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Activity of the anterior cingulate cortex and ventral hippocampus underlie increases in contextual fear generalization

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ABSTRACT

Memories for context become less specific with time resulting in animals generalizing fear from training contexts to novel contexts. Though much attention has been given to the neural structures that underlie the long-term consolidation of a context fear memory, very little is known about the mechanisms responsible for the increase in fear generalization that occurs as the memory ages. Here, we examine the neural pattern of activation underlying the expression of a generalized context fear memory in male C57BL/6J mice. Animals were context fear conditioned and tested for fear in either the training context or a novel context at recent and remote time points. Animals were sacrificed and fluorescent in situ hybridization was performed to assay neural activation. Our results demonstrate activity of the prelimbic, infralimbic, and anterior cingulate (ACC) cortices as well as the ventral hippocampus (vHPC) underlie expression of a generalized fear memory. To verify the involvement of the ACC and vHPC in the expression of a generalized fear memory, animals were context fear conditioned and infused with 4% lidocaine into the ACC, dHPC, or vHPC prior to retrieval to temporarily inactivate these structures. The results demonstrate that activity of the ACC and vHPC is required for the expression of a generalized fear memory, as inactivation of these regions returned the memory to a contextually precise form. Current theories of time-dependent generalization of contextual memories do not predict involvement of the vHPC. Our data suggest a novel role of this region in generalized memory, which should be incorporated into current theories of time-dependent memory generalization. We also show that the dorsal hippocampus plays a prolonged role in contextually precise memories. Our findings suggest a possible interaction between the ACC and vHPC controls the expression of fear generalization.

1. Introduction

The formation of a new memory involves the reorganization of information across the neural systems that encode and store memory as the memory trace is transferred from temporary to more permanent locations within the brain, a process known as consolidation (Dudai, 2004; Frankland et al., 2006; Kim & Fanselow, 1992; McGaugh, 1966; Squire & Alvarez, 1995; Vetere et al., 2011; Zola-Morgan & Squire, 1990). Specifically, it has been demonstrated that expression of recent contextual memories are dependent on activity of the hippocampus whereas expression of remote contextual memories involves increased activity of the infralimbic (IL) and prelimbic (PL) areas of the medial prefrontal cortex (mPFC) and becomes dependent on activity of the anterior cingulate cortex (ACC) (Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004; Gafford, Parsons, & Helmstetter, 2013; Goshen et al., 2011; Kim & Fanselow, 1992). These results suggest that as a context memory ages, it is transferred from the hippocampus to a distributed cortical network for long-term storage. While studies on the neural structures involved in the long-term storage and consolidation of contextual 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 as a result of long-term consolidation.

In the case of context fear conditioning, placing animals in a novel context (i.e., distinctly different from the training context) shortly following training results in little expression of learned fear (lower levels of freezing behavior) (Feinberg & Riccio, 1990; Jasnow, Cullen, & Riccio, 2012; Riccio, Ackil, & Burch-Vernon, 1992; Ruediger et al., 2011; Wiltgen & Silva, 2007; Wiltgen et al., 2010; Zhou & Riccio, 1996). However, as the retention interval between training and testing increases, there is a loss of memory precision (i.e., fear responses to the training context generalize over time) such that animals exhibit equivalent levels of fear expression in both the training context and the novel context

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(Gisquet-Verrier & Alexinsky, 1986; McAllister & McAllister, 1963; Metzger & Riccio, 2009; Perkins & Weyant, 1958; Richardson, Williams, & Riccio, 1984; Zhou & Riccio, 1996). A similar, yet distinct process occurs when animals are trained to discrete cues, or undergo discrimination training (eg., Jasnow et al., 2012; Thomas & Burr, 1969; Thomas & Lopez, 1962; Thomas et al., 1985). As with generalization to contextual cues, generalization gradients to discrete cues flatten as the retention interval increases. To account for the increase in contextual fear generalization, Winocur, Moscovitch, and Sekeres (2007) proposed that as the memory trace is transferred from the hippocampus to the cortex over time, it is transformed into a schematic-like memory. Thus, the memory becomes stored as a "gist-like" memory trace that lacks context-specificity. According to this hypothesis, the expression of a generalized fear memory results from activation of the general or schematic-like cortical fear memory by the novel contextual cues. Several studies using post-training inactivation or hippocampal lesions have supported this hypothesis demonstrating that the retrieval of generalized context memories do not involve activity of the hippocampus (Wiltgen et al., 2010; Winocur, Frankland, Sekeres, Fogel, & Moscovitch, 2009). As a result, it is generally concluded that at remote time points, animals retrieve a hippocampus-independent memory that lacks context specificity.

Although a few studies have implicated a more prolonged role of the hippocampus in the expression of contextually precise memories (Biedenkapp & Rudy, 2007; Ruediger et al., 2011), several studies suggest 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, other than the hippocampus, it remains unclear what neural structures are involved in the expression of a generalized fear memory. Further, most existing literature on consolidation focus solely on the dorsal hippocampus, overlooking the potential role of the ventral hippocampus. Here we directly investigate the involvement of the ventral hippocampus and prefrontal cortex in the loss of context specificity that occurs at remote time points.

