NEUROBIOLOGICAL MECHANISMS OF FEAR GENERALIZATION

A dissertation submitted
To Kent State University partial
fulfillment of the requirements for the
degree of Doctor of Philosophy

by

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August 2013
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>STUDY 1</td>
<td>20</td>
</tr>
<tr>
<td>Methods</td>
<td>22</td>
</tr>
<tr>
<td>Results</td>
<td>32</td>
</tr>
<tr>
<td>Discussion</td>
<td>71</td>
</tr>
<tr>
<td>STUDY 2</td>
<td>83</td>
</tr>
<tr>
<td>Methods</td>
<td>85</td>
</tr>
<tr>
<td>Results</td>
<td>92</td>
</tr>
<tr>
<td>Discussion</td>
<td>94</td>
</tr>
<tr>
<td>GENERAL DISCUSSION</td>
<td>105</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>108</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Contexts and apparatus</td>
<td>30</td>
</tr>
<tr>
<td>2.</td>
<td>Time-dependent loss of context specificity</td>
<td>34</td>
</tr>
<tr>
<td>3.</td>
<td>Fluorescent in situ hybridization figure</td>
<td>38</td>
</tr>
<tr>
<td>4.</td>
<td>Prefrontal cortex expression of Arc following recent or remote memory tests</td>
<td>42</td>
</tr>
<tr>
<td>5.</td>
<td>Dorsal hippocampus expression of Arc following recent or remote memory tests</td>
<td>44</td>
</tr>
<tr>
<td>6.</td>
<td>Ventral hippocampus expression of Arc following recent or remote memory tests</td>
<td>46</td>
</tr>
<tr>
<td>7.</td>
<td>ACC inactivation experiment</td>
<td>59</td>
</tr>
<tr>
<td>8.</td>
<td>Hippocampus inactivation experiment</td>
<td>66</td>
</tr>
<tr>
<td>9.</td>
<td>Novel object recognition/location protocol</td>
<td>89</td>
</tr>
<tr>
<td>10.</td>
<td>Effects of GABA_{B1(a)} receptor knock out on context specificity</td>
<td>94</td>
</tr>
<tr>
<td>11.</td>
<td>Effects of GABA_{B1(a)} receptor knock out memory precision</td>
<td>97</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

I would like to thank my advisors, Dr. David Riccio and Dr. Aaron Jasnow, for their guidance and support throughout this project. I am grateful for their time and work they have put into helping me develop my skills as a researcher and scientist. I would also like to thank my committee members, Drs. Stephen Fountain, Jeffrey Ciesla, John Johnson, and Sean Veney for their time and helpful comments on this project. I am grateful to my colleagues in the Riccio and Jasnow Labs for their help and collaboration on this project. I would like to thank my undergraduate research assistants (in order of appearance) Sydney Trask, Brooke Dulka, Samantha Ortiz, Dina Dejanovic, and Jessica Mulvany for their tireless hard work and dedication to research, without which this project would never have been completed. Lastly, I would like to thank my family and friends for their continued love and support.
INTRODUCTION

Neurobiological mechanisms of fear generalization

Posttraumatic stress disorder (PTSD) is the fourth most common psychiatric disorder in the United States, affecting approximately 24 million Americans (Yehuda, 2002). Memory alterations are a key component of PTSD, with trauma patients generalizing their fear from the event to other situations in their day-to-day lives. Specifically, patients suffering from PTSD often re-experience the traumatic event and come to associate the fear of that event with other stimuli in their environment, which can lead to the development of fear to stimuli that were not present at the time of the trauma and a generalization of trauma reminders (Lissek et al., 2008; Yehuda, 2002). Fear generalization represents a substantial obstacle to the treatment of the disorder, as it is difficult for afflicted patients to learn strategies for coping with the wealth of reminders in their everyday lives (Layton & Krikorian, 2002). To develop more effective treatments for PTSD, the mechanisms through which generalization of the traumatic event takes place must be understood. This can be accomplished through the use of animal models of learning and memory.

While current animal models of PTSD (i.e. learned helplessness and Fanselow’s stress-induced enhancement of learning model) have provided important insight into behavioral and physiological responses to traumatic stress
(Minor & Saade, 1997; Rau, DeCola, & Fanselow, 2005), they have not directly assessed memory impairment of contextual cues (i.e. generalization between contexts). Such failure to investigate fear generalization between contextual cues in current models creates a substantial gap in our understanding of how these memory impairments develop and are maintained over time. The current study directly investigates memory for contextual cues in order to gain an understanding of how fear generalization develops and is maintained as a result of fear learning. A better understanding of the etiology of generalized fear can aid in the development of more effective treatments or prevention strategies for the memory impairments observed in PTSD.

One animal model/task that allows for the examination of these contextual memory processes is Pavlovian context fear conditioning. Contextual fear conditioning involves pairing a novel context (conditioned stimulus) with several footshocks (unconditioned stimulus) that serve to condition fear (assessed as freezing behavior) to that context. Many studies have demonstrated that shifting contextual cues following context fear conditioning results in reduced freezing, (i.e., the context shift effect). At early time points following context fear conditioning, rodents can discriminate between a training and novel context (they express fear only in the training context). However, these shifts in contextual cues become less disruptive as the retention interval between training and testing increases (context specificity is lost 14-36 days post-training) (Jasnow, Cullen, & Riccio, 2012; Wiltgen & Silva, 2007; Winocur, Frankland, Sekeres, Fogel, & Moscovitch, 2009; Winocur,
Moscovitch, & Sekeres, 2007; Y. Zhou & Riccio, 1996). In other words, the fear memory is no longer precise or context-specific, but has generalized to novel contexts. To date, the neurobiological mechanisms that underlie the time-dependent loss of context specificity are not well understood. Further, this phenomenon is similar to the generalization observed in patients suffering from PTSD, in which the victim comes to associate fear with cues not present at the time of trauma (Golub, Mauch, Dahlhoff, & Wotjak, 2009; Layton & Krikorian, 2002).

The investigation of memory generalization relies heavily on the process of fear consolidation. Specifically, when a learning event occurs, such as learning to associate a specific context with footshock, several processes occur that result in the creation of a memory trace for that event. The first process to occur is known as acquisition, which involves learning to associate two stimuli together, such as learning that certain contextual cues (e.g., visual, olfactory, tactile, and auditory cues) predict shock. To retain this new learned association, this information then needs to be converted to a long-term memory trace than can be stored and retrieved. A brief overview of memory consolidation, its underlying neuronal processes, and how consolidation processes are thought to relate to memory generalization will be discussed below.

**Memory Consolidation**

It has been widely recognized that closed head injury or brain trauma result in the forgetting of more recent events while sparing memory for older events (Ribot, 1882; Russell & Nathan, 1946). This temporal gradient for retrograde
amnesia (RA) has led to the theory that memories become consolidated over time. This theory posits that a memory remains labile for a certain period of time after a learning event, but over time becomes resistant to disruption. When the memory trace strengthens to become resistant to disruption, the memory is said to have consolidated. Using a number of amnesic agents, such as electroconvulsive shock (ECS), CO$_2$ exposure, concussion, and hypothermia, early lab studies discovered that these agents were only effective if administered shortly after the learning episode (Duncan, 1949; Kesner & Conner, 1972; Palfini & Chillag, 1971; Paolino, Quartermain, & Miller, 1966; Mactutus & Riccio, 1978; Riccio, Hodges, & Randall, 1968; Zhou & Riccio, 1995). The amnesic agent loses its ability to produce amnesia if administered beyond a certain time-window.

The term consolidation has been credited to Muller and Pilzecker who studied the phenomenon in the late 19th century (Muller & Pilzecker, 1900). Their study involved 40 experiments in which they attempted to study the formation and retrieval of memory using a list of nonsense syllables (Lechner, Squire, & Byrne, 1999). During their experimentation, they had participants learn a list of nonsense syllables that were presented as paired associates. During the test phase, participants were cued with the first syllable of each pair and asked to recall the second syllable of the pair. They discovered that the brain continued to process the information even after the training session ended as participants reported that the nonsense syllables continued to “come to mind” following training (Lechner, Squire, & Byrne, 1999). Muller and Pilzecker referred to this as perseveration, which they
speculated was the result of the brain continuing to process the information as short-term memory as well as a method to strengthen the association between the syllables. This led to additional experiments in which Muller and Pilzecker tried to disrupt memory by disrupting perseveration. Participants were given a list of paired syllables to learn (list A). They were then given a second list of paired syllables (List X) to read several times to prevent perseveration of list A. They discovered that participants remembered a much higher percentage of correct paired syllables if they did not have the second list following training. That is, when administered immediately following list A, list X was a source of retroactive interference resulting in a lower retention of list A. However, when list X was given after a longer interval, participants had a higher retention of list A (Lechner, Squire, & Byrne, 1999; Muller & Pilzecker, 1900). Muller and Pilzecker inferred that the longer the interval between the two lists, the more time the brain had to strengthen the memory of the first list. From their experiments, Muller and Pilzecker concluded that certain physiological processes continue to process information following training and that any additional material/information given soon after, but not long after, disrupts this consolidation process.

The findings of Muller and Pilzecker have been extended by early work using animal models of amnesia. One of the first studies to look at retrograde amnesia in animals (albino rats) used ECS to disrupt the consolidation process. In his study, Carl Duncan (1949) trained animals to avoid the black side of a black/white shuttle box after shocking animals in the black compartment. Animals were administered
ECS treatments at various times following training. He discovered that the animals that received ECS close to the training event (within 60 seconds) exhibited amnesia for the training shock. When tested, the animals did not avoid the black compartment as compared to control animals that did not receive ECS. More importantly, animals that received ECS 1-14 hours following training exhibited the proper avoidance response (they had identical avoidant levels as the control animals) (Duncan, 1949). This study provided some of the first evidence of a time-dependent consolidation process in animals. Numerous other labs and investigations showed similar time-dependent processes that were reviewed in McGauh’s seminal paper in Science (McGauh, 1966). In his review paper, Mcgaugh (1966) points out that Duncan’s (1949) study as well as number of other ECS experiments provide evidence supporting the hypothesis that memory consolidation is time-dependent. This idea is further supported by studies employing different learning paradigms, such as step-down avoidance and passive avoidance, and various amnesic agents, such as hypothermia, CO2 exposure, anesthesia, and protein synthesis inhibitors (Agranoff & Klinger, 1964; Duncan, 1949; Flexner, Flexner, & Stellar, 1963; Kesner & Conner, 1972; Palfini & Chillag, 1971; Paolino, Quartermain, & Miller, 1966; Mactutus & Riccio, 1978; Riccio, Hodges, & Randall, 1968). These findings, as Mcgauh (1966) points out, suggest that the memory trace is not initially fixed or consolidated at the time of training. Consolidation of a memory trace is a time-dependent process in which a memory
becomes stabilized and encoded over time, at which point the memory becomes resistant to disruption by amnesic agents.

These early studies led to the search for the underlying neural mechanisms responsible for the consolidation of memory. This search yielded two complimentary neurobiological mechanisms for consolidation: synaptic consolidation and systems consolidation (Dudai, 2004). Synaptic consolidation takes place during the first several minutes to hours following the training/learning event and appears to be a ubiquitous process occurring across species and memory tasks (Dudai, 2004). Synaptic consolidation refers to the cellular consolidation process that involves the strengthening of synapses and/or an increase in intracellular signaling (Dudai, 2004). Specifically, the increase in transcription factor activity, de novo protein synthesis, increases in protein kinases, and changes in receptor excitability appear to underlie the consolidation of memory (Alberini, 2009; Debiec, LeDoux, & Nader, 2002; Guzowski & McGaugh, 1997; Kida, et al., 2002; Kinney, et al., 2009; Levenson, et al., 2004; Nader, Schafe, & LeDoux, 2000a; Nader, Schafe, & LeDoux, 2000b; Tully, 1997; Yeh, Lin, & Gean, 2004). These processes help explain the temporal characteristic of consolidation as evidenced by the finding that once these cellular processes occur, the memory trace becomes resistant to disruption.

The second underlying neurobiological mechanism responsible for the consolidation of memory is referred to as systems consolidation (Dudai, 2004).
Systems consolidation occurs over days to weeks or even months and involves the reorganization of information or the brain systems that encode memory and may transfer the memory trace to new locations within the brain (Dudai, 2004). Specifically, it has been proposed that memory is initially processed by the hippocampus and structures in the medial temporal lobe for a short period of time before moving to more permanent stores in the neocortex (Bayley, Hopkins, & Squire, 2003; Dudai, 2004; Kim & Fanselow, 1992; Squire & Alvarez, 1995; Squire, Clark, & Knowlton, 2001). The evidence for this systems consolidation comes from both human and animal studies. For instance, human patients with damage to the hippocampal region exhibit temporally graded retrograde amnesia (Kapur & Brooks, 1999; Squire & Alvarez, 1995). Specifically, these patients typically display amnesia for more recent declarative memory while having more remote memories spared (Dudai, 2004). Conversely, human patients with damage to the neocortex typically display a flat or ungraded retrograde amnesia (Kapur, 1993; Squire & Alvarez, 1995; Squire, Clark, & Knowlton, 2001). Further, lesions to the hippocampus or entorhinal cortex in rats, mice, rabbits, or monkeys result in a temporally graded retrograde amnesia for various types of memory (Cho, Beracochea, & Jafford, 1993; Clark, Broadbent, Zola, & Squire, 2002; Dudai, 2004; Kim & Fanselow, 1992, Kim, Clark, & Thompson, 1995; Kubie, Sutherland, & Miller, 1999; Winocur, 1990; Zola-Morgan & Squire, 1990). Both synaptic and systems consolidation theories nicely explain the time-dependent nature of temporally graded retrograde amnesia. Both forms of consolidation require a period of time in
which the memory trace is strengthened to the point at which it becomes resistant to disruption by amnesic agents. Consolidation theory also assumed and was for the most part accepted, though there were some contrasting findings reported (for example: Young & Galluscio, 1971), that once the memory trace goes through the consolidation process, it becomes permanently stored in the brain and can no longer be disrupted.

A recent study by Frankland et al. (2004) directly investigated the neural structures involved in the long-term systems consolidation (i.e. transfer of memory from temporary hippocampal stores to more permanent cortical stores) of a context fear memory in mice. Using activity-dependent gene expression analysis, they looked at activity levels of a number of areas thought to be involved in consolidation at a recent time point, 24 hours, or a remote time point, 36 days following training. Retrieval of a recent context fear memory resulted in activation of the CA1 region of the hippocampus, but little to no cortical activation. However, reactivation of a remote memory resulted in little to no activation of the hippocampus but did result in increased activity of the infralimbic (IL) and prelimbic (PL) areas of the medial prefrontal cortex (mPFC), as well as the anterior cingulate cortex (ACC). Further, pharmacological inactivation of the ACC, but not PL, only disrupted the contextual memory at remote time points, but had no effect on the memory at recent time points. These results suggest that as a context memory ages, it is transferred from the hippocampus to a distributed cortical network for long-term storage. Further, these data suggest that once the context memory trace leaves the hippocampus, it is
heavily dependent upon the ACC area of the neocortex. However, it should be noted that the authors only tested animals in the same context they were trained in. Frankland, et al. (2004) did not investigate the precision, or context specificity, of a context memory (i.e. they did not test animals in a shifted context at either recent or remote time points). While studies on the neural structures involved in the long-term storage and consolidation of memory provide valuable insight into the time-dependent role of the hippocampus and neocortex, they do not explain the loss of context-specificity (i.e. increase in generalization) that occurs over time.

