Clark et al., 2005a), and with extended interval between training and lesioning (Clark et al., 2005b). Even memory of simpler versions of the water maze with minimal navigational demands such as the dry-land version of the water maze (oasis maze) and a version consisting of a circular corridor (the annular maze) has been found to be ungraded following hippocampal lesioning (Clark et al., 2005b). It is only in tasks with very limited navigational choices, such as those where extra-maze cues are used to discriminate between arms in a maze (Ramos, 2000; Maviel et al., 2004), that temporal gradient of retrograde amnesia has been found following manipulations of the hippocampus.

In summary, there is strong evidence from animal studies that disruptions of the hippocampus leads to temporally graded retrograde memory impairments in a variety of tasks known to depend on the hippocampus (Table 1). Studies finding flat gradients seem to be limited to one of two conditions: first, either using excitotoxic lesioning, which has

been suggested to lead to tissue damage in distal sites and may thus not reflect the involvement of the hippocampus; and second, testing memory using the water maze paradigm (Table 2). Take together, the evidence strongly suggest that the participation of the hippocampus in mediating memory changes with time, in as much as memory can be expressed without the structure as memory ages. It is important to note that the evidence does not support the conclusion that the hippocampus no longer participates in mediating memory retrieval of remote memory under normal circumstances, or that the memory becomes independent of the hippocampus.

Temporally Graded Activity: From the Hippocampus to the Cortex

Findings that hippocampal lesions impair recent memories more severely than remote ones have suggested that the hippocampus plays a time-limited role in the retrieval of memory. In the past decade, a number of studies have used brain imaging techniques to identify cortical regions that might play a role in mediating remote memory (Frankland et al., 2004a; Maviel et al., 2004; Ross and Eichenbaum, 2006; Gusev and Gubin). In one of the first paper that compared post-retrieval activity throughout the brain at a recent and remote time points, Bontempi and associates (1999) trained animals on a hippocampus-dependent spatial discrimination task and then monitored brain activity using (14C) 2-deoxyglucose uptake. They identified a number of structures that activity changed with the age of the memory. Whereas retrieval of recent memory produced elevated hippocampal activation, retrieval of remote memory was not. In contrast, the opposite pattern was found for activity in cortical regions such as the frontal, temporal and anterior cingulate cortices. These results suggested, for the first time, that specific cortical structures are more active in the retrieval of more remote memories.

Since then, a number of studies have identified similar network changes in hippocampal and cortical activity following the retrieval of recent (1-3 day old) and remote (~30 day old) memories by looking at immediate-early gene expression as a measure of neural activity in a number of different tasks. For example, in addition to reduced hippocampal activity, retrieval of spatial discrimination task increased zif268 activity in the retrosplenial and anterior cingulate cortices (Maviel et al., 2004), retrieval of remote contextual fear memory increased c-Fos and zif268 activity in a number of prefrontal cortical areas, such as the prelimbic, infralimbic, and anterior cingulate cortices (Frankland et al., 2004a), and retrieval of remote socially transmitted food preference increased c-Fos activation of the orbitofrontal cortex (Ross and Eichenbaum, 2006; Lesburgueres et al., 2011). Studies on water maze memory have found somewhat different pattern of activity. One study found similar c-Fos activity in the hippocampus at both recent and remote time-points in rats, but increased activity in the ACC (Teixeira et al., 2006). Two other studies, however, comparing Arc mRNA expression at recent and remote time-points in mice found reduced activity in the hippocampus (Gusev et al., 2005), several cortical areas, including the anterior cingulate cortex (Gusev and Gubin, 2010). Although these findings seem contradictory at first, the difference may be due to a number of factors that varied between the studies, such as differences between gene expression in rats and mice (Snyder et al., 2009), differences in training and testing protocols, different types of control groups, different type of mapping methods (in situ hybridization vs. immunohistochemistry) and differences in the characteristics of c-Fos and Arc as measures of activity.

In a similar fashion, several recent papers have described changes in dendritic spine density following training. Restivo et al. (2009) first described how at one day following contextual fear conditioning dendritic spine growth in mice was increased in CA1 of the hippocampus but not the ACC. At 36 days, however, spine growth had fallen back to control levels in the hippocampus and increased in the ACC. A later study showed that a marked increase in spine growth in the ACC could be detected at 8 days, but not at 1 day following conditioning (Vetere et al., 2011). Another study using the socially transmitted food preference task found that dendritic spine growth in the orbitofrontal cortex of rats was increased relative to controls as soon as one day after training, and was further elevated on day 30 (Lesburgueres et al., 2011).

Several caveats to the interpretation of imaging studies should be noted. First, to control for activity that is not task-specific many studies have used different kinds of "non-learning" controls to subtract activity that is unrelated to memory retrieval. However, as pointed out by Guzowski and associates (Kubik et al., 2007), matching controls for all behavioural parameters except the "memory signal" is problematic. For example, studies examining hippocampus activity using a control group exposed to the training context / task without acquiring the specific response under study run the risk of controlling not only for handling and exposure to the context, but also latent learning (Packard and McGaugh, 1996) and ongoing automatic contextual encoding known to occur in the hippocampus (Frey and Morris, 1997), which may preclude detection of a memory signal. Such comparison of two different types of memories, instead of subtracting non-memory related activity from the memory signal, may become a confounding factor in studies comparing activity at two different time-points, especially

if the two types of memories undergo systems consolidation at a different rate. Second, reorganization within a structure may be reflected in different neurons being active rather than overall increase or decrease in activity. For example, zif268 activity in the parietal cortex changes from deep to more superficial layers following the retrieval of remote special discrimination memory compared to a recent one (Maviel et al., 2004). However, the overall activity in the parietal cortex does not change following the retrieval of recent and remote memories.

Temporally Graded Retrograde Amnesia and the Cortex

Methodological issues notwithstanding, findings of elevated activity in specific cortical structures following remote memory retrieval have been supported by demonstrations that post-training disruptions of the same areas primarily affect remote memory retrieval. Combined lesions of the anterior cingulate and prelimbic cortices resulted in notably more trace eye-blink memory impairments when made 4 weeks after training than at 1 day (Takehara et al., 2003). Lesions of the pre- and infralimbic cortices did not affect auditory trace fear memory expression when made 1 day after training, while leading to impairments at 200 days (Quinn et al., 2008). With regards to pharmacological inactivation, pre-test infusions of the sodium channel blocker lidocaine impaired memory expression at 4 weeks but not 1 day after training for spatial discrimination when infused into the prelimbic cortex (Maviel et al., 2004), and when infused to the ACC, contextual fear memory (Frankland et al., 2004a) and Morris water maze memory (Teixeira et al., 2006). Pre-test infusions of the sodium channel blocker TTX into the orbitofrontal cortex impair expression of socially transmitted food preference memory at 4 weeks but not at 1 day after training (Lesburgueres et al., 2011). Together, these findings suggest that

discrete cortical areas become more critical for memory expression in a number of different paradigms, as the memory ages.

Involvement of the ACC in Memory Acquisition and Consolidation

There are, however, recent studies that have found that manipulations of the ACC can also impair the expression of recent memories of tasks that are initially hippocampus-dependent. For example, intra-ACC infusions of a protein synthesis inhibitor immediately after inhibitory avoidance training impairs memory when tested one day later, but not after 2 hours, consistent with a cellular consolidation impairment (Zhang et al., 2011). Similarly, pre-test infusions of the $GABA_A$ receptor agonist muscimol into the ACC, impairs the expression of inhibitory avoidance memory 1, 4 and 7 days, but not at 2 or 6 hours after training (Liu et al., 2009), and pre-test infusions of the ERK inhibitor UO126 into the ACC impaired Morris water maze memory expression 1 day after training (Leon et al., 2010). In addition, a recent study found that increasing the function of the transcription factor myocyte enhancer factor 2 with a viral vector in the ACC, known to disrupts neuronal spine growth, impaired contextual fear memory at 7 days post-training if started at day 1, whereas the same treatment 42 days post-training had no effect (Vetere et al., 2011). Thus, although a number of studies suggest that cortical structures such as the ACC play a critical role in remote memory expression only, recent studies targeting the same structure, using the same tasks (e.g. context fear and water maze), but targeting different processes, suggest that the structure is critical for expression as soon as 1 day after training. In support of an earlier role for the ACC, local infusions of the NMDA-NR2B subunit antagonist Ro25-6981 impair the formation of contextual fear memory (Zhao et al., 2005). The critical difference seems to be that studies using lesioning or

pharmacologically blocking neural transmission find impaired remote memory expression impairment (Takehara et al., 2003; Frankland et al., 2004a; Teixeira et al., 2006), whereas studies using methods more likely to affect synaptic plasticity also find impairments at more recent time points (Liu et al., 2009; Leon et al., 2010; Vetere et al., 2011). Together, these studies suggest that although cortical structures such as the ACC become increasingly more important for memory expression as systems consolidation progresses, they play a key role much earlier with regards to the underlying synaptic plasticity required for memory expression.

Reengagement of the Hippocampus: Cellular and Systems Reconsolidation of Remote Memory

Although animal models of retrograde amnesia have provided compelling evidence for the transient role of the hippocampus in retrieving certain kinds of memories, they do not necessarily rule out the participation of the hippocampus in remote memory in intact animals. A few studies have suggested that although the hippocampus becomes dispensable for retrieving remote contextual fear memory, the structure still plays an important role in processing the memory once it has been retrieved (Land et al., 2000; Debiec et al., 2002; Winocur et al., 2009). In a study by Debiec et al. (2002), hippocampal lesions were ineffective when performed 45 days after training, indicating that at that time point systems consolidation was complete. However, if the remote memory was reactivated immediately before applying the lesion, the animals were amnesic when tested later. Lesions of the hippocampus were only effective if performed up to two days after reactivation. Thus, an apparent hippocampus-independent memory became again transiently hippocampus-dependent, suggesting the existence of a *systems*

reconsolidation process. Furthermore, local infusion of the protein-synthesis inhibitor anisomycin into the hippocampus at either recent or remote time-points after training also resulted in memory impairment on a later test. Similarly to the lesioning effect, impairment following anisomycin infusions was contingent on prior memory reactivation. Together, these findings suggest that systems consolidation, like cellular consolidation, may not be a unidirectional process of memory fixation.

System Consolidation and Increased Memory Generalization Over Time

As with other types of memory, memory for context is known to change qualitatively with the passage of time through forgetting. Typically, forgetting is reflected in impaired retrieval under the same cuing conditions that were effective for retrieval at an earlier occasion. However, another type of forgetting is manifested in stimuli generalization increase over time, where cuing conditions that did not evoke conditioned responses at earlier time-points, will do so when tested later (Riccio et al., 1992). With regards to contextual information, a number of studies have documented how animals are proficient at discriminating between a training context and a novel context sharing some features with the original training context, such that testing in the training context evokes stronger conditioned responding than the novel context. However, as the interval between training and testing is increased, animals start to respond similarly to testing in the original training context and a novel one (Mcallister and Mcallister, 1963; Feinberg and Riccio, 1990; Zhou and Riccio, 1996; Anderson and Riccio, 2005). In this type of forgetting, there is no loss in the ability of the original cuing condition (training context) to evoke conditioned responding, rather, other conditions that share some features with the cuing condition also evoke the conditioned response. This enhanced generalization with time

has been suggested to reflect a decay of the original memory trace where animals forget some stimuli attributes from the original experience, leading them to perceive related cues as similar to the original ones (Riccio et al., 1992).

In the past few years, a number of studies have started to characterize the relationship between generalization enhancement and systems consolidation in rodents using contextual fear conditioning. A number of studies have found the timing of the change from precise context-specific expression to a more generalized expression parallels systems consolidation, with good memory precision 1 day after training, decreasing precision ~15 days post-training and marked context generalization at 30-40 days (Biedenkapp and Rudy, 2007; Wiltgen and Silva, 2007; Winocur et al., 2007; Wiltgen et al., 2010; Ruediger et al., 2011). Recently, two studies have provided strong evidence suggesting that the hippocampus is critical in maintaining detailed contextual fear memory. First, Wiltgen (2010) and associates found that around 14 days post-training, some mice expressed generalized contextual fear memory, whereas others still maintained detailed fear memory expression evident by low generalization to a novel context. The authors then showed how pharmacological inactivation of the dorsal hippocampus before testing in the original training context impaired memory retrieval of mice that had previously been found to express detailed memory, whereas mice previously expressing generalized memory were unaffected by the same manipulation. This suggests that the dorsal hippocampus plays a specific role in mediating the expression of detailed context memory, but not that of generalized memory mediated by extra-hippocampal structures following systems consolidation. A second more recent study by Ruediger et al (2011) suggests that the expression of detailed contextual fear

memory is mediated by the levels of filopodial synapses on fast-spiking interneurons that trigger feed-forward inhibition in mossy fibre complexes in the hippocampus. The authors found that increased filopodial growth and feed-forward inhibition connectivity in mice was negatively correlated with freezing to a novel context. Using mutant mice, first, the study further described how Rab3a -/- mice, which lack LTP at mossy fibres in the hippocampus, showed generalized memory expression one day after training and no increase in filopodia or feed-forward inhibition after training. Second, using adducin 2 knockout mice, which exhibit early LTP but are unable to stabilize new synapses, the authors found the same pattern of finding described in Rab3a -/-. However, by re-expressing adducin 2 in the dentate gyrus with viral transduction before training, the authors were able to rescue the defective filopodial growth and feed-forward inhibition, as well as maintain the precision of the contextual fear memory and inhibit generalization to novel contexts. These findings suggest that memory precision is maintained by feed-forward inhibitory connectivity in the mossy fibres of hippocampus of which decay leads to context generalization, consistent with earlier notions of generalization being due to decay of the memory trace (Riccio et al., 1992).

Interestingly, Ruediger et al also found that a reminder session in the training context 15 days after training restored context discrimination and filopodal growth for another 10 days. At 40 days post-training, a reminder session only partially restored context discrimination when tested two days later without renewed filopodial growth. This consistent with an earlier behavioural study showing renewed discrimination following a reminder session 35 days post-training (Wiltgen and Silva, 2007). Together, findings of renewed discrimination and its neural correlates in the hippocampus

following the reactivation of a remote memory (Ruediger et al., 2011), and findings consistent with cellular and systems reconsolidation of remote memories in the hippocampus (Debiec et al., 2002), strongly suggest that the structure plays a long-lasting role in the network supporting the contextual fear memory trace, specifically with regards to maintaining memory precision.

Theories of Systems Consolidation

The three arguably most dominant models accounting for of findings of temporally graded retrograde amnesia in both humans and animals are the trace reactivation theory, the standard model and the multiple-trace theory / transformation model.

The Trace Reactivation Theory of Memory Consolidation

McClelland and associates (McClelland et al., 1995; McClelland and Goddard, 1996) proposed a connectionist model, building on ideas initially formulated by Marr (1971). The model posits that there are two complementary learning systems in the brain: a hippocampal system that rapidly stores new episodes, later "replaying" them to a slower learning cortical system, interleaving the new episodes with previous knowledge stored in the cortex. This type of hippocampus-driven memory replay, which in turn reinstates the memory in the cortical memory system, can take place either in task-relevant situations or off-line, i.e., through rehearsal or reactivation during sleep. With time, as the memory undergoes multiple rounds of reinstatement, the cortical system becomes capable of supporting the memory without hippocampal contribution. The model assumes that all hippocampus dependent memories undergo a time-dependent consolidation process and thus makes no distinction between detailed episodic memories and schematic ones. The model further proposes that reinstatement of a pattern in the hippocampal system can

strengthen the hippocampal representation itself, as well as the representation in the cortex, thereby impeding the decay of the memory in both systems, even to the extent that the memory can remain in both systems throughout life (McClelland and Goddard, 1996). It is not clear which system or which representation is expressed under such conditions as the theory does not posit competition of any kind between the systems. The authors note that such reinstatement would most likely take place in task-relevant situations and not during off-line reinstatement, i.e., during some stages of sleep when hippocampal synaptic plasticity has found to be suppressed (Leonard et al., 1987; Diekelmann et al., 2011). However, if the memory were stored in both systems, it follows that lesioning of the hippocampus should not impair memory expression of the cortical representation. This leads to methodological issues for the theory, as lesioning of the hippocampus with unimpaired memory expression does not distinguish between a memory representation that is stored in the hippocampus and one that is not. It can only demonstrate that such a representation is now stored in the cortex.

The model can accommodate memory reconsolidation in as much as it assumes that memory reactivation can reinstate both hippocampal and cortical representations, implying post-retrieval memory lability. However, the theory can not account for findings of memory reconsolidation in the hippocampus *following* systems consolidation (Land et al., 2000; Debiec et al., 2002; Winocur et al., 2009), as the cortical representation is independent of the hippocampus - from which the original memory is posited to be decayed.

The Standard Model of Systems Consolidation

The standard model of consolidation posits that the hippocampus and related structures in the medial temporal lobe play only a temporary role in memory storage for declarative memory (semantic, episodic and spatial) (Squire, 1992; Squire and Alvarez, 1995). Through a gradual process of consolidation by hippocampus-driven reactivation of the memory in the cortex, the contribution of the hippocampal system gradually diminishes and the cortex alone becomes capable of supporting permanent memory storage and retrieval. The standard model shares many features with the reactivation theory (McClelland et al., 1995) with regards to hippocampal replay gradually modifying cortical networks. However, the standard model differs from the reactivation model on the fate of information stored in the hippocampus following acquisition, as the standard model does not posit any strengthening or modification of the hippocampus stored memory following memory reactivation, but rather that the hippocampus stored memory gradually decays as consolidation progresses. This position of hippocampal plasticity limited to the encoding of new memories is at odds with several findings of reconsolidation of recent and remote memory in the hippocampus (Land et al., 2000; Debiec et al., 2002; Winocur et al., 2009).

Multiple-Trace Theory / Transformation Hypothesis

The multiple-trace theory was developed as a response to some of the main tenets of the standard model of systems consolidation, specifically the limited memory lability posited to occur in the hippocampus after memory acquisition (Nadel and Moscovitch, 1997; Moscovitch and Nadel, 1998). Similarly to the standard model, multiple trace theory posits that information is sparsely encoded in a hippocampal-neocortical memory trace

where the hippocampus contains representations that bind together memory content in neocortical areas. However, multiple-trace theory posits that reactivation of the memory trace can create a new trace in the hippocampus as well as facilitate the creation of semantic memory in the cortex. Furthermore, the theory posits that spatial and detailed contextual information, which conveys episodic quality to memory, always depends on the hippocampal involvement. Thus, detailed episodic and spatial memory is assumed to always depend on the hippocampus.