2. Materials and methods

2.1. Subjects

All experiments were conducted on male C57BL/6J mice generated from a breeding colony in the Department of Psychological Sciences at Kent State University. Animals were 6 weeks of age or older before they were used for experimentation/surgical cannulation and were group housed (3–4 animals per cage) with free access to food and water in a room maintained on a 12:12 light/dark cycle. All procedures were conducted in a facility accredited by the Association for Assessment and Accreditation and Laboratory Animal Care. All animal procedures were carried out in accordance with the National Institutes of Health guidelines and were approved by Kent State University Institutional Animal Care and Use (IACUC) Guidelines.

2.2. 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). cDNA clones containing the coding sequence for mouse Arc were 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 h 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 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 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 (+1.78 mm A/P) regions of the medial prefrontal cortex, the anterior cingulate cortex (+0.6 mm A/P), the CA1, CA3, and dentate gyrus regions of the ventral hippocampus (−3.16 mm A/P), and the CA1, CA3, and dentate gyrus regions of the dorsal hippocampus (−2.18 mm A/P).

Analysis of Arc mRNA expression involved calculating a percent change score compared to home cage control animals. A homecage control activity score was calculated by averaging activation levels for each area of interest across all animals. For each region of interest, a "percent homecage" was calculated for each animal in each group (i.e. 1 Day Training, 1 Day Novel, 14 Day Training, 14 Day Novel). We used 3 bilateral consecutive sections for each region and averaged the scores together, giving each animal one score per region of interest.

2.3. Surgery and cannulation

Animals were anesthetized with an intraperitoneal injection of a Ketamine (75 mg/kg) + Dexdomitor (dexmedetomidine) (1.5 mg/kg) cocktail. Following administration of anesthesia, mice were mounted on a stereotaxic apparatus (Kopf Instruments). A single guide cannula (Plastics One, Inc) was inserted into the skull above the anterior cingulate cortex for midline infusions using a 14° angle. Two guide cannulae were surgically implanted above the CA1 region of either the ventral hippocampus or dorsal hippocampus for bilateral infusions. Cannulae were positioned in the following coordinates using a 14° angle with respect to bregma including a 1 mm protrusion for the infusion needle: +0.8 mm A/P, 0.7 mm M/L, 1.75 mm D/V for ACC; −3.16 mm A/P, 4.2 mm M/L, 3.6 mm D/V for CA1 region of the ventral hippocampus (vCA1); −2.5 mm A/P, 2.5 mm M/L, 1.6 mm D/V for CA1 region of the dorsal hippocampus (dCA1).

Following the completion of behavioral experiments, we verified guide cannula placement by injecting 0.2 μL 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 was not observed in the proper place, behavioral data from that mouse was excluded from analyses.

2.4. Drug infusions

The sodium channel blocker lidocaine HCl (Sigma) was used to suppress neuronal firing, thereby temporarily inactivating brain regions of interest. For all infusion experiments, lidocaine HCl was dissolved in phosphate-buffered saline (PBS) to a concentration of 4% (w/v) lidocaine and adjusted to a pH of ~7.0 (Frankland et al., 2004). 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 min at a rate of 0.2 μL per minute. Injectors were left in place for 2 min 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 housing cage and allowed to walk freely and were only briefly restrained during injector insertion/removal.

2.5. Context fear conditioning apparatus

Behavioral procedures were performed in 4 identical conditioning chambers (7″W × 7″D × 12″H) containing 2 Plexiglas walls (front and back), 2 aluminum sidewalls and a stainless steel shock-grid floor (Coulbourn Instruments, Allentown, PA). The Training context consisted of the context chamber (2 Plexiglas and 2 aluminum walls), with a polka-dot insert attached to the rear Plexiglas wall, white noise (65 db), dim illumination (house light), and stainless steel grid floors cleaned with 70% ethanol. The Novel context consisted of identical chambers, without the polka dot wall background, was illuminated only by infrared lights and contained no white noise. In addition, a flat-brown Plexiglas floor replaced the grid floor and was washed with 50% Quatricide.

2.6. Procedure

All animals were handled for 5 min on two consecutive days prior to fear conditioning. Following handling all animals received two 5-min pre-exposures to the Training context on the two days prior to fear conditioning. This pre-exposure served to introduce animals to the Training context and allowed animals to encode the contextual cues. Fear conditioning occurred in the Training context, which consisted of 5 footshocks (1 s, 0.8 mA) separated by 90 s ITIs. Testing occurred in either the Training context or the Novel context. Testing consisted of a 5-min exposure to either Training or Novel context, during which freezing was measured using FreezeFrame software (Actimetrics, Wilmette, IL). A behavioral control group (No Shock) received handling and context pre-exposures as the Training and Novel context groups but did not receive shocks during the training trial. These animals were placed in the Training context during the training period to control for context exposure, but did not receive foot shocks. An additional control group was not handled or exposed to the context. This homecage group was removed from the colony and sacrificed for baseline Arc mRNA activity for analysis.