While the transfer of memory theory of systems consolidation nicely accounts for the temporal gradient of RA seen in hippocampally lesioned animals and humans, it does not however account for changes in the specificity of the memory (i.e. increased rates of fear generalization). As mentioned previously, shifting the contextual cues away from the training context results in a reduction of conditioned responding. For instance, in the case of context fear conditioning, placing animals’ in a novel context (one with different visual, auditory, and olfactory cues from those of the training context) shortly following training reduces expression of learned fear (lower levels of freezing behavior)(Feinberg & Riccio, 1990; Jasnow et al., 2012; Riccio, Ackil, & Burch-Vernon, 1992; Ruediger et al., 2011; Wiltgen & Silva, 2007; Wiltgen et al., 2010; Y. Zhou & Riccio, 1996). However, as the retention interval between training and testing increases, shifts in contextual cues become less disruptive. In other words, fear conditioned to the training context generalizes over time to the point that animals exhibit equivalent levels of fear
expression in both the training context and the novel context. This shift effect and time-dependent increase in generalization has been well documented in a number of cases using rats, mice and birds using passive avoidance, appetitive conditioning, conditioned taste aversion, and latent inhibition (Gisquet-Verrier & Alexinsky, 1986; W. R. McAllister & McAllister, 1963; Metzger & Riccio, 2009; PERKINS & WEYANT, 1958; Richardson, Williams, & Riccio, 1984; Y. Zhou & Riccio, 1996). In a recent attempt to explain the loss of context specificity, or increase in generalization of contextual cues, (Winocur et al., 2007) proposed that this phenomenon results from the completion of systems consolidation. Specifically, (Winocur et al., 2007) proposed that as the memory trace (in this case a context fear memory) is transferred from the hippocampus to the cortex over time, it is transformed into a schematic-like memory. In other words, the memory is not stored in its original form, but is stored as a “gist-like” memory that lacks context-specificity. This proposal nicely explains the gradual increase in context generalization over time. This also suggests that the expression of a generalized fear memory (fear expression in a novel context) results from activation of the general or schematic-like cortical fear memory by the novel contextual cues. To investigate the transformation hypothesis, Winocur et al. (2009) demonstrated that the hippocampus was not involved in the expression of a generalized memory by conducting post-training lesions of the hippocampus. Lesioning the hippocampus at a remote time point (28 days following fear conditioning) did not disrupt fear expression (i.e., freezing behavior) in either the training or a novel context. (Winocur et al., 2009) concluded
that at remote time points, animals are retrieving a hippocampus independent schematic-like cortical memory that lacks context specificity.

Further supporting Winocur, et al.’s (2007) transformation of memory hypothesis, Wiltgen et al. (2010) reported that the hippocampus plays a time-limited role in the retrieval of detailed, context-specific memories. Specifically, Wiltgen et al. (2010) found that for animals that had lost the ability to discriminate between contexts at a remote time point, hippocampal inactivation did not affect performance on subsequent tests. However, animals that were able to discriminate at the remote time-point exhibited impaired fear expression on subsequent tests following hippocampal inactivation. This suggests that while the memory trace is dependent on the hippocampus, context specificity is retained. When the contextual memory trace is transferred/transformed into a hippocampal-independent memory, context specificity is lost. These data along with data from Winocur et al., (2007) and Winocur et al., (2009) suggest that hippocampal activity underlies expression of a specific or precise context memory and that the precision of that memory is lost once the trace is transformed from a hippocampus-dependent memory to a hippocampal-independent cortical memory.

The transformation of memory hypothesis (Winocur et al., 2007; 2009) nicely accounts for the time-dependent loss of context specificity (i.e. gradual increase in fear generalization) observed when contextual cues are shifted away from the training context. It also fits with the neural pattern of activation that Frankland, et al. (2004) discovered underlying the transfer of a recent context fear
memory to a remote memory. That is, at remote time points, when the memory is transferred from a hippocampal-dependent context memory to a hippocampal-independent cortical memory, it is also transformed from a context-specific memory to a general representation of the context that lacks context specificity or precision. The resulting increase in generalized fear responding in the presence of the novel context is a result of the novel contextual cues, which are different but similar from those of the training context, activating the schematic-like cortical memory resulting in subsequent fear expression.

In addition to the transformation hypothesis (Winocur et al., 2007; 2009), an alternative explanation for the time-dependent loss of context specificity was proposed by Rudy, Biedenkapp, & O’Reilly (2005) and is referred to as the trace-decay hypothesis. In their 2005 commentary, the authors propose that instead of a transfer/transformation of the original memory trace, the memory remains in the hippocampus but decays over time. In other words, the strength or quality of the hippocampal trace decays and becomes difficult to retrieve. In an effort to aid in the retrieval, several cortical regions, in particular areas of the medial prefrontal cortex, send a signal to boost activity of the hippocampus and allow the memory to be retrieved (Rudy, 2005). This suggests that the gradual loss of context specificity is a result of forgetting and that the cortex serves as a compensatory mechanism to aid in the retrieval of the forgotten or degraded memory trace. Biedenkapp and Rudy (2007) provided evidence for this trace decay theory by showing that preexposures to the training context prior to fear conditioning prevented the loss
of context specificity. Rats were given three consecutive days of a 5-minute exposure to the training context during which animals were allowed to explore the context. Animals then received context fear conditioning and were tested for fear in either the same context or a novel context at a remote time point (15 days). Animals that received the pre-exposures to the training context failed to generalize conditioned fear to the novel context compared to animals that did not receive the pre-exposures. In other words, the pre-exposures prevented the forgetting of the contextual details and prolonged the specificity of the memory. Biedenkapp & Rudy (2007) argue that the pre-exposures to the training context create a stronger memory trace or representation of the context within the hippocampus that doesn’t decay or decays at a slower rate than the memory trace created under “normal” conditions (i.e. no pre-exposures).

It is clear from a number of studies that the hippocampus plays a time-limited role in the consolidation and expression of a contextually precise memory. Once the memory becomes independent of the hippocampus, the precision or specificity of that memory is lost resulting in increased rates of fear generalization. However, outside of the hippocampus, it remains unclear what neural structures are involved in the expression of a generalized fear memory. It may be the case that certain cortical areas, such as the ACC and prelimbic and infralimbic cortices, regulate the expression of a generalized, or non-specific, context memory once the hippocampus is no longer involved in the expression of the memory. It may also be the case that other neural structures not involved in the original memory are
activated during expression of a generalized memory. Currently, there have been no
direct assessments, with the exception of the hippocampus, of the neural structures
involved in the loss of context specificity.

Study 1 will attempt to address this gap in our understanding of the neural
processing that underlies the loss of context specificity, or increase in fear
generalization, that occurs as the memory ages. Specifically, using a similar method
employed by Frankland, et al. (2004), Study 1 will utilize activity-dependent gene
expression analyses to investigate the neural pattern of activation underlying the
expression of a contextually precise and a contextually imprecise memory (i.e. a
generalized memory). It should be noted that the two competing theories proposed
to explain the gradual loss of context specificity make two very different predictions
as to what the neural of activation should look like. The transformation of memory
hypothesis states that the loss of context specificity (increase in generalization) over
time is the result of the memory being transformed as it is transferred to the cortex
for long-term storage from a context specific memory to a more schematic-like
memory that lacks contextual detail. Therefore, this hypothesis would predict that
expression of a generalized or imprecise memory involves activity of the medial
prefrontal cortex and the anterior cingulate cortex independent of the hippocampus
(i.e. little to no hippocampal activity). However, the trace-decay hypothesis states
that the loss of context-specificity results from decay in the strength or quality of the
hippocampal memory trace and requires a compensatory cortical response to aid in
the retrieval of the memory, thereby losing specifics of the contextual cues. The
trace-decay hypothesis would predict that expression of a generalized memory involves activity of both the hippocampus and medial prefrontal cortex.

*Synaptic mechanisms of generalization*

Similar to investigations of memory precision taking a systems approach, not much attention has been given to mechanisms underlying the precision of memory at the cellular/synaptic level. One of the few studies that have investigated cellular mechanisms underlying the ability to discriminate between stimuli has identified activity of the inhibitory receptor GABA_B as being necessary. GABA_B receptors are G protein-coupled receptors that exist as heterodimers containing two subunits, GABA_B1 and GABA_B2 (Gassmann & Bettler, 2012). The GABA_B1 receptor exists in two isoforms, GABA_B(1a) and GABA_B(1b), with the isoforms being localized to presynaptic and postsynaptic terminals, respectively (Gassmann & Bettler, 2012; Kulik et al., 2003; Vigot et al., 2006). GABA_B(1a) act through voltage-gated calcium channels to inhibit neurotransmitter release, typically glutamate, resulting in reduced excitation of the post-synaptic terminal (Gassmann & Bettler, 2012; Guetg et al., 2009; Pan et al., 2009; Vigot et al., 2006).

Recently, GABA_B1(a) receptors have been shown to play a role in cued-fear discrimination (Shaban et al., 2006). Shaban, et al. (2006) discovered that mice lacking the presynaptic GABA_B1 receptor exhibit an increase in non-associative cortico-amygdala LTP and a loss of cued discrimination. Blocking presynaptic GABA_B receptors resulted in homosynaptic LTP at cortical inputs onto the lateral amygdala using a stimulation protocol that normally would not result in this type of
LTP induction. In addition to this increase in amygdala potentiation, blocking $\text{GABA}_{\text{B1(a)}}$ receptors also resulted in an increase in generalization between two cues in a cued discrimination task. In cued-discrimination training, animals are presented with a particular tone that is always paired with a footshock (CS+) and a second tone that is never paired with shock (CS-). The result of this training is that animals learn to fear the tone that was always paired with shock, meaning they will freeze in the presence of that tone, and will exhibit very little fear, or freezing, to the tone that was never paired with shock. However, when $\text{GABA}_{\text{B1(a)}}$ receptors were genetically ablated, animals were unable to discriminate between the two cues and fear from the CS+ generalized to the CS- (i.e. animals froze equivalently to both stimuli)(Shaban et al., 2006).

In addition to cued-fear discrimination, $\text{GABA}_{\text{B1(a)}}$ receptors have also been implicated in more general spatial discrimination tasks as well. For instance, Jacobson, et al. (2007) discovered impaired novel object discrimination, a task heavily dependent upon the hippocampus (Broadbent, Squire, & Clark, 2004), in animals lacking presynaptic $\text{GABA}_{\text{B1(a)}}$ receptors. Novel object discrimination involves presenting animals with a particular object (in this case a plastic disc) for several trials to allow the animal to explore and learn the object. During testing, the familiar object is replaced with a novel object (in this case a plastic cone) and the time spent exploring the novel object is recorded. If the animals can discriminate between the familiar object and the novel object, they will typically spend more time exploring the novel object due to their curious nature (Kim, et al., 2005). Jacobson,
et al. (2007) found that control wild-type (non-mutant) mice were able to
discriminate between the novel and familiar object. However, GABA$_{B1(a)}$ knock out
mice exhibited impaired ability to discriminate between the two objects as these
animals exhibited less exploration of the novel object compared to control animals.
These data suggest that in addition to playing a role in cued-fear discrimination,
GABA$_{B1(a)}$ receptors are also involved in the discrimination of novel objects,
suggesting that these presynaptic inhibitory receptors may play a general role in
discrimination processes.

Shaban et al.’s (2006) study is among the first to identify a potential cellular
mechanism for the discrimination/generalization of both LTP within the amygdala
and fear expression. However, these generalization mechanisms were limited to
cortical afferent inputs to the lateral amygdala. It is unclear whether the loss of
GABA-mediated presynaptic inhibition plays a role in reducing memory precision as
a function of time within the framework of systems consolidation theory. In other
words, it is unknown whether these receptors are important in maintaining the
context-specificity prior to the transfer of the memory from a context-specific
hippocampal memory to a schematic-like cortical memory or prior to decay of the
hippocampal trace.

Study 2 will investigate the role of presynaptic GABA$_{B1(a)}$ receptors in the
discrimination/generalization of contextual cues. Using the same genetic knock out
mice lacking GABA$_{B1(a)}$ receptors that were used in both Shaban, et al. (2006) and
Jacobson, et al. (2007), Study 2 will determine what role, if any, GABA$_{B1(a)}$ receptors
play in the context-specificity surrounding context fear conditioning. Based on the previous data discussed above, it is reasonable to predict that animals lacking \( \text{GABA}_{B1(a)} \) receptors will exhibit impairments in context discrimination. In other words, the \( \text{GABA}_{B1(a)} \) knock out animals should show increased fear generalization when tested in a novel context compared to non-mutant control animals.
Study 1

The purpose of this experiment is to investigate the neural pattern of activation underlying expression of a generalized context fear memory. As discussed above, a number of studies have suggested that the consolidation of a context fear memory involves the transfer of a recent context memory from a context-specific hippocampal-dependent memory to a cortical-dependent memory that lacks context specificity. Several cortical structures have been implicated in this transfer of memory, including the prelimbic (PL) and infralimbic (IL) cortices of the medial prefrontal cortex (mPFC) and the anterior cingulate cortex (ACC). Therefore, we will investigate the neural pattern of activation underlying expression of a context-specific fear memory and a generalized fear memory focusing on these structures in addition to the hippocampal formation. To achieve this, we used context fear conditioning and tested animals at recent (1 day) and remote (14 days) time points in either the same or a novel context. Following testing, we extracted brains and ran activity-dependent gene activation analyses on the IL, PL, ACC, the CA1, CA3, and dentate gyrus (DG) regions of the dorsal and ventral hippocampus. The activity analysis involves probing for expression of Arc mRNA, which is a member of the immediate early gene family and has been used as an indicator of cellular
of the brain were recently active during expression of a context fear memory.
Following the activity experiment, we conducted inactivation experiments using the sodium channel blocker lidocaine to confirm the activity of certain neural structures implicated in activity-dependent analyses.
METHOD

Subjects

The subjects were 120 male C57BL/6 mice. The C57BL/6 mice were generated from a breeding colony in the Department of Psychology at Kent State University. Animals were be 6 weeks of age or older before they were used for experimentation/surgical cannulation and were be group housed (3-4 animals per cage) with free access to food and water in a room maintained on a 15/9 light/dark cycle. All procedures were conducted in a facility accredited by the Association for Assessment and Accreditation and Laboratory Animal Care. Kent State University’s Institutional Animal Care and Use Committee approved all protocols in this paper.

