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Hippocampal $GABA_{B(1a)}$ Receptors Constrain Generalized Contextual Fear

Joseph F Lynch, III[1], Patrick Winiecki[1], T Lee Gilman[1], Jordan M Adkins[1] and Aaron M Jasnow*[,1]

[1]*Department of Psychological Sciences, Kent State University, Kent, OH, USA*

Many anxiety disorders are characterized by generalization of fear responses to neutral or ambiguous stimuli. Therefore, a comprehensive understanding of the mechanisms contributing to generalized fear is essential for formulating successful treatments for anxiety disorders. Previous research shows that GABA-mediated presynaptic inhibition has a critical role in cued fear generalization, as animals with genetically deleted presynaptic $GABA_{B(1a)}$ receptors cannot discriminate between CS+ and CS− tones. Work from our laboratory has further identified that $GABA_{B(1a)}$ receptors are necessary for maintaining contextual memory precision, thereby constraining generalized contextual fear. We previously found that $GABA_{B(1a)}$ KO mice show generalized fear to a neutral context 24 h after training, but not 2 h after training. A similar pattern was observed with object location and recognition, suggesting that this receptor subtype affects consolidation and/or retrieval of precise contextual and spatial memories. Here we sought to specifically examine the involvement of $GABA_{B(1a)}$ receptors in consolidation or retrieval of a precise fear memory. To do so, we infused a selective $GABA_{B(1a)}$ receptor antagonist, CGP 36216, intracerebroventricularly (ICV), or locally into the dorsal hippocampus, ventral hippocampus, or anterior cingulate cortex (ACC), during consolidation and retrieval of context fear training. Blockade of $GABA_{B(1a)}$ receptors through ICV, dorsal hippocampal, or ventral hippocampal infusions 'after' training (consolidation) resulted in fear generalization to the neutral context when mice were tested 24, but not 6 h after training. Post-training infusions of CGP into the ACC, however, did not promote generalized fear. In addition, ICV, dorsal hippocampal, ventral hippocampal, or ACC infusions immediately 'before' testing (retrieval) did not result in context fear generalization. These data suggest that GABA-mediated presynaptic inhibition is not critical for retrieval of precise contextual memory, but rather has an important role in the long-term consolidation of precise contextual memories and constrains generalized fear responses. *Neuropsychopharmacology* advance online publication, 14 December 2016; doi:10.1038/npp.2016.255

INTRODUCTION

Anxiety disorders are the most prevalent class of mental disorders in the United States, emphasizing the importance of identifying neural mechanisms contributing to their etiology to facilitate better treatments (Kessler *et al*, 2005). One characteristic of many anxiety disorders is generalization of fear responses to neutral or ambiguous stimuli and contexts. For example, post-traumatic stress disorder (PTSD) patients show heightened fear responses to contextual cues compared with healthy controls (Grillon and Morgan, 1999). Further, PTSD patients display fearful responses to discrete stimuli or cues that are never paired with an aversive stimulus (CS−) and that should not elicit fearful responding during discrimination learning (Bittencourt and Sawchenko, 2000; Grillon and Morgan, 1999; Lissek *et al*, 2008). Similarly, high symptom PTSD patients are unable to discriminate between danger and safety cues in a fear potentiated startle discrimination procedure (Jovanovic *et al*, 2012; Jovanovic *et al*, 2010). In addition, a common finding across laboratory studies of people with anxiety disorders is the generalization of fear responses to CS− cues. These data are also supported by a clinical conceptualization of anxiety disorders, in which significant spreading of fear cues across contexts leads to sustained anxiety symptoms (Lissek, 2012; Lissek *et al*, 2005).

Fear generalization can be interpreted as a 'loss of memory precision' for specific information including discrete CSs and contextual cues; thus, mechanisms contributing to the establishment, maintenance, and recall of contextual memory are implicated in this process. Considerable research indicates that contextual fear generalization naturally increases over time (For review, see Jasnow *et al*, 2012). However, generalization can also occur under time-independent mechanisms, such as those that modulate the retrieval of contextual cues (Lynch *et al*, 2013; Lynch *et al*, 2016; Lynch *et al*, 2014). Despite the importance of fear generalization as a fundamental component underlying many anxiety disorders (Brewin, 2001; Grillon and Morgan, 1999; Jovanovic *et al*, 2009), we do not fully understand how this phenomenon occurs. However, some recent evidence from our laboratory has uncovered specific brain regions (Cullen *et al*, 2015) and neural mechanisms underlying generalization (Cullen *et al*, 2014), and this evidence implicates a critical role for the GABAergic system.