2.6.1. Experiment 1

Fear conditioning began when animals were between 60 and 80 days of age. All animals were fear conditioned to the Training context as described above. Animals were divided into two context groups. Half of the animals were tested in the Novel context (Recent $n = 8$; Remote $n = 8$), whereas the other half of the animals were tested in the Training context (Recent $n = 9$; Remote $n = 10$). The Training context groups serve as context controls (to ensure fear conditioning was successful) and to provide benchmarks of retention for comparison. We used 2 separate retention intervals to assess the ability to discriminate between the Training context and the Novel context. Half of the animals were tested for fear at a recent time point (24 h after training) and the other half were tested at a remote time point (14 days after training). While a number of studies that have investigated remote context memory use a later time point, such as 28–36 days, for their remote test (Frankland et al., 2004; Wiltgen & Silva, 2007; Wiltgen et al., 2010), a number of published studies investigating the loss of context discrimination over time have used the14 day interval as the remote test and have shown that animals consistently lose the ability to discriminate by 14 days post-training (Land & Riccio, 1998; MacArdy & Riccio, 1991; Riccio, Richardson, & Ebner, 1984; Zhou & Riccio, 1996). To be more consistent with these previous investigations, we refer to a memory that is 14 days or older as a remote memory. We added a home cage control group that allowed us to assess changes in gene activation following testing compared to non-trained animals. This group allowed 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, Recent $n = 6$; Remote $n = 6$). This No Shock group was included to assess whether the contexts used for training and testing were inherently aversive.

Thirty minutes following testing in either the Training or Novel contexts at Recent or Remote time points, animals were sacrificed and brains were extracted. This post-test interval was chosen due to findings suggesting that Arc mRNA expression peaks at around 30 min following fear retrieval (Lonergan, Gafford, Jarome, & Helmstetter, 2010; Ressler, Paschall, Zhou, & Davis, 2002). Extracted brains were flash frozen using dry ice and stored at −80 °C until processed.

2.6.2. Experiment 2

All mice underwent surgical cannulation as described above and were given 1 week to recover before behavioral experimentation began. Following recovery, all animals underwent handling, pre-exposure, and context fear conditioning in the Training context as described above. Each drug group receiving lidocaine and vehicle were divided into two context groups. Half of the animals were tested in the Novel context (Novel groups). The other half of the animals were tested in the same context where they received training (Training groups). Vehicle groups served as a control group and allowed us to assess the behavioral effects of temporarily inactivating specific brain regions with lidocaine. We used two separate retention intervals to assess the ability to discriminate between the Training context and the Novel context. Animals were tested for fear 24 h (Recent) or 21 days (Remote) following fear conditioning. Ten minutes prior to fear conditioning, animals received an infusion of either lidocaine or PBS in the ACC or the ventral hippocampus. Sample size for each group is indicated in Table 1.

2.7. Statistical analysis

Freezing responses during contextual fear testing were analyzed using a factorial analysis of variance (ANOVA). Statistically significant ANOVAs were followed up with Tukey HSD post hoc comparisons. Changes in Arc mRNA expression following recent

Table 1
Treatment groups and sample sizes for inactivation experiments.

Region	Recent (1 Day)		Remote (21 Days)	
	Training	Novel	Training	Novel
ACC	Vehicle ($n = 7$) Lidocaine ($n = 7$)	Vehicle ($n = 7$) Lidocaine ($n = 7$)	Vehicle ($n = 8$) Lidocaine ($n = 8$)	Vehicle ($n = 7$) Lidocaine ($n = 8$)
vCA1	Vehicle ($n = 6$) Lidocaine ($n = 8$)	Vehicle ($n = 7$) Lidocaine ($n = 6$)	Vehicle ($n = 6$) Lidocaine ($n = 7$)	Vehicle ($n = 6$) Lidocain ($n = 6$)
dCA1			Vehicle ($n = 8$) Lidocaine ($n = 8$)	Vehicle ($n = 6$) Lidocaine ($n = 10$)

and remote time points were analyzed using a factorial ANOVA and followed up with Tukey HSD post hoc comparisons.

3. Results

3.1. Memory for contextual cues becomes less specific over time

To investigate the increase in context fear generalization, we tested fear-conditioned animals in either the Training context or a Novel context at recent (1 day) or remote (14 days) time points (Fig. 1a). We found a statistically significant Context by Retention interaction, $F(2, 41) = 8.76$, $p < .01$. At the recent time point, animals in the Novel context froze less than animals in the Training context ($p < .001$). This difference indicates animals express a contextually precise memory at a recent time point following fear training. However, at a remote time point post-training (14 days), animals in both the Novel context and the Training context froze at equivalent levels indicating animals exhibited a contextually imprecise memory and had generalized their fear from the Training context to the Novel context.

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 when tested at a recent time point. This context specificity was lost by 14 days post-training as animals generalized their fear from the Training context to the Novel context.

3.2. Activity of the ventral hippocampus and ACC underlies the increase in fear generalization

In order to investigate the neural pattern of activation that underlies the gradual loss of context specificity, a randomly selected subset of animals (4 animals from each group) from the previous behavioral experiment were sacrificed and fluorescent in situ hybridization was performed on brains extracted from these