Fluorescent in situ Hybridization

Fluorescent in situ hybridization was performed on 16 μm sections sliced on a cryostat and mounted on Superfrost Plus slides (Fisher Scientific). Our hybridization procedure included creating riboprobes using cDNA clones containing the coding sequence for the mouse Arc gene. cDNA was linearized with appropriate restriction enzymes and fluorescently labeled riboprobes were generated with T3 RNA polymerase and digoxigenin (DIG) labeling. Following our prehybridization procedure, the sections were hybridized with DIG-labeled arc probes at 55°C for 16 hours and then underwent a series of rigorous washes. Sections were then
incubated with anti-DIG-POD, Fab fragments, followed by fluorescent amplification using TSA Plus Cyanine 5 Fluorescent System (Perkin Elmer). Sections were then be incubated with Hoechst solution (Sigma) for nuclear staining followed by another series of washes and finally coverslipped with MOWIOL mounting medium.

Imaging was conducted on an Olympus 70X inverted microscope. The software program ImageJ (NIH) was used to quantify the hybridization signal intensities of brain regions of interest. Background was subtracted from the images using a rolling bar radius of 5.0. Each image was despeckled in order to filter image noise. The auto threshold feature was used (to remove potential subjective thresholding by the experimenter) to set the upper and lower signal threshold for each image and then converted the image to a binary image. Binary images were then further adjusted using watershed segmentation to automatically separate particles that were touching. The brain regions of interest, with coordinates in relation to bregma that were analyzed, include the infralimbic and prelimbic (+1.78mm) regions of the medial prefrontal cortex, the anterior cingulate cortex (+0.6mm), the CA1, CA3, and dentate gyrus regions of the dorsal hippocampus (-2.18mm), and the CA1, CA3, and dentate gyrus regions of the ventral hippocampus (-3.16mm).

Analysis of Arc mRNA expression involved calculating difference scores by subtracting baseline activity levels in home cage control animals from the activity levels of experimental animals. A home cage control activity score was calculated by averaging activation levels for each area of interest across all animals. For each
region of interest, a difference score was calculated for each animal in each group (i.e. 1 Day Same, 1 Day Shift, 14 Day Same, 14 Day Shift). We used 3 bilateral consecutive sections for each region and averaged difference scores together, giving each animal one difference score per region of interest. For example, for activity levels of the ACC, 6 difference scores were calculated for each animal: 3 consecutive sections of the ACC each with a right and a left side results in 6 images.

*Surgery and Cannulation*

All animals in the inactivation experiments underwent surgical cannulation procedures approved by the Kent State Institutional Animal Care and Use Committee. Animals were anesthetized with an intraperitoneal injection of a Ketamine (75 mg/kg) + Dexdormitor (dexmedetomidine) (1.5 mg/kg) cocktail. Following administration of anesthesia, mice were mounted on a stereotaxic apparatus designed for mouse use (Kopf Instruments). A single guide cannula (Plastics One, Inc) was be inserted into the skull above the anterior cingulate cortex for midline infusions. Two guide cannulae were surgically implanted above the CA1 region of either the dorsal hippocampus or ventral hippocampus for bilateral infusions. Cannulae were positioned in the following coordinates with respect to bregma: +0.8mm A/P, + 0.7mm M/L, 1.75mm D/V for ACC; -2.5mm A/P, +/-2.5mm M/L, 1.6mm D/V for CA1 region of the dorsal hippocampus; -3.16mm A/P, +4.2mm M/L, 3.6mm D/V for CA1 region of the ventral hippocampus.

Following the completion of behavioral experiments, we verified guide cannula placement by injecting 0.2μl of India ink through guide cannulae. Brains
were removed and flash frozen using dry ice for storage in -80°C freezer. Brains were sectioned on a cryostat at a thickness of 25 μm. If the dye is not observed in the proper place, behavioral data from that mouse will be excluded from analyses. No animals were excluded from the ACC inactivation experiments. Animals in the hippocampal inactivation experiments were excluded from analyses if ink was not found in the proper place on both sides. No animals were excluded from the 1 Day dCA1 inactivation experiments. Four animals were excluded from analyses from the 21 Day dCA1 inactivation experiments. Four animals were excluded from the 1 Day vCA1 inactivation experiments. Six animals were excluded from analyses from the 21 Day dCA1 inactivation experiments.

Drug Infusions

The sodium channel blocker lidocaine hydrochloride (Sigma) was used to suppress neuronal firing, thereby temporarily inactivating brain regions of interest. For all infusion experiments, lidocaine HCl will was dissolved in phosphate-buffered saline (PBS) to a concentration of 4% lidocaine and adjusted to a pH of ~7.0. Mice received midline or bilateral infusions (0.5 μL/side) of either lidocaine HCl (4%) or PBS from a 5 μL Hamilton syringe operated by a micro-infusion pump (Harvard Apparatus). Infusions were administered over 2.5 minutes at a rate of 0.2 μL per minute. Injectors were left in place for 2 minutes following infusion to ensure diffusion of the drug/vehicle into the brain. Bilateral infusions were administered simultaneously using a two-syringe micropump. During the infusion, mice were
placed in a clean home cage shoebox and allowed to walk freely and were only be briefly restrained during injector insertion/removal.

*Apparatus and Contexts*

Behavioral procedures were performed in 4 identical conditioning chambers (7" W x 7" D x 12" H) containing 2 Plexiglas walls (front and back) and 2 aluminum sidewalls and a stainless steel shock-grid floor (Coulbourn Instruments, Allentown, PA). All fear conditioning chambers are placed inside of sound attenuating chambers and contain cameras mounted on top of each chamber that record training and testing sessions. Context A consists of the context chamber (2 Plexiglas and 2 aluminum walls), with polka-dots on the back wall, white noise projected through a wall speaker, dim illumination (house light), and the grid floors were cleaned with 70% ethanol. Context B consists of identical chambers, minus the polka dots, was not illuminated and contains no white noise. A flat-brown Plexiglas floor replaced the grid floor and was washed with 70% Quatricide (see Figure 1). Scrambled footshocks (0.8mA) were generated by a precision shock generator (Coulbourn Instruments) and were delivered through the grid floor in Context A.

*Procedure*

All animals were handled for 5 minutes on two consecutive days prior to fear conditioning. This serves to acclimate the animals to the presence of the experimenter. All animals received two 5-minute pre-exposures to Context A on the two days prior to fear conditioning. This pre-exposure serves to introduce the animal to the training context and allows the animal to encode the contextual cues.
Fear conditioning occurred in Context A, which consisted of 5 footshocks (1 sec, .8 mA) separated by 90 sec ITIs. Testing occurred in either Context A (Same group) or Context B (Shift group) at various intervals following training. Testing consists of a 5 minute exposure to either Context A or Context B, during which freezing, defined as the absence of all movement minus the movement associated with breathing, is measured using FreezeFrame software (Actimetrics, Wilmette, IL). A behavioral control group (Control), received handling, context pre-exposures as the Same and Shift groups but did not receive shocks during the training trial. These animals were placed in the context during the training period to control for context exposure, but did not receive foot shocks.

**Experiment 1:** Fear conditioning began when animals were between 60-80 days of age. All animals were fear conditioned in Context A in which they learn to fear the specific cues of the context, as described above. Animals were divided into two context groups. Half of the animals received training in Context A and were tested in Context B (Shift groups; 1 Day n = 8; 14 Days n = 8). The other half of the animals were tested in the same context they received training in (Same groups; 1 Day n = 9; 14 Days n = 10). The Same groups serve as context controls (to ensure fear conditioning was successful) and to provide benchmarks for comparison. We used 2 separate retention intervals to assess the ability to discriminate between Context A and Context B. Half of the animals were tested for fear at a recent time point, 24 hours following training and the other half were tested at a remote time point, 14 days following fear conditioning. A number of studies have used these two intervals
and show that animals can discriminate well at 1 day post-training but lose the
ability to do so by 14 days post-training (Land & Riccio, 1998; MacArdy & Riccio,
Figure 1: Fear Conditioning Apparatus and Contexts. Context A (left) consists of 2 aluminum sidewalls, a polka-dot back wall, shock-grid floor, white noise projected from wall speaker, illumination provided by a house light, and is be cleaned with 70% ethanol to provide an olfactory cue.

Context B (right) consists of 2 aluminum sidewalls, 2 Plexiglas walls (no polka-dots), no illumination, no white noise, and a flat brown Plexiglas floor cleaned with 70% Quatricide.
Figure 1
We will also added a home cage control group that allows us to assess changes in gene activation following testing compared to non-trained animals. Again, this group allows us to assess changes in gene expression induced by fear conditioning following reactivation of fear in the two different contexts. We also added a group that received identical procedures to the animals described above with the exception that no shocks were delivered (No Shock group, 1 Day n = 6; 14 Days n = 6). This No Shock group will allow us to assess whether the contexts used for training and testing are inherently aversive.

Thirty minutes following testing in either the same or shifted context at 1 or 14 days post-training, animals were be sacrificed and brains were extracted. This post-test interval was chosen due to findings suggesting that Arc mRNA expression peaks at around 30 minutes following fear expression (Lonergan et al., 2010; Ressler et al., 2002). Extracted brains were flash frozen using dry ice and stored in a -80°C freezer.

Experiment 2: All mice underwent surgical cannulation as described above and given 1 week to recover before behavioral experimentation began. Following their one-week recovery period, all animals underwent handling, pre-exposure, and context fear conditioning in Context A as described above. Each drug group receiving lidocaine and vehicle were divided into two context groups. Half of the animals received training in Context A and were tested in Context B (Shift groups). The other half of the animals were tested in the same context they received training in (Same
groups). The vehicle groups serve as a control group and allow us to assess the behavioral effects of temporarily inactivating specific brain regions. We used several separate retention intervals to assess the ability to discriminate between Context A and Context B. Animals were tested for fear 24 hours, 14 days, or 21 days following fear conditioning. Ten minutes prior to fear conditioning, animals received an infusion of either lidocaine or PBS in the anterior cingulate cortex, dorsal hippocampus, or the ventral hippocampus. Sample size was as follows: 1 Day ACC inactivation: Same PBS n = 7, Same Lidocaine n = 7, Shift PBS n = 7, Shift Lidocaine n = 7; 14 Day ACC inactivation: Same PBS n = 6, Same Lidocaine n = 10, Shift PBS n = 9, Shift Lidocaine n = 5; 21 Day ACC inactivation: Same PBS n = 8, Same Lidocaine n = 8, Shift PBS n = 7, Shift Lidocaine n = 8; 1 Day dCA1 inactivation: Same PBS n = 4, Same Lidocaine n = 5, Shift PBS n = 4, Shift Lidocaine n = 7; 21 Day dCA1 inactivation: Same PBS n = 3, Same Lidocaine n = 6, Shift PBS n = 5, Shift Lidocaine n = 5; 1 Day vCA1 inactivation: Same PBS n = 4, Same Lidocaine n = 6, Shift PBS n = 6, Shift Lidocaine n = 5; 21 Day vCA1 inactivation: Same PBS n = 3, Same Lidocaine n = 5, Shift PBS n = 4, Shift Lidocaine n = 3.
RESULTS

Memory for contextual cues becomes less specific over time.

To investigate the neural pattern of activation underlying the time-dependent loss of context-specificity (or increase in fear generalization), we contextually fear conditioned C57BL/6 mice and tested for fear in either the same (Same) or a novel (Shift) context at 1 or 14 days following training. Figure 2 shows grand mean (+SEM) freezing scores for animals in all context groups and retention intervals. At 1 day post-training, animals in the Shift condition froze (exhibited less fear) less than animals in the Same condition. This difference indicates the context shift effect is present at 1 day following fear training. In other words, animals tested in the novel context are exhibiting a contextually precise memory and are not expressing fear in the presence of the novel cues. However, at 14 days post-training, animals in both the Shift context and the Same context are freezing at equivalent levels. In other words, animals in the Shift context are exhibiting a contextually imprecise memory and have completely generalized their fear from the training context to the novel context. Animals in the no shock control condition (Control) exhibited very low levels of freezing in the training context at both 1 and 14 days following exposure to the training context. This group shows that at either time
Figure 2: Grand mean (+ SEM) percent time spent freezing for C57BL/6 mice in context specificity experiment. Groups labeled as Same were tested in the same context they were trained in. Groups labeled Shift were tested in a novel context. Groups labeled as Control received the same context exposures in the training context but received no shocks. Animals in the Shift condition are exhibiting a contextually precise fear memory 1 day following training. However, this context specificity is lost by 14 days as animals in the Shift condition have completely generalized their fear from the training context to the novel context. Animals in the no shock Control condition at either time point are exhibiting close to zero fear expression, indicating the training context itself is not aversive.
Figure 2

The graph shows the percentage of freezing behavior over time for three groups: Same, Shift, and Control. The x-axis represents time in days (1 Day and 14 Days), and the y-axis represents the percentage of freezing. The bars indicate the data points with error bars for each time point.
point, the training context itself is not aversive. Therefore, we can conclude that any fear expression exhibited in the training context (and the novel context at a remote time point) is due to the association formed between the context and the shock (i.e. learned fear).

A 3 (context) X 2 (retention interval) analysis of variance (ANOVA) was conducted on freezing scores (percent time spent freezing) and yielded a statistically significant main effect of Context (Same vs. Shift vs. Control), \( F(2, 41) = 54.97, p < .001 \), indicating that overall animals in the shift and control conditions froze significantly less than animals in the same condition. There was also a statistically significant main effect of Retention (1 Day vs. 14 Days), \( F(1, 41) = 10.66, p < .01 \), indicating that overall animals froze significantly more at 14 days post-training than at 1 day post-training. The analysis also yielded a statistically significant Context by Retention interaction, \( F(2, 41) = 8.76, p < .01 \). Tukey HSD post hoc comparisons showed that animals in the Shift condition froze significantly less than animals in the Same condition at 1 day following training (\( p < .001 \)). Further, animals in the no shock Control condition froze significantly less than animals in either the Same context or the Shift context at either time point (\( p < .001 \)). Freezing levels in both the Same and Shift condition at 14 days post-training did not differ significantly.

These data replicate the basic context shift effect and time-dependent loss of context specificity using Pavlovian context fear conditioning in mice. Animals were able to discriminate between the novel context and the training context 1 day
following training (i.e. exhibited a contextually precise memory). However, this context specificity was lost by 14 days post-training as animals completely generalized their fear from the training context to the novel context. Further, animals in the no-shock Control condition froze at equivalently low levels in the training context at both time points indicating that the animals did not find the context/apparatus naturally aversive. Therefore we can conclude the freezing exhibited by animals in the Same context at both time points and animals in the Shift condition at the remote time point is due to the retrieval of learned fear from training.