*Correspondence: Dr AM Jasnow, Department of Psychological Sciences, Kent State University, 600 Hilltop Drive, 230 Kent Hall, Kent, OH 44242, USA, Tel: +1 330 672 2137, Fax: +1 330 672 3786, E-mail: ajasnow@kent.edu

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Responses to GABA neurotransmission occurs through two distinct receptor populations: $GABA_A$ or $GABA_B$ receptors. $GABA_A$ receptors are ionotropic, fast acting, and primarily postsynaptic (Enna, 2007; Rudolph *et al*, 2001; Sieghart, 2006), whereas $GABA_B$ receptors are metabotropic receptors formed by heterodimers of $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits (Jacobson *et al*, 2007b). Within the $GABA_{B1}$ family, receptors can be classified as $GABA_{B(1a)}$ or $GABA_{B(1b)}$ based on the presence of two sushi domains within the N-terminus (Biermann *et al*, 2010; Gassmann and Bettler, 2012). $GABA_{B(1b)}$ is predominately postsynaptic whereas the $GABA_{B(1a)}$ subunit is predominately presynaptic and located on glutamatergic terminals (Jacobson *et al*, 2007b), contributing to presynaptic inhibition of glutamate release. The first evidence for the involvement of GABAergic signaling through $GABA_{B(1a)}$ receptors in fear generalization comes from the finding that global $GABA_{B(1a)}$ knockout (KO) mice freeze more to a CS− than wild-type (WT) littermates (Shaban *et al*, 2006). These mice also exhibit a form of presynaptic non-associative long-term potentiation (LTP) at cortico-amygdala synapses, suggesting that presynaptic mechanisms can contribute to generalized fear. Shaban *et al* (2006) found that $GABA_{B(1a)}$ KO mice were able to discriminate between CS+ and CS− cues at lower shock intensities but generalized at higher shock intensities. In fact, if the shock intensity was raised high enough, KO and WT mice generalized to the CS−. Given these findings, the authors concluded that KO mice shifted the threshold, or shock requirement, to a lower intensity compared with WT mice. However, short-term tests of memory function and non-aversive memory tests were not performed during these experiments, leaving open the possibility of alternative interpretations. In a recent study, Cullen *et al* (2014) found that $GABA_{B(1a)}$ KO mice were able to discriminate between a training context and novel context 2 h after contextual fear training, but generalized fear to a novel context 24 h later compared with WT mice that were able to maintain memory precision for up to 5 days after training. In addition, $GABA_{B(1a)}$ KO mice showed memory precision deficits in location and object discrimination, at a 24-h test, but not at a 2 h test compared with WTs (Cullen *et al*, 2014). Overall, these data suggest that impaired GABA-mediated presynaptic inhibition does not affect early contextual discrimination, but produces an inability to store or retrieve precise contextual memories, ultimately contributing to generalized fear. These findings suggest that the shifted threshold of generalization for KO mice is not due to shock intensity (Shaban *et al*, 2006); $GABA_{B(1a)}$ KO mice have the ability to discriminate contexts at short intervals and display generalization for non-aversive stimuli.