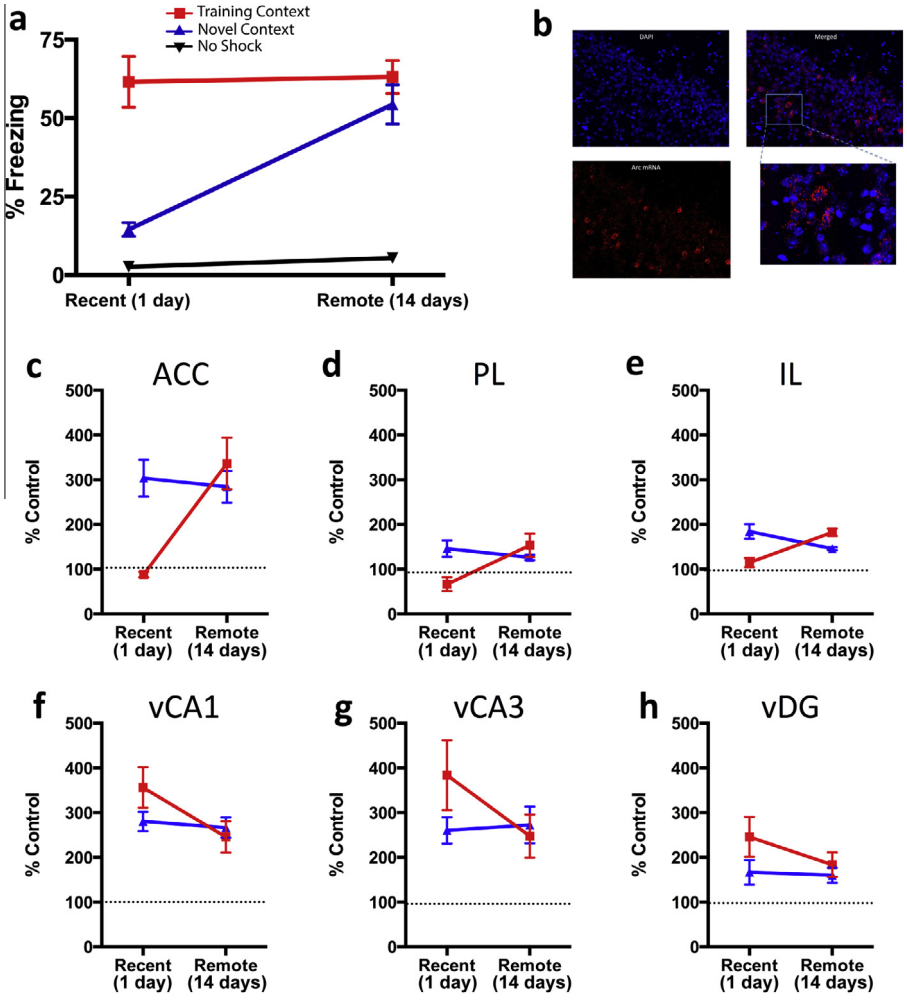

Fig. 1. Activity of the mPFC and ventral hippocampus underlie the increase in context fear generalization. (a) Average freezing levels for C57BL/6 during recent and remote retrieval tests. In this experiment, recent indicates 1 day post training and remote indicated 14 days post training. Mice tested in the Novel context (blue) exhibit a contextually precise fear memory at the recent time point. Mice tested in the Novel context at the remote time point generalized fear from the Training context (red) to the Novel context. (b) Representative sample of Arc mRNA expression in a section from the CA1 region of the ventral hippocampus. (c–h) Arc expression following retrieval of a recent or remote context memory in either the Training or Novel contexts expressed as a percentage relative to No Shock controls. Mice tested in the Training context exhibit increased ACC (c), PL (d), or IL (e) Arc activity above baseline when tested at remote, but not recent, time point. The ACC, IL, and PL exhibit Arc activity above baseline during expression of both a contextually precise and a contextually imprecise context fear memory as indicated by high activity at both time points in the Novel context. ACC, IL, and PL Arc expression was elevated following recent and remote memory retrieval in the Novel context. Arc activity was elevated in the vCA1 (f), vCA3 (g) and vDG (h) above baseline when tested at both time points. Arc activity was similarly elevated when animals were tested in the Novel context at both recent and remote time points. vHPC activity decreased as a function of time when animals were tested in the Training context, however activity remained relatively stable in animals that were tested at recent and remote time points in the Novel context.

animals (Fig. 1b). Animals tested in the Training context exhibit little to no ACC (Fig. 1c), PL (Fig. 1d), or IL (Fig. 1e) activity relative to controls (homecage control animals) when tested 1 day following fear conditioning. However, ACC, IL, and PL activity greatly increased when animals were tested in either the Training context or the Novel context at the remote time point (14 days). These data are similar to the pattern of activity in the prefrontal cortex observed after remote memory activation in a previous study (Frankland et al., 2004). Memory precision, however, was not examined that study. Here, we show that the ACC, IL, and PL appear to be highly active at both time points in the Novel context relative to controls, suggesting that the prefrontal cortex may be recruited or involved in both discrimination and generalization of contextual stimuli (statistically significant Context x Retention interaction for ACC: $F(1, 15) = 13.19$, $p < .01$; IL: $F(1, 15) = 20.71$, $p < .001$; PL: $F(1, 14) = 7.83$, $p < .05$).

To examine activity of the ventral hippocampus in the expression of both a contextually precise and imprecise fear memory, we analyzed Arc mRNA activity in the CA1 (vCA1), CA3 (vCA3), and dentate gyrus (vDG) regions of the ventral hippocampus. Animals tested in the Training context exhibited high Arc activity in the vCA1 (Fig. 1f), vCA3 (Fig. 1g) and vDG (Fig. 1h) relative to controls when tested at recent and remote time points following fear conditioning. Animals tested in the Novel context similarly exhibited elevated activity of the vCA1, vCA3, and vDG regions of the hippocampus at both recent (1 day) and remote (14 days) time points (Context $\times$ Retention interaction for vCA1: $F(1, 12) = 3.08$, p = ns; vCA3: $F(1, 12) = 9.65$, $p < .01$; and vDG: $F(1, 12) = 0.59$, p = ns). In general, vHPC activity decreased as a function of time when animals were tested in the Training context, however activity remained relatively stable in animals that were tested at recent (1 day) and remote time points (14 days) in the Novel context. Although, activity was consistently above control levels in the Training context regardless of time of testing, this reduction in activity associated with the Training context may indicate reduced involvement of the vHPC in remote contextually precise memory retrieval. The sustained activity in the Novel context suggests that activity of the ventral hippocampus may regulate, in part, expression of generalized fear memories.