The loss of context specificity is due to an interaction between the hippocampus and cortical regions. In order to investigate the neural pattern of activation that underlies the gradual loss of context specificity, animals from the previous behavioral experiment were sacrificed and activity-dependent gene activation analysis was performed on brains extracted from these animals (see Figure 3). Each area of interest, including the infralimbic cortex (IL), prelimbic cortex (PL), the anterior cingulate cortex (ACC), the CA1 (dCA1), CA3 (dCA3), and dentate gyrus (dDG) regions of the dorsal hippocampus, and the CA1 (vCA1), CA3 (vCA3), and dentate gyrus (vDG) regions of the ventral hippocampus, can be found in Figure 4 and will be discussed individually below. A 2 (Context) X 2 (Retention) factorial ANOVA was conducted for each area of interest on difference scores (activity levels of each experimental group was compared to activity levels of home cage control animals). Though statistical analyses were conducted on difference
Figure 3: Representative sample Arc mRNA expression in a section from the CA1 region of the ventral hippocampus. Cell nuclei were stained with Hoescht solution (DAPI; upper left panel). DIG-labeled probe was amplified with Cyanine 5 Fluorescent System (lower left panel). The merged image (upper right panel) shows Arc activity surround cell nuclei indicating recent activation of those cells.
Figure 3
scores, the data in Figure 4 are presented as percent change relative to baseline controls. The data are presented this way in the figure to make interpretation of the activity analysis easier to visualize and comprehend. It should be noted that the no shock control (context exposure) group served as a behavioral control to assess potential aversive qualities of the training context. Brains from these animals were not analyzed for activity.

Figure 4A shows grand mean (+SEM) difference scores for Arc mRNA activity in the ACC for animals in all context groups and retention intervals. Animals tested in the Same context exhibit little to no ACC activation above baseline when tested 1 day following fear conditioning. However, ACC activity greatly increases when animals are tested in the Same context at 14 days post-training. This replicates Frankland, et al.’s (2004) finding indicating that the ACC is heavily active in the expression of a remote, but not recent, context fear memory. Interestingly, the ACC appears to be highly active during expression of both a contextually precise and a contextually imprecise context fear memory as evidenced by high Arc activity at both time points in the Shift context. This suggests that the ACC region of the cortex is recruited or involved in both discrimination and generalization of contextual stimuli.

A 2 (Context) X 2 (Retention) factorial ANOVA was conducted on difference scores for the ACC and yielded a non-significant main effect of Context, \( F(1, 15) = 3.89, p = .06 \), indicating no overall differences in ACC activity between the Same and Shift contexts. There was a statistically significant main effect of Retention, \( F(1, 15) \)
40

= 10.38, p< .01, indicating that ACC activity was overall higher at 14 days than at 1
day post-training. There was a statistically significant Context by Retention
interaction, F(1, 15) = 13.19, p< .01, indicating that ACC activity was significantly
lower at 1 day in the Same context than at 14 days in the Same context. ACC activity
did not significantly differ between 1 and 14 days post-training
in the Shift context. These data suggest that ACC activity underlies expression of
both a contextually precise and a contextually imprecise fear memory.

Figure 4B shows grand mean (+SEM) difference scores for Arc mRNA activity
in the IL for animals in all context groups and retention intervals. Animals tested in
the Same context exhibit little IL activation above baseline when tested 1 day
following fear conditioning. However, IL activity greatly increases when animals are
tested in the Same context at 14 days post-training. In addition to the ACC activity
data, this replicates Frankland, et al.’s (2004) finding indicating that the IL is heavily
active in the expression of a remote, but not recent, context fear memory. The
reverse pattern emerges when animals are tested in a novel context. Animals tested
in the Shift context 1 day following training exhibited an increase in IL activity.
However, this activity appeared to decrease when animals were tested 14 days
following training in the Shift context. This suggests that the IL region of the mPFC is
involved in expression of a contextually precise fear memory and that activity of this
cortical structure, though there appears to be a reduction in activity, may also be
involved in expression of a contextually imprecise memory.
Figure 4: Prefrontal cortex expression of activity-dependent Arc following recent or remote memory tests in either the Same or Shift context. To assess changes in neural activation following memory expression, the data is expressed as percent change compared to home cage control baseline activity (baseline is represented as the dashed line at 100%).

a) Arc expression within the ACC was elevated following the recent and remote memory test in the Shift context. Arc expression was elevated following remote, but not recent memory expression in the Same context. b) Arc expression within the IL was elevated following the recent and remote memory test in the Shift context. Arc expression was elevated following remote, but not recent memory expression in the Same context. c) Arc expression within the IL was elevated following the recent and remote memory test in the Shift context.
Arc Activity in the Prefrontal Cortex

Figure 4

Arc’Activity’in the Prefrontal Cortex

a
ACC

b
IL

C
PL

% Control

Shift
Same

1 Day
14 Days

% Control

Shift
Same

1 Day
14 Days

% Control

Shift
Same

1 Day
14 Days
Figure 5: Dorsal hippocampal expression of activity-dependent Arc following recent or remote memory tests in either the Same or Shift context. To assess changes in neural activation following memory expression, the data is expressed as percent change compared to home cage control baseline activity (baseline is represented as the dashed line at 100%). Arc expression within the CA1 (a), CA3 (b) and dentate gyrus (DG) (c) regions of the dorsal hippocampus were elevated following the recent and remote memory test in the Same context. Arc expression was elevated following recent, but not remote memory expression in the Shift context.
Figure 5

Arc Activity in the Dorsal Hippocampus

(a) dCA1
(b) dCA3
(c) dDG
Figure 6:  Ventral hippocampal expression of activity-dependent Arc following recent or remote memory tests in either the Same or Shift context. To assess changes in neural activation following memory expression, the data is expressed as percent change compared to home cage control baseline activity (baseline is represented as the dashed line at 100%). Arc expression within the CA1 (a), CA3 (b), and dentate gyrus (DG) (c) regions of the ventral hippocampus were elevated following the recent and remote memory test in both the Same and Shift context.
Arc Activity in the Dorsal Hippocampus

Figure 6
A 2 (Context) X 2 (Retention) factorial ANOVA was conducted on difference scores for the IL and yielded a non-significant main effect of Context, $F(1, 15) = 0.01, p = .99$, indicating no overall differences in IL activity between the Same and Shift contexts. There was a statistically significant main effect of Retention, $F(1, 15) = 4.44, p < .05$, indicating that IL activity was overall higher at 14 days than at 1 day post-training. There was a statistically significant Context by Retention interaction, $F(1, 15) = 20.71, p < .001$, indicating that IL activity was significantly lower at 1 day in the Same context than at 14 days in the Same context. IL activity did not significantly differ between 1 and 14 days post-training in the Shift context. These data suggest that IL activity underlies expression of both a contextually precise and a contextually imprecise fear memory.

Figure 4C shows grand mean (±SEM) difference scores for Arc mRNA activity in the PL for animals in all context groups and retention intervals. Animals tested in the Same context exhibit PL activation lower to that of baseline controls when tested 1 day following fear conditioning. However, PL activity greatly increases when animals are tested in the Same context at 14 days post-training. Again, this replicates Frankland, et al.’s (2004) finding indicating that the PL is only active in the expression of a remote, but not recent, context fear memory. Animals tested in the Shift context at both 1 day and 14 days following training exhibited an increase in PL activity. This suggests that the PL region of the mPFC is involved in expression of both a contextually precise and contextually imprecise fear memory.
A 2 (Context) x 2 (Retention) factorial ANOVA was conducted on difference scores for the PL and yielded a non-significant main effect of Context, $F(1, 14) = 3.58$, $p = .08$, indicating no overall differences in PL activity between the Same and Shift contexts. There was also no statistically significant main effect of Retention, $F(1, 14) = 3.73$, $p = .07$, indicating that PL activity was equivalent across both post-training intervals. There was a statistically significant Context by Retention interaction, $F(1, 14) = 7.83$, $p < .05$, indicating that PL activity was significantly lower at 1 day in the Same context than at 14 days in the Same context. PL activity did not significantly differ between 1 and 14 days post-training in the Shift context. These data suggest that PL activity underlies expression of both a contextually precise and a contextually imprecise fear memory.

Figure 5A shows grand mean (+SEM) difference scores for Arc mRNA activity in the dCA1 for animals in all context groups and retention intervals. Animals tested in the Same context exhibit very high dCA1 activation above baseline when tested both 1 day and 14 days following fear conditioning. This finding contrasts Frankland, et al.’s (2004) results indicating that the hippocampus is only active following the expression of a recent, but not remote, context fear memory. Animals tested in the Shift context 1 day following training exhibited a substantial increase in dCA1 activity. Interestingly, this activity appeared to decrease when animals were tested 14 days following training in the Shift context. This suggests that the dCA1 region of the hippocampus is involved in expression of a contextually precise fear
memory and that a reduction of activity in this region appears to underlie the loss context specificity or increase in fear generalization.

A 2 (Context) X 2 (Retention) factorial ANOVA was conducted on difference scores for the dCA1 and yielded a non-significant main effect of Context, $F(1, 14) = 0.01$, $p = .91$, indicating no overall differences in dCA1 activity between the Same and Shift contexts. There was a statistically significant main effect of Retention, $F(1, 14) 11.72$, $p< .01$, indicating that dCA1 activity was overall higher at 1 day than at 14 days post-training. There was a statistically significant Context by Retention interaction, $F(1, 14) = 38.87$, $p< .001$, indicating that dCA1 activity was significantly higher at 1 day in the Shift context than at 14 days in the Shift context. dCA1 activity did not significantly differ between 1 and 14 days post-training in the Same context. These data suggest that dCA1 activity underlies expression of a contextually precise fear memory and that a contextually imprecise fear memory involves the reduction of activity in this hippocampal region.

Figure 5B shows grand mean ($\pm$SEM) difference scores for Arc mRNA activity in the dCA3 for animals in all context groups and retention intervals. Animals tested in the Same context exhibit very high dCA3 activation above baseline when tested both 1 day and 14 days following fear conditioning. Again, this finding contrasts Frankland, et al.’s (2004) data indicating that the hippocampus is only active following the expression of a recent, but not remote, context fear memory. Animals tested in the Shift context 1 day following training exhibited a substantial increase in dCA3 activity. Mirroring dCA1 activity, activity of the dCA3 appeared to decrease
when animals were tested 14 days following training in the Shift context. This suggests that the dCA3 region of the hippocampus is involved in expression of a contextually precise fear memory and that a reduction of activity in this region appears to underlie the loss context specificity or increase in fear generalization.

A 2 (Context) X 2 (Retention) factorial ANOVA was conducted on difference scores for the dCA3 and yielded a non-significant main effect of Context, $F(1, 12) = 0.21, p = .65$, indicating no overall differences in dCA3 activity between the Same and Shift contexts. There was a statistically significant main effect of Retention, $F(1, 12) 7.32, p< .05$, indicating that dCA3 activity was overall higher at 1 day than at 14 days post-training. There was a statistically significant Context by Retention interaction, $F(1, 12) = 12.01, p< .01$, indicating that dCA3 activity was significantly higher at 1 day in the Shift context than at 14 days in the Shift context. dCA3 activity did not significantly differ between 1 and 14 days post-training in the Same context.

These data suggest that dCA3 activity underlies expression of a contextually precise fear memory and that a contextually imprecise fear memory involves the reduction of activity in this hippocampal region.

Figure 5C shows grand mean (+SEM) difference scores for Arc mRNA activity in the dDG for animals in all context groups and retention intervals. Animals tested in the Same context exhibit high dDG activation above baseline when tested both 1 day and 14 days following fear conditioning. Along with the dCA1 and dCA3 activity data, this result contrasts Frankland, et al.’s (2004) finding that the hippocampus is only active following the expression of a recent, but not remote, context fear
memory. Animals tested in the Shift context 1 day following training exhibited an increase in dDG activity. As with dCA1 and dCA3 activity, the dDG activity appeared to decrease when animals were tested 14 days following training in the Shift context. This suggests that the dDG region of the hippocampus is involved in expression of a contextually precise fear memory and that a reduction of activity in this region appears to underlie the loss context specificity or increase in fear generalization.

A 2 (Context) X 2 (Retention) factorial ANOVA was conducted on difference scores for the dDG and yielded a non-significant main effect of Context, $F(1, 13) = 2.74, p = .12$, indicating no overall differences in dDG activity between the Same and Shift contexts. There was no significant main effect of Retention, $F(1, 13) = 3.33, p = .09$, indicating no overall differences in dDG activity between 1 day and 14 days post-training. There was a statistically significant Context by Retention interaction, $F(1, 13) = 29.01, p < .001$, indicating that dDG activity was significantly higher at 1 day in the Shift context than at 14 days in the Shift context. dDG activity did not significantly differ between 1 and 14 days post-training in the Same context. These data suggest that along with the activity data from the dCA1 and dCA3 that dorsal hippocampus activity underlies expression of a contextually precise fear memory and that a contextually imprecise fear memory involves the reduction of activity in dorsal hippocampus.

Figure 6A shows grand mean (+SEM) difference scores for Arc mRNA activity in the vCA1 for animals in all context groups and retention intervals. Animals tested
in the Same context exhibit very high vCA1 activation above baseline when tested both 1 day and 14 days following fear conditioning. Animals tested in the Shift context similarly exhibit elevated activity of the vCA1 region of the hippocampus at both 1 and 14 days post-training. This suggests that the vCA1 region of the hippocampus is involved in expression of a contextually precise and imprecise fear memory.

A 2 (Context) X 2 (Retention) factorial ANOVA was conducted on difference scores for the vCA1 and yielded a non-significant main effect of Context, F(1, 12) = 1.38, p = .26, indicating no overall differences in vCA1 activity between the Same and Shift contexts. There was a statistically significant main effect of Retention, F(1, 12) 5.61, p< .05, indicating that vCA1 activity was overall higher at 1 day than at 14 days post-training. There was not a significant Context by Retention interaction, F(1, 12) = 3.08, p=.11, indicating that vCA1 activity remains high in both Context groups at both Retention time points. These data suggest that vCA1 activity underlies expression of both a contextually precise fear memory as well as a contextually imprecise fear memory.

Figure 6B shows grand mean (+SEM) difference scores for Arc mRNA activity in the vCA3 for animals in all context groups and retention intervals. Animals tested in the Same context exhibit very high vCA3 activation above baseline when tested both 1 day and 14 days following fear conditioning. Animals tested in the Shift context similarly exhibit elevated activity of the vCA3 region of the hippocampus at both 1 and 14 days post-training. This suggests that the vCA3 region of the
hippocampus is involved in expression of a contextually precise and imprecise fear memory.

A 2 (Context) X 2 (Retention) factorial ANOVA was conducted on difference scores for the vCA3 and yielded a non-significant main effect of Context, $F(1, 12) = 0.01$, $p = .91$, indicating no overall differences in vCA3 activity between the Same and Shift contexts. There was a statistically significant main effect of Retention, $F(1, 12) = 7.25$, $p < .05$, indicating that vCA3 activity was overall higher at 1 day than at 14 days post-training. However, there was a significant Context by Retention interaction, $F(1, 12) = 9.65$, $p < .01$, indicating that vCA3 activity was significantly higher at 1 day in the Same context than at 14 days in the Same context. vCA3 activity did not significantly differ between 1 and 14 days post-training in the Shift context. These data suggest that vCA3 activity underlies expression of both a contextually precise fear memory as well as a contextually imprecise fear memory.