These previous studies utilized global $GABA_{B(1a)}$ KO mice, inherently limiting investigations of which learning and memory process is affected by $GABA_{B(1a)}$ transmission in order to induce fear generalization. This is important for understanding the development of, and improving potential treatments for, anxiety disorders. Although Shaban *et al* (2006) demonstrated that presynaptic inhibition in the lateral amygdala was important for cued fear discrimination, the role of presynaptic inhibition in contextual discrimination is likely localized to additional brain regions. For example, Arc mRNA activity increases in the anterior cingulate cortex (ACC) and ventral hippocampus during expression of a generalized fear, implicating activity of these structures in contextual fear generalization. Inactivation of the ACC or ventral hippocampus before retrieval at a long retention interval significantly attenuates generalized contextual fear (Cullen *et al*, 2015). $GABA_{B(1a)}$ receptors are located at glutamatergic terminals in neocortical and hippocampal pyramidal neurons (Jacobson *et al*, 2007b; Shaban *et al*, 2006; Vigot *et al*, 2006). Given the involvement of these structures both in processing contextual information (Bannerman *et al*, 2002; Bannerman *et al*, 1999; Kjelstrup *et al*, 2002; Moser *et al*, 1993; Moser and Moser, 1998), and in fear generalization (Cullen *et al*, 2014; Cullen *et al*, 2015), the current study was designed to explore when presynaptic $GABA_{B(1a)}$ receptors contribute to contextual discrimination, thereby constraining generalized fear. In order to assess this, C57BL/6 male mice received cannula implants aimed at the lateral ventricle and were infused with a $GABA_{B(1a)}$ receptor-specific antagonist CGP 36216. To further refine the brain regions involved in contextual fear generalization, additional mice received cannula implants aimed at the dorsal hippocampus, the ventral hippocampus, or the ACC and were infused with CGP 36216. Given the findings with global $GABA_{B(1a)}$ KO mice, in which animals displayed immediate memory precision, but no precision within 24 h (Cullen *et al*, 2014), we hypothesized that infusions of CGP 36216 during the consolidation period (ie, infusions given just before or immediately after training) would induce generalized fear 24 h later. In addition, because of the presumed effect on consolidation, we did not expect CGP 36216 to have an effect on memory retrieval, although the data from KO mice was inconclusive on what specific process may be affected. Therefore, animals were given infusions before training, immediately after training, or before testing.

MATERIALS AND METHODS

Animals and Housing Conditions

All experiments were conducted with male C57BL/6 mice generated from a breeding colony in the Department of Psychological Sciences at Kent State University. Breeders for this colony were purchased from Jackson Laboratories. Animals were 6–9 weeks at the time of surgery and were group housed (3–5 animals per cage) with *ad libitum* 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 (AAALAC). 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.

Surgical Procedures

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 lateral ventricle at the

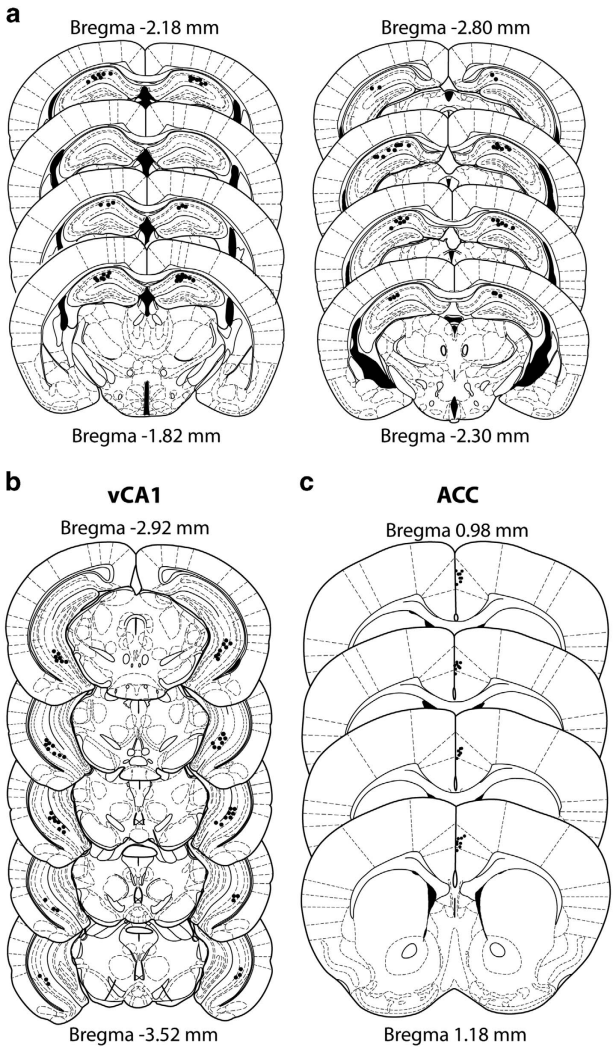

Figure 1 Infusion site verification for the dCA1, vCA1 and ACC. Local infusion site verification for all animals having cannula aimed at (a) the dCA1, (b) the vCA1, or (c) the ACC. For the ACC site verification is depicted unilaterally for ease, but the hemisphere for guide cannula implantation was varied across animals. Black dots represent the site of infusion for one or more animals. Drawings are adapted from Paxinos and Franklin, 2004.