3.3. ACC inactivation at remote time points reduces generalized fear expression and returns context-specificity to the memory

The data indicated that the ACC is active in the Novel context at a recent time point when animals do not exhibit a fear response, but is also active during expression of a contextually imprecise fear memory at a remote time point. To determine whether this cortical structure is required for expression of recent contextually precise and remote contextually imprecise fear memory, we temporarily inactivated the ACC using lidocaine infusions. Even though the ACC was highly active at a recent time point in the Novel context (when animals are not freezing), we hypothesized that ACC inactivation at a recent time point would have no effect on expression of a contextually precise fear memory because it has been previously demonstrated that recent context memories are hippocampal-dependent (Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998; Ruediger et al., 2011; Wiltgen et al., 2010; Winocur et al., 2007, 2009). However, we predicted that inactivating the ACC at remote time points would result in a reduction of both remote memory expression and generalized fear expression.

At the recent time point, animals infused with either vehicle or lidocaine and tested in the Novel context exhibited a contextually precise memory and did not express high levels of fear in the presence of the novel cues ($p < .05$). Both Vehicle and Lidocaine animals tested in the Training context exhibited significantly higher levels of freezing (main effect of Context (Same vs. Shift), $F(1, 24) = 65.04$, $p < .001$) (Fig. 2a). These data suggest that temporary inactivation

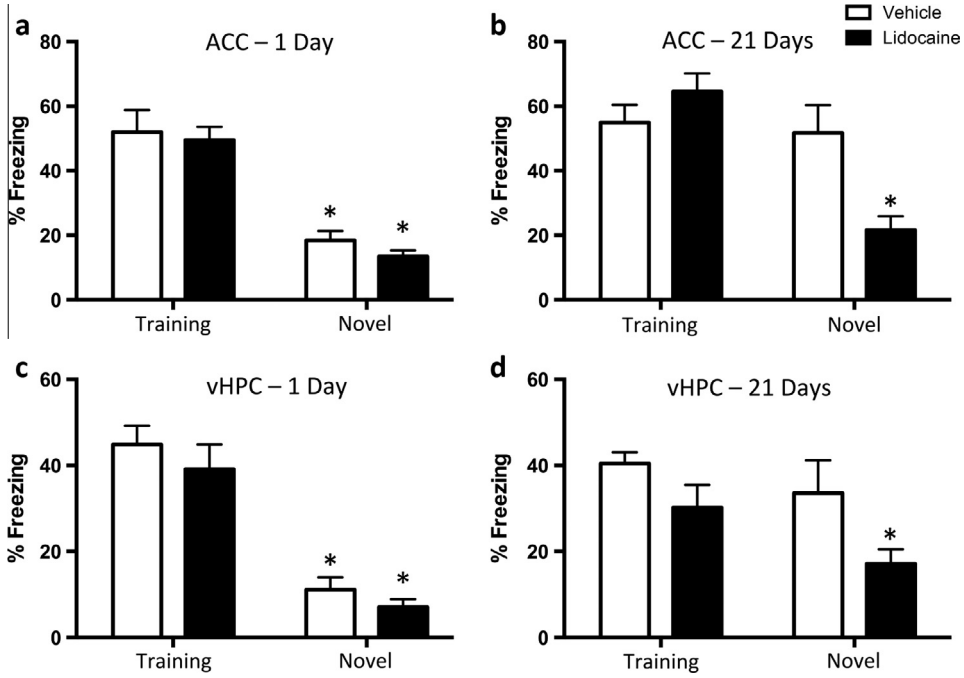

Fig. 2. Inactivation of the ACC or vCA1 returns memory to a contextually precise form. Recent memory test = 1 day post training; Remote memory test = 21 days post training. (a) ACC inactivation at recent time point: infusion of lidocaine into the ACC had no effect on expression of a contextually precise fear memory in the Novel context nor did it reduce freezing in the Training context. (b) ACC inactivation at remote time point: Infusion of lidocaine into the ACC significantly reduced freezing in the Novel, but not Training, context. (c) vHPC inactivation at recent time point: Infusion of lidocaine into the CA1 region of the vHPC had no effect on expression of a contextually precise fear memory in the Novel context nor did it reduce freezing in the Training context. (d) vHPC inactivation at remote time point: Infusion of lidocaine into the CA1 region of the vHPC significantly reduced freezing in the Novel, but not Training, context. $^{*}p < .05$.

of the ACC did not alter expression of a contextually precise fear memory at the recent time point. When animals were tested for context fear at the remote time point (21 days post-training), vehicle animals in the Novel context froze at equivalent levels as vehicle control animals in the Training context (Fig. 2b). Thus, at 21 days post-training, control animals exhibited a complete loss of context specificity and 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 Novel context compared with the vehicle animals in the Novel context (Context x Drug interaction, $F(1, 27) = 10.67$, $p < .01$). Mice infused with lidocaine into the ACC and tested in the Training context did not show a reduction in freezing (Fig. 2b). Further, lidocaine animals in the Novel context had lower freezing scores than lidocaine animals in the Training context ($p < .01$). These data suggest that the ACC activity is involved in expression of a generalized context fear memory and that inactivation of this cortical structure returned the context memory back to a more precise state.