Figure 6C shows grand mean (+SEM) difference scores for Arc mRNA activity in the vDG for animals in all context groups and retention intervals. Animals tested in the Same context exhibited elevated vDG activation above baseline when tested both 1 day and 14 days following fear conditioning. Animals tested in the Shift context similarly exhibited elevated activity of the vDG region of the hippocampus at both 1 and 14 days post-training. This suggests that the vDG region of the hippocampus is involved in expression of both a contextually precise and imprecise fear memory.
A 2 (Context) X 2 (Retention) factorial ANOVA was conducted on difference scores for the vDG and yielded a statistically significant main effect of Context, $F(1, 12) = 4.82, p< .05$, indicating that the vDG was more active during expression of fear in the Same context than during expression of fear in the Shift Context. There was a statistically significant main effect of Retention, $F(1, 12) = 6.5, p< .05$, indicating that vDG activity was overall higher at 1 day than at 14 days post-training. However, there was no significant Context by Retention interaction, $F(1, 12) = 0.59, p=.46$.

These data suggest that vDG activity underlies expression of a contextually precise fear memory and that the reduction of vDG activity underlies a contextually imprecise fear memory.

Taken together, these data suggest that expression of a contextually precise fear memory involves activity of both the hippocampus (dorsal and ventral) and areas of the cortex. Expression of a contextually imprecise, or a generalized memory, appears to involve activity of the mPFC and the ACC. However, surprisingly we found that expression of a generalized memory appears to involve a decrease in the activity of the dorsal hippocampus and elevated activity of the ventral hippocampus. This suggests that the hippocampus is involved in the expression of a remote context memory and that the ventral hippocampus may be involved in the loss of context-specificity. To investigate the potential interaction between the cortex and the hippocampus indicated by the activation analyses, we next inactivated several neural structures to verify their activity in a precise and imprecise context memory and to better assess their role in this process.
**ACC inactivation at remote time points returns context-specificity to the memory.** The activity data indicated that the ACC is active during expression of both a contextually precise memory at 1 day post-training and during expression of a contextually imprecise fear memory at 14 days post-training. To determine whether this cortical structure is required for expression of both types of memory, we temporarily inactivated it by infusing the sodium channel blocker lidocaine into the region. Even though the ACC appeared highly active at a recent time point in the novel context (in which case the animal is not freezing), it was hypothesized that ACC inactivation at a recent time point would have no effect on expression of a contextually precise fear memory. In other words, ACC inactivation would not change the already low freezing in the novel context at a recent time point. Though the ACC was found to be highly active during expression of a precise memory, the hippocampus was also highly active and has been shown to be capable of expressing a context-specific memory ([Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998; Ruediger et al., 2011; Wiltgen et al., 2010; Winocur et al., 2007; 2009]). However, we did predict that inactivating the ACC at remote time points would result in a reduction of generalized fear expression. In other words, we predicted that the inactivation of this cortical structure would reduce fear expression in the novel context at remote time points. To test these hypotheses, we contextually fear conditioned mice and a recent or remote time point and tested for learned fear in
either the same or a shifted context. Ten minutes prior to testing, animals were infused with either lidocaine or vehicle (PBS) directly into the ACC.

Figure 7A shows grand mean (+SEM) freezing scores for animals in both context groups (Same vs. Shift) and both drug conditions (lidocaine vs. PBS). At 1 day post-training, drug control (PBS) animals in the Shift condition froze less than control animals in the Same condition. This difference indicates the context shift effect is present at 1 day following fear training. In other words, animals infused with PBS and tested in the novel context are exhibiting a contextually precise memory and are not expressing fear in the presence of the novel cues. Animals infused with lidocaine into the ACC exhibited a similar pattern of fear behavior. Lidocaine animals in the Shift condition had lower freezing scores than lidocaine animals in the Same condition. Temporary inactivation of the ACC did not alter expression of a contextually precise fear memory at 1 day post-training. Further, it is not surprising that inactivation of the ACC did not disrupt fear expression in the Same context since our activation data and that of Frankland, et al.’s (2004) data suggest that this cortical region is not active or involved in expression of a recent context memory.

A 2 (context) X 2 (drug) ANOVA was conducted on freezing scores and yielded a statistically significant main effect of Context (Same vs. Shift), $F(1, 24) = 65.04, p< .001$, indicating that overall animals in the Shift condition froze significantly less than animals in the Same condition. There was not a significant main effect of Drug (1 lidocaine vs. PBS), $F(1, 24) = 0.76, p = .39$, indicating that
overall there were no differences in freezing scores between lidocaine and vehicle (PBS). The analysis yielded a non-significant Context by Drug interaction, $F(1, 24) = 0.09$, $p = .77$. This lack of significant interaction indicates that inactivating the ACC at 1 day post-training had no effect on freezing behavior in either context. Regardless of the drug condition, animals froze equivalently high in the Same context and equivalently low in the Shift context.

Figure 7B shows grand mean (+SEM) freezing scores for animals in both context groups (Same vs. Shift) and both drug conditions (lidocaine vs. PBS). At 14 days post-training, drug control (PBS) animals in the Shift condition froze slightly less than control animals in the Same condition. While it appears some context specificity was lost, the control animals did completely generalize their fear from the training context to the novel context. In other words, animals infused with PBS and tested in the novel context are in the process of gradually losing precision of the context memory, but are still exhibiting some context specificity. However, animals infused with lidocaine into the ACC exhibited a reduction in freezing in the presence of the Shift context compared the PBS animals in the Shift context. Further, lidocaine animals in the Shift condition had lower freezing scores than lidocaine animals in the Same condition. These data suggest that inactivation of the ACC returns the context memory back to its precise state.

A 2 (context) X 2 (drug) ANOVA was conducted on freezing scores at 14 days post-training and yielded a statistically significant main effect of Context (Same vs. Shift), $F(1, 26) = 12.20$, $p < .01$, indicating that overall animals in the Shift condition
Figure 7: Grand mean (+ SEM) percent time spent freezing for C57BL/6 mice in ACC inactivation experiment. Groups labeled as Same were tested in the same context they were trained in. Groups labeled Shift were tested in a novel context. 

a) 1 Day post-training: inactivating the ACC with lidocaine had no effect on expression of a contextually precise fear memory in the Shift context nor did it reduce freezing in the Same context. 

b) 14 Days post-training: Infusing lidocaine into the ACC did alter fear expression in animals tested in the Same context. Inactivation of the ACC reduced freezing in the Shift context compared to PBS infused animals. 

c) 21 Days post-training: Infusing lidocaine into the ACC did alter fear expression in animals tested in the Same context. Inactivation of the ACC significantly reduced freezing in the Shift context. ACC inactivation at remote time points returned context specificity to the fear memory. * p< .05
Figure 7

(a) 

PBS  

Lidocaine

Same Context  

Shift Context

(b)  

(c)
froze significantly less than animals in the Same condition. There was not a significant main effect of Drug (1 lidocaine vs. PBS), $F(1, 26) = 1.68, p = .21$, indicating that overall there were no differences in freezing scores between lidocaine and vehicle (PBS). The analysis yielded a non-significant Context by Drug interaction, $F(1, 24) = 1.06, p = .31$. This lack of significant interaction indicates that inactivating the ACC at 14 days post-training had no effect on freezing behavior in either context. Regardless of the drug condition, animals froze equivalently high in the Same context and equivalently low in the Shift context. While it appears as though ACC inactivation reduced generalized freezing at 14 days, there was not a significant interaction. This lack of an effect may be due to the PBS animals not having completely generalized their fear to the novel context by 14 days, thereby masking any potential effects lidocaine may have had on freezing behavior. Therefore, we chose to run an additional inactivation experiment at 21 days to ensure a complete loss of context-specificity is achieved in the vehicle Shift animals.

Figure 7C shows grand mean (+SEM) freezing scores for animals in both context groups (Same vs. Shift) and both drug conditions (lidocaine vs. PBS). At 21 days post-training, drug control (PBS) animals in the Shift condition froze at equivalent levels as vehicle control animals in the Same condition. Thus, at 21 days post-training, control animals exhibited a complete loss of context specificity and have generalized their fear from the training context to the novel context. However, animals infused with lidocaine into the ACC exhibited a reduction in freezing in the presence of the Shift context compared the PBS animals in the Shift context. Further,
lidocaine animals in the Shift condition had lower freezing scores than lidocaine animals in the Same condition. These data are further evidence that inactivation of the ACC returns the context memory back to its precise state.

A 2 (context) X 2 (drug) analysis of variance (ANOVA) was conducted on freezing scores at 21 days post-training and yielded a statistically significant main effect of Context (Same vs. Shift), $F(1, 27) = 14.45, p< .01$, indicating that overall animals in the Shift condition froze significantly less than animals in the Same condition. There was not a significant main effect of Drug (1 lidocaine vs. PBS), $F(1, 27) = 2.80, p= .10$, indicating that overall there were no differences in freezing scores between lidocaine and vehicle (PBS). However, the analysis yielded a statistically significant Context by Drug interaction, $F(1, 27) = 10.67, p< .01$. This significant interaction indicates ACC inactivation resulted in a significantly lower level of freezing in the Shift condition compared to animals in the Same condition and PBS animals in the Shift condition. These data, along with the 14 day data, suggest that the ACC is involved in expression of a contextually imprecise memory and that inactivation of this cortical structure returns the context memory back to its precise state. It should be noted that we did not impair expression of a remote context fear memory when animals were tested in the Same context when we inactivated the ACC. This suggests that the ACC may not be required for the expression of a remote context fear memory.
A switch from dorsal hippocampal to ventral hippocampal processing may underlie the time-dependent loss of context-specificity.

The activity data indicated that the dCA1 region of the hippocampus is active during expression of a contextually precise memory at a recent time point but not during expression of a contextually imprecise fear memory at a remote time point. Since we found animals had not completely generalized fear by 14 days in the ACC inactivation experiments, we chose to run a 21-day test to ensure that mice would generalize their fear in the control condition. Further, the activation data indicated that the vCA1 region of the hippocampus is active during expression of both a contextually precise and a contextually imprecise memory. To further investigate the role of the hippocampus in the precision of a context fear memory, we temporarily inactivated either the dorsal or ventral CA1 regions of the hippocampus by infusing the sodium channel blocker lidocaine into the regions. Based on our activity data, it was hypothesized that dCA1 and vCA1 would reduce fear expression in the Same context at 1 day following fear conditioning. However, we predicted that inactivating either hippocampal region would have no effect on the typical low levels of fear expressed in the Shift condition at 1 day. We also predicted that inactivating the dCA1 would reduce freezing in the Same, but not Shift, context at a remote time point since this area was only active in the Same context at the remote time point. Further, since the vCA1 region was active in both contexts at remote time points, we predicted that inactivation of the vCA1 would reduce fear expression in both the Same and Shift contexts at time 21 days post-training.
Figure 6A shows grand mean (+SEM) freezing scores for animals in both context groups (Same vs. Shift) and both drug conditions (lidocaine vs. PBS) for animals in the 1 day dCA1 inactivation experiment. At 1 day post-training, drug control (PBS) animals in the Shift condition froze less than control animals in the Same condition. This difference indicates the context shift effect is present at 1 day following fear training. In other words, animals infused with PBS and tested in the novel context are exhibiting a contextually precise memory and are not expressing fear in the presence of the novel cues. Animals infused with lidocaine into the dCA1 exhibited a similar pattern of fear behavior. Lidocaine animals in the Shift condition had lower freezing scores than lidocaine animals in the Same condition. Temporary inactivation of the dCA1 did not alter expression of a contextually precise fear memory at 1 day post-training. However, we did not see a decrease in fear expression in the same context at 1 day post-training. Numerous studies over the last several decades have shown a recent context fear memory is dependent upon hippocampal functioning/activity (Anagnostaras, Gale, & Fanselow, 2001; Clark, Broadbent, Zola, & Squire, 2002; Fanselow, 2000; Frankland et al., 1998; Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004; Holland, P.C. & Bouton, 1999; J. J. Kim & Fanselow, 1992; McDonald & White, 1993; Squire, 2006; Squire & Alvarez, 1995; Squire, Slater, P.C., & Chace, 1976; Wiltgen et al., 2010; Zola-Morgan & Squire, 1990)). Therefore, it is likely that somehow, perhaps due to behavioral variability or some unknown confounding variable, we were unsuccessful in inactivating the dCA1 of the hippocampus and consequently did not disrupt fear expression.
A 2 (context) X 2 (drug) ANOVA was conducted on freezing scores and yielded a statistically significant main effect of Context (Same vs. Shift), $F(1, 16) = 60.04, p< .001$, indicating that overall animals in the Shift condition froze significantly less than animals in the Same condition. There was not a significant main effect of Drug (lidocaine vs. PBS), $F(1, 16) = 0.29, p=.58$, indicating that overall there were no differences in freezing scores between lidocaine and vehicle (PBS). The analysis yielded a non-significant Context by Drug interaction, $F(1, 16) = 0.01, p=.91$. This lack of significant interaction indicates that inactivating the dCA1 at 1 day post-training had no effect on freezing behavior in either context. Regardless of the drug condition, animals froze equivalently high in the Same context and equivalently low in the Shift context. Again, the finding that inactivation of the dCA1 region of the hippocampus did not reduce fear expression in the Same context is both unexpected and troubling. These data would either suggest that the lidocaine did not work, or the hippocampus is not required for expression of a recent context fear memory.

Figure 8B shows grand mean (+SEM) freezing scores for animals in both context groups (Same vs. Shift) and both drug conditions (lidocaine vs. PBS) for animals in the 21-day dCA1 inactivation experiment. At 21 days post-training, drug control (PBS) animals in the Shift and Same conditions froze at equivalent levels, indicating that control animals exhibited a loss of context specificity and had generalized their fear from the training context to the novel context. Lidocaine animals in the Same condition exhibited lower freezing scores than vehicle animals
Figure 8: Grand mean (+ SEM) percent time spent freezing for C57BL/6 mice in dorsal and ventral hippocampus inactivation experiment. Groups labeled as Same were tested in the same context they were trained in. Groups labeled Shift were tested in a novel context. a) 1 Day post-training dCA1 inactivation: inactivating the dCA1 with lidocaine had no effect on expression of a contextually precise fear memory in the Shift context nor did it reduce freezing in the Same context. b) 21 Days post-training dCA1 inactivation: Infusing lidocaine into the dCA1 did lower fear expression in animals tested in the Same context. Inactivation of the dCA1 did not alter freezing in the Shift context compared to PBS infused animals. c) 1 Day post-training vCA1 inactivation: inactivating the vCA1 with lidocaine had no effect on expression of a contextually precise fear memory in the Shift context nor did it reduce freezing in the Same context. d) Infusing lidocaine into the vCA1 lowered fear expression in animals tested in the Same context. Inactivation of the vCA1 also significantly reduced freezing in the Shift context. * p < .05
Figure 8

(a) 1 Day Dorsal CA1
(b) 21 Day Dorsal CA1
(c) 1 Day Ventral CA1
(d) 21 Day Ventral CA1

PBS
Lidocaine

% Freezing

Same
Shift

*
in the Same condition. This suggests that expression of a remote context memory in the Same context requires dorsal hippocampal activity. However, inactivating the dCA1 did not reduce freezing levels in the Shift condition, suggesting that expression of a contextually precise fear memory is independent of the dorsal hippocampus.