Building on the multiple-trace theory, the more recent transformation hypothesis similarly states that episodic and context-bound memory always depends on the hippocampus (Moscovitch et al., 2005; Winocur et al., 2009; Winocur et al., 2010). With time and trace reactivation, the hippocampus dependent memory supports the development of a less integrated schematic version in the cortex. However, the transformation hypothesis additionally proposes that episodic and schematic memory representations can co-exist and compete for dominance of which memory is expressed behaviourally, depending on the circumstances at retrieval. Furthermore, the hypothesis emphasizes that the nature of the respective memory is determined by the structures that mediate them, where a hippocampus-based memory is necessarily episodic and context-specific and cortex-based memory is schematic and context independent.

According to this view, cuing circumstances control whether either detailed context-specific memory or a schematic memory is retrieved. Following retrieval, the dominant memory undergoes reconsolidation (Winocur et al., 2009). However, the assumption that schematic neocortical memory can co-existed with a hippocampus based is not consistent with findings of systems reconsolidation (Land et al., 2000; Debiec et

al., 2002; Winocur et al., 2009) where challenges to the hippocampus following remote memory retrieval (in the training context) result in later memory impairments for both types of memory.

The three models described above are not wholly mutually exclusive as they overlap in several respects, most notably on the mechanism of systems consolidation (e.g. hippocampal replay) and cortical plasticity following memory reactivation (by replay or direct memory retrieval). The models differ most notably on the role of the hippocampus following memory reactivation: both the trace reactivation theory and the transformation hypothesis posit lability for a hippocampal stored representation, whereas the standard model assumes no lability in the hippocampus. However, all of the models suggest the existence of separate neocortical and hippocampal memory systems that are independent following systems consolidation. Thus, as noted above, they are all challenged by findings of hippocampal systems reconsolidation of remote memory that seems to impair memory representations in both systems.

The Questions

As stated in the beginning of this introduction, the aim of this thesis is to study the involvement of the ACC in cellular consolidation and reconsolidation of contextual fear memory, and how the contribution of the structure changes over time with regards to reconsolidation and retrieval over time as the memory is thought to undergo systems consolidation. To this end, three questions are posed: 1) Is the ACC involved in cellular consolidation and reconsolidation of contextual fear memory, and if so, does the involvement of the ACC change over time? 2) Studies have found that following retrieval, remote contextual fear memories become once again transiently sensitive to

manipulations of the hippocampus, suggesting renewed involvement of the hippocampus. However, with systems consolidation, the ACC has become more critical for memory retrieval as the hippocampus becomes disengaged. This poses the question, if the ACC has become critical for remote memory retrieval, what becomes of the ACC contribution when the hippocampus is re-engaged? 3) A number of studies have found an inverse relationship between the involvement of the hippocampus and the ACC in supporting memory expression as systems consolidation progresses. Similarly, as the hippocampus becomes less critical for memory retrieval and the ACC more involved, context generalization increases. As the hippocampus has been found to specifically mediate precise context memory, does the ACC mediate context generalization? The study of these questions is presented in two manuscripts. The first manuscript 'The involvement of the anterior cingulate cortex in the acquisition, consolidation and reconsolidation of contextual fear memory' studies the first two questions, whereas the second manuscript 'The involvement of the anterior cingulate cortex in the expression of generalized contextual memory' examines the third question.

Behavioural Model: Contextual Fear Conditioning

The behavioural model used in this thesis is contextual fear conditioning. In this task, a rodent is placed in a distinctive chamber (context) with a grid floor. After a brief exploration period for encoding the context the animal receives a mild electric shock through the grid floor, leading to the animal learning an association between the context and the aversive shock. When placed back into the same context after a delay, rodents show a number of conditioned fear responses, such as immobility (freezing), indicating a

memory for the association (Fanselow, 1980). Such one-trial fear conditioning is slowly forgotten and can last over the adult lifespan of the rodent (Gale et al., 2004).

Contextual fear conditioning is normally hippocampus dependent, as memory acquisition is impaired by pre- (Sutherland and McDonald, 1990; Kim et al., 1993), and post- training lesions to the structure (Anagnostaras et al., 1999), pre-training infusion of NMDA receptor antagonist (Young et al., 1994; Stiedl et al., 2000; Bast et al., 2003), as well as post-training infusions of GABA receptor agonist (Maren and Holt, 2004), sodium channel blocker (Daumas et al., 2005) and protein-synthesis inhibitor (Fischer et al., 2004; Suzuki et al., 2008). However, pre-training lesions of the hippocampus reduces, but does not abolish, contextual conditioning suggesting that other structures can encode contextual representations, albeit somewhat weaker and less detailed (Frankland et al., 1998; Wiltgen et al., 2006). As previously described, systems consolidation of contextual fear memory has been demonstrated with a number of methods targeting the hippocampus at different time-points (Table 1). Similarly, contextual fear memory has been found to depend on the ACC for its formation and cellular consolidation (Zhao et al., 2005), and as the memory ages, increasingly for memory retrieval. Another structure considered critical for contextual fear conditioning is the basolateral amygdala (Maren, 1999; Ponnusamy et al., 2007). However, contrary to the involvement of the hippocampus and ACC, participation of the basolateral amygdala does not seem to show any temporal gradient, as disrupting the structure impairs memory expression both at recent and remote time-points (Gale et al., 2004).

Table 1. Studies finding temporal retrograde gradient following hippocampal lesion/inactivation in rodents

Task	Brain target	Method	Amnesia (d)	Spared (d)	Refs
Socially acquired food preference	DH	Electrolytic	2	5, 10	(Winocur, 1990)#
	HC	Neurotoxic	2	5, 10	(Winocur et al., 2001)#
	HC & subiculum	Electrolytic	1	30	(Clark et al., 2002)
	HC	Electrolytic	1	21	(Ross and Eichenbaum, 2006)
	DH	TTX	1	30	(Lesburgueres et al., 2011)
Context fear	DH	Electrolytic	1	7, 14, 28	(Kim and Fanselow, 1992)
	DH	Electrolytic	1	50	(Anagnostaras et al., 1999)
	DH	Electrolytic	1, 7	14, 28	(Ward et al., 1999)*
	DH	CNQX	1	28	(Wiltgen et al., 2010)
	DH	TTX	1	28	(Kitamura et al., 2009)
	HC	Neurotoxic	1	28	(Winocur et al., 2009)
Odour fear learning	DH	Muscimol	7, 28	42	(Parsons and Otto, 2010)*
Spatial multiple choice discrimination	DH	Lidocaine	1	30	(Maviel et al., 2004)
	DH	Electrolytic	1, 16, 32	64	(Ramos, 1998)*
Trace-eye-blink	HC	Aspiration	1	30	(Kim et al., 1995)
	DH	Aspiration	1	28	(Takehara et al., 2002)
	DH	Aspiration	1	7, 14, 30	(Takehara et al., 2003)
Inhibitory avoidance	DH	CNQX	1	31, 60	(Quillfeldt et al., 1996)
	DH	CNQX	1	31, 60	(Izquierdo et al., 1997)
Trace-auditory	DH	Neurotoxic	1	200	(Quinn et al., 2008)
Object location task	HC	Neurotoxic	,1-3	21	(Gaskin et al., 2009)

*Experimental group improves with time (past 4 d)
Experimental group does not improve (past 4 d)

Table 2. Studies finding flat temporal retrograde gradient following hippocampal lesion/inactivation in rodents

Task	Brain target	Method	Amnesia (d)	Spared (d)	Refs
Water maze	DH	Neurotoxic	98	-	(Bolhuis et al., 1994)
	HC & subiculum	Neurotoxic	98	-	(Mumby et al., 1999)
	DH	CNQX	16	-	(Riedel et al., 1999)
	HC	Neurotoxic	98	-	(Sutherland et al., 2001)
	DH / HC	Neurotoxic	42	-	(Martin et al., 2005)
	DH / HC	Thermo-coagulation	1, 56, 98	-	(Clark et al., 2005b)
	DH	Lidocaine	30	-	(Broadbent et al., 2006)
	DH	Lidocaine	30	-	(Teixeira et al., 2006)
	HC	Thermo-coagulation	100	-	(Clark et al., 2005a)
Oasis maze	HC / DH	Thermo-coagulation	1, 56	-	(Clark et al., 2005b)
Annular maze	HC / DH	Thermo-coagulation	56, 98		(Clark et al., 2005b)
Modified WM	HC	Thermo-coagulation	60	-	(Clark et al., 2007)
Spatial multiple choice discrimination	DH	Neurotoxic	1, 70	-	(Ramos, 2009)
Spatial task cross maze	HC	Neurotoxic	270	-	(Winocur et al., 2005a)
Spatial task complex maze	HC	Neurotoxic	98	-	(Winocur et al., 2005b)
	DH	Neurotoxic	1, 28	-	(Haijima and Ichitani, 2008)
Object discrimination	HC	Neurotoxic	98	-	(Sutherland et al., 2001)
Two-choice visual discrimination	HC	Neurotoxic	1, 60	-	(Epp et al., 2008)
Object-fear	HC	Neurotoxic	1, 14	-	(Lehmann et al., 2006)
Contextual fear	DH	Neurotoxic	1, 28, 100	-	(Maren et al., 1997)
	DH, VH, HC	Neurotoxic	1-3, 84	-	(Sutherland et al., 2008)
	DH, HC	Neurotoxic	7, 90, 180	-	(Lehmann et al., 2007)
	DH	Neurotoxic	1, 200	-	(Quinn et al., 2008)

Chapter 2

Involvement of the Anterior Cingulate Cortex in the Acquisition, Consolidation and Reconsolidation of Contextual Fear Memory

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Background: The standard view of systems-consolidation posits that the hippocampus plays a temporary role in memory retrieval, after which the memory is supported by cortical structures such as the anterior cingulate cortex (ACC). A number of recent studies, however, have suggested that manipulations of the hippocampus immediately post-retrieval can impair older memories, suggesting that retrieval can re-engage the hippocampus for systems reconsolidation of remote memories. Currently, little is known about how the reactivation of remote memories affects the involvement of the ACC, nor whether the structure contributes to persistence of new or recent memories.

Results: The current experiments address this by testing the involvement of the ACC in the acquisition, consolidation, and reconsolidation of contextual fear memory. We report that intra-ACC infusions of an NMDA-NR2B antagonist impair memory acquisition. Similarly, ACC infusions of the protein-synthesis inhibitor anisomycin immediately following conditioning, or retrieval of a recent or remote memory, disrupted later memory expression, consistent with consolidation and reconsolidation impairments. Furthermore, we found that pre-test ACC inactivation impairs remote memory retrieval. However, if retrieved 6 hours earlier, memory expression is only impaired if both the ACC and dorsal hippocampus are inactivated, while at 24 hours ACC inactivation impaired expression again.

Conclusions: These findings suggest that the ACC is not limited to mediating remote memory as the hippocampus disengages. Rather, the ACC is involved in the formation and consolidation, as well as reconsolidation at both recent and remote time-points, and, following the retrieval of remote memory, is transiently re-engaged with the hippocampus.

Memory consolidation refers to the process of gradual stabilization new memories must undergo in order to persist [1]. Two types of consolidation have been proposed to exist: cellular and systems consolidation [2]. The first type, cellular consolidation, is thought to be a ubiquitous property of neurons and occurs over minutes to hours and is thought to involve RNA transcription and transient protein synthesis in localized circuits [3-5]. The second type, systems consolidation, occurs over a much longer time frame and involves additional changes at the level of brain regions, where memories that are initially dependent upon the hippocampus become independent of that structure, a process hypothesized to reflect neocortical traces becoming sufficiently strong to maintain the memory and support its retrieval [6-8]. Such temporally graded retrograde amnesia of hippocampal-dependent knowledge has been modeled in a number of animal behavioral paradigms, one being contextual fear conditioning [9-11] but see [12]. In this task an animal receives a mild foot shock in a distinct chamber (context) that subsequently becomes associated with the shock. Both cellular and systems consolidation hypotheses assume that once consolidation ends, memories become permanently stabilized, thus implying that memories undergo consolidation at both levels only once. Recently, a number of studies have provided evidence that the ACC mediates retrieval of remote memories as the hippocampus is thought to become disengaged [7]. For example, pre-test reversible pharmacological inactivation of the ACC impairs the expression of remote, but not recent five-arm discrimination [13], spatial [14], and contextual fear memories [15]. Thus, it has been suggested that the ACC may play a similarly integrative role in the expression of remote memory as the hippocampus does for recent memory [7].

However, evidence for cellular and systems reconsolidation of hippocampus-

dependent memories have questioned the standard view of consolidation [10, 16]. In line with previous findings of temporally graded retrograde amnesia, lesions of the DH in rats produced amnesia for contextual fear memory when made recently after training, but were ineffective when performed at a more remote time-point [10, 17, 18]. However, if the remote memory was reactivated immediately before the lesioning, the animals now became amnesic on a later test [10, 18]. Thus, reactivation of an apparent DH-independent memory caused the memory to transiently become DH-dependent once more, a process of systems reconsolidation. Moreover, memory reactivation followed by local infusion of the protein synthesis inhibitor anisomycin into the DH, also resulted in memory impairments for recent and remote memories [10]. Together, these findings suggest that, following memory retrieval, the DH plays a more dynamic role in mediating remote memory than the standard systems consolidation hypothesis posits. Furthermore, these findings raise the question of whether the ACC also plays a more dynamic role than hypothesized by the standard systems consolidation view of primarily being recruited to support remote memory. Thus, in the present study, we examined the involvement of the ACC in acquisition of contextual fear memory, cellular consolidation and reconsolidation of recent and remote memory, and systems reconsolidation of remote memory.

First, we tested whether contextual fear memory acquisition involves NMDA-receptor NR2B subunit synaptic plasticity, and protein-synthesis dependent cellular consolidation in the ACC by infusing a pharmacological NR2B subunit antagonist before training and the protein synthesis inhibitor anisomycin post-training, respectively. Second, we asked whether the reactivation of either a recent (3 days) or a remote (30 days) contextual fear memory induces cellular reconsolidation in the ACC by infusing

anisomycin immediately thereafter. Third, as reactivation of a remote memory has previously been shown to return the memory to a hippocampus-dependent state akin to a recent memory [10], we asked if such a reactivation would similarly make the memory independent of the ACC. To test this idea we used two complementary approaches, first by inactivating the ACC using AMPA receptor antagonists at different time points after reactivation, and second, by assaying neuronal activity in the CA1 of the dorsal hippocampus and ACC at the same time points by measuring the immediate early gene c-Fos by western blotting.

Results

Involvement of the ACC in acquisition and cellular consolidation of contextual fear memory

Although the ACC is suggested to be primarily involved in mediating the expression of remote memories that have undergone systems consolidation [7], a number of studies have shown that the structure can also be involved in the acquisition phase of hippocampus-dependent memory tasks [19, 20]. In testing whether the ACC contributes to the formation of contextual fear memory we infused the NMDA receptor NR2B subunit antagonist Ro25-6981 immediately before contextual fear conditioning with 8 unsignaled foot shocks, a treatment previously found to impair the acquisition of one-shock contextual fear conditioning [20]. When tested 3 days later, animals receiving Ro25-6981 showed significantly lower freezing than animals receiving the saline vehicle infusions ($t(12) = 2.5$, $p < 0.05$; Figure 1A). This confirms previous findings of Zhao and associates [20] that NMDA-NR2B subunit activity in the ACC is critical for the

acquisition of contextual fear memory in the ACC.

In order to test whether the ACC is involved in cellular consolidation of contextual fear memory, we infused the protein synthesis inhibitor anisomycin immediately following conditioning. While anisomycin, a broad-spectrum translation inhibitor, is thought to produce its amnesic effects via inhibition of protein synthesis, it has recently been suggested to also work by changing neurotransmitter levels [21]. For our purposes, the exact mechanism by which anisomycin induces amnesia is not important, but rather whether it can impair cellular consolidation and reconsolidation in the ACC, as it has been found to do in other brain structures [16]. Rats infused with anisomycin immediately after conditioning showed no impairment compared to vehicle infused animals when tested 4 hours later for short-term memory (STM), whereas when tested a day later for long-term memory (LTM), they showed a reduction in freezing (drug × memory test interaction $F(1, 13) = 7.07$, $p < 0.05$; post hoc STM-test, $p > 0.05$, post hoc LTM-test, $p < 0.05$; Figure 1B). This suggests that the ACC is involved in the cellular consolidation of contextual fear memories.

Involvement of the ACC in cellular reconsolidation of recent and remote contextual fear memory

To test the involvement of the ACC in cellular reconsolidation of recent memories, animals were re-exposed to the conditioning context for memory reactivation 3 days after training for 90 sec, followed by intra-ACC infusions of anisomycin or its vehicle. Anisomycin and vehicle groups exhibited similar levels of freezing during reactivation ($t(17) < 1$; Figure 2A). Repeated measures ANOVA showed a significant drug × memory test interaction ($F(1, 17) = 19.5$, $p < 0.001$). Post hoc comparison showed similar

freezing during the PR-STM test ($p > 0.05$), but impaired freezing by the anisomycin group compared to the vehicle group on the PR-LTM test ($p < 0.05$). This suggests that the ACC is involved in the cellular reconsolidation of 3 day old contextual fear memory.

To examine cellular reconsolidation of remote memory in the ACC we re-exposed animals to the conditioning context 30 days after training using the same protocol as above. Both vehicle and anisomycin groups demonstrated equivalent freezing during reactivation ($t (20) < 1$; Figure 2B). A repeated-measures ANOVA comparing drug (anisomycin vs. vehicle) with memory test (PR-STM vs. PR-LTM) revealed a significant interaction ($F (1, 20) = 6.49$, $p < 0.05$). Post hoc comparisons showed that on the PR-STM test both groups were again comparable ($p > 0.05$), while on the PR-LTM test the anisomycin-treated animals were significantly impaired compared to the vehicle group ($p < 0.05$), consistent with a reconsolidation impairment. Similar infusions without a reactivation trial at 30 days did not cause any memory impairment when tested 4 and 24 hours later (drug × test interaction: $F (1, 8) < 1$, $p > 0.05$; Figure 2C). Thus, the sensitivity of the memory trace to anisomycin was predicated on memory reactivation. To test for anatomical specificity of the effects of infusion into the ACC, a separate group received anisomycin infusions into the adjacent primary/secondary motor cortex following a reactivation trial 30 days after training. During reactivation both groups showed comparable freezing ($t (15) < 0$; Figure 2D), and similarly showed no difference at PR-STM and PR-LTM testing (drug × memory test interaction: $F (1, 15) = 0.22$, $p > 0.05$). This suggests that the effects of anisomycin infused into the ACC were not due to lateral diffusion into these neighboring areas.