3.4. Inactivation of the ventral hippocampus reduces expression of fear generalization

Our Arc mRNA expression data indicated that the CA1 region of the ventral hippocampus is active during expression of both a contextually precise and a contextually imprecise memory. To further investigate the role of the ventral hippocampus in the expression of a context-specific fear memory and generalized fear memory, we temporarily inactivated the CA1 region of the ventral hippocampus by infusing lidocaine into this region. Based on our activity data, it was hypothesized that vCA1 inactivation would reduce fear expression in the Training context, but have no effect on low levels of freezing in the Novel context at the recent 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 Training and Novel contexts at the remote time point. However, because vHPC activity decreased as a function of time when animals were tested in the Training context it was equally possible that this region would not be involved in remote context-specific memories.

At the recent time point, both vehicle and lidocaine animals in the Novel context froze less than animals in the Training context (main effect of Context: $F(1, 23) = 57.57$, $p < .001$; post hoc comparison: $p < .05$) (Fig. 2c). In other words, animals infused with either vehicle or lidocaine and tested in the Novel context exhibit a contextually precise memory and express little fear in the presence of the novel cues. Temporary inactivation of the vCA1 did not alter expression of a contextually precise fear memory at 1 day post-training (Context × Drug interaction, $F(1, 23) = 0.85$, $p = .ns$). When animals were tested for context fear at the remote time point (21 days post-training), vehicle-treated animals tested in the Novel context froze at equivalent levels as vehicle-treated control animals in the Training context, demonstrating that control animals generalized their fear from the Training to the Novel context. Inactivation of the vCA1 resulted in a reduction of generalized freezing (main effect of drug: $F(1, 21) = 6.74$, $p < .05$; post hoc comparison between Same Vehicle and Shift Lidocaine $p < .05$) but only in the Novel context (Fig. 2d). Inactivation of vCA1 did not significantly reduce fear expression in the Training context. These data indicate that although the vCA1 remains active during remote context fear expression regardless of context, its activity may only be necessary when animals generalize fear responses.

3.5. Activity of the dorsal hippocampus is required for remote memory expression

Because we observed a reduction of generalized freezing when the vCA1 was inactivated at a remote testing point, we next investigated whether inactivation of the dorsal CA1 would have a similar effect at a remote retention interval. Several recent studies have indicated that the dorsal hippocampus is involved in context memory regardless of the age of the memory (Goshen et al., 2011; Wiltgen et al., 2010). To examine activity of the dorsal hippocampus in the expression of both a contextually precise and imprecise fear memory, we analyzed Arc mRNA activity in the CA1 (dCA1), CA3 (dCA3), and dentate gyrus (dDG) regions of the dorsal hippocampus. Animals tested in the Training context exhibit high Arc activity in the dCA1 (Fig. 3a), dCA3 (Fig. 3b) and dDG (Fig. 3c) relative to controls (homecage control) when tested both recent and remote time points. Animals tested in the Novel context exhibit elevated activity of the dCA1, dCA3, and dDG regions of the hippocampus at the recent time point. However, dorsal hippocampal activity greatly diminishes when animals are tested in the Novel context at the remote time point (Context × Retention interaction for dCA1: $F(1, 14) = 38.87$, $p < .001$; dCA3: $F(1, 12) = 12.01$, $p < .01$; and dDG: $F(1, 13) = 29.01$, $p < .001$). These data suggest that the dorsal hippocampus is active during expression of both a recent and remote context fear memory, which supports recent evidence that the hippocampus underlies the expression of a context memory regardless of the age of that memory (Goshen et al., 2011; Ruediger et al., 2011; Wiltgen et al., 2010). Further, these data suggest that dorsal hippocampus activity underlies expression of a contextually precise fear memory. However, the drastic