A 2 (context) X 2 (drug) ANOVA was conducted on freezing scores and yielded non-significant main effect of Context (Same vs. Shift), $F(1, 15) = .38, p = .55$, indicating that overall animals in the Shift condition froze at equivalent levels compared to animals in the Same condition. There was not a significant main effect of Drug (lidocaine vs. PBS), $F(1, 15) = 1.88, p = .19$, indicating that overall there were no differences in freezing scores between lidocaine and vehicle (PBS). The analysis yielded a statistically significant Context by Drug interaction, $F(1, 15) = 7.62, p < .05$. This significant interaction indicates that inactivating the dCA1 at 21 days post-training reduced freezing in only the Same context. Inactivating the dCA1 at 21 days had no effect on fear expression in the Shift context (i.e. a contextually imprecise memory was expressed in the absence of the dCA1). This supports the activation data that showed a significant reduction in dCA1 activity when animals were tested in the Shift condition at a remote time point.

Figure 8C shows grand mean (±SEM) freezing scores for animals in both context groups (Same vs. Shift) and both drug conditions (lidocaine vs. PBS) for animals in the 1 day vCA1 inactivation experiment. At 1 day post-training, drug control (PBS) animals in the Shift condition froze less than control animals in the Same condition. This difference indicates the context shift effect is present at 1 day
following fear training. In other words, animals infused with PBS and tested in the novel context are exhibiting a contextually precise memory and are not expressing fear in the presence of the novel cues. Animals infused with lidocaine into the vCA1 exhibited a similar pattern of fear behavior. Lidocaine animals in the Shift condition had lower freezing scores than lidocaine animals in the Same condition. Temporary inactivation of the vCA1 did not alter expression of a contextually precise fear memory at 1 day post-training. However, we did not see a decrease in fear expression in the same context at 1 day post-training. Again, as with the dCA1 1 day data, this is surprising since a large number of studies have demonstrated the necessity of the hippocampus for expression of a context memory at recent time points. Therefore, it is likely that along with the 1-day dCA1 groups, there was some confounding variable that resulted in the lidocaine not having an effect on freezing behavior.

A 2 (context) X 2 (drug) ANOVA was conducted on freezing scores and yielded a statistically significant main effect of Context (Same vs. Shift), $F(1, 17) = 53.99$, $p < .001$, indicating that overall animals in the Shift condition froze significantly less than animals in the Same condition. There was not a significant main effect of Drug (lidocaine vs. PBS), $F(1, 17) = 0.21$, $p = .65$, indicating that overall there were no differences in freezing scores between lidocaine and vehicle (PBS). The analysis yielded a non-significant Context by Drug interaction, $F(1, 17) = 0.07$, $p = .79$. This lack of significant interaction indicates that inactivating the vCA1 at 1 day post-training had no effect on freezing behavior in either context. Regardless of
the drug condition, animals froze equivalently high in the Same context and equivalently low in the Shift context.

Figure 8D shows grand mean (+SEM) freezing scores for animals in both context groups (Same vs. Shift) and both drug conditions (lidocaine vs. PBS) for animals in the 21-day vCA1 inactivation experiment. At 21 days post-training, drug control (PBS) animals in the Shift and Same conditions froze at equivalent levels, indicating that control animals exhibited a loss of context specificity and had generalized their fear from the training context to the novel context. Lidocaine animals in the both the Same and Shift contexts exhibited lower freezing scores than vehicle animals in the Same and Shift contexts. This suggests that expression of a remote context memory in either context requires ventral hippocampal activity. These data suggest that the ventral hippocampus plays an extended role the expression of a remote context memory and a contextually imprecise memory.

A 2 (context) X 2 (drug) ANOVA was conducted on freezing scores and yielded non-significant main effect of Context (Same vs. Shift), $F(1, 11) = 3.45$, $p = .09$, indicating that overall animals in the Shift condition froze at equivalent levels compared to animals in the Same condition. There was a significant main effect of Drug (lidocaine vs. PBS), $F(1, 11) = 25.69$, $p < .001$, indicating that overall animals in the lidocaine condition froze significantly less than animals in the PBS condition. The analysis yielded a non-significant Context by Drug interaction, $F(1, 11) = .13$, $p = .72$. These results indicate that expression of a remote context fear memory and a contextually imprecise fear memory requires activity of the vCA1 of the
hippocampus. These data support what was found in the activation analyses above indicating that expression of fear in both the Same and Shift contexts at remote time points involves increased activity of the ventral hippocampus.
DISCUSSION

The current study investigated the neural pattern of activation following expression of a contextually precise fear memory and a contextually imprecise fear memory. Using C57BL/6 mice and context fear conditioning, we replicated the basic context shift effect and gradual loss of context specificity, or gradual increase in fear generalization, over time. Specifically, we found that mice that were tested in a novel context 24 hours following fear conditioning exhibited significantly lower levels of freezing, or fear expression, compared to animals tested in the training context. However, when animals were tested at a remote time point, or 14 days following fear conditioning, they exhibited equivalent levels of freezing in both the novel and training contexts indicating they had completely generalized their fear from the training context to the novel context. In other words, animals exhibited a contextually imprecise memory at the remote time point. Brains from these animals were extracted 30 minutes following testing and were probed for Arc mRNA expression to investigate the neural structures that were active during expression of either a contextually precise or imprecise fear memory.

Firstly, we partially replicated Frankland, et al.’s (2004) finding that the hippocampus, but not specific regions of the cortex, is highly active during
expression of a recent context fear memory (1-day post-training) when animals are tested in the training context. Specifically, we found little to no activity (compared to home cage controls) in the IL and PL regions of the mPFC as well as little activity in the ACC. Both the dorsal hippocampus (CA1, CA3, and DG) and ventral hippocampus (CA1, CA3, DG) are highly active when animals are placed back into the training context 1-day following training. We did fail to replicate Frankland, et al.’s (2004) finding that the cortex, but not hippocampus, is highly active during expression of a remote context fear memory. Our data indicate that not only are there increases in activity of the ACC, IL, and PL regions of the cortex, but both the dorsal and ventral regions of the hippocampus were active during expression of a remote context memory. These data suggest that the expression of a remote context memory involves a potential interaction, or at the very least increased activity, between the hippocampus and the cortex.

Our primary interest in Study 1 was to investigate the neural pattern of activation underlying expression of both a contextually precise and imprecise memory fear memory. During expression of a contextually precise memory (animals tested at 1-day post-training in the novel context), the ACC, IL, and PL were highly active along with both dorsal and ventral regions of the hippocampus. Further, with the exception of the dorsal hippocampus, which was significantly less active, this pattern of activation remained during expression of a contextually imprecise memory, during which animals expressed generalized fear to the novel context at 14 days post-training. These data suggest that an interaction between the cortex and
hippocampus underlies expression of both a contextually precise and a contextually imprecise fear memory. In an attempt to dissect this cortical-hippocampal interaction, we inactivated the ACC and hippocampus at recent and remote time points prior to testing. Inactivating the ACC at a recent time point had no effect on expression of fear in the training context nor did it have any effect on expression of a contextually precise memory. However, inactivating the ACC at remote time points had no effect on expression of fear in the training context, but did reduce fear expression in the novel context. In other words, inactivating the ACC returned context-specificity to the memory and reduced expression of generalized fear.

Inactivation of the dorsal and ventral CA1 regions of the hippocampus present a less clear picture of what neural processes may be underlying the expression of context specificity. Specifically, we found that inactivating both the dorsal and ventral hippocampus had no effect on behavior when animals were tested for fear 1 day following training. Temporarily inactivating either hippocampal region failed to reduce freezing in the presence of the training context and did not alter the animals’ low levels of freezing in the novel context 1 day following training. These data would suggest that the hippocampus is not required for the expression of a recent context memory. However, this result was unexpected since a recent memory has been shown countless times to involve processing of the hippocampus. It is likely that some unknown variable out of our control contributed to this result. These 1-day experiments need to be conducted again in order to determine whether the hippocampus is actually involved in expression of a recent
contextually precise fear memory. However, the 21-day hippocampal inactivation did support what the Arc activity indicated. Inactivating the dorsal CA1 region of the hippocampus reduced freezing the training context, but not in the novel context. These data suggest that the dorsal hippocampus is involved in the expression of a remote context memory, but not involved in the expression of a contextually imprecise generalized fear memory. This finding supports the Arc data, which showed increased dorsal hippocampus activity when animals were tested for fear in the training context at the remote time point. However, we found a significant decrease in dorsal hippocampal activity when animals were tested for generalized fear in the novel context at the remote time point. In addition to the dorsal hippocampus, we discovered that inactivating the ventral CA1 region of the hippocampus at remote time points similarly reduced freezing in the training context. We also discovered that inactivating this region of the hippocampus reduced freezing in the presence of the novel context at the remote time point. In other words, inactivating the ventral hippocampus reduced generalized fear expression and appeared to return the memory back towards a contextually precise state.

The data presented in Study 1 provide evidence against the transformation of memory hypothesis ((Winocur et al., 2007)) proposed to account for the gradual reduction in context-specificity that occurs following context fear conditioning. This hypothesis states that the gradual increase in generalization between the training context and a novel context results from the transformation of the memory trace
from a context-specific hippocampus-dependent memory to a hippocampal-independent cortical memory that lacks context specificity. In other words, the context memory is stored in the cortex not in its original form, but as a general or schematic-like representation that gets reactivated in the presence of similar contexts. This hypothesis states that expression of a contextually imprecise generalized fear memory results from activation of a cortical memory and therefore we should only see increased activation of the cortex. This hypothesis would predict that the hippocampus would not be active during expression of a contextually imprecise schematic-like cortical memory. However, our activation data suggest that the hippocampus is active during expression of a remote generalized memory. We even found that expression of a remote context memory (animals tested in the training context) involved activity of both the dorsal and ventral hippocampus. The finding that the hippocampus is active at remote time points suggests that the memory trace may not be “leaving” the hippocampus over time. Further, inactivating the ACC at remote time points returned the context memory back to its precise state. If the memory was transformed away from a contextually precise memory, it should not be able to return to its precise state. One could argue that the reduction in freezing exhibited in ACC-inactivated animals in the novel context is due to a general impairment of the context memory. In other words, you would expect to see a reduction in freezing if the cortical memory is being pharmacologically inhibited or impaired. However, ACC-inactivated animals did not exhibit reduced freezing when tested at remote time points in the training context.
The effect was specific to the novel context, suggesting that the original context memory is not being transformed into a generalized cortical memory. At the very least, the data suggests that the animals are not using one memory or even the same neural mechanism to retrieve a contextually imprecise fear memory.

The data from the current study partially support the trace-decay theory ((Rudy, 2005)) that states the loss of context specificity is due to a decay in the strength or quality of the hippocampal memory trace. According to this theory, the mPFC comes online at remote time points to aid in the retrieval of the decayed hippocampal trace. This theory states that expression of a contextually imprecise generalized fear memory results from an interaction between the cortex and hippocampus and therefore would make the prediction that both cortical and hippocampal activation underlies expression of a generalized memory. We did find that expression of a contextually imprecise fear memory involved activation of the mPFC, ACC, and the ventral hippocampus. Further, a contextually imprecise memory involved a reduction of activity of the dorsal hippocampus. This reduction in dorsal hippocampal activity may be a result of the hippocampal trace decaying at remote time points. The IL, PL, and/or ACC regions of the cortex are then activated in an attempt to aid in the retrieval of the decayed hippocampal trace, resulting in expression of generalized fear. The ventral hippocampal activity fits nicely with the trace-decay theory, as the ventral CA1 region is the primary output region of the hippocampus. The activity of this region may be sending projections either to the ACC and other cortical regions to recruit cortical compensation or may be sending
projections to the amygdala, resulting in fear expression. The activation data following expression of fear in the training context at the remote time point also fits with the trace-decay hypothesis. Firstly, the cortex was not active during testing 1-day following training. This is expected since the trace has not had time to decay and therefore activity of the mPFC is not required. Additionally, the finding that both the dorsal and ventral hippocampus were active during expression of a remote context memory (tested in the training context), and that the inactivation of these structures impaired fear expression at remote time points, fits with the trace-decay theory as well. Placing an animal back into the training context reminds the animal that they are in the particular context they were shocked and re-strengthens the hippocampal trace, thereby increasing hippocampal activity. However, the cortical data following expression of a remote context fear memory in the training context does not fit with the trace decay hypothesis. If the hippocampal trace is re-strengthened by re-exposure to the training context, there should be no need for cortical activation (the hippocampus can express the memory on its own). We found high cortical activity along with the increased hippocampal activity following expression of a remote context memory. Further, the cortical activation, assuming it is related to memory retrieval/expression, following expression of a contextually precise memory do not fit with the trace-decay theory. At a recent time point, the hippocampal trace is still strong and capable of expressing context-specificity and therefore should not require cortex activity. Another puzzling finding is that inactivating the ACC at a remote time point, presumably when the cortex is needed to help retrieve the
decayed memory trace, results in returning the memory back to its precise state.

This suggests that the hippocampus is capable of expressing a precise remote fear memory and that recruitment of the ACC reduces the context-specificity, which contradicts the trace-decay theories’ account for the loss of memory precision. Though are data strongly support the trace-decay hypothesis, they do slightly contradict some of the predications made. It is possible that the hippocampal trace decays as the memory ages, however it does not explain why blocking cortical involvement returned the memory back to a precise state.