A test of system's reconsolidation: remote contextual fear memory expression becomes transiently insensitive to pre-test ACC inactivation following memory reactivation

We tested whether remote ACC-dependent contextual fear memory undergoes systems reconsolidation, that is, whether following its reactivation, the memory can transiently be expressed while the ACC is pharmacologically inactivated. Different groups were trained and tested at 3 or 30 days after receiving local infusions of the AMPA-receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) or its vehicle in the ACC. Animals tested at 30 days were tested again in a 2 × 2 repeated measures counterbalanced design where the opposite treatment was given on one of two tests; rats receiving vehicle on test 1 received CNQX before test 2, and those that received CNQX on test 1 received vehicle before test 2. First we tested performance with 24 hours inter-test interval, as a previous study had showed that reactivating remote contextual fear memory re-engages the DH for 1-2 days [10], suggesting that the memory should once again be dependent on the ACC if systems reconsolidation is completed at that time. We found that inactivating the ACC before testing 3 days after training did not affect the expression of the memory ($t(18) = 0.56$, $p > 0.05$; Figure 3A). At 30 days, a repeated measures ANOVA comparing drug (CNQX / Vehicle vs. Vehicle / CNQX) with the test (test 1 vs. test 2) revealed a significant interaction ($F(1, 11) = 19.5$, $p < 0.05$; Figure 3B). Post hoc comparison confirmed that rats receiving CNQX froze less than those receiving the vehicle at test 1 ($p < 0.05$). Similarly, 24 hours later, rats receiving CNQX showed impaired freezing compared to those receiving vehicle ($p < 0.05$). This demonstrates that a functional ACC is required for the initial retrieval of the remote memory and for a second recall 24 hours

later. Furthermore, the reduced conditioned response of freezing displayed by the group receiving CNQX before test 2, was not due to memory extinction as a separate group of animals receiving vehicle infusions before both tests at 30 days and 24 hours later, did not show reduced fear response between tests (Figure S1A).

In order to further test for systems reconsolidation from the ACC, the inter-test interval was reduced to 6 hours. As before, CNQX inactivation of the ACC impaired memory expression of a remote memory at test 1, whereas, 6 hours later CNQX inactivation did not disrupt expression of the remote memory (drug × test interaction: $F(1, 22) = 4.9$, $p < 0.05$; post hoc drug at test 1, $p = 0.05$; drug at test 2, $p > 0.05$; Figure 3C), indicating that 6 hours after reactivation the ACC is no longer critical for memory retrieval. Together, these results suggest that the ACC is critical for the initial retrieval of 30 day old contextual fear memory and again 24, but not 6 hours later. These results were replicated in separate experiments 45 days after training (Figure S1B), the interval used in a previous study demonstrating systems reconsolidation involving the DH [10].

Only dual inactivation of the ACC and the DH impairs expression of a 30 day old contextual fear memory reactivated 6 hours earlier

Reactivation of remote contextual fear memory can make the memory transiently susceptible to manipulations of the DH, suggesting re-engagement of the DH [10]. We therefore tested whether memory expression 6 hours after memory reactivation without the participation of the ACC could be mediated by the DH. To this end we inactivated both the ACC (CNQX) and DH (GABA-receptor agonist muscimol), either structure, or neither one before test 2. Furthermore, to control for anatomical specificity of our DH infusions, we inactivated the cortical area overlying the DH in one group (medial parietal

association cortex) with the ACC inactivated. For consistency with previous experiments all groups received vehicle infusions into the ACC before test 1. This gave us five groups (ACC infusion/DH infusion): CNQX/muscimol, vehicle/muscimol, CNQX/vehicle, vehicle/vehicle, CNQX/dorsal-control muscimol. A 5 × 2 repeated measures ANOVA contrasting drug with test revealed a significant interaction ($F(4, 28) = 6.8$, $p = 0.01$) (Figure 4). Post hoc comparison showed that only group CNQX/muscimol froze significantly less than the other groups on test 2 ($p < 0.05$), which did not differ between themselves ($p > 0.05$). Thus, inactivating either the DH or ACC, 6 hours after memory reactivation, does not block memory expression, but inactivating both structures does. The fact that only 1 out of 5 groups showed a deficit, demonstrates that these infusions did not cause a significant lesions of either structure. In addition, the finding that the infusions dorsal to the DH did not impair memory expression demonstrates that this combination of drugs does not generally lead to a behavioral impairment. Given that infusions in, but not dorsal to, the hippocampus suggest that the DH is most likely the site of action of the second infusion. This indicates that the expression of remote contextual fear memory 6 hours after a previous memory reactivation requires either a functional ACC or DH.

A second reactivation of remote memory maintains elevated c-Fos activity in the ACC while increasing activity in the DH

The expression of immediate-early genes such as the transcription factor c-Fos correlates with neuronal firing, and is used extensively as a putative marker for previously activated neurons [22]. In order to test whether changes in functional dependence on the ACC and DH described above is reflected in neural activity, we measured c-Fos expression in the

ACC and CA1 region of the DH by Western blot analysis of nuclear extracts 90 minutes after memory reactivation at four time-points after training: at 3 days, 30 days, 6 hours following a retrieval at 30 days, and at 24 hours following a retrieval at 30 days (Figure 5A). Groups reactivated at 3 and 30 days were compared to control groups that were trained but not re-exposed before being sacrificed at the same time as re-exposed animals (home cage control). For animals reactivated for a second time, the control group underwent the same first exposure on day 30, but not the second one at either 6 or 24 hours later. In the ACC, c-Fos expression changed over time ($F(3, 32) = 4.66$, $p < 0.01$; Figure 5B), with post-hoc analysis showing c-Fos levels increasing from 3 to 30 days, and remaining elevated following a second retrieval at 6 and 24 hours after a reactivation trial on day 30, relative to 3 day levels (all comparisons: $p < 0.05$). In the CA1 of the DH, c-Fos levels also varied ($F(3, 35) = 3.66$, $p < 0.05$; Figure 5C), with post hoc tests showing a c-Fos expression at 30 days to be lower than at 3 days, and 30 days + 6 hours, and 30 days + 24 hours (all comparisons: $p < 0.05$). Thus, while c-Fos is greater in the ACC following the retrieval of a remote memory than recent one, the opposite pattern is observed in the CA1. However, with a second retrieval either 6 or 24 hours after the first trial on day 30, CA1 c-Fos is increased relative to the first 30 day retrieval. ACC c-Fos remained similar at these two later time points. These changes in c-Fos at the remote time-point, from the first to the retrieval trial at 6 hours, mirrors changes in functional dependence at same time points as revealed by our pre-test inactivation where either the DH or ACC are capable of supporting memory expression without the other. However, somewhat surprisingly, c-Fos in both structures remained elevated after a second retrieval trial at 24 hours, although inactivation of the ACC alone was sufficient to impair memory

expression at that time-point.

Discussion

In summary, our findings suggest that the ACC is not limited to mediating remote retrieval as posited by the standard view of systems-consolidation [23]. We find that the ACC is involved in the formation, cellular consolidation as well as reconsolidation at recent and remote time points, and following retrieval of remote memory, is transiently re-engaged with the dorsal hippocampus.

Our data demonstrate that the circuits supporting contextual fear memory involve the ACC right from the formation of the memory as pre-training pharmacological inhibition of NMDAR-NR2B subunit activity in the structure impairs memory acquisition, consistent with the findings of Zhao and associates [20]. Similarly, local infusions of the protein-synthesis inhibitor anisomycin immediately following conditioning lead to delayed memory impairments, suggesting that contextual fear memory undergoes cellular consolidation in the ACC. The standard view of systems consolidation posits that context memory is encoded in a hippocampal-cortical network, initially driven by the hippocampus in concert with weak sparsely coded traces in the cortex [23]. With systems consolidation, the cortical traces become stronger and capable of driving memory expression, demonstrated, for example, by pre-test pharmacological inactivation of specific structures impairing retrieval. Our findings suggest, however, that cortical areas, such as the ACC, play a larger role in memory acquisition and cellular consolidation than the standard model suggests.

Previous studies have shown that contextual fear memory can undergo cellular

reconsolidation in the DH following retrieval at recent [10, 24-26] and remote [10] time-points. Here we present the first demonstration that such memory undergoes anisomycin-sensitive cellular reconsolidation in the ACC, at both recent and remote time points. Similar post-retrieval infusions in the primary/secondary motor cortex had no effect on later memory expression, suggesting that impairments following infusions into the ACC were not due to lateral diffusion of the drug. Thus, similar to a previous study using the same task and training parameters but focusing on the DH [10], we find that anisomycin infusions into the ACC following retrieval of recent and remote memory leads to ungraded retrograde amnesia. Contrary to our findings, however, a recent study did not find any evidence of recent or remote contextual fear memory undergoing cellular reconsolidation in the ACC in mice [26], in addition to finding evidence for DH involvement in cellular reconsolidation of recent, but not remote memory, contrary to Debiec et al. [10]. It is possible that the parameters of that study were not adequate to induce cellular reconsolidation known to not occur under certain boundary conditions [16]. For example, the susceptibility of contextual fear memory to systemic pharmacological disruption of reconsolidation has been found to depend on factors such as the strength of training and duration of the reactivation session [27, 28], as well as how stressed animals are before training [29]. With regards to contextual fear memory age, there are conflicting reports on reduced susceptibility to systemic anisomycin infusions, with Suzuki and associates [27] reporting more resistance, while Frankland et al. [26] found no more resistance with age. Similarly, conflicting findings have been reported on susceptibility to local DH infusions being reduced with time, Frankland et al. finding reduced susceptibility [26], while Debiec and colleagues did not [10].

With regards to consolidation and reconsolidation on a systems level, we found that contextual fear memory expression is not sensitive to inactivation of the ACC 3 days after training, whereas at 30 days inactivation impaired memory expression, indicating that the memory had by that time undergone systems consolidation to becoming dependent on the ACC for retrieval. However, 6 hours after the reactivation of a 30 day old remote memory, subsequent memory expression was unaffected if either the ACC or DH were inactivated, but severely impaired if both were. When the interval from reactivation to test was increased to 24 hours, memory expression was again sensitive to inactivation of the ACC. Similarly, we found that following retrieval c-Fos levels increased from 3 to 30 days in the ACC, and remained elevated following the second retrieval at 6 hours, whereas c-Fos levels decreased in the DH from 3 to 30 days and increased again following the second retrieval at 6 hours. Thus, levels of c-Fos activity in both the DH and ACC are in line with findings of DH being critical for retrieval of recent memories [9, 11], and the ACC being critical for retrieval of remote memories [15], as well as both structures being able to support remote memory expression 6 hours following retrieval. Interestingly, c-Fos levels following retrieval at 24 hours remained elevated in both the ACC and the DH, a time-point where memory expression was once again impaired by ACC inactivation. This suggests that although both structures are activated by retrieval at this point, the ACC is now once more critical for expressing the memory.

The standard view of systems consolidation states that memories which initially require the hippocampus for acquisition and retrieval can eventually be expressed without the participation of the structure, as they are by that time stored in distributed cortical

regions and dependent on specific cortical structures, such as the ACC, for memory expression [23]. However, contrary to the systems consolidation hypothesis, it has been found that once reactivated, such memories can return to rely transiently on the DH for expression before again becoming independent of that structure, a process of systems reconsolidation [10, 18]. Our finding that either the DH or ACC are sufficient in supporting memory expression at the 6 hr time-point is inconsistent with the systems consolidation hypothesis which assumes such consolidation to be unidirectional. However, our finding is also inconsistent with the straightforward systems reconsolidation hypothesis, which predicts that while the DH can support memory expression the memory should be independent of the ACC.

Our finding of such pervasive involvement of the ACC in memory processing contrasts with findings of pre-test pharmacological inactivation of the ACC showing temporally-graded retrograde amnesia [15]. This suggests that a brain structure can play an important role in processing a new or retrieved memory, despite not being critical for the retrieval of the memory (Figure 7). In other words, findings of apparent normal performance despite lesion or inactivation of a brain structure only shows that the structure is not critical for expression of the memory; it does not necessarily mean that the structure is no longer involved in processing the memory. Apparent normal performance could be due to compensation by other intact brain structures or memory systems. One example of such a systems-compensation is demonstrated by studies showing that excitotoxic lesioning of the DH [30-32] or pharmacological inactivation with the GABA receptor agonist muscimol [33, 34] prior to training does not block contextual fear conditioning, whereas blocking NMDA [35] or muscarinic cholinergic

[36] receptors will. One possible factor in determining whether such systems compensation takes place may be to what extent interactions between the DH and other structures are affected. A complete incapacitation of the DH (lesions or blocking of neural transmission) could allow other systems to compensate without interference from the DH, while pharmacological agents that impair neuronal plasticity without affecting synaptic transmission (such as inhibition of NMDA or muscarinic cholinergic receptors) could allow the DH to dominate during initial memory formation, but failing to retain a long-term memory trace. Such compensation could explain the different effects we found with 3 day old memory where pre-test AMPA receptor inactivation did not affect memory expression, but post-retrieval anisomycin infusions led to delayed memory impairments.

One function that could be served by having the ACC undergo cellular reconsolidation prior to being required for retrieval might be to allow new information mediated by the hippocampus to be incorporated into the hippocampal-cortical representation, which retrieval will later be mediated by the ACC (Figure S3). Furthermore, the finding that either the ACC or the DH can transiently support the expression of remote contextual fear memory raises the question of whether memory expression that relies on only one of these structures is qualitatively different. The standard view of systems consolidation makes no distinction between different types of memories [6, 37]. An alternative view to systems consolidation is the transformation hypothesis of systems consolidation reflecting a transformation of memory from being detailed and context-dependent to being more gist like and schematic, and is context-independent in the sense that animals come to generalize their fear response to novel

contexts with time [38, 39]. According to this view, detailed memory always depends on the hippocampus and transformed schematic memory on cortical structures. With regards to our results, this would suggest that the first retrieval of contextual fear memory 30 days after training could be schematic in nature as it requires an intact ACC, and not DH, but the second retrieval is detailed and context-dependent as the DH can now support the expression of the memory without the ACC. Thus, retrieval of a remote schematic memory could serve to re-instate the detailed DH-dependent memory, which could then be transformed by the subsequent retrieval context [40].

Furthermore, it has recently been shown that the addition of new information to an existing consolidated memory can occur very quickly, within one day of learning new information [41]. This time course is similar to what has been found here and previously [10] on systems reconsolidation. It may very well be that in order to add new learning onto an existing remote memory, the hippocampus would have to become functional for a short period of time to add on this new information to the hippocampal-cortical trace, after which it is no longer necessary for expression. Such a putative process would fit well with cognitive-oriented studies of human memory that model memory to be a constructive rather than a reproductive process [42-44].

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Methods

Subjects

Adult male Sprague-Dawley rats (Charles River, Saint-Constant, PQ) were housed individually and maintained on a 12/12 light/dark cycle (lights on at 7 a.m.) with food and water provided *ad libitum*. The rats were handled on three consecutive days for ~3 min before start of training.

Surgery and histology

Under Ketamine (55 mg/kg), Xylazine (3.33 mg/kg) and Domitor (27 mg/kg) anesthesia, 26-gauge stainless steel cannulae were implanted bilaterally into the ACC (injector co-ordinates: AP: 2.6 mm relative to bregma; ML: ± 2.4 mm; DV: -1.6 mm to dura surface). For DH cannulation, 24-gauge stainless steel cannulae were also implanted bilaterally (injector co-ordinates: AP: -3.6 mm relative to bregma; ML: ± 3.1 mm; DV: -3.1 mm to dura surface, and -1.6 to dura surface for the control group receiving infusions dorsally to DH infusion site). For motor cortex cannulation, 24-gauge were implanted bilaterally (injector co-ordinates: AP: 2.6 mm relative to bregma; ML: ± 0.7 mm; DV: -1.6 mm to dura surface). Rats were given a week to recover. In experiments testing recent memory (3d post-conditioning) rats were operated on before conditioning. In all other experiments testing remote memory (30 and 45 d post-conditioning) rats were operated on between conditioning and testing. At the end of the experiment, animals were transcardially perfused with physiological saline followed by 10% formal-saline. Brains were sectioned at 50 μm thickness and stained with formol-thionin and examined by light microscopy for verification of cannula placement in the ACC and DH. All procedures were in accordance with the CAA Guide, and were approved by the McGill University Animal Care and Use

Committee.

Drugs and infusions

In order to use a within design where the same animals receive an inactivation agent before one trial and its vehicle before another 6 hours later, we used 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) for inactivation, a drug that has been shown to reverse rapidly within a few hours after cortical infusions [45, 46]. Other commonly used drugs, such as muscimol, are known to inactivate neurons for much longer. In one experiment involving the DH, we used muscimol for inactivation as the drug's effects and diffusion have been extensively characterized in that structure. A previous study [47] targeting the DH, using the same dose as in this study, and using autoradiographic analysis of the spread of [3H]muscimol, found that the drug did not bind to other subcortical structures, although it did diffuse to more dorsal cortical regions. To further test the functional effects of the dorsal cortical diffusion we included a group that received muscimol infusions into cortical regions dorsal to the DH to rule out that inactivation of those areas might be responsible for memory impairments we observed when muscimol was infused into the DH (Figure 4). With regards to anatomical specificity of anisomycin infusions targeting the ACC, we found that post-reactivation infusions in the primary/secondary motor cortices did not lead to later memory impairments (Figure 2C).

Drugs were infused slowly via infusion pump at a rate of 0.25µl/min. Following drug infusion, injectors were left in place for an additional minute to allow diffusion of the drug away from the injector tip. Ro25-6981 (Tocris, Ellisville, MO) was dissolved in artificial cerebrospinal fluid (ACSF) and infused at 2 µg / 0.5 µl per side. Anisomycin

(Sigma, St. Louis, MO) was dissolved in equimolar HCl, diluted with artificial cerebrospinal fluid (ACSF), and adjusted to pH 7.4 with NaOH. The dose was 62.5 µg / 0.5 µl per side. CNQX disodium salt (Tocris, Ellisville, MO) was dissolved in nanopure dH2O and infused at 2.5 µg / 0.5 µl per side. Muscimol was dissolved in artificial cerebrospinal fluid (ACSF) and injected at 0.5 µg / 0.5 µl per side.

Apparatus

Conditioning was conducted in a Plexiglas rodent conditioning chamber with a metal grid floor (Model E10-10, Coulbourn Instruments) that was enclosed within a sound attenuating chamber (Model E10-20). The chamber was dimly lit with a single house light and scented with diluted vanilla in order to create a distinct context.