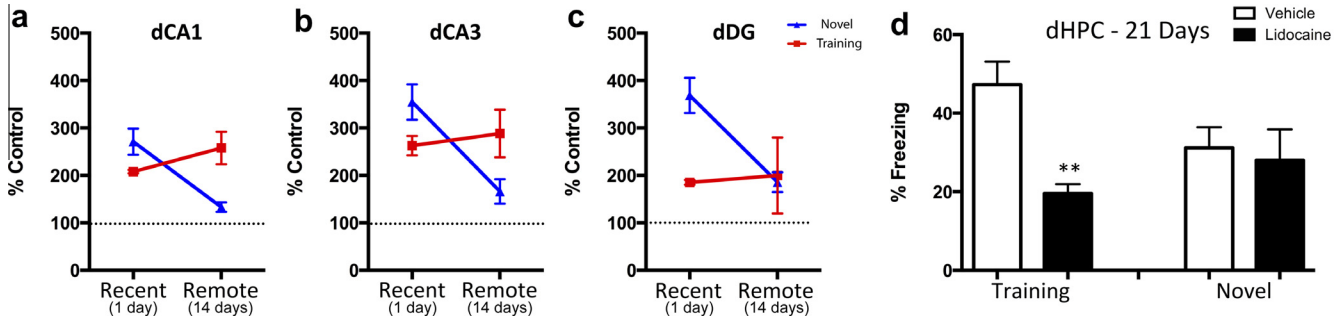

Fig. 3. Activity of the dorsal hippocampus is necessary for expression of remote contextually precise fear memory. (a–c) Arc expression following retrieval of a recent (1 day) or remote (14 days) context memory in either the Training (red) or Novel (blue) contexts expressed as a percentage relative to No Shock controls. Mice tested in the Novel context exhibit increased dCA1 (a), dCA3 (b), or dDG (c) Arc activity when tested at a recent time point, but activity is greatly reduced at a remote time point. The dCA1, dCA3, and dDG exhibit sustained Arc activity above baseline during expression of both a recent and remote context fear memory as indicated by high activity at both time points in the Training context. (d) dHPC inactivation at remote time point (21 days post training): Infusion of lidocaine into the dCA1 region of the HPC significantly reduced freezing to the Training, but not Novel, context. $^{**}p < .01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reduction in dorsal hippocampal activity following expression of a generalized fear memory suggests that the dorsal hippocampus may not be involved in context fear generalization.

To determine whether the dorsal hippocampus is involved in the expression of a generalized context fear memory, we temporarily inactivated the CA1 region of the dorsal hippocampus by infusing lidocaine into this region prior to retrieval at a remote time point. Based on our activity data, we hypothesized that dCA1 inactivation would reduce fear expression in the Training context, but have no effect on the high levels of freezing in the Novel context at the remote time point.

At the remote time point (21 days post-training), animals infused with either vehicle or lidocaine and tested in the Novel context exhibit a contextually imprecise generalized fear memory as evidenced by equivalent levels of freezing in the Novel context (Fig. 3d). Animals infused with lidocaine and tested in the Training context froze significantly less ($p < .001$) than animals infused with vehicle (Context by Drug interaction, $F(1, 28) = 3.8$, $p = .06$). However, we found no statistically significant differences between animals infused with vehicle and tested in the Training context compared to vehicle animals tested in the Novel context. We also found no statistically significant differences between animals infused with lidocaine and tested in the Novel context compared to vehicle animals tested in the Training context. These data suggest that dorsal hippocampus activity is involved in expression of a 21 day old remote context fear memory, which supports recent findings (Goshen et al., 2011; Ruediger et al., 2011; Wiltgen et al., 2010), but not a generalized fear memory as we predicted.

4. Discussion

The current study investigated the pattern of activity-dependent neural activation following expression of both a contextually precise and generalized context fear memory. Using C57BL/6J mice and context fear conditioning, we replicated the gradual increase in fear generalization that occurs over time (Land & Riccio, 1998; Ruediger et al., 2011; Wiltgen & Silva, 2007; Zhou & Riccio, 1996). We found that expression of a contextually precise memory was associated with activity of the ACC, IL, and PL. The ventral hippocampus was also consistently active during recent contextually precise memory recall, but the activity declined as a function of time. Activity of the mPFC and ventral hippocampus remained more stable over time when animals were tested in a novel context suggesting that activity of the cortex and ventral hippocampus underlies expression of contextually imprecise fear memory. In an attempt to dissect critical regions involved in the expression of generalized fear, we inactivated the ACC and ventral CA1 at recent and remote time points as well as the dorsal CA1 at a remote time point prior to testing. Inactivating the ACC at a recent time point had no effect on expression of a contextually precise memory. Similarly, inactivating the ACC at a remote time point had no effect on expression of fear in the training context, but significantly reduced fear expression in the Novel context. In other words, inactivating the ACC returned the memory to a contextually precise state and reduced expression of generalized fear. A recent study has shown that the ACC is involved in generalized fear responding after reactivation in the training context (Einarsson, Pors, & Nader, 2014). The present data demonstrate that the ACC is involved in generalized contextual fear memory in the absence of any reactivation episode. Inactivation of the ventral CA1 region of the hippocampus also had no effect on behavior when animals were tested for fear 1 day following training, suggesting that the ventral hippocampus is not required for the expression of a recent context memory. However, inactivation of the ventral CA1 reduced generalized fear expression in the Novel context at the remote time point. Further, inactivation of the dorsal CA1 at a remote time point reduced freezing, but only to the training context. These data are consistent with the sustained activity we observed in the dorsal CA1 when animals were tested in the training context and suggest that the dorsal CA1 plays a critical role in the retrieval of contextually precise memories, but is not involved in generalized memory recall. These data are also consistent with a lasting role of the dorsal hippocampus in contextually precise memories (Goshen et al., 2011; Wiltgen et al., 2010). A previous study demonstrated that successful discrimination between training and novel contexts is dependent upon activity of the hippocampus. Further, this study showed that when rodents generalize fear, the memory is no longer dependent upon the hippocampus suggesting that the cortex supports generalized contextual fear memories (Wiltgen et al., 2010). To the best of our knowledge, the present study is the first to demonstrate that the ventral hippocampus controls, in part, generalized contextual fear memory. Thus, our data build upon these prior conclusions suggesting that over time, generalized contextual memories may be regulated by an interaction between the ventral hippocampus and ACC.