Given that the transformation and trace-decay hypotheses cannot completely account for the results found in this study, an alternative explanation is needed. One possible alternative explanation, though not the simplest, is that there are multiple processes underlying expression of fear in either the training context or the novel context and which process takes over is dependent upon the context. It is possible that when animals are tested in a novel context, additional processing is required. It may be the case that when an animal is unsure of the environment, such as in the case of being placed in a similar but different context from that of training, they require higher order cortical processing in order to determine whether or not they are in the “danger” context. This could work in conjunction with the trace-decay hypothesis. For instance, at an early time point, the decision is made to not freeze but at later time points, perhaps as the strength of memory trace decays and the animal becomes more unsure of the context, the decision is made to freeze.
There is evidence provided by Winocur, et al. (2009) that at some level the animals can perceive differences between the training context and the novel context at remote time points even though they are freezing equally in both. Specifically, Winocur et al. (2009) administered a reactivation/reminder session by briefly exposing animals to the training context prior to hippocampally lesioning the animals. It has been well established that administering a reminder in the training context at remote time points briefly re-sharpens the generalization gradient ((Gisquet-Verrier & Alexinsky, 1986; Jasnow et al., 2012; Moye & Thomas, 1982; Ruediger et al., 2011; Wiltgen & Silva, 2007; Winocur et al., 2009; Y. L. Zhou & Riccio, 1994)). The exposure to the training context reminds animals of the specifics of the context and if tested shortly after the reminder, exhibit a contextually precise memory and do not freeze in the presence of the novel context even at remote time points. Winocur, et al., (2009) administered the reminder and then lesioned the hippocampus. They discovered that the non-lesioned controls exhibited a context-specific memory following the reminder. However, hippocampal lesions following the reminder eliminated freezing in both the training and novel context. However, when the animals were given a reactivation/reminder treatment with the novel context (a context they were now freezing in as if it were the training context), no subsequent sharpening of the generalization gradient was discovered. Further, hippocampal lesions following a reminder in the novel context failed to reduce freezing in either the training or novel context. These data suggest that even though the animals were treating the two contexts the same behaviorally (i.e. freezing
equivalently in both the training and novel context), they were not being treated equivalently at the neural level. If the animals could not perceive or could not discriminate between the training context and the novel context, a reminder in the novel context should have had the same effect as a reminder in the same context. The finding that it did not have the same effect suggests that at some level, the animals can still perceive differences in the two contexts or even potentially discriminate between the novel context and the same context. This would suggest that even though they can perceive a difference in the two contexts at the neural level, something is driving fear expression in the novel context at remote time points. Based on the data presented in this study, the ACC may be the overriding mechanism driving freezing behavior at remote time points. When the ACC was inactivated at remote time points the animals reduced their freezing in the novel context, suggesting that by blocking this higher-order cortical involvement the memory is expressed as contextually precise. However, why the animals do not freeze at a recent time point but do at a remote time point when in a novel context is unclear.

Data from the current study, along with the reminder data from Winocur, et al., (2009), suggest that expression of fear in the training context and expression of fear in a novel context involve separate processes that ultimately decide whether fear will be expressed in the presence of the contextual cues. It is clear that expression of a contextually precise memory involves the interaction between the hippocampus and the prefrontal cortex. However, what is not well understood is the
functional relationship between the cortex, in particular the ACC, and the hippocampus. For instance, it is possible that the ACC is somehow driving hippocampal activity and subsequent freezing in the novel context at remote time points. It is also possible that the hippocampus is driving ACC activity/recruitment that somehow results in expression of a contextually imprecise memory. To gain a better understanding of the direction of this relationship, follow up studies should be conducted in which hippocampal and ACC inactivation are followed by *in situ* hybridization and Arc mRNA analysis to determine if inactivating one changes activity in the other. For instance, does inactivating the dorsal or ventral hippocampus at either recent or remote time points reduce ACC activity and impair subsequent fear expression? Or it may be possible that inactivating the ACC reduces activity in the hippocampus, suggesting the ACC may be driving expression of a contextually precise fear memory. In addition to inactivation studies, subsequent experiments could be aimed at artificially driving fear expression in the novel context to gain a better understanding of the functional relation between the hippocampus and prefrontal cortex. For instance, would driving activity in areas of the cortex, such as the ACC, either through optogenetic stimulation or through administration of a glutamatergic agonist, increase freezing in the novel context at a recent time point? This could potentially shed light on the directionality and functional relationship between the hippocampus and prefrontal cortex and help determine which neural structures drive expression of a contextually imprecise fear memory.
In addition to manipulating activity levels in different neural regions, an investigation into the type of cells that are active during expression of either a contextually precise versus imprecise memory may help shed light on the somewhat complicates pattern of neural activation discovered in the current study. For instance, we found that the ACC was highly active at 1-day post-training, but inactivating this structure did not alter expression of a contextually precise memory. However, inactivating the ACC at a remote time point did reduce generalized freezing. It may be the case that this change in ACC depdencey may reflect a change in inhibitory to excitatory activity. In other words, inhibitory GABAergic cells may be active during expression of a contextually precise memory and excitatory glutamatergic cells may be active during expression of a contextually imprecise memory. To investigate this possibility across all neural areas of interest, a dual fluorescent in situ hybridization could be conducted in which the tissue is probed for both Arc activity as well as glutamatergic activity (probing for vglut – the glutamatergic vesicular transporter). This could potentially shed light onto whether the hippocampus and cortical regions are expressing excitatory or inhibitory activity. This in turn could be informative as to what type of activity underlies expression of a contextually precise and imprecise fear memory in addition to what areas are involved.
Study 2

The purpose of this study was to investigate the role of presynaptic GABA\textsubscript{B(1a)} receptors in context discrimination. It has been previously demonstrated that these receptors are required for cued discrimination (Shaban et al., 2006). However it remains unclear what role, if any, these receptors play in the discrimination, or memory precision, of contextual cues. Therefore, we used a strain of mice that have had GABA\textsubscript{B(1a)} receptors selectively ablated to investigate the role the receptors play in the consolidation and expression of a context fear memory. To achieve this, we used context fear conditioning and tested animals at different time points (2 hours, 1 day, or 5 days following fear conditioning) in either the same or a novel context. We used both GABA\textsubscript{B(1a)} knock out animals and wild-type litter-mate controls, which are animals that do not express the GABA\textsubscript{B(1a)} knock out. Using the three time points, we were be able to determine whether mice lacking GABA\textsubscript{B(1a)} receptors can discriminate between two contexts and allowed us to track the loss of context-specificity over time in these animals.

In addition to context discrimination, we were also interested in using a non-fearful discrimination task to gain a better understanding for the role of GABA\textsubscript{B(1a)} receptors in the discrimination process. Based on findings from the context fear conditioning study, we chose to run both novel object recognition and novel object location procedures and use different retention intervals (2 hours and 1 day
following training) to investigate the role of presynaptic inhibition in the precision of a non-fearful spatial memory.
METHOD

Subjects

All experiments were conducted on male GABA_B1a knock out (GABA_{B1a}^{+/−}) mice (n = 52) and male wild type (WT) mice (n = 52) (a generous gift from Dr. Bernhard Bettler and Dr. Martin Gassmann, University of Basel). Briefly, GABA_{B1a}^{+/−} mice were created on a BALB/c background in which the translation of GABA_{B1a} proteins was prevented by mutating initiation codons of mRNA into stop codons. This mutation does not affect the normal transcription of GABA_{B1a} mRNA; it merely blocks the translation of mRNA to functional protein. GABA_{B1a} knock out mice expressed a global deletion of the presynaptic GABA_{B1} receptor, while WT mice exhibit normal expression of the receptor and are littermates of the GABA_{B1a}^{+/−} animals, both of which are on a BALB/c background.

Context fear conditioning apparatus

Behavioral procedures were performed in 4 identical conditioning chambers (7”W x 7”D x 12”H) containing 2 Plexiglas walls (front and back) and 2 aluminum sidewalls and a stainless steel shock-grid floor (Coulbourn Instruments, Allentown, PA). Context A consisted of the context chamber (2 Plexiglas and 2 aluminum walls), with a polka-dot insert attached to the rear Plexiglas wall, white noise, dim
illumination (house light), and stainless steel grid floors cleaned with 70% ethanol. 

Context B consisted of identical chambers, minus the polka dots, was not illuminated and contained no white noise. A flat-brown Plexiglas floor replaced the grid floor and was washed with 50% Quatricide.

**Novel Object Recognition/Location**

The novel object recognition and location procedures were conducted in 4 identical open field arenas (46 cm x 46 cm x 39 cm) containing 4 Plexiglas sidewalls (Coulbourn Instruments, Allentown, PA). The chambers were placed in a dimly lit room (red lighting) with cameras (Coulbourn Instruments, Allentown, PA) mounted on the wall above each chamber to record activity. Chamber floors were cleaned with 70% ethanol to remove potential olfactory cues left by other animals.

The familiar objects used in the novel object procedure were 200mL glass beakers (7.5 cm circumference X 9 cm height) placed 12 inches away from each other and were cleaned with 70% ethanol to remove potential olfactory cues. The novel objects presented to animals in the novel object recognition experiment were plastic powder funnels (9 cm circumference X 9 cm height) cleaned with a 60% Quatricide solution.

**Contextual fear conditioning procedure**

All animals were handled for 5 minutes for two days prior to fear conditioning. All animals received fear conditioning in Context A, which consisted of 5 footshocks (1 sec, .8 mA) separated by 90 sec ITIs. Testing occurred in either Context A or Context B either 2 hrs, (Context A, WT n= 6, GABA_{B1a}−/− n = 6; Context B,
WT n = 6, GABA
B1a
−/− n = 6), 1 day (Context A, WT n= 6, GABA
B1a
−/− n = 6; Context B, WT n = 6, GABA
B1a
−/− n = 6), or 5 days (Context A, WT n= 6, GABA
B1a
−/− n = 6; Context B, WT n = 6, GABA
B1a
−/− n = 6) following training. All animals received a 5 minute test during which freezing, defined as the absence of all movement minus the movement associated with breathing, was measured using FreezeFrame software (Actimetrics, Wilmette, IL).

**Novel object discrimination/location procedure**

All animals were handled for 5 minutes for two days prior to exposure to the novel object arena (WT n= 15, GABA
B1a
−/− n = 15). Animals were habituated to the open field arena with a 5-minute exposure to the chamber in the absence of the objects for two days prior to object training. During training, each animal was placed in the arena on 3 consecutive days with two identical objects (200mL glass beakers) and allowed to explore for 10 minutes. Two hours following the third and final day of object exposure, all animals were placed back into the arena for 3 minutes in the presence of one familiar object and one novel object (plastic funnel) (novel object recognition test). The location of the novel object was counterbalanced so that half of the animals were presented with the novel object on the right side of the apparatus and half of the animals saw the novel object on the left side of the apparatus. Following the 3-minute novel object recognition test, the animals were removed from the arena, the arena was cleaned, and the animals were placed back into the arena for 3 minutes in the presence of two familiar objects (novel object location test). One of the familiar objects was moved to a new location within the
Figure 9: Novel object recognition/location procedure. **Top panel:** novel object recognition: during training animals are presented with two identical objects and allowed to explore. During testing, one of the objects is replaced with a novel object and the time spent exploring each object is recorded. If animals remember the familiar object from training, they will spend more time investigating the novel object. **Lower panel:** novel object location: during training animals are presented with two identical objects and allowed to explore. During testing, one of the objects is moved to a new location within the arena and the time spent exploring each object is recorded. If animals remember the familiar location from training, they will spend more time investigating the object in the novel location.
Figure 9

Training | Testing

Object 1 | Object 2

Familiar | Novel

Training | Testing

Object 1 | Object 2

Familiar | Novel
arena. The object that was moved was counterbalanced so that half of the animals saw the left object moved and half of the animals saw the right object moved. The order of testing was counterbalanced such that half of the animals received the novel object recognition test first and half of the animals received the novel object location test first.

Twenty-four hours following the third and final day of object exposure, all animals were placed back into the arena for 5 minutes for an additional novel object recognition test as described above. All animals also received an additional novel object location test for 5 minutes as described above. Again, the order of testing was counter balanced such that half of the animals received the novel object recognition test and half received the novel object location test (see Figure 9).

All training and test trials were recorded via digital camera mounted above the open field arena and scored by two observers blind to the condition/genotype of the animals. A mouse was considered exploring the object when any part of the body (minus the tail) was touching it or when the animal’s head was facing the object within 1 inch of the object (as outlined in Tang, et al.16). The amount of time spent exploring the novel object/location and familiar object/location was expressed as a discrimination index using Stefanko et al.’s (2009) equation D.I. = \((t_{\text{novel}} - t_{\text{familiar}})/(t_{\text{novel}} + t_{\text{familiar}})\) X 100%. Discrimination indexes were calculated for each animal and averaged for each group. Higher values on the D.I. indicate more time spent exploring the novel object/location.
Both WT and GABA<sub>B1a</sub>-/- animals were tested at two time points, at 2 hours and 24 hours post-training. The power of this approach is that we can observe within-subject degradation of memory precision as a function of time. Test durations were 3 minutes for the 2-hour time point, which is not long enough for the animals to form a long-term memory of the objects<sup>17</sup>. Thus, initial exposures did not affect performance at the 24-hour time point. Therefore, any differences in performance at 24 hours is likely due to the lack of presynaptic GABA<sub>B(1a)</sub> receptors and not due to habituation to the novel object from the previous day.
RESULTS

Presynaptic GABA\textsubscript{B(1a)} receptors are required for maintaining a contextually precise fear memory. To test the involvement of GABA\textsubscript{B(1a)} receptors in context memory precision, we used mice lacking the GABA\textsubscript{B(1a)} isoform (GABA\textsubscript{B(1a)}^{-/-}) (Vigot et al., 2006). All animals underwent fear conditioning in the training context (Context A) and were tested for conditioned fear in either the same or a novel context (Context B) 24 hours following training. At twenty-four hours post-training, wild type mice exhibited significantly more freezing in Context A than in Context B ($t(10) = 3.04, p<.05$), illustrating normal context discrimination (i.e. the context shift was present). Wild-type mice and GABA\textsubscript{B(1a)}^{-/-} mice also froze equivalently in Context A. However, GABA\textsubscript{B(1a)}^{-/-} mice exhibited a loss of context discrimination, as they exhibited significantly higher levels of freezing in Context B than wild-type mice ($t(12) = -2.19, p<.05$) (Figure 10A). Further, this loss of discrimination was relatively stable as GABA\textsubscript{B(1a)}^{-/-} mice exhibited a complete loss of discrimination at 5 days post-training compared to wild type animals that continued to exhibit lower freezing levels in the novel context (i.e. more context discrimination) ($t(12) = -2.47, p<.05$) (Figure 10B). These data suggest that presynaptic GABA\textsubscript{B} receptors are required for the discrimination of contextual cues and support earlier findings that these receptors are important in the precision of fear memory (Shaban et al., 2006).
Presynaptic GABA\textsubscript{B(1a)} receptors are required for the maintenance, but not initial encoding, of a contextually precise memory. a) GABA\textsubscript{B(1a)}\textsuperscript{−/−} mice freeze at equivalently high levels compared to wild-type (WT) mice when tested in the Same context (Context A) 24 hours following fear conditioning. When tested in the Shift context (Context B), GABA\textsubscript{B(1a)}\textsuperscript{−/−} mice exhibit significantly more freezing (P < .05) than WT mice. Thus, WT mice exhibit a normal contextually precise memory, whereas GABA\textsubscript{B(1a)}\textsuperscript{−/−} mice exhibit an imprecise context fear memory. b) At 5 days post-training, WT animals retain a contextually precise memory (significantly lower levels of freezing in the Shift context compared to the Same context). GABA\textsubscript{B(1a)}\textsuperscript{−/−} mice exhibit a contextually imprecise memory as evidenced by significantly more freezing in the Shift context compared to WT animals. c) Both WT and GABA\textsubscript{B(1a)}\textsuperscript{−/−} mice exhibit a contextually precise memory when tested in the Shift context 2 hours post-training. * p < .05.
Figure 10

(a) and (b) Show comparison of freezing behavior in Same Context and Shift Context for WT and GABA<sub>B1a</sub>−/− mice.

(c) Displays no significant difference (ns) in freezing behavior between Same Context and Shift Context for WT mice.