General behavioral procedures

Animals were trained using a contextual fear conditioning paradigm that has been shown to demonstrate a temporally graded retrograde amnesia after lesions of the DH, and be sensitive to DH cellular and systems reconsolidation of recent and remote memories [10]. In all of the experiments, rats were habituated to the conditioning chamber for 5 min one day before training. Rats were placed in the chamber and after 2 min received 8 unsignaled foot-shocks (1 sec, 1.5 mA) at 62 sec interval. The rats were left in the chamber for 30 sec after the termination of the procedure. For all tests, freezing (defined as the complete absence of movement, except that of respiration) was scored with an instantaneous time-sampling procedure in which each animal was observed as either freezing or not every 5 sec. These observations were then averaged to yield an estimate of the percentage time freezing. In experiments involving anisomycin, rats were infused immediately following training or a 90 seconds reactivation trial. In experiments

involving CNQX or muscimol, rats were infused 15 min prior to testing where they were placed in the conditioning chamber and observed for 3 min, after which the animals were returned to their home cage. Subsequent testing was then performed either 6 or 24 h later, 15 min after a second infusion.

SDS-Page and western blotting

Animals were sacrificed 90 minutes after memory retrieval testing, their brain rapidly removed and stored at -80 °C. Tissue punches (1 mm in diameter) were taken from the ACC, and the CA1 region of the dorsal hippocampus was microdissected from 1mm thick coronal slices. Nuclear extracts were supplemented with one volume of 6× Laemmli buffer (300 mM Tris–HCl pH 6.8, 12% SDS, 0.6% bromophenol blue, 60% glycerol, 6% dithiothreitol) and heated at 95 °C for 6 min. Equal quantities of total protein (30 µg per lane) were separated by 10% SDS-Page and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline solution containing 0.1% Tween 20 (TBST) for 1 hr at room temperature. After brief washing, blots were incubated with primary antibody (c-Fos, 1:500; Cell Signaling Technology) in 5% BSA in TBST overnight at 4 °C, washed and incubated with HRP-conjugated goat anti-rabbit IgG (1:2000; Cell Signaling Technology) in TBST for 1 hr at room temperature. Following additional washes, the blots were incubated with chemiluminescent substrate (ECL Plus kit; Amersham) which was then captured on a Storm 860 scanner (Amersham).

Subcellular fractionation

For nuclear fractionation, tissue from either the CA1 region of the dorsal hippocampus or the ACC were gently homogenized with an iso-osmotic extraction buffer (20 mM HEPES

– pH 7.2, 0.25 M sucrose, 1 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche). The homogenized sample was centrifuged for 10 min at 2000×g and the supernatant was separated from the pelletized material by pipetting. The pelletized material was thoroughly re-suspended in a second extraction buffer (10 mM HEPES – pH 7.2, 1.5 mM MgCl2, 10 mM KCl, supplemented with protease and phosphatase inhibitors [Roche]) and incubated for 5 min on ice. The sample was then centrifuged for 10 min at 4000×g. The supernatant was removed and discarded. The remaining pellet was sonicated on ice and suspended in RIPA buffer. Protein concentrations were determined by BCA protein assay, and their concentration standardized.

Statistical analysis

Paired and independent groups t-tests were performed, in addition to one-way independent groups and two-way mixed-factor ANOVAs that were followed up with *Post hoc* tests where appropriate.

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Figure legends

Figure 1. The involvement of the ACC in the acquisition and cellular consolidation of contextual fear memory. (A) Animals receiving ACC infusions of the NR2B subunit antagonist Ro25-6981 (n = 7) before conditioning showed impaired levels of freezing relative to the vehicle control group (n = 7) when tested three days later. ***** $p < 0.05$. (B) Animals receiving anisomycin (n = 8) infusions immediately after conditioning showed similar freezing to those receiving vehicle (n = 7) 4 hours later (STM test), whereas at 24 hours after conditioning anisomycin animals showed impaired freezing (LTM test) (drug × test interaction, $F(1, 13) = 7.07$, $p < 0.05$), suggesting impaired memory consolidation. ***** $p < 0.05$ compared to vehicle group. Data presented as group means ± SEM.

Figure 2. ACC involvement in cellular reconsolidation: infusions of anisomycin following memory reactivation impair 3 and 30 day old contextual fear memory. (A) Animals received intra-ACC infusions of anisomycin (n = 9) or its vehicle (n = 10) immediately after memory reactivation 3 days after conditioning. Both groups showed similar levels of freezing 4 hours later (PR-STM test), while at 24 hours the anisomycin group showed impaired memory expression (PR-LTM) (drug × test interaction , $F(1, 17) = 19.5$, $p < 0.001$), indicating a reconsolidation impairment. ***** $p < 0.05$ relative to the vehicle group. (B) Post-reactivation intra-ACC infusions 30 days after training found anisomycin (n = 11) and vehicle groups (n = 11) to show similar levels of freezing at PR-STM and anisomycin group with impaired memory expression at PR-LTM (drug × test interaction , $F(1, 20) = 6.49$, $p < 0.05$), demonstrating a reconsolidation impairment. ***** $p < 0.05$ relative to the vehicle group. (C) Intra ACC infusions of anisomycin (n = 5) or its

vehicle (n = 5) 30 days after training in the absence of memory reactivation did not impair memory reconsolidation. (D) Post-reactivation infusions of anisomycin (n = 9) or its vehicle (n = 8) into the *primary/secondary motor cortices* which neighbors the ACC 30 days after training did not impair memory reconsolidation. Data presented as group means ± SEM.

Figure 3. The ACC is necessary for memory retrieval of a 30 day old contextual fear memory, and at subsequent test at 24, but not 6, hr later. (A) Three days after conditioning, rats receiving intra-ACC infusions of CNQX (n = 10) or its vehicle (n = 10) into the ACC prior to testing showed similar levels of freezing. (B) Thirty days after conditioning, rats were infused with either CNQX or its vehicle into the ACC prior to testing. Twenty-four hours later the animals received a second infusion of the opposite compound and were tested again (first infusion / second infusion: CNQX / vehicle: n = 6; vehicle / CNQX: n = 7). Intra-ACC infusion of CNQX before testing impairs the expression of 30 day remote memory, as well as 24 hr after reactivation (drug × test interaction, $F(1, 11) = 19.5$, $p < 0.05$). ***** $p < 0.05$ relative to the vehicle group. (C) Pre-test infusions of CNQX or its vehicle into the ACC 30 days post-training impair memory expression, but not if reactivated 6 hours prior (CNQX / vehicle: n = 11; vehicle / CNQX: n = 13; drug × test interaction, $F(1, 22) = 4.9$, $p < 0.05$). ***** $p < 0.05$ relative to the vehicle group. Data presented as group means ± SEM.

Figure 4. Expression of a 30 day old contextual fear memory 6 hours after retrieval can be supported by either the ACC or the DH. Thirty days after training rats were

infused with vehicle into the ACC prior to test 1. Six hours later the animals received a second round of infusions into both the ACC and DH before test 2. This made four groups in addition to a control group that received infusions dorsal to the DH (ACC infusion/DH infusion, respectively): CNQX/muscimol (n = 6), vehicle/muscimol (n = 6), CNQX/vehicle (n = 7), vehicle/vehicle (n = 6), and CNQX/dorsal-control muscimol (n = 6). Inactivation of either the ACC or DH does not block the expression of 30 day old memory, but inactivating both does (drug × test interaction, $F(4, 28) = 6.8$, $p = 0.01$) . ***** $p < 0.05$ all groups compared to CNQX/muscimol group. Data presented as group means ± SEM.

Figure 5. c-Fos expression in the ACC and CA1 following reactivation of a contextual fear memory at 3 and 30 days after training, and a second reactivation of a 30 day old memory, 6 and 24 hours later. (A) Separate groups of animals were conditioned and then re-exposed to the training context at 3 days, 30 days, at 30 days and again 6 hours later, and at 30 days and again 24 hours later. All groups were then sacrificed 90 minutes later and samples collected for Western blot analysis from the ACC (for both reactivated and cage control: 3 d, n = 8; 30 d, n = 7; 30 d + 6 hr, n = 8; 30 d + 24 hr, n = 13) and CA1 are of the DH (for both reactivated and cage control: 3 d, n = 9; 30 d, n = 9; 30 d + 6 hr, n = 8; 30 d + 24 hr, n = 13). Groups reactivated at 3 and 30 days had their c-Fos levels standardized to control groups that were trained but had no memory reactivation (home cage control). Animals reactivated for a second time (at 6 or 24 hours) were standardized to a control group that had the first reactivation session, but not the second one, thus controlling for c-Fos activity due to the first reactivation session. (B) In

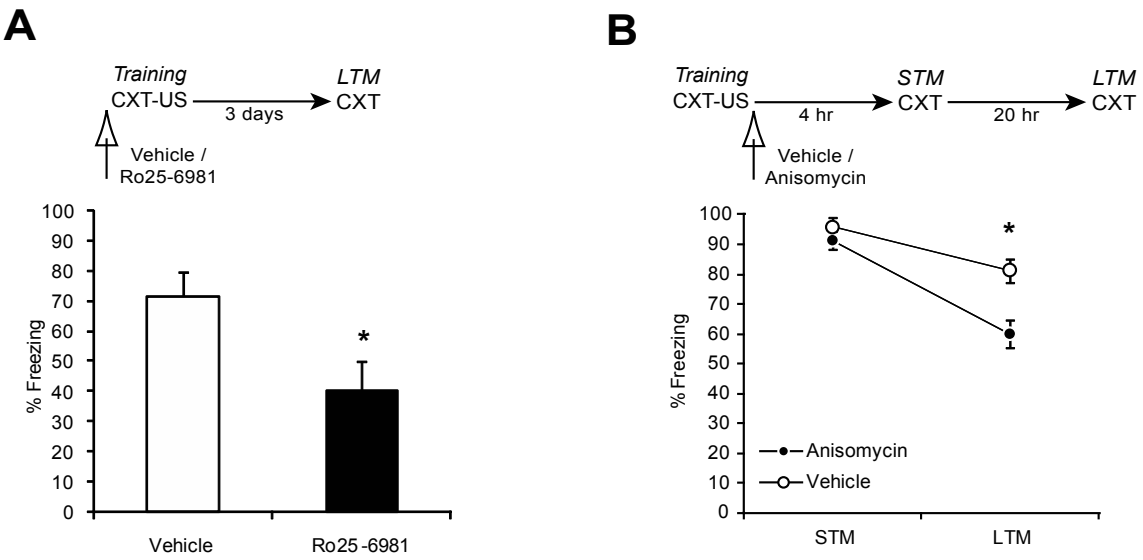
the ACC, c-Fos activity increased following retrieval at 3 to 30 days and remained elevated following a second retrieval at 6 or 24 hours after a first one on day 30 ($F(3, 32) = 4.66$, $p < 0.01$). * $p < 0.05$ compared to 3 day group. (C) In the CA1, c-Fos expression levels decreased following retrieval at 3 to 30 days, but increased again following a second retrieval at 6 or 24 hours after a previous one on day 30 ($F(3, 35) = 3.66$, $p < 0.05$). * $p < 0.05$ compared to 30 day group. Data presented as group means ± SEM.

Figure 6. Retrieval dynamically regulates the roles of DH and ACC within a hippocampal-cortical network. Results from studies using pharmacological agents blocking neuronal plasticity (e.g. NMDA receptor activity; anisomycin protein synthesis inhibition) and inactivating synaptic transmission (e.g. AMPA receptor [AMPAR] activity) indicate that contextual fear memory is encoded in a broad hippocampal-cortical network where the ACC and DH are crucial nodes. (A) Both the DH [35] and ACC [20] (current study) play a role in the formation and cellular consolidation of newly formed memories as suggested by impairments due to NMDA receptor antagonists or anisomycin. (B) When tested recently after training, the DH, but not the ACC, is critical for memory retrieval as AMPA receptor inactivation of the DH [11], but not AMPAR inactivation of the ACC (current study;) or sodium channel inhibition[15], impair memory expression. (C) However, once the memory is retrieved it enters a transient plastic state in both the DH [10, 26] and ACC (current study), from which it undergoes cellular reconsolidation, as revealed by sensitivity to the protein-synthesis inhibitor anisomycin. (D) As the memory ages, control over its retrieval shifts from the DH to the ACC as inactivating AMPAR in the DH does not impair remote memory retrieval [11],

whereas AMPAR inactivation (current study) or sodium channel inhibition [15] in the ACC leads to an impairment. (E) However, similar to a recent memory, a remote memory can undergo cellular reconsolidation in both the DH [10] and ACC (current study). Furthermore, following the retrieval of a remote memory trace, both the ACC and DH can transiently support subsequent retrieval of the memory as inactivating either structure 6 hours after the first retrieval does not block the expression of the memory on a subsequent test, but inactivating both does (current study).

Figure 7. The ACC is involved in cellular reconsolidation of recent and remote contextual fear memory, but is only necessary for remote memory retrieval: a summary. (A) At 3 days after training, pre-test inactivation of the ACC by CNQX does not impair memory expression (data from Figure 1B), whereas post-retrieval ACC infusion of anisomycin impairs memory expression tested 24 hr later (post-reactivation LTM test; data from Figure 5C). (B) At 30 days after training, pre-test inactivation now impairs memory expression (data from Figure 2BC), in addition to post-retrieval ACC infusion of anisomycin impairing memory expression when tested 24 hr later (post-reactivation LTM test; data from Figure 5D). ***** $p < 0.05$ relative to the vehicle group. Data presented as group means ± SEM.

Figure 1


A
Training
CXT-US
3 days
LTM
CXT
Vehicle /
Ro25-6981
% Freezing
100
90
80
70
60
50
40
30
20
10
0
*
Vehicle
Ro25-6981
B
Training
CXT-US
4 hr
STM
CXT
20 hr
LTM
CXT
Vehicle /
Anisomycin
% Freezing
100
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Anisomycin
Vehicle
STM
LTM

Figure 2

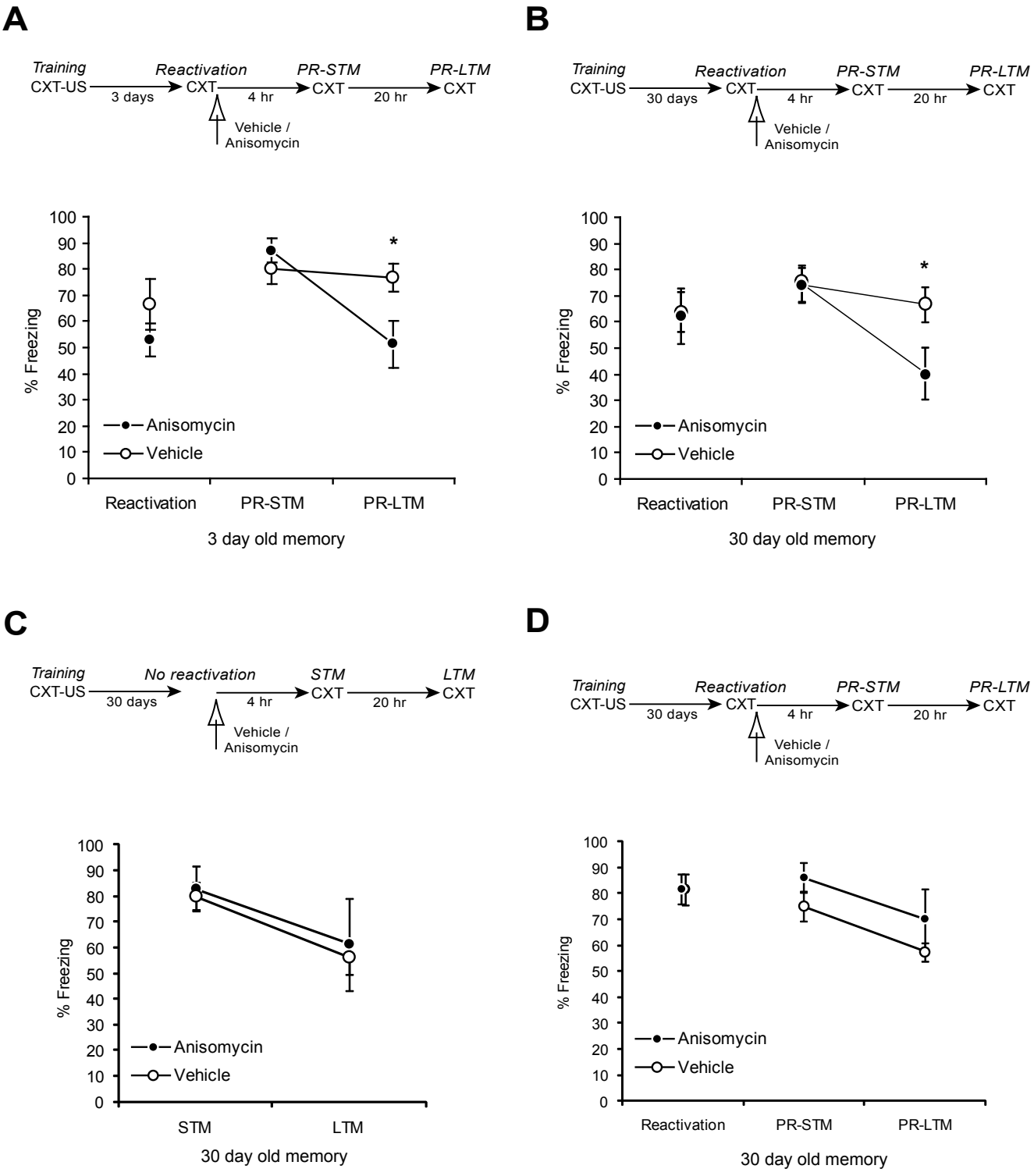

A
Training
CXT-US
3 days
Reactivation
CXT
4 hr
PR-STM
CXT
20 hr
PR-LTM
CXT
Vehicle /
Anisomycin
% Freezing
Anisomycin
Vehicle
Reactivation
PR-STM
PR-LTM
3 day old memory
B
Training
CXT-US
30 days
Reactivation
CXT
4 hr
PR-STM
CXT
20 hr
PR-LTM
CXT
Vehicle /
Anisomycin
% Freezing
Anisomycin
Vehicle
Reactivation
PR-STM
PR-LTM
30 day old memory
C
Training
CXT-US
30 days
No reactivation
4 hr
STM
CXT
20 hr
LTM
CXT
Vehicle /
Anisomycin
% Freezing
Anisomycin
Vehicle
STM
LTM
30 day old memory
D
Training
CXT-US
30 days
Reactivation
CXT
4 hr
PR-STM
CXT
20 hr
PR-LTM
CXT
Vehicle /
Anisomycin
% Freezing
Anisomycin
Vehicle
Reactivation
PR-STM
PR-LTM
30 day old memory