The data presented in the current study are in agreement with some aspects of the transformation of memory hypothesis (Winocur et al., 2007) proposed to account for the gradual increase of fear generalization that occurs following context 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. Central to the transformation hypothesis is the assumption that context-specific memories, represented by the hippocampus, and context-independent memories, represented by the cortex can exist simultaneously (Winocur, Sekeres, Binns, & Moscovitch, 2013). Our Arc mRNA and inactivation data do support the idea that context-specific memories are regulated by the dorsal hippocampus. This has also been supported by recent evidence demonstrating that the dorsal hippocampus is involved in the expression of remote context memories (Goshen et al., 2011) and in the long term precision of contextual memories (Ruediger et al., 2011), suggesting that expression of a context memory, regardless of the age, is dependent on hippocampal activity. Likewise, inactivation of the ACC returned the contextual memory back to its precise state, consistent with generalized memories being stored in the cortex. Thus, inactivating the ACC enabled the dorsal hippocampus to express the contextually precise fear memory. However, our data also suggest that the ventral hippocampus is active during remote time points and this activity is associated with a generalized fear memory-inactivation of the ventral hippocampus returned memory to a context-specific form. Therefore, the present data suggest that the dorsal hippocampus supports remote contextually precise memories and the ACC supports generalized contextual fear memories, consistent with the transformation hypothesis. Yet, the finding that activity of the ventral hippocampus is associated with generalized contextual fear memory does not fit within the transformation hypothesis. It is possible that the activity of the ventral hippocampus is a result of its projections either to the ACC and other cortical regions to recruit cortical compensation or its connections with the amygdala, resulting in fear expression. Alternatively, the ACC may activate the ventral hippocampus either directly or through the nucleus reuniens resulting in generalized fear (Davoodi, Motamedi, Akbari, Ghanbarian, & Jila, 2011; Dolleman-Van der Weel, Lopes da Silva, & Witter, 1997; Loureiro et al., 2012; Xu & Sudhof, 2013).

The findings from the current study are not fully consistent with the trace-decay theory (Rudy, 2005) because inactivating

the ACC at a remote time point, presumably when the cortex is needed to help retrieve the decayed memory trace, resulted 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. In our study, inactivation of the ACC did not reduce freezing to the training context at remote testing points as has been previously demonstrated (Frankland et al., 2004; Xu et al., 2012). However, there are several notable differences between the current set of experiments and previous experiments (e.g., pre-testing lidocaine *vs* pre-training Tet-tox or SytK1 KD infusions) that could account for the reason why we only observed a reduction in the novel context and not also in the training context at remote time points. One difference between the current study and previous experiments (Frankland et al., 2004) that could potentially explain this discrepancy is the post-training interval used for the remote test. The current study inactivated the ACC at 21 days post-training to test remote/generalized memory, whereas Frankland et al. (2004) inactivated the ACC 36 days following training. It is possible that the longer time point is required to recruit the involvement of the ACC during remote context specific memory retrieval and we therefore did not see an effect of ACC inactivation on remote memory expression in the training context due to a shorter training-to-test interval. However, Frankland et al. (2004) also tested the effects of ACC inactivation on memory retrieval at several intermediate time points and reported that inactivating the ACC at 18 days following training resulted in a significant reduction in remote memory expression, which is a shorter time interval than the current study employed. Therefore, it is unlikely that the time of test can account for the discrepancy between the current study and Frankland et al. (2004).

An interesting recent finding is that pre-training inactivation of the mPFC, including the ACC, induced generalization of fear responses to novel contexts at recent time points, but impaired contextual fear in both the training and novel contexts at remote time points (Xu et al., 2012). Furthermore, pre-training inactivation of neurons within the nucleus reuniens receiving synaptic input from the mPFC induced fear generalization at a recent time point (Xu & Sudhof, 2013), but post-training infusions had no effect on fear memory. These data suggest that the ACC is involved in encoding the specificity of contexts and relays that information to the hippocampus through the nucleus reuniens. If the ACC is involved in encoding specificity of the context, why activity of the ACC then becomes associated with generalized fear at remote time points is not currently clear. One possibility is that this effect is due to direct connectivity of the ACC with the ventral hippocampus rather than through the nucleus reuniens at remote time points.

Given that the transformation and trace-decay hypotheses cannot completely account for the results found in this study, one possible alternative explanation 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 itself. Winocur et al. (2009) provided evidence that at some level the animals can perceive differences between the training context and a novel context at remote time points even though they freeze equally in both. This would suggest that even though they can perceive a difference in the two contexts, animals express fear in novel contexts at remote time points. Based on the data presented in this study, the ACC and its potential interaction with the ventral hippocampus may be the primary mechanism driving freezing behavior at remote time points. When the ACC or the ventral CA1 were inactivated at remote time points animals reduced their freezing in the novel context, suggesting that by blocking this higher-order cortical involvement the memory can be expressed as contextually precise. This contextually precise memory is controlled by the dorsal hippocampus.

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 determine whether fear is 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, the functional relationship between the cortex, in particular the ACC, and the hippocampus is not well understood. To gain a better understanding of the direction of this relationship between these structures, further study should investigate whether inactivation of one structure alters activity of the other. Additionally, subsequent experiments aimed at artificially driving fear expression, through the use of optogenetic or chemogenetic stimulation, in the novel context would provide a better understanding of the functional relation between the hippocampus and prefrontal cortex. This could potentially shed light on the directionality and functional relationship between the hippocampus and ACC and help determine which neural structures drive expression of a contextually imprecise fear memory.

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