* indicates significant difference.
However, since GABA$_{B(1a)}^{-/-}$ mice failed to discriminate at 1 day or 5 days post-fear conditioning, it is plausible that the knock out of presynaptic GABA$_B$ receptors resulted in an inability to perceive differences in the contextual stimuli. Therefore, we conducted a 2-hour post-training test to determine whether GABA$_{B(1a)}^{-/-}$ mice could perceive differences in the two contexts at an early time-point. At 2-hours post-training, GABA$_{B(1a)}^{-/-}$ mice exhibited normal context discrimination, as they showed similarly low levels of freezing behavior in Context B compared to Context A ($t(19) = .23$, ns) (Figure 10C). These data suggests that mice lacking GABA$_{B(1a)}$ receptors can initially discriminate between contextual cues, indicating a precise memory at an early time point. Previous electrophysiological and behavioral data suggested that the loss of presynaptic inhibition shifted the threshold for generalization (Shaban, et al., 2006). If this was the case however, GABA$_{B(1a)}^{-/-}$ mice should not be able to distinguish contexts at any time point. Rather, our data suggest that presynaptic inhibition is more likely involved in the maintenance, but not the initial encoding of contextually precise memories.

**Presynaptic GABA$_{B1a}$ receptors are required for the maintenance of spatial and object discrimination memory.** Because we used a fear memory task, the lack of discrimination over time could be due mainly to the absence of presynaptic inhibition within the lateral amygdala (Shaban et al., 2006). However, the rapid time dependent decline of memory precision observed in our experiments suggested that presynaptic inhibition plays an important role in the long-term consolidation or
Figure 11: Presynaptic GABA$_B$(1a) receptors are required for the maintenance, but not initial encoding, of an object recognition and location memory. a) Wild-type (WT) and GABA$_B$(1a)$^{-/-}$ mice were habituated to the open field arena and then exposed to two identical objects. Both groups of animals spent equal amounts of time exploring both objects during training. b) WT and GABA$_B$(1a)$^{-/-}$ mice exhibit equivalently high preference for the novel object when tested for novel object recognition 2 hours following the last training session. When tested 24 hours post-training, GABA$_B$(1a)$^{-/-}$ mice exhibited an impaired ability to discriminate between the novel and familiar object compared to WT animals ($p < .05$). c) WT and GABA$_B$(1a)$^{-/-}$ mice exhibit equivalently high preference for the object moved to the novel location when tested for novel object location 2 hours following the last training session. However, GABA$_B$(1a)$^{-/-}$ mice exhibited an impaired ability to discriminate between the novel and familiar object location compared to WT animals ($p < .05$) when tested 24 hours following training. Data in b and c are presented using a discrimination index \(^* p < .05\); \(^{**} p < .01\).
Figure 11

(a) Time spent (%)

(b) Discrimination Index

(c) Discrimination Index
maintenance of contextual memories. Thus, we next investigated time dependent changes in memory precision using GABA_{B(1a)}^{-/-} mice and using amygdala independent memory tasks. We tested GABA_{B(1a)}^{-/-} mice on novel object discrimination and novel location discrimination tasks and demonstrate that GABA_{B(1a)}^{-/-} mice failed to discriminate between a familiar and novel object when tested for novel object discrimination 24-hours following training (a 2 (genotype) X 2 (retention interval) repeated measures analysis of variance (RM ANOVA) for discrimination index: \( F_{1,28} = 4.32, P < .05 \) (Figure 11b). Further, we show that mice lacking GABA_{B(1a)} receptors are also impaired in a novel object location task 24-hours following training (a 2 (genotype) X 2 (retention interval) RM ANOVA for discrimination index: \( F_{1,28} = 11.09, P < .01 \) (Figure 11c). However, consistent with the context discrimination data, GABA_{B(1a)}^{-/-} mice show normal discrimination immediately following training in both the novel object recognition and location tasks (Figure 11b and 11c). These data again suggest that GABA_{B(1a)} receptors are required for the maintenance of discrimination between stimuli, but not the initiation of discrimination.
DISCUSSION

In the current study, we were interested in investigating the role of presynaptic GABA\(_B(1a)\) receptors in the discrimination of both contextual stimuli following context fear conditioning as well as a non-aversive discrimination task, novel object recognition and location. Here we have demonstrated that mice lacking presynaptic GABA\(_B(1a)\) receptors exhibit impaired discrimination of context, novel objects, and object location at 24 hours following training, but not 2 hours post-training. These data suggest that GABA-mediated presynaptic inhibition is likely involved in the maintenance of a precise memory. However, the loss of GABA\(_B(1a)\) receptors had no effect on the acquisition or initial consolidation of the precise memory. These results provide novel insight into a potential synaptic mechanism involved in the precision of a memory. Further, these results also provide one of the first synaptic mechanisms involved in the maintenance of memory specificity.

Previous physiological and behavioral work with GABA\(_B(1a)\) knock out mice suggested that the loss of presynaptic inhibition shifts the threshold for fear generalization (Shaban et al., 2006). When given a cued fear discrimination task, GABA\(_B(1a)\) knock out animals trained with a 0.6mA amplitude footshock were able to discriminate between the excitatory cue (cue paired with shock during training) and the inhibitory cue (cue never paired with shock). However, when the shock intensity
was increased to 0.9mA, GABA<sub>B(1a)</sub> /- mice generalized their fear from the excitatory cue to the excitatory cue. Thus, it was concluded that the lack of presynaptic inhibition in GABA<sub>B(1a)</sub> /- mice resulted in a shift in the threshold for generalization of fear responses to lower US intensities. (Shaban et al., 2006). However, if this were the case, it would be expected that GABA<sub>B(1a)</sub> knock out animals trained using a high shock intensity would fail to discriminate contextual cues at any post-training interval. In the present experiments, we observed a rapid time dependent decline in memory precision in which GABA<sub>B(1a)</sub> knock out animals were able to discriminate between contexts at 2 hours post-training, but not at 1 or 5 days (Note, that in WT mice, this decline in memory precision occurs over 14-36 days,(Wiltgen & Silva, 2007; Y. Zhou & Riccio, 1996)). Furthermore, a similar rapid time dependent decline in memory precision was observed using non-fear tasks providing additional support against a threshold shift phenomenon and suggesting a potential interaction with systems consolidation of memory. Taken together, our data suggest that presynaptic inhibition via GABA<sub>B(1a)</sub> receptors help maintain the discrimination and/or the precision of the memory, but are not required for the initial encoding of this information.

The rapid decline in memory precision (or rapid increase in fear generalization) observed in the present study suggests that presynaptic inhibition may play an important role in limiting systems consolidation of the memory. Hippocampal-dependent memory, such as memory for context, undergoes a transfer a hippocampal-dependent memory to a hippocampal-independent cortical memory,
a process known as systems consolidation (Anagnostaras et al., 2001; Jasnow et al., 2012; J. J. Kim & Fanselow, 1992; Wiltgen, 2006). It is possible that the lack of presynaptic GABA$_B^{(1a)}$ receptors, which are heavily populated in the CA3 and CA1 regions of the hippocampus (Kulik et al., 2003), and resultant increased excitatory drive, may have enhanced the transfer of the memory from a hippocampus-dependent memory to the more long-term cortical-dependent memory, resulting in a loss of memory precision.

Alternatively, this phenomenon may be the result of disruption specifically within hippocampal circuits. This is because we observe a loss of memory precision in both the contextual fear task as well as novel object recognition and novel object location tasks in GABA$_B^{(1a)}$ knockout mice. Both of these novel object tasks are independent of fear responses and rely heavily on hippocampal functioning (Ambrogi Lorenzini, Baldi, Bucherelli, Sacchetti, & Tassoni, 1997; Baarendse et al., 2007; Best & Orr, 1973; Lorenzini, Baldi, Bucherelli, Sacchetti, & Tassoni, 1996). Moreover, dysfunction of presynaptic inhibition and synaptic potentiation within the Mossy fiber pathway (axonal projections from the dentate gyrus region of the hippocampus to neurons within the CA3 region of the hippocampus) and Schaffer commissural pathway (axonal projections from pyramidal neurons of the CA3 region to the CA1 region of the hippocampus) is well documented in these mice (Gassmann & Bettler, 2012; Guetg et al., 2009; Vigot et al., 2006), as is the importance of the Mossy fiber pathway in memory precision (Jasnow et al., 2012; Ruediger et al., 2011). Thus, as discussed above, the loss of context specificity over
time may result from a decay of the hippocampal memory trace (Biedenkapp & Rudy, 2007; Rudy, 2005). In other words, the quality of the contextual memory decays, while the fear trace remains strong, and becomes reactivated by novel contextual stimuli. Presynaptic inhibition may be necessary within the hippocampus for retaining information about context specificity. In other words, presynaptic inhibition may be a mechanism responsible, in part, for the maintainence of the quality or strength of the memory trace. The lack of presynaptic inhibition within hippocampal circuits in GABA\textsubscript{B(1a)} knockout mice would then cause context specificity to rapidly decay as we observed in the present study.

Supporting the decay interpretation of the loss of context-specificity, recent work demonstrates that feed-forward inhibition within the hippocampus is necessary for maintaining contextually precise memories (Ruediger et al., 2011). Specifically, it was discovered that a learning event, such as context fear conditioning and spatial learning, results in an increase in mossy fiber filopodial contacts onto both interneurons (feed-forward inhibition) and excitatory pyramidal neurons (feed-forward excitation) within the CA3 region of the hippocampus. Increases in feed-forward inhibition were associated with expression of a contextually precise memory and that the gradual decrease in these filopodial contacts overtime was associated with the gradual loss of context specificity. A lack of inhibition on feedforward excitatory contacts may result in enhanced excitatory drive within the hippocampus and negate the effects of feed-forward inhibition.
within the CA3 region of the hippocampus, resulting in a rapid decline of memory precision.

To test whether the loss of presynaptic inhibition results in a quickening of the systems consolidation and resulting loss of context specificity, a follow up study could look at the neural pattern of activation in the knock out animals during expression of a 2 hour old precise memory and a 24 hour old imprecise memory. Using a method similar to that used in Study 1, Arc mRNA expression should be investigated in the cortex and hippocampus to determine if the pattern of activation underlying a precise versus imprecise memory in the knock out mice match the pattern found in non-mutant mice from Study 1 that naturally lose context specificity over time. Further, it would be interesting to see if inactivation of the ACC at 1 day following training when the knock out animals are generalizing returns the memory back to a precise state. This would help determine whether GABA$_{B(1a)}$ mediated presynaptic inhibition is a mechanism maintaining context-specificity at recent time points prior to the shift in hippocampal activity we found underlying the loss of context specificity in Study 1. Additionally, another study could investigate potential changes in GABA$_{B(1a)}$ receptor expression at recent and remote time points following context fear conditioning in non-mutant animals that gradually lose context specificity over time. If GABA$_{B(1a)}$ receptors are involved in maintaining memory precision at recent time points, it would be expected they are down regulated or become deactivated as the memory ages and loses it context specificity. This would further enhance our understanding of discrimination processes and tie
together synaptic and systems mechanisms working in conjunction with each other to regulate the specificity of memory.

In conclusion, GABA_B(1a) receptors appear to be required for the maintenance of the precision of memory, but not for the initial encoding of that memory. Mice lacking these presynaptic receptors demonstrated an initially precise discrimination of stimuli that rapidly degraded within 24 hours. These findings provide novel insight into one of the synaptic mechanisms underlying the preservation of memory precision and provide a potential mechanism for the transformation of initially precise contextual memories into those that lack context specificity.
Study 1 investigated the neural pattern of activation underlying the gradual loss of context-specificity or increase in fear generalization. We discovered that an interaction between areas of the prefrontal cortex and the hippocampus underlie expression of both a contextually precise fear memory as well as a contextually imprecise generalized fear memory. Inactivation of the ACC and CA1 region of both the dorsal and ventral hippocampus provided further evidence that regions of the cortex and hippocampus interact at remote time points, and that a generalized fear memory results from this interaction. Study 2 investigated GABAB(1a) receptors as a potential cellular mechanism for the regulation of both context novel object discrimination. We discovered that GABAB(1a) receptors are required for the maintenance, but not acquisition or consolidation, of memory precision.

From the studies presented in this document, we hope to gain a better understanding of how a fear memory loses context-specificity and generalizes across contexts. Currently, very little attention has been given to the loss of context discrimination over time. Due to the lack of attention that this phenomenon has received, we currently know very little about the neural mechanisms, at the systems level or at the synaptic level, that are involved in or responsible for the generalization of a fear memory. At the neural systems level, determining which
structures are involved in the generalization of a fear memory will allow us to 1) better understand what neural structures are even involved in the expression of a generalized memory and 2) allow us to hone in on particular areas and dissect the neural circuit underlying the generalization of fear. We have identified a number of hippocampal and cortical regions that appear to interact during the expression of both a contextually precise and imprecise memory. However, the nature of this interaction and the functional relationship between these neural areas are not well understood. Much more work needs to be done on this topic in order to better understand how the hippocampus and cortex work together, or not together, in the creation of generalized memories. At the synaptic level, demonstrating that the activity of GABA$_B^{1(a)}$ receptors is required for the discrimination between contexts provides a cellular mechanism underlying context fear generalization. With the exception of Shaban et al.’s (2006) demonstration that GABA$_B^{1(a)}$ receptors are required for cued discrimination, no one has investigated the role of these receptors in acquisition, consolidation, or expression of a fear memory. By studying the role of GABA$_B^{1(a)}$ receptors at different time points following fear conditioning, we were able to gain an understanding of their involvement in maintenance of a contextually-specific fear memory.

In addition to providing an understanding of neural mechanisms underlying discrimination and generalization of both fearful and non-fearful stimuli, the current study and potential follow up studies proposed in this document may help direct investigations into ways in which the precision of a memory may be maintained
longer. In other words, by shedding light on mechanisms of generalization, we hope to also highlight potential mechanisms in which generalization can be mitigated or prevented. For instance, we provided strong evidence that the loss of presynaptic $\text{GABA}_B(1\alpha)$ receptors results in enhanced generalization responses. One question this raises is whether activating or driving expression of $\text{GABA}_B(1\alpha)$ receptors may prevent generalization. This can easily be tested by administering a $\text{GABA}_B(1)$ receptor agonist, such as Blacofen, at different intervals post-training or pre-testing to determine if activating these receptors continually maintains context specificity. Results from experiments like this could aid in the identification of novel therapeutic targets to aid in the treatment or prevention of psychological disorders that have fear generalization as a major symptom.

Taken together, these studies provide valuable insight into the neural mechanisms underlying the generalization of fear. A better understanding of these mechanisms will provide a deeper insight into not only the storage of context fear memories, but also the mechanisms responsible for the loss of context-specificity surrounding a fear memory. Further, a better understanding of fear generalization mechanisms may also provide insight into the mechanisms underlying the memory impairments observed in anxiety disorders such as PTSD and potentially lead to new therapeutic targets for the treatment or prevention of such disorders.
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