Figure 3

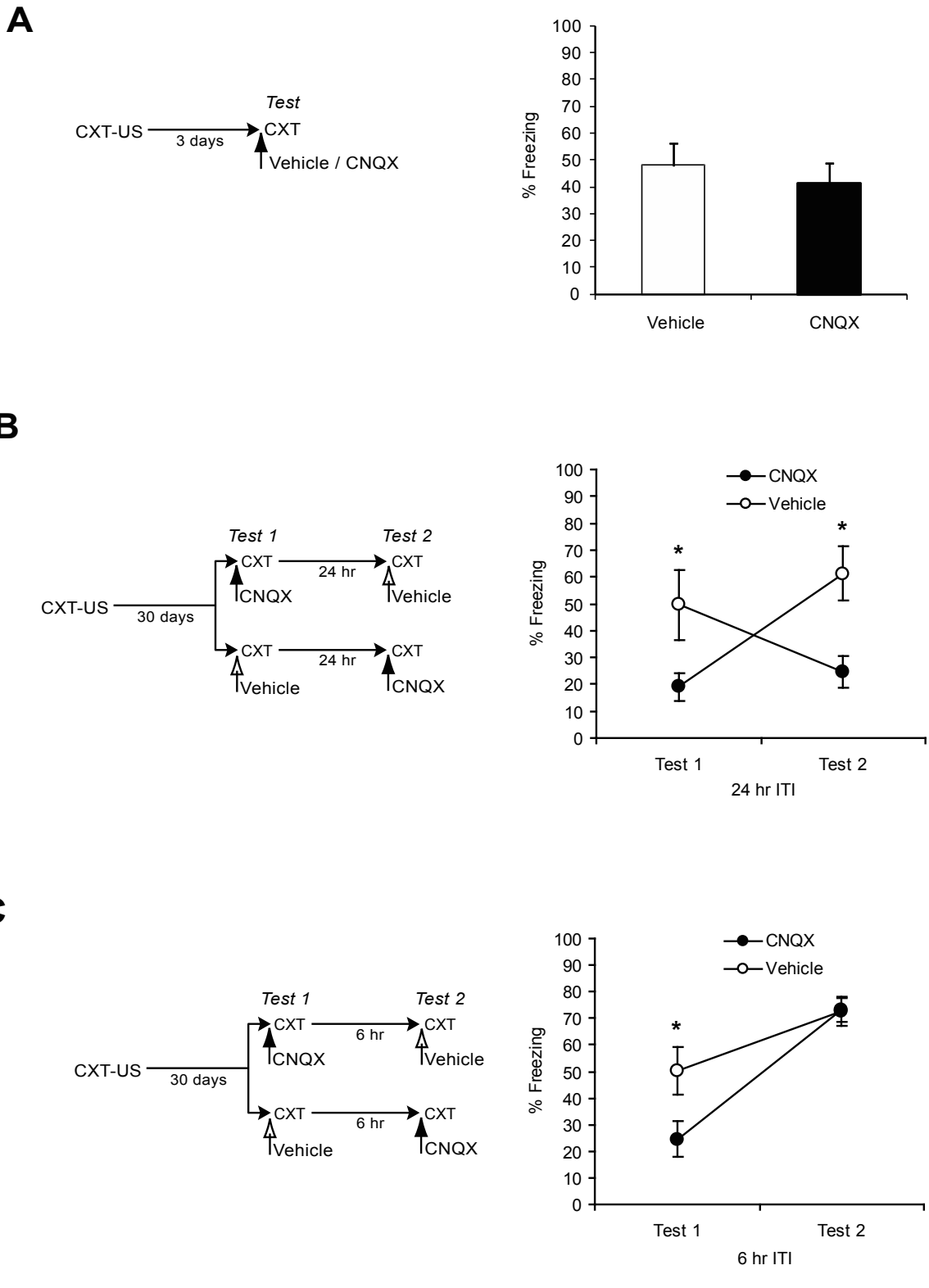

A
Test
CXT-US
3 days
CXT
Vehicle / CNQX
% Freezing
Vehicle
CNQX
B
Test 1
Test 2
CXT-US
30 days
CXT
CNQX
24 hr
CXT
Vehicle
CXT
Vehicle
24 hr
CXT
CNQX
CNQX
Vehicle
% Freezing
Test 1
Test 2
24 hr ITI
C
Test 1
Test 2
CXT-US
30 days
CXT
CNQX
6 hr
CXT
Vehicle
CXT
Vehicle
6 hr
CXT
CNQX
CNQX
Vehicle
% Freezing
Test 1
Test 2
6 hr ITI

Figure 4

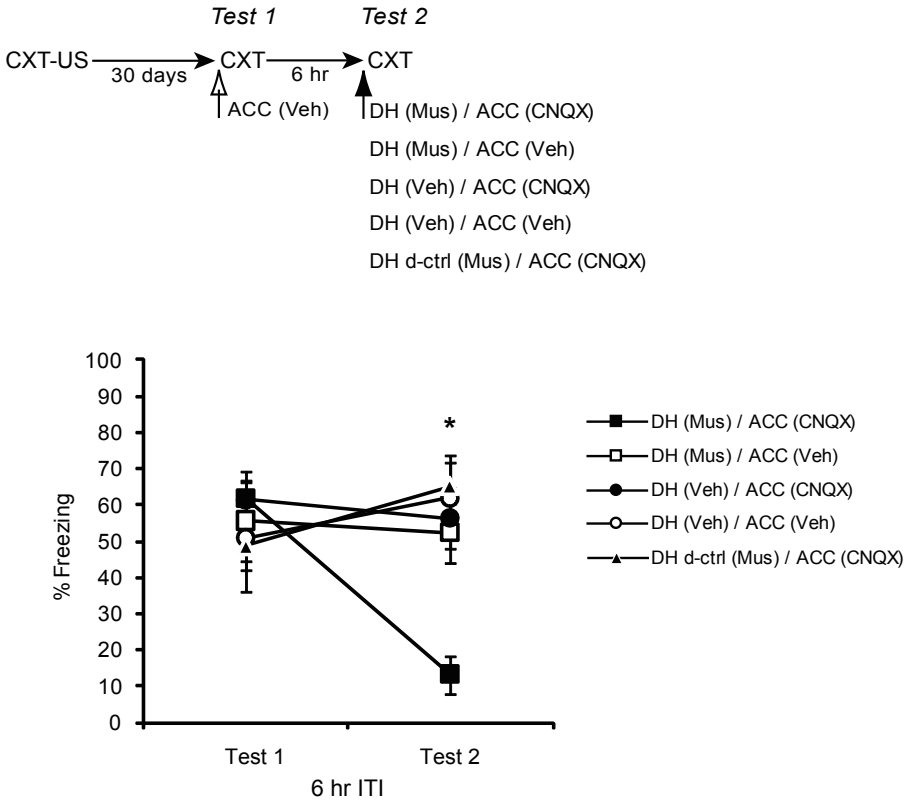
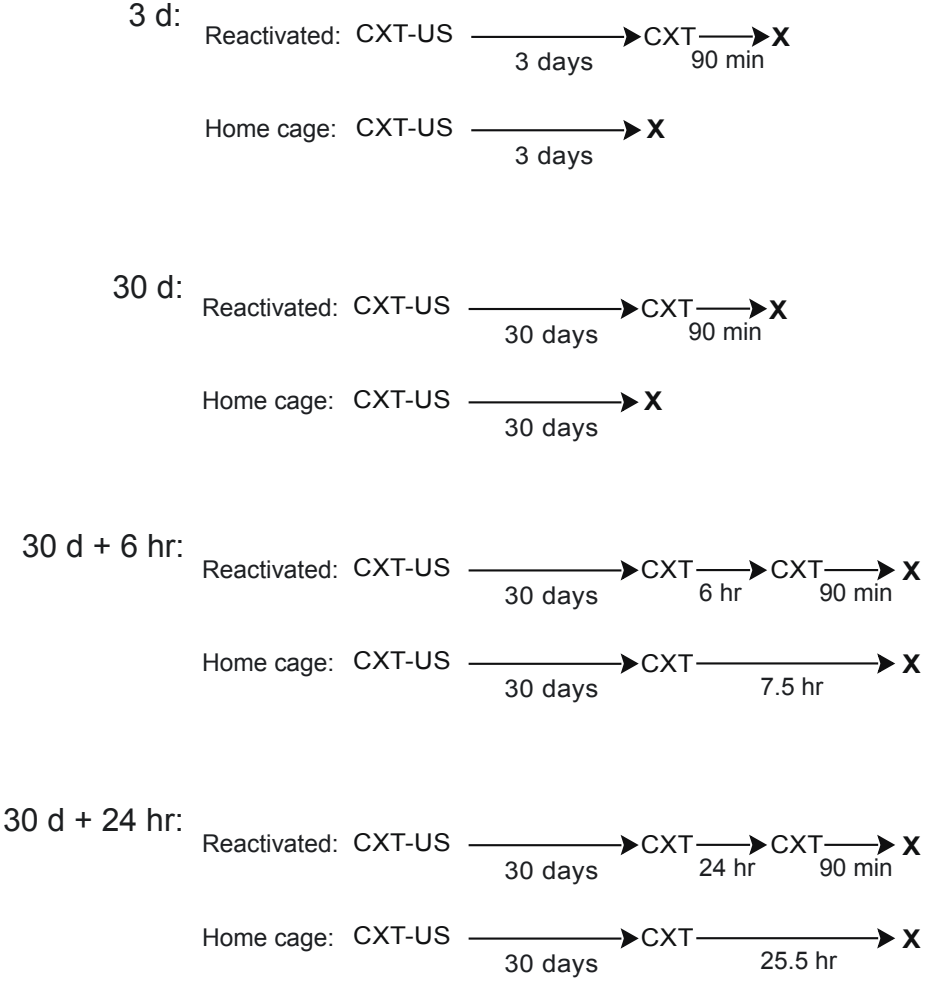
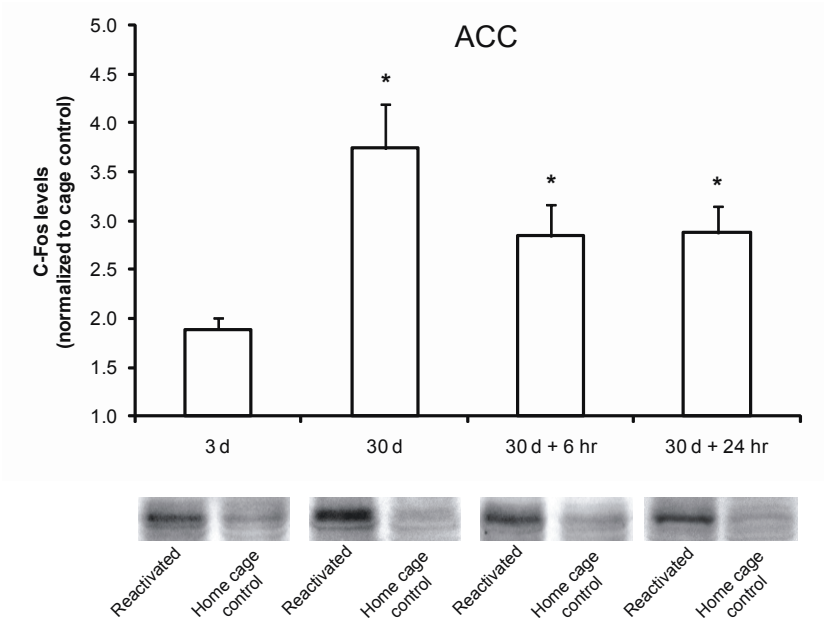

Test 1
Test 2
CXT-US
30 days
CXT
6 hr
CXT
ACC (Veh)
DH (Mus) / ACC (CNQX)
DH (Mus) / ACC (Veh)
DH (Veh) / ACC (CNQX)
DH (Veh) / ACC (Veh)
DH d-ctrl (Mus) / ACC (CNQX)
100
90
80
70
60
50
40
30
20
10
0
% Freezing
*
Test 1
Test 2
6 hr ITI
DH (Mus) / ACC (CNQX)
DH (Mus) / ACC (Veh)
DH (Veh) / ACC (CNQX)
DH (Veh) / ACC (Veh)
DH d-ctrl (Mus) / ACC (CNQX)

Figure 5

A


3 d:
Reactivated: CXT-US → 3 days → CXT → 90 min → X
Home cage: CXT-US → 3 days → X
30 d:
Reactivated: CXT-US → 30 days → CXT → 90 min → X
Home cage: CXT-US → 30 days → X
30 d + 6 hr:
Reactivated: CXT-US → 30 days → CXT → 6 hr → CXT → 90 min → X
Home cage: CXT-US → 30 days → CXT → 7.5 hr → X
30 d + 24 hr:
Reactivated: CXT-US → 30 days → CXT → 24 hr → CXT → 90 min → X
Home cage: CXT-US → 30 days → CXT → 25.5 hr → X

B


ACC
C-Fos levels (normalized to cage control)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
*
*
*
3 d
30 d
30 d + 6 hr
30 d + 24 hr
Reactivated
Home cage control
Reactivated
Home cage control
Reactivated
Home cage control
Reactivated
Home cage control

C

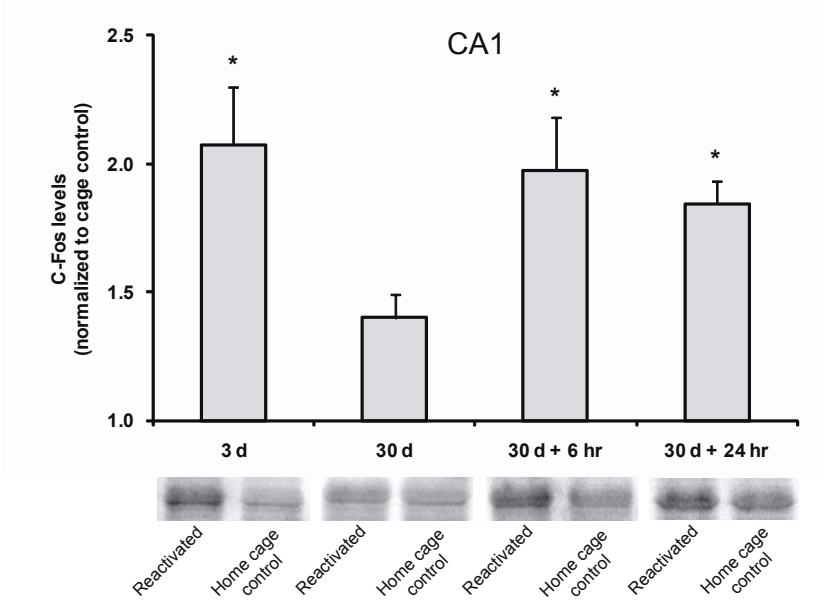

CA1
C-Fos levels (normalized to cage control)
2.5
2.0
1.5
1.0
*
*
*
3 d
30 d
30 d + 6 hr
30 d + 24 hr
Reactivated
Home cage control
Reactivated
Home cage control
Reactivated
Home cage control
Reactivated
Home cage control

Figure 6

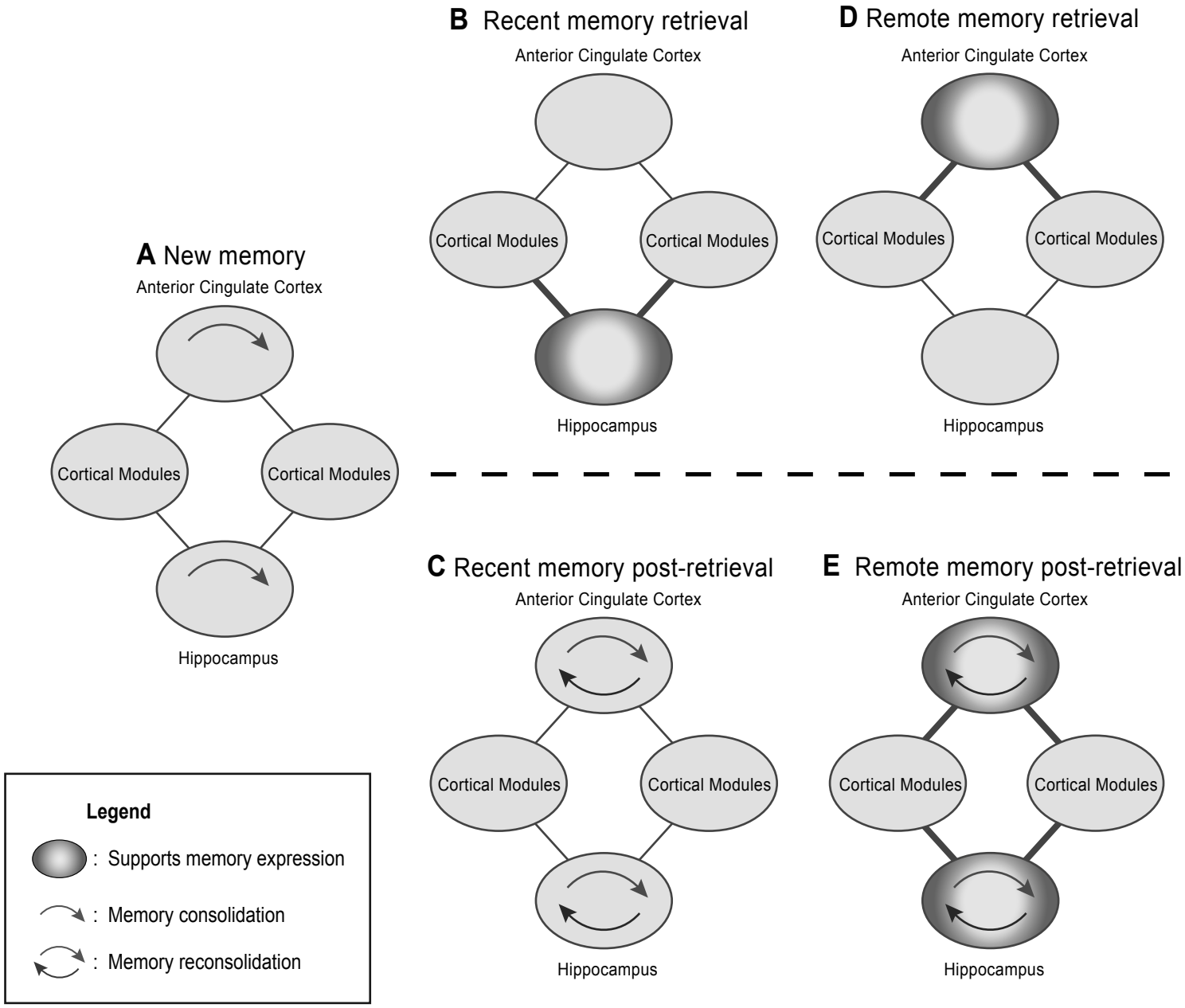
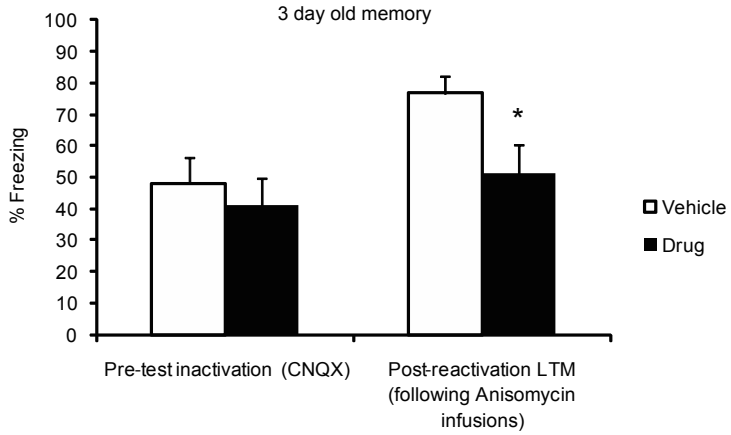
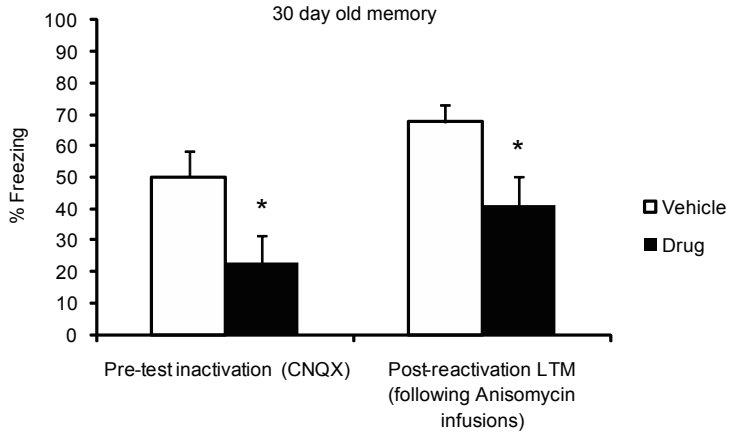