following coordinates with respect to bregma (−2.10 mm D/V, − 0.34 mm A/P, +1 mm M/L) including a 1 mm protrusion for the infusion needle. Cannulations into specific brain regions were completed as described previously (Cullen *et al*, 2015). Briefly, for insertion into the dorsal CA1, bilateral guide cannulae were surgically implanted using a 14° angle (−1.6 mm D/V, − 2.5 mmA/P, +2.5 mm M/L) with a 1 mm infusion needle. For insertion into the ventral CA1, bilateral guide cannulae were surgically implanted using a 14° angle (−3.6 mm D/V, − 3.16 mm A/P, +4.2 mm M/L) with a 3 mm infusion needle. For insertion into the ACC, a single guide cannula was implanted using a 14° angle (−1.75 mm D/V, +0.8 mm A/P, +0.7 mm M/L) with a 1 mm infusion needle. Other studies from our lab and others have used unilateral cannula within the ACC successfully to assess the involvement of the ACC in long term fear memory and fear generalization (Cullen *et al*, 2014; Frankland *et al*, 2004).

Site Verification

Cannula placement was verified for intracerebroventricular (ICV) infusions, the dCA1, vCA1, and ACC (Figure 1) using 0.5 μl infusions of xylene cyanol FF at 0.25% in saline followed by rapid decapitation. Brains were fresh frozen on powdered dry ice and sliced on a cryostat and slices were mounted and observed for correct placement using an inverted microscope. Any animal with a misplaced cannula was not included in the final analysis. No ICV animal was excluded due to misplaced cannula. For site-specific placement, 7% of animals were excluded across experiments for misplaced cannula (13 animals total).

Drug Administration

The $GABA_{B(1a)}$ receptor antagonist, CGP 36216 (Sigma) was used to block presynaptic $GABA_{B(1a)}$ receptors within specific brain regions during the learning and memory process of contextual fear generalization. CGP 36216 shows no activity for postsynaptic $GABA_{B1}$ receptors and is selective for presynaptic $GABA_{B1}$ receptors (Ong *et al*, 2001). For the initial experiment in which animals received infusions into the lateral ventricle, CGP 36216 was dissolved in 0.9% saline at 1 mM, 2 mM, or 3 mM concentrations. Subsequent experiments used a 3 mM concentration. Animals received infusions (1 μl infused at a rate of 0.2 μl per min for ICV; and 0.5 μl at a rate of 0.1 μl per min for site-specific infusions) of CGP 36216 or vehicle (0.9% saline from a 5 μl Hamilton syringe operated by a micro-infusion pump (Harvard Apparatus). Infusion needles were left in place for 1 min following infusion completion to ensure diffusion into the brain. During the infusion, mice were placed in a clean housing cage and allowed to walk freely with only brief restraint to place and remove infusion needles. Animals received infusions either 30 min before training, immediately following training, or 30 min before testing for ICV experiments. For experiments in which CGP 36216 or vehicle was locally infused into the hippocampus or ACC, animals received infusions immediately after training or 5 min before context fear testing. The timing for behavior following infusions was chosen to allow enough time for circulation throughout the brain (ICV) or to assess near-immediate effects locally within the hippocampus or ACC (site-specific infusions).

Fear Training Apparatus

Behavioral procedures were performed in four identical training chambers (7" W × 7" D × 12" H) containing two Plexiglas walls (front and back), two aluminum sidewalls and a stainless steel shock-grid floor (Coulbourn Instruments, Allentown, PA). The training context consisted of the context chamber (two Plexiglas and two 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 neutral context did not have a polka dot background, was illuminated only via infrared lights, did not have white noise,

had a solid surface floor, and was cleaned with 50% Quatricide.