A New memory
Anterior Cingulate Cortex
Cortical Modules
Cortical Modules
Hippocampus
B Recent memory retrieval
Anterior Cingulate Cortex
Cortical Modules
Cortical Modules
Hippocampus
D Remote memory retrieval
Anterior Cingulate Cortex
Cortical Modules
Cortical Modules
Hippocampus
C Recent memory post-retrieval
Anterior Cingulate Cortex
Cortical Modules
Cortical Modules
Hippocampus
E Remote memory post-retrieval
Anterior Cingulate Cortex
Cortical Modules
Cortical Modules
Hippocampus
Legend
: Supports memory expression
: Memory consolidation
: Memory reconsolidation

Figure 7

A


3 day old memory
% Freezing
100
90
80
70
60
50
40
30
20
10
0
*
Vehicle
Drug
Pre-test inactivation (CNQX)
Post-reactivation LTM (following Anisomycin infusions)

B


30 day old memory
% Freezing
100
90
80
70
60
50
40
30
20
10
0
*
*
Vehicle
Drug
Pre-test inactivation (CNQX)
Post-reactivation LTM (following Anisomycin infusions)

Supplementary Figure Legends

Supplementary Figure 1. (**a**) A retention test 30 and 31 days after training does not induce significant extinction. Thirty days after conditioning, rats were infused into the ACC with vehicle to CNQX before test 1, and again 24 hours later (n = 7). Vehicle/Vehicle control tested with 24 hours interval did not show any extinction between tests (t (6) = -1.05, p > 0.05). **(b)** To test the generality of our findings of the effects of inactivation of the ACC on memory expression of 30 d old remote contextual fear memories we repeated the experiment with 45 day old memories. As with 30 d old memories, we found a significant interaction between treatment and test with a 24 hour inter-test interval (F (1, 9) = 12.7, p < 0.05). *Post-hoc* comparison did not reveal a significant difference between treatment groups on test 1 (p < 0.05), most likely due to a lack of power in our analysis to detect a true difference. However, more importantly, ACC inactivation 24 hours after retrieval was effective in blocking memory retrieval on test 2 (p < 0.05). When animals were tested with a 6 hour interval there was again an interaction between treatment and test (F (1, 9) = 6.5, p < 0.05). *Post-hoc* analyses showed that ACC inactivation blocked the retrieval of 45 day old memory (p < 0.05), but was ineffective 6 hours after memory reactivation (p > 0.05). Thus, we replicated our results from 30 day old memories with 45 day old memories, in that ACC inactivation blocks the expression of a remote memory 24, but not 6, hours after last retrieval. Data presented as group means ± SEM.

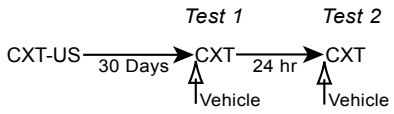
Supplementary figure 2. Cellular reconsolidation in the ACC at a time when it is not required for retrieval could add new information to an existing HC-cortical memory trace. (**a**) During acquisition the memory is encoded in a broad HC-cortical network, where the ACC and the DH are crucial in establishing new connections between neural

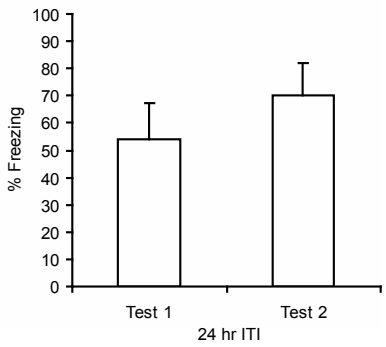
units of the memory trace (solid black nodes/lines). (**b**) The retrieval of a recent memory requires the DH that is strongly connected (thick gray lines) to the cortical modules. At this age the ACC, however, has weaker connections (thin gray lines) to the cortical modules and cannot support retrieval of the memory. (**c**) Retrieval of a recent memory induces cellular reconsolidation where the memory trace becomes labile, which possibly allows for new information to be integrated into the existing HP-cortical memory trace. (**d**) As the memory ages, connections between the ACC and cortical modules strengthen while connections between the HC and cortical modules may weaken. At this more remote time point the ACC has become crucial for retrieving the original (black in A) and new experiences (black in C), but the DH has ceased to be critical for this response. In this manner reconsolidation in the ACC could serve to incorporate new experiences into an existing HC-cortical memory trace.

Supplementary Figure 3. Representative photomicrographs of animals infused with 4% methylene-blue solution in the DH (a) or the ACC (b) giving an indication of the diffusion of drugs and vehicle infused. (b) Dotted lines mark the ACC.

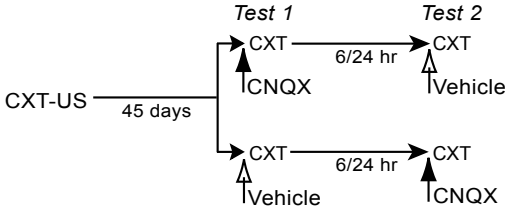
Figure S1

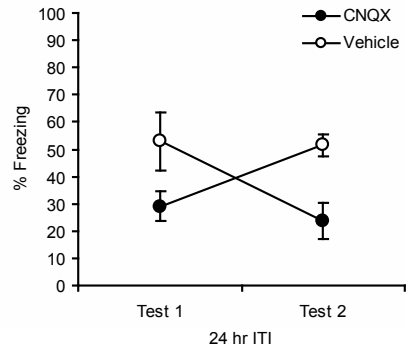
A


Test 1
Test 2
CXT-US
30 Days
CXT
24 hr
CXT
Vehicle
Vehicle


% Freezing
100
90
80
70
60
50
40
30
20
10
0
Test 1
Test 2
24 hr ITI

B


Test 1
Test 2
CXT-US
45 days
CXT
6/24 hr
CXT
CNQX
Vehicle
CXT
6/24 hr
CXT
Vehicle
CNQX


CNQX
Vehicle
% Freezing
100
90
80
70
60
50
40
30
20
10
0
Test 1
Test 2
24 hr ITI

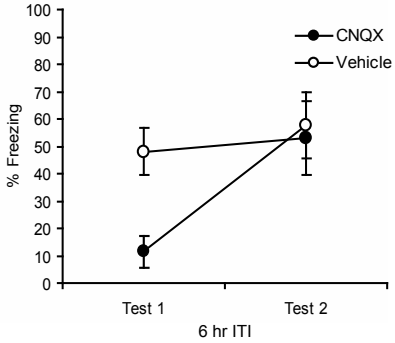

CNQX
Vehicle
% Freezing
100
90
80
70
60
50
40
30
20
10
0
Test 1
Test 2
6 hr ITI

Figure S2

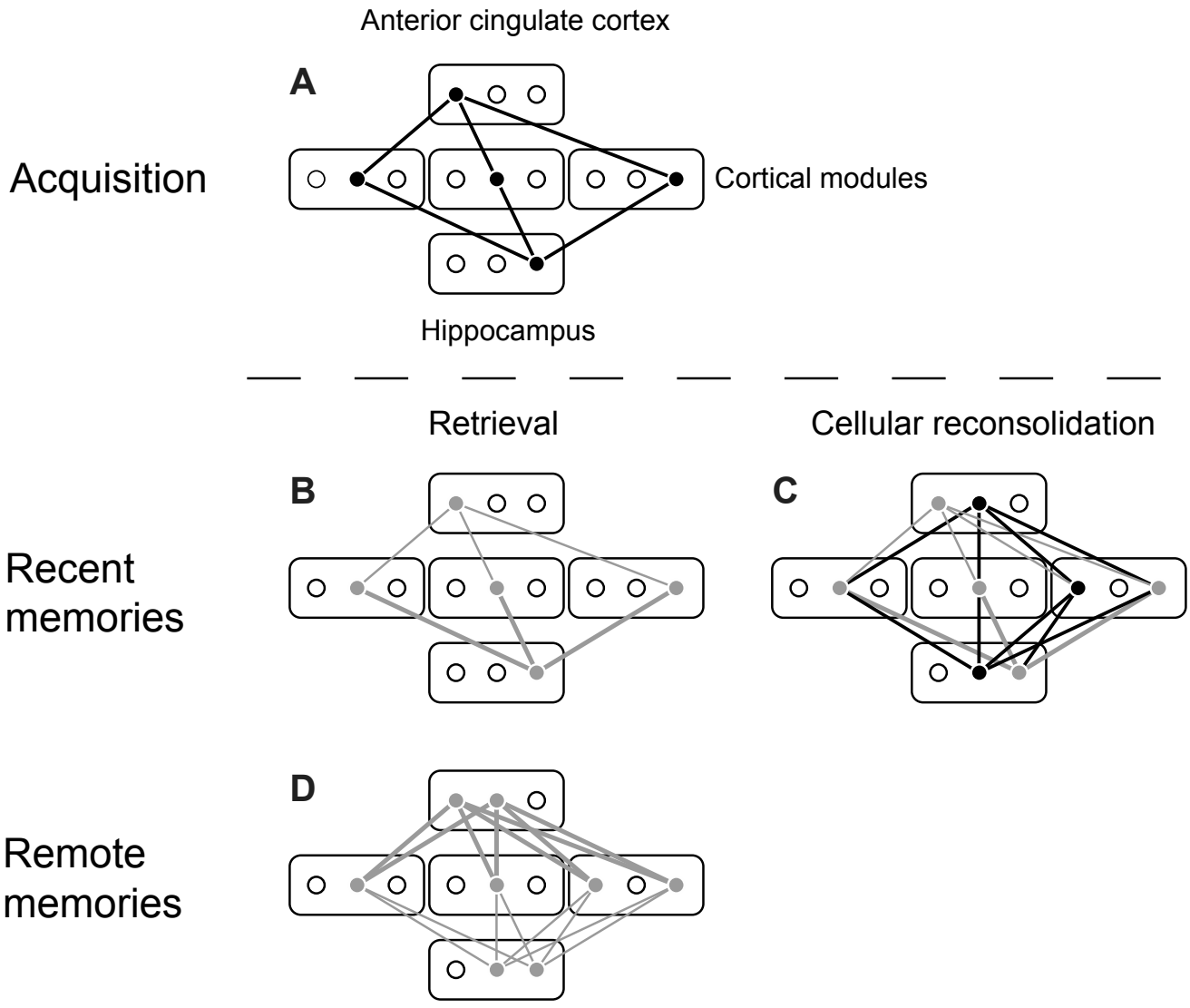
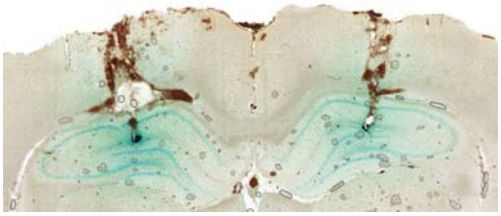
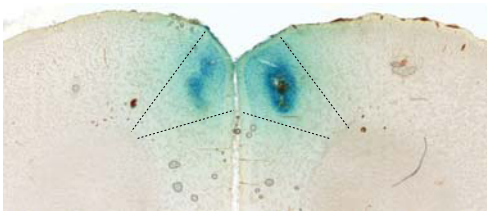

Anterior cingulate cortex
A
Acquisition
Cortical modules
Hippocampus
Retrieval
Cellular reconsolidation
B
C
Recent memories
D
Remote memories

Figure S3

A



B



Bridge Between Chapter 2 and 3

In chapter 2, results suggests that 6 hours following remote memory retrieval, only simultaneous inactivation of both the ACC and dorsal hippocampus is effective in impairing contextual fear memory expression, while inactivating either structure is ineffective. At 24 hours following the first retrieval, however, memory expression is once more impaired by inactivation of the ACC. This finding raises the question whether the ACC and dorsal hippocampus have a similar role in memory expression, or whether they mediate different aspects of memory expression not tested in our study. Consequently, in the next chapter we examine the effect of remote memory reactivation on context fear generalization, and the effects of pharmacological inactivation of the ACC on memory generalization 6 hours following a reminder session.

Chapter 3

The anterior Cingulate Cortex Mediates the Expression of Generalized Contextual Fear Memory

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Department of Psychology, McGill University, 1205 Dr. Penfield Avenue, Montreal, Quebec, Canada, H3A 1B1

Contextual generalization of fear memory expression is known to increase with time, coinciding with the decreased dependence on the dorsal hippocampus, and increased dependence on the anterior cingulate cortex (ACC) for memory expression. We report that 3 d after training rats show a high level of discrimination between the original training context and a novel distinctly different context, whereas 30 d after training, rats show similar conditioned responding to both contexts, demonstrating contextual fear generalization. However, following the retrieval of a 30 d old memory, context discrimination is restored 24 hours later, but not at 6 or 48 hours. Moreover, we find that AMPA receptor inactivation of the ACC 6 hours after the reminder selectively impairs responding to the novel context, but not the original training context, thus restoring context discrimination. These results suggest that the expression of generalized contextual fear is specifically mediated by the ACC.

Introduction

Memories can undergo a prolonged period of reorganization in the brain after learning. A number of studies have found that the hippocampus plays a time-limited role in the retrieval of certain memories, being critical at more recent time-points but not at remote ones (Frankland and Bontempi, 2005). One task of which memory has been found to undergo such reorganization is contextual fear conditioning, a task where an animal receives a mild foot-shock in a distinct environment. Later, when placed in the same environment, animals will show memory of the experience by displaying fear responses, most notably immobility, or freezing (Blanchard and Blanchard, 1969). In rodents, pharmacological inactivation (Wiltgen et al., 2010) or lesioning (Kim and Fanselow, 1992; Anagnostaras et al., 1999; Ward et al., 1999) of the dorsal hippocampus has been found to lead to marked memory impairments in the first days after training, but not after a few weeks (but see)(Sutherland et al., 2008). Conversely, inactivation of the anterior cingulate cortex (ACC) has been found to show the reverse gradient; impairing older memories while sparing new ones (Chapter 2; Frankland et al., 2004).

Contextual-related memories are known to change in other ways. Shortly after a conditioning experience animals are good at discriminating between the original training context and a novel context that might share only some of the features of the training context. However, with time, animals will increasingly generalize the conditioned response to the novel context, and eventually show similar conditioned responding to both contexts (Riccio et al., 1992). Recently, a number of papers have demonstrated this effect with the contextual fear conditioning task in rodents (Biedenkapp and Rudy, 2007; Wiltgen and Silva, 2007; Winocur et al., 2007), where the enhanced generalization

gradient parallels the gradient of hippocampal dependence for memory retrieval, suggesting that enhanced generalization may reflect diminished participation of the hippocampus.

However, if preceded by a reminder treatment consisting of a re-exposure to the training context, older fear memories are subsequently expressed with renewed contextual discrimination (Wiltgen and Silva, 2007; Winocur et al., 2009), as well as undergoing systems reconsolidation where the memory returns to being sensitive to lesioning of the dorsal (Debiec et al., 2002) or greater part of the hippocampus (Winocur et al., 2009). These findings suggest that retrieval may be re-engaged the hippocampus in the expression of context discriminative memories. In a previous study, we showed that following a reminder trial, only the inactivation of both the ACC and the dorsal hippocampus would impair conditioned freezing response in the training context 6 hours later, while at 24 hours inactivating the ACC was sufficient to impair the response (Einarsson and Nader, 2011). Having established time-points where the memory can be supported by either the dorsal hippocampus or the ACC in our previous study, we now test whether the engagement of the dorsal hippocampus and ACC relates to the expression of either a discriminative or generalized expression of the fear memory, respectively.

Materials and methods

Subjects

Subjects were male Sprague-Dawley rats (Charles River, Saint-Constant, PQ) weighing at least 250 g at the start of training. Animals were housed individually and maintained

on a 12/12 light/dark cycle (lights on at 7 a.m.) with food and water provided *ad libitum*. The rats were handled on three consecutive days for ~2 min before start of training. All procedures were in accordance with the CAA Guide, and were approved by the McGill University Animal Care and Use Committee.

Surgery

Under Ketamine (55 mg/kg), Xylazine (3.33 mg/kg) and Domitor (27 mg/kg) anesthesia, animals were mounted in a stereotaxic frame, the scalp incised to expose the skull, bregma and lamda aligned on the same horizontal plane. Small holes were drilled into the skull and infusion guide cannulae (26-gauge) implanted bilaterally into the ACC with injector cannulae (33-gauge) coordinates as 2.6 mm anterior to bregma, 0.7 mm lateral to the midline, and 1.6 mm ventral to dura surface. Guide cannulae were fixed to the skull with dental cement and stainless steel screws. Dummy cannulae were inserted into the guide cannulae after surgery. Rats were given a week to recover before testing during which they were habituated to the restraint required for infusion.

Infusions

Fifteen min before testing, dummy cannulae were replaced by infusion cannulae connected to microsyringers in a microinfusion pump via flexible polyvinyl chloride tubing. CNQX disodium salt (Tocris, Ellisville, MO) was dissolved in nanopure dorsal hippocampus$_2$O and injected at 0.75 µg / 0.5 µl per side at a rate of 0.25µl / min after which the injectors were left in place for an additional minute to allow diffusion of the drug away from the injector tip.

Histology

Animals were deeply anaesthetized with urethane (0.25 g/0.5 mL/kg i.p.) and were

decapitated. Brains were fixed in a mixture of formal saline and 20% sucrose, sectioned at 50 μm thickness and stained with formol-thionin and examined by light microscopy for verification of cannula placement in the ACC.

Apparatus

Conditioning and testing was conducted in two windowless rooms, each containing 4 conditioning cages. The training context consisted of rodent conditioning cages with clear Plexiglas walls and a metal grid floor (Coulbourn Instruments) that was enclosed within a sound-attenuating chamber in a well lit room. The cages were dimly lit with a single house light, had a fan on to provide background noise, and were scented with diluted vanilla. The novel context was in a dark room and consisted of a distinctly different rodent conditioning cage (Med Associates, St. Albans, VT) where the front of the cage was a striped Plexiglas wall, the sides were aluminum, top was clear Plexiglas, and the back was made curved with a gray plastic insert, forming a semi-curved enclosure. The floor was a plastic insert covered with bedding. The cage was housed in a sound attenuating chamber with a slow blinking white LED light above the conditioning chamber.

Behavioral procedures

In all of the experiments, rats were habituated to the conditioning chamber for 5 min one day before training. For conditioning, after 2 min in the chamber rats received 8 unsignaled foot-shocks (1 sec, 1.5 mA) at 62 sec interval. The rats were left in the chamber for 30 sec after the termination of the procedure. For all tests, freezing (defined as the complete absence of movement, except that of respiration) was scored with an instantaneous time-sampling procedure in which each animal was observed as either

freezing or not every 5 sec. These observations were then averaged to yield an estimate of the percentage time freezing. All tests were 5 min long except reminder trials that were 3 min long. All reminder trials were in the training context.

Experiment 1: Generalization of freezing response over time. Rats were tested in either the training context or the novel context at either 3 (training CXT, n = 8; novel CXT, n = 8) or 30 days (training CXT, n = 11; novel CXT, n = 11) following contextual fear conditioning.

Experiment 2: The effects of training context reminder on subsequent context discrimination. For memory reactivation, rats were returned to the conditioning cage 30 days after training for a reminder trial. Different groups were then tested in either the training context or the novel context 6 (training CXT, n = 11; novel CXT, n = 11), 24 (training CXT, n = 11; novel CXT, n = 13) or 48 hours (training CXT, n = 12; novel CXT, n = 13) later.

Experiment 3: The effects of AMPAR antagonism inactivation of the ACC on contextual discrimination 6 hours following a training context reminder. Six hours after a reminder trial four groups of rats received local infusions to the ACC of CNQX (training CXT, n = 6; novel CXT, n = 7) or its vehicle (training CXT, n = 7; novel CXT, n = 7) and were then tested 15 min later in either the training context or the novel context.

Statistical analysis

Experiment 1 was analyzed with a two-way independent-group ANOVA, while all other comparisons were done by a two-way mixed ANOVA with test interval as within-subjects factor and type of context tested as between-subject factor. Significant effects were further analyzed *post-hoc* with Fischer's protected LSD t-test with Bonferroni's

corrected significance set at $p < 0.05$.

Results

Changes in contextual fear discrimination over time

In establishing our context fear discrimination protocol, rats were trained using the same contextual fear protocol as before (Chapter 2), and then different groups were tested either 3 or 30 days later, in the training or a novel context (Fig. 1A). Consistent with previous studies, the animals fear response contexts generalized over time (Fig 1B; test × context interaction, F (1, 34) = 7.4 p > 0.01); animals showed freezing response in the training context at 3 days, but less so to the novel context (*post-hoc* analysis, $p > 0.001$) thus demonstrating their ability to discriminate between the contexts, while at 30 days the animals responded similarly to both contexts (*post-hoc* analysis, $p > 0.05$).

A reminder restores context discrimination when tested 24, but not 6 or 48 hours later

A number of studies have demonstrated that exposure to a reminder (typically the training context) before discrimination testing, at a time-point when animals show a generalized response, can restore their ability to discriminate between contexts (Zhou and Riccio, 1994; Rosas and Bouton, 1997; Wiltgen and Silva, 2007). Previously, we found both the ACC and the dorsal hippocampus to be involved in the expression of a remote contextual fear memory when tested 6 hours after a reminder and the ACC also at 24 hours (Chapter 2). The dorsal hippocampus has been shown to be sensitive to lesioning at 24 hours, but not at 48 hours (Debiec et al., 2002). In testing the effects of a reminder treatment on renewing context discrimination, we exposed the animals to the training

context 30 days following conditioning, and tested separate groups 6, 24, or 48 hours later in the training context or a novel context (Fig. 2A). At 6 hours (Fig. 2B), rats showed reduced freezing relative to the reminder trial (test main effect, F (1, 20) = 17.16, $p < 0.001$), but did not show renewed discrimination as they responded similarly to both contexts (context main effect, $p > 0.05$; test × context interaction, $p > 0.05$). At 24 hours after a reminder (Fig. 2C), however, rats responded differently to the contexts (test × context interaction, F (1, 22) = 6.63, $p < 0.05$), while freezing similarly during the reminder session (*post-hoc* analysis, $p > 0.05$), the animals froze markedly less to the novel context and thus showed renewed discrimination (*post-hoc* analysis, $p < 0.0001$). When tested 48 hours after the reminder (Fig. 2D), rats showed reduced freezing relative to the reminder trial (test main effect, F (1, 23) = 36.05, $p < 0.001$), and once more showed comparable freezing to both contexts and thus returned to generalized memory expression (context main effect, $p > 0.05$; test × context interaction, $p > 0.05$).

AMPA receptor inactivation in the ACC restores context discrimination 6 hours following a reminder

Having found that a reminder treatment does not restore context discrimination when tested 6 hours later, a time-point where context fear memory expression can be mediated by either the ACC or the dorsal hippocampus (Chapter 2), led us to ask whether the involvement of the ACC were enhancing generalization to a novel context which dominated the more discriminative memory expression mediated by the dorsal hippocampus. In order to test this, we inactivated the ACC with the AMPA receptor antagonist CNQX or its vehicle before testing for context discrimination 6 hours after a

reminder session (Fig. 3A). Rats receiving the vehicle infusion showed comparable freezing to both contexts and similar freezing as during the reminder trial (Fig. 3B; main effect of interval and context, $p > 0.05$; interval × context interaction, $p > 0.05$). Animals receiving CNQX infusions, however, showed reduced freezing in the novel context (Fig. 3C; interval × context interaction, $F(1, 11) = 10.33$, $p < 0.01$), both relative to the novel context groups' own freezing during the reminder trial (*post-hoc* analysis, $p < 0.01$) and to the training context groups' freezing at 6 hours (*post-hoc* analysis, $p < 0.01$). Both groups showed comparable freezing during the reminder trial (*post-hoc* analysis, $p > 0.05$). CNQX infusions did not impair animals freezing to the training context as they demonstrated similar freezing at the reminder and 6 hours test in the training context (*post-hoc* analysis, $p > 0.05$). Thus, inactivating the ACC impairs fear memory expression in the novel context, while leaving fear memory expression in the training context seemingly intact.

Discussion

The objective of this study was to determine whether the involvement of the dorsal hippocampus and ACC following a reminder trial related to the expression of either a discriminative or generalized fear memory expression, respectively. We evaluated this question by establishing a contextual discrimination protocol where the animals discriminated between the training context and a novel context with 3 day old memories, but generalized the fear response to a novel context with 30 day old memoires, a finding in line with previous studies describing how contextual fear conditioning memory becomes less specific with time (Biedenkapp and Rudy, 2007; Wiltgen and Silva, 2007;

Winocur et al., 2007). Following a reminder trial, we find that animals' ability to discriminate between the training context and a novel context changed in a non-monotonic fashion; from context generalization at 6 hours after the reminder, to context discrimination at 24 hours, and back to context generalization at 48 hours. These findings are consistent with a previous study showing renewed context discrimination to a novel context 24 hours following a reminder, 35 days after training (Wiltgen and Silva, 2007).

When we inactivate the ACC before testing at 6 hours, however, the pattern changed to that of renewed discrimination; inactivation disrupted memory expression in the novel context while performance in the training context was unaffected. Using a similar protocol, we previously showed that following a reminder trial, contextual fear memory expression could be supported by either the ACC or the dorsal hippocampus 6 hours later, as inactivating either structure did not impair memory expression, while inactivating both structures did (Chapter 2). Thus, in the absence of a functional ACC, the dorsal hippocampus was able to compensate, and vice versa. However, as memory was only tested in the training context, we were not able to test if our manipulation affected the specificity of the memory expression. This suggests the renewed discrimination we find here with the ACC inactivated is dependent on the dorsal hippocampus.

Our findings are consistent with the transformation hypothesis of systems consolidation of memory (Winocur et al., 2010). According to this view, the nature of context memories is determined by the structure that mediates their retrieval, where hippocampus-based memories are detailed and context-specific, whereas memories mediated by key cortical structures such as the ACC are schematic and context-general.

Further support for the posited role of the dorsal hippocampus by the transformation view comes from a recent study (Wiltgen et al., 2010) demonstrating how the dorsal hippocampus can be critical for the expression of detailed contextual fear memories, but not that of generalized memories.

Our findings however do not address another aspect of the transformation hypothesis, which is that detailed context-specific memory co-exists and competes with schematic context-general memory for behavioral expression. Renewed discrimination following either inactivation of the ACC, or a reminder session 24 hours before testing in our study, could either reflect the renewed dominance of an inhibited context-specific memory or the expression of a new detailed memory of the reminder session.

An alternative account of memory schematization of older memories and increased engagement of pre-frontal cortical areas has been proposed by Rudy and associates (Rudy et al., 2005; Biedenkapp and Rudy, 2007). Specifically, they propose that as memory ages, it degrades and becomes more difficult to retrieve, requiring additional activation of pre-frontal cortical areas, such as the ACC, for retrieval. One example of such memory degradation is the enhancement of fear generalization over time, reflecting a weakening of the memory trace. According to this view, involvement of the ACC does not represent a cortical component of the memory trace that has been consolidated into the region, but a signal to boost weak memory retrieval from areas that do contain the memory trace. Conversely, new memories do not require such boosting for retrieval as the memory trace is still strong and maintaining a detailed representation. Our finding of renewed discrimination following inactivation of the ACC, however, is not consistent with this position. That is, it is not clear how the inactivation of a structure posited to be critical for

the expression of a weak memory trace can lead to the expression of a strong detailed memory trace.

When taken together with our previous findings that memory retrieval cued by the training context relies on the dorsal hippocampus when the ACC is inactivated (Chapter 2), our findings suggest that the ACC is critical for the expression of generalized context memory, and the dorsal hippocampus mediates expression of detailed discriminative context memory.

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Zhou YL, Riccio DC (1994) Pretest cuing can alleviate the forgetting of contextual stimulus attributes. Learning and Motivation 25:233-244.

Figure Legends

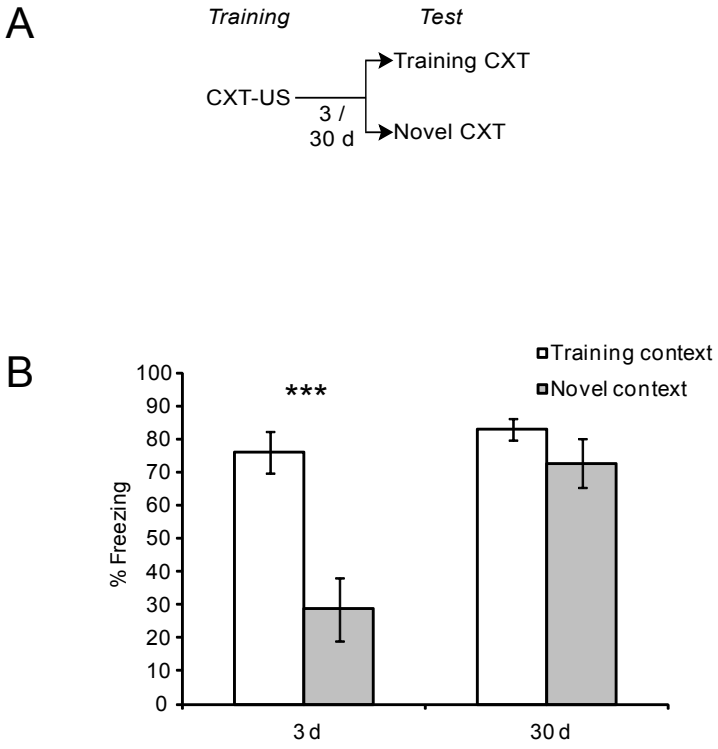
Figure 1. Contextual generalization of fear memory expression increases with time. ***A***, The experimental design. ***B***, Three days after training, rats are able to discriminate between the training context (training CXT) and a novel context (novel CXT), as levels of conditioned freezing were greater in the training context than in the novel one. At thirty days post-training, animals have lost their ability to discriminate between the contexts, as the conditioned fear response in the novel context has increased to the same level of freezing as that of the training context. *** $p < 0.001$. Data presented as group means ± SEM values.

Figure 2. A reminder restores context discrimination when tested 24, but not 6 or 48 hours later. ***A***, The experimental design. ***B***, Following a reminder trial in the training context, memory expression remains generalized 6 hours later, with some lower overall freezing, indicating extinction from the reminder trial. ***C***, when tested 24 hours after a reminder, however, memory expression returns to context discriminative state, where freezing to a novel context is lower than that to the training context, as well as to the novel contexts' group own freezing during the reminder trial. ***D***, At 48 hours post-reminder, memory expression returned once more generalizing the fear response to the novel context, again with reduced overall freezing from the reminder trial. *** $p < 0.001$. Data presented as group means ± SEM values.

Figure 3. Inactivating the ACC restores context discrimination 6 hours following a reminder. ***A***, The experimental design. ***B***, Control group receiving vehicle infusions

before the 6 hour discrimination test, showed generalized fear memory expression as conditioned freezing was similar in both the training context and a novel context. ***C***, In contrast, animals receiving CNQX infusions expressed renewed context discrimination as freezing to the novel context was reduced, while freezing to the training context was unaffected. ** $p < 0.01$. Data presented as group means ± SEM values.

Figure 1


A
Training
Test
CXT-US
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Training CXT
Novel CXT
B
Training context
Novel context
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% Freezing
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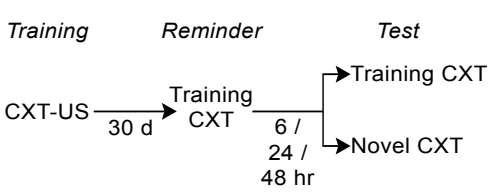
Figure 2

A



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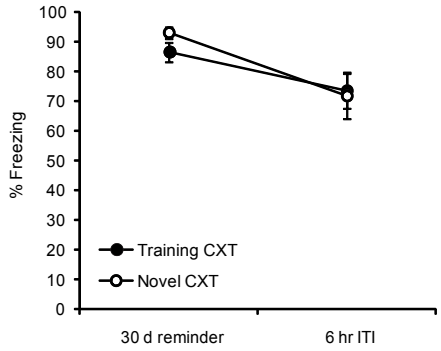

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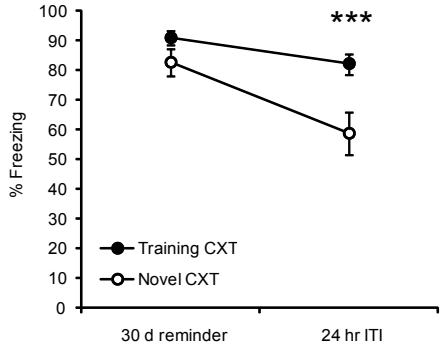

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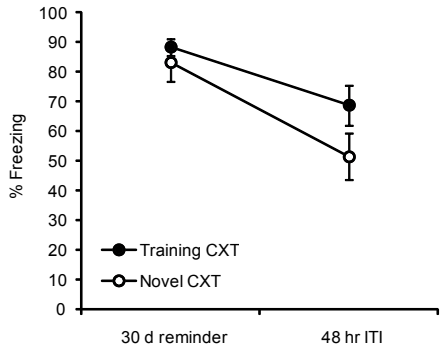
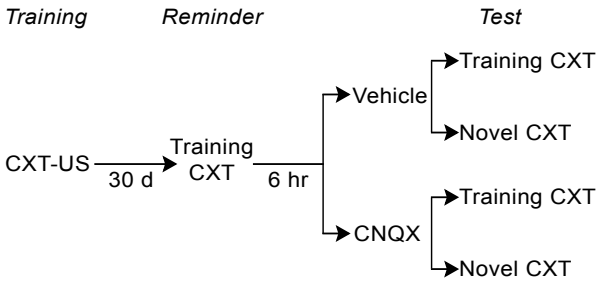
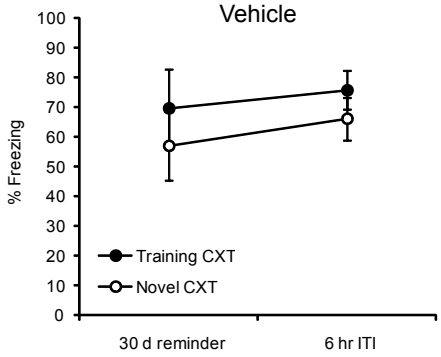


Figure 3

A


Training
Reminder
Test
CXT-US
30 d
Training CXT
6 hr
Vehicle
CNQX
Training CXT
Novel CXT
Training CXT
Novel CXT

B


Vehicle
% Freezing
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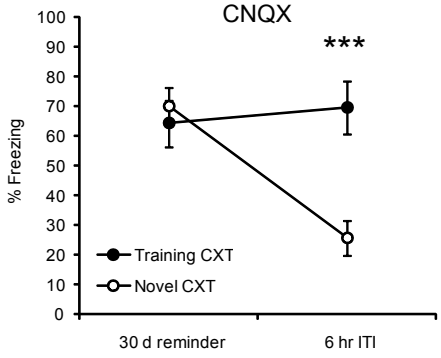
Training CXT
Novel CXT
30 d reminder
6 hr ITI

C


CNQX
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Training CXT
Novel CXT
30 d reminder
6 hr ITI

General Discussion

The aim of this thesis was to examine the following questions: (1) whether the ACC is involved in cellular consolidation and reconsolidation of contextual fear memory, and if involved in reconsolidation, if that involvement changes with time; (2) how a remote memory's dependence on the ACC is affected by retrieval, previously found to transiently re-engage the dorsal hippocampus (Debiec et al., 2002), on a subsequent second testing; and (3) if increased contextual generalization over time is specifically mediated by the corresponding increased involvement of the ACC in memory retrieval.

Tests of the first two questions were presented in chapter 2 and suggests, first, that the ACC is involved in the consolidation and reconsolidation of recent and remote memory, and second, that following the retrieval of remote memory, a subsequent second retrieval 6 hours later can transiently be supported by either the ACC or DH, with only simultaneous inactivation of both structures blocking memory expression. The study of the third question in chapter 3 reveals that the ACC specifically supports generalized contextual fear memory, with inactivation of the ACC before a second test, 6 hours following a reminder trial, only affecting conditioned responding to a novel context. In the following discussion I address these results in more detail before reviewing the structural connectivity between the ACC and DH. I then discuss the possible functional memory networks involving the ACC and DH and evaluate the findings of this thesis from a network perspective.

Cellular consolidation and reconsolidation in the ACC

Research presented in chapter 2 described how the ACC plays an extended role in the stabilization of memory in its plastic state, from memory formation and cellular consolidation to cellular reconsolidation of recent and remote memories. More specifically, contextual fear memory formation is blocked by pharmacologically inhibiting NMDA-NR2B subunit receptors in the ACC before conditioning, while infusions of a protein-synthesis inhibitor immediately following conditioning disrupts cellular consolidation, and similarly disrupts cellular reconsolidation when given immediately after retrieval on day 3 or 30.

These findings are in line with a number of recent studies describing a role for the ACC in the formation and maintenance of memory for a number of hippocampus-dependent tasks. For example, intra-ACC infusions of a protein-synthesis inhibitor disrupt cellular consolidation of inhibitory avoidance memory (Zhang et al., 2011), pre-conditioning infusions of NMDA-NR2B inhibitor into the ACC impair the formation of contextual fear memory (Zhao et al., 2005), and that disruption of neuronal spine growth in the ACC by increasing the function of the transcription factor MEF2, impairs the expression of 7 day old contextual fear memory when started 6 days earlier (Vetere et al., 2011).

Our findings of cellular consolidation and reconsolidation in the ACC, described in chapter 2, and that of previous studies showing contextual fear memory being dependent on the DH for cellular consolidation (Motanis and Maroun, 2011) and cellular reconsolidation at recent and remote time points (Debiec et al., 2002), suggest that the memory trace is encoded in a hippocampal-cortical network where the ACC and

hippocampus serve as key nodes in stabilizing the memory following encoding or retrieval.

However, in contrast to the continuing role of both the ACC and DH in cellular reconsolidation, a number of studies have found an inverse relationship between the involvement the structures in supporting memory expression at different ages of the memory, where the DH is critical for retrieval of recent memory (e.g. Wiltgen et al., 2010) and the ACC for that of remote memory (e.g. Frankland et al., 2004a). Taken together, this pattern of findings suggests that the memory is encoded and maintained within a hippocampal-ACC/cortical network, in which the information is reorganized and transformed (see below) over time.

Remote memory retrieval transiently engages both the ACC and DH

Second, chapter 2 describes how remote memory retrieval transiently re-engages the DH in addition to the ACC. More specifically, in line with previous studies (Frankland et al., 2004), pharmacological inactivation of the ACC impairs the retrieval of remote, but not recent contextual fear memory. However, when tested 6 hours after a remote memory retrieval session, only simultaneous inactivation of the ACC and the DH impaired memory expression. However, 24 hours after the initial retrieval session the same effect was achieved by inactivating the ACC alone, suggesting that the role of the DH in retrieval had diminished at that time. Similarly, an examination of the activity-modulated immediate-early gene c-Fos showed that following the second retrieval at 6 hours, c-Fos activity in the ACC remained at similar levels as after the first reactivation, whereas activity in the CA1 area of the DH increased, mirroring the functional re-engagement of the structure. However, at 24 hours, c-Fos activity in both structures remained elevated

although pharmacological inactivation of the ACC alone sufficed to impair memory expression. Thus, following a second retrieval trial at 24 hours, the DH could not support memory expression without a functioning ACC, although DH neural activity remained elevated. These findings suggest that remote memory retrieval can induce rapid global reorganization of the hippocampal-cortical network supporting the memory, where the network partly reverts to a state akin to that of a recently acquired memory with DH re-engagement possibly reflecting an updating of the memory trace.

Further support for the notion of remote memory retrieval returning the memory trace to a network state similar to that of a recently acquired memory comes from electrophysiological studies on the synchronization between the CA1 area of the DH and the lateral amygdala: High theta-phase synchronization between the lateral amygdala and the CA1 that was apparent during retrieval of a 1-day-old contextual fear memory, but not during the retrieval of a 30-day-old one (Narayanan et al., 2007b), reappeared when mice were tested again 24 hours later on day 31 (Narayanan et al., 2007a). This re-emergence of high theta-phase synchronization one day after the retrieval of the 30-day-old memory coincides with the time-point in our study when c-Fos activity in the CA1 was still elevated relative to that following 30-day-old retrieval, and at a similar level as that of following the retrieval of a recent 3-day-old memory. Together, our findings and those of Narayanan and associates suggest that the memory trace network returns to a state that resembles that of a recent memory following remote memory retrieval.

In sum, our findings and that of others (Debiec et al., 2002; Narayanan et al., 2007b; Narayanan et al., 2007a) suggest that the retrieval of remote contextual fear memory induces transient global reorganization in the network of structures supporting

the memory. This reorganization comprises cellular reconsolidation in key structures such as the ACC and DH, transient re-engagement of the DH in supporting memory expression with the ACC, re-engagement of retrieval associated c-Fos activity the CA1 of the DH, and delayed re-emergence of high theta-phase synchronization between the lateral amygdala and the CA1.

Comparison with Debiec et al. 2002

A previous study by Debiec et al. (2002) found that electrolytic lesioning of the DH at 4 or 24 hours after remote memory reactivation impaired memory expression when tested 7 days later. As described in chapter 2, we found that pharmacologically inactivating the DH 6 hours after memory retrieval did not impair the expression of remote memory tested immediately thereafter. Although these findings might seem contradictory at first, there are notable differences between the experiments that might account for the different results, most importantly, different reactivation/manipulation-testing interval. One possibility is that the different intervals assay for different memory processes, with acute inactivation of the DH assessing the importance of the structure for memory expression, whereas permanent lesioning with testing delayed by 7 days, assaying both importance for memory expression and the effect of the lesion on reconsolidation at the time of lesioning. Thus, while memory expression can still be mediated by the ACC when the DH is inactivated (chapter 2), it is possible that a more permanent inactivation/lesioning of the DH would lead to memory impairments when tested at later time-points.

The ACC plays a selective role in the expression of generalized contextual fear memory

Expanding on findings presented in chapter 2 that remote memory expression can be mediated by either the ACC or the DH 6 hours after retrieval, chapter 3 further examined the qualitative nature of memory expression following remote memory retrieval, and the role of the ACC in that expression.

First, we found that contextual generalization of fear memory increases with time: while 3 days after training animals showed robust freezing in the original training context, but not in a distinctly different novel context, 30 days after training, the animals showed similar levels of robust freezing to both contexts. Furthermore, after a reminder trial for 30 day old memory, context discrimination was restored 24 hours later, but not at 6 or 48 hours. Previous studies have found that a reminder session can restore context discrimination of mice when 35 day old memory is tested 1 day later (Wiltgen and Silva, 2007), and when 40 day old memory is tested 2, but not 5 days later (Ruediger et al., 2011). The apparent difference between our finding of generalization at 2 days after a reminder for a 30 day old memory, and that of discrimination with a 40 day old memory in Ruediger et al (2011) could be due to a number of factors, such as different design in testing in the training and novel context (within vs. between groups comparisons), different species, different ages of the memory, and intensity of training protocol. These differences notwithstanding, our findings and that of others (Wiltgen and Silva, 2007; Ruediger et al., 2011) demonstrate that a reminder session can transiently restore the precision of contextual fear memory.

Interestingly, renewed contextual fear discrimination 24 hours after a reminder trial coincides with the time point when memory expression is once more impaired by inactivation of the ACC, described in chapter 2. This suggests that the ACC may have a role in mediating detailed contextual fear memory at this time point, possibly reflecting rapid integration of new information into an en existing schema mediated by cortical areas, as previously found in a paired-associate flavour-place task (Tse et al., 2007).

Second, we find that pharmacologically inactivating the ACC before the 6-hour test impairs freezing to the novel but not to the training context, thus restoring context discrimination. Our previous finding presented in chapter 2 of remote contextual fear memory expression requiring either the ACC or DH when tested 6 hours after a reminder, suggests that the DH is mediating renewed context discrimination during inactivation of the ACC. Thus, our findings suggest that the ACC selectively mediates the expression of generalized contextual fear memory, and provide further evidence for the DH being more critical for the expression of detailed discriminative memory expression (Wiltgen and Silva, 2007; Ruediger et al., 2011). As discussed in chapter 3, this finding is consistent with the transformation hypothesis of systems consolidation of memory (Winocur et al., 2010), which states that hippocampus-based memory is detailed and context-specific, whereas memory mediated by cortical structures, such as the ACC, are schematic and context-general.

Our findings of renewed discrimination, either following a reminder treatment or pre-test inactivation of the ACC could reflect either a renewed dominance of a disinhibited hippocampus-mediated context-specific memory over a cortical-based generalized memory or the expression of a new hippocampus mediated context-specific

memory of the reminder session. One experiment that could possibly test the latter interpretation would be to give the reminder session in a novel context, and then later test for discrimination between the original training context and the same reminder context. If memory expression 1 day after training reflects a new hippocampus mediated context-specific memory (now a memory of a fear response in the reminder context only), then that new memory should dominate behavioural expression over the generalized neocortical memory - and result in more fear expression in the reminder context than in the original training context.

Both previous suggested interpretations, of disinhibited hippocampus memory trace or a new hippocampus memory, are made within a dual memory system framework that assumes that a memory can become truly hippocampus-independent as posited by the main theoretical positions discussed in chapter 1. However, as noted in chapter 1, such dual memory system theories are inconsistent with findings of hippocampal systems reconsolidation (Land et al., 2000; Debiec et al., 2002; Winocur et al., 2009).

Structural connectivity between the ACC and hippocampus

The interactions between the hippocampus and ACC have been implicated in various aspects of memory of the same tasks in rodents (Frankland and Bontempi, 2005). The structures are thought to interact through a set of connections via the parahippocampal region while having no major direct pathways between themselves (Jones and Witter, 2007). Connections between the ACC and hippocampus have been proposed to follow two parahippocampal-hippocampal parallel processing pathways running through either the lateral or medial entorhinal cortex (van Strien et al., 2009). The pathway running through the lateral entorhinal cortex includes the rostral dorsal ACC, prelimbic-,

infralimbic-, and the perirhinal cortices, whereas the medial entorhinal cortex pathway includes the caudal dorsal ACC and ventral ACC, presubiculum, parasubiculum, and the retrosplenial-, and postrhinal cortices (Jones and Witter, 2007). Based on the regions involved, the lateral entorhinal cortex pathway has been posited to support non-spatial learning and memory, in contrast to the medial entorhinal cortex pathway, which is suggested to mediate spatial learning and memory (Burwell, 2000; Hargreaves et al., 2005). The mid- dorsal ACC (the area targeted in chapters 2 and 3) is extensively connected to both pathways (Jones and Witter, 2007), suggesting that this region is especially well positioned to integrate information from both pathways, as is the hippocampus.

Functional networks between the ACC and DH

In light of the structural connectivity between the ACC and the hippocampus, and results showing both ACC and DH are involved in memory acquisition in some tasks, (Zhao et al., 2005; Zhang et al., 2011) it seems that memory is embedded in a broad hippocampal-cortical network in which both the ACC and DH play a key role.

However, to date, little is known about how this network's structure shapes its function. Complex networks analysis (Bullmore and Sporns, 2009) presents one promising new approach in the study of brain network organization. Within this framework, networks are described in graphs consisting of nodes and links that serve as an abstract representation of elements and their interactions in real-world systems. The study of networks as diverse as invertebrate nervous systems, power grids, and social networks have revealed that such different complex systems are built on shared organizational principles (Watts and Strogatz, 1998). One such example is the small-

world phenomenon, which has been found to be near ubiquitous in real-world networks, where most links are between neighbouring nodes, while a few links extend to distant nodes serving as shortcuts across the network. Another example is the finding that many such small-world complex networks have highly skewed links-per-node distribution, where the vast majority of nodes have few links, while a few nodes have a large number of links (e.g. scale-free networks). Such nodes with a large number of links are called hubs and are typically major conduits for the flow of activity or information in a network and exert a major influence on the state of more peripheral nodes. An important property of such small-world networks, and one possible reason for why they are so prevalent, is their high resilience for the deletion of nodes; if a random set of nodes is deleted from a large network, it will most likely be nodes with few links. However, this robustness comes at a cost as such networks are extremely vulnerable to elimination of network hubs (Albert et al., 2000). Simulations of cat and macaque cortical networks have been found to behave just as such: exhibiting robustness towards random deletion of nodes and high vulnerability towards damage of highly connected hubs (Kaiser et al., 2007).

Within this framework, the ACC has been suggested to function as a network hub for remote memory, as inactivation of this structure blocked the retrieval of remote memory (Frankland, 2005). Moreover, findings showing the ACC and DH being required for the memory acquisition of some of the same tasks (Zhao et al., 2005; Zhang et al., 2011) suggest that those types of memory are encoded in structural brain networks in which the ACC and DH serve as network hubs, networks that most likely involve the two parallel processing pathways running through either the lateral or medial entorhinal cortex (van Strien et al., 2009). When the memory is in an active state, either

during/following acquisition or retrieval, a subset of nodes within the structural network is engaged in a functional network of interacting cell populations, possibly through phase locking or synchronization (Fries, 2005) allowing for synchronized activity-dependent plasticity (Womelsdorf et al., 2007; Benchenane et al., 2010; Battaglia et al., 2011).

One exception to the resilience of complex systems to random node failure is the occurrence of cascading failure in a network, where the failure of one node causes failure in other nodes, leading to network-wide disruption (Carlson and Doyle, 2002). For this to occur, first, the originally failed node has to be in a position to spread the corrupt information in the network, and second, other nodes have to be sensitive to input, i.e. labile. Findings of systems reconsolidation (Debiec et al., 2002) may be an example of disruption of this nature: Retrieval of remote memory (that does not require the DH) involves recreation of a brain-wide functional network of interacting synchronized neuronal populations representing the memory. Although lesioning or pharmacologically inhibiting the DH does not impair retrieval of the remote memory (Anagnostaras et al., 1999; Wiltgen et al., 2010), lesioning or infusions of protein-synthesis inhibitor into the structure shortly *after* memory retrieval leads to later memory impairments (Debiec et al., 2002). This pattern of findings suggests that although the DH does not play a critical role as a network hub for the retrieval of the memory, the structure is recruited to a functional network supporting the active memory once the memory is retrieved. Memory impairments following disruption of DH function during this period suggest that the local DH disruption affects information stored in a wider network, which would not be affected by the same manipulations without being preceded by memory reactivation. In other

words, disrupting DH function during this state of plastic network synchronization leads to network-wide disruption akin to a cascading failure found in complex networks.

A network perspective

As previously mentioned, the main models of systems consolidation, which assume independent neocortical and hippocampal memory systems, are inconsistent with findings of systems reconsolidation. An alternative interpretation from a complex network perspective may be more successful in reconciling findings of systems consolidation, functional specialization of the DH and ACC with reconsolidation. The main findings from this thesis within a network perspective are as follows:

Cellular and systems reconsolidation. Local infusions of a protein-synthesis inhibitor into either the DH (Debiec et al., 2002) or the ACC (chapter 2) following the retrieval of recent and remote memory lead to memory impairments, even at time-points when pre-test disruption of the structure does not impair memory expression (Anagnostaras et al., 1999; chapter 2; Frankland et al., 2004a; Wiltgen et al., 2010). This suggest that the DH and ACC may be hubs in interdependent networks with overlapping neural populations, where network-wide disruptions in one network cause disruptions in the other (e.g. findings of systems reconsolidation).

Systems consolidation. Inactivation or lesioning of the DH (Anagnostaras et al., 1999; Wiltgen et al., 2010) and ACC (Frankland et al., 2004a; chapter 2) impair the expression of recent and remote memory, respectively. Furthermore, following the reactivation of remote memory 6 hours earlier, only dual inactivation of the DH and ACC immediately before a second retrieval trial impairs memory expression (chapter 2),

suggesting that either structure can support memory retrieval without the other. These findings suggest that the DH and ACC can function as independent hubs in brain networks involved in memory retrieval.

Functional specialization. Inactivation of DH specifically affects the expression of discriminative contextual fear memory as mice with high generalization to a novel context are not affected (Wiltgen et al., 2010). Conversely, inactivation of the ACC specifically affects the expression of generalized contextual fear memory as memory expression changes from one that of high generalization to a novel context, to that of discriminating between the training and novel context (chapter 3). This also provides indirect support to the idea that the DH mediates detailed memory expression as we previously found that memory expression in the absence of a functioning ACC under these conditions were supported by the DH (chapter 2). These findings suggest that the DH and ACC act as network hubs with different functional specialization: the DH supports detailed context memory expression, while the ACC supports generalized context memory.

Thus, the data suggest that the hippocampal and neocortical memory systems consist of networks with the DH and ACC as network hubs, which in turn consist of partly overlapping neural populations, making them interdependent. With time, the neocortical network becomes strengthened and the hippocampal network weakened, to the point where expression of memory can be supported without participation of the hippocampus. This change in network support for the memory is reflected in the quality of the memory expression, where the memory goes from being detailed and supported by the DH, to a more schematic and imprecise one supported by the ACC. However, it

should be emphasized that despite this transition between networks, retrieval causes the memory to be reactivated and reinstated in the hippocampal network, during which both networks are sensitive to input through their shared components.

Summary

Findings presented in this dissertation support the notion that memory is a dynamic phenomenon, one that is possibly never fully 'fixed' or consolidated. Rather, memory undergoes a prolonged period of reorganization in the brain after learning, and can remain malleable to new information upon retrieval. Results presented in chapter 2 indicate, first, that contextual fear memory undergoes cellular consolidation in the ACC, as well as cellular reconsolidation at recent and remote time-points. Second, results in chapter 2 indicate that the retrieval of remote memory, which depends on the ACC, transiently re-engages the DH to the extent that later memory retrieval can be supported by either structure. Findings from chapter 3 suggest that remote memory expression that has become more generalized over time, will, following a reminder, return to that of context discrimination when tested 24, but not at 6 or 48 hours later. Following up on our previous finding that memory expression 6 hours after a reminder can be mediated by either the DH or the ACC, we found that inactivating the ACC before this test led to restored context discrimination, suggesting that generalized context memory expression is mediated by the ACC. Together, findings presented in this thesis suggest that the ACC plays a key role in brain networks in which contextual fear memory is encoded, consolidated and reconsolidated. Moreover, as memory ages and becomes more schematic and generalized, the ACC assumes a critical role in memory retrieval.

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