Procedure

Animals were acclimated to handling for 5 min per day for 2 consecutive days before context exposure. Following handling, animals were exposed to the training context for 5 min per day for 2 days. Twenty-four hours after the last context exposure, animals were fear conditioned in the training context by being placed into the context and 120 s later, they received five, 0.8 mA footshocks separated by 90 s ISIs. Testing occurred at distinct time points following fear training (6 h or 24 h) in either the training context or a neutral context. Testing consisted of a 10 min exposure to the training or neutral context, during which freezing was measured during minutes 2 through 6 in order to allow for contextual recognition, acclimation to the chamber and to avoid any extinction effects using FreezeFrame 3 software (Actimetrics, Wilmette, IL). Freezing was used as an indicator of fear behavior, and defined as the absence of any movement except that required for respiration.

Open Field

To test for effects on locomotor activity, 1 week after testing for context fear, a subset of animals were given infusions of CGP 36216 or vehicle 30 min before placement in an open-field chamber. Animals were allowed to explore the open field for 10 min (46 cm × 46 cm × 39 cm; Coulbourn Instruments). Locomotor activity was measured via Any-Maze 4.99 software (Stoelting, Wood Dale, IL).

Statistical Analyses

Freezing responses during context fear testing were analyzed using independent *t*-tests to statistically verify *a priori* defined criteria for generalization by comparing freezing in drug-treated mice across contexts and also comparing vehicle and drug-treated mice in the neutral context. A Student's *t*-test was used to analyze locomotor behavior. Statistical significance for analyses were set at $p \leqslant 0.05$.

RESULTS

Central Inhibition of $GABA_{B(1a)}$ Receptors Promotes Generalized Contextual Fear

Before any experiments were conducted, we established criteria for assessing sufficient generalization based on our previous assessments of fear generalization (Lynch *et al*, 2013; Lynch *et al*, 2016; Lynch *et al*, 2014). Specifically, in order for the CGP-treated animals to display sufficient generalization, animals tested in the neutral context must display similar levels of freezing to those animals tested in the training context. In addition, the CGP-treated animals must demonstrate significantly higher freezing compared with vehicle-treated animals when tested in the neutral context. To assess these specific directional hypotheses, independent *t*-tests were conducted to assess differences in fear among vehicle-treated animals tested in either context, drug-treated animals tested in either context, and animals tested in the neutral context.

Animals were administered infusions of different doses of CGP 36216 into the lateral ventricle 30 min before training in context fear. Animals were then tested 24 h later in either the training context or a neutral context (Figure 2a). Independent *t*-tests revealed that animals given vehicle infusions displayed significant contextual memory precision, displaying little freezing in the neutral context compared with the training context ($t(23) = 4.33$, $p < 0.001$) (Figure 2b). Animals given 1 mM of the $GABA_{B(1a)}$ antagonist, CGP 36216, also displayed significant contextual memory precision, (1 mM: $t(8) = 3.41$, $p < 0.01$). However, animals administered 2 mM of CGP 36216 displayed similar freezing levels in the training context and neutral context suggesting impaired contextual memory precision, ($t(9) = 1.77$, ns). In addition, animals given 3 mM of CGP 36216 also displayed significantly impaired memory precision, ($t(9) = 0.96$, ns). To determine if animals given 2 mM or 3 mM of CGP 36216 displayed significantly more fear in the neutral context to vehicle-treated animals, we compared freezing between vehicle and drug treated animals tested in the neutral context. Animals given a 2 mM dose did not display significantly more fear in the neutral context compared with vehicle-treated animals, ($t(15) = 1.58$, ns). However, the animals given a 3 mM dose displayed significantly more fear in the neutral context compared with vehicle-treated animals, ($t(17) = 4.16$, $p < 0.001$). Taken together, these results suggest that a 3 mM dose of CGP 36216 given before training into the lateral ventricle impairs contextual memory precision and induces generalized fear to the neutral context 24 h later. These data replicate effects that we have previously observed in global $GABA_{B(1a)}$ KO mice (Cullen *et al*, 2014).

Previously, with $GABA_{B(1a)}$ KO mice, the impaired memory precision seen at 24 h was not seen during a 2 h test, suggesting that presynaptic inhibition was not required for short-term memory precision, but was necessary for the establishment, or maintenance of long-term memory precision (Cullen *et al*, 2014). To further test that hypothesis, and because a 2-hour retention interval could still be within the consolidation phase, animals were given 3 mM CGP 36216 ICV infusions 30 min before context fear training and were tested 6 h later (Figure 2c). All subsequent experiments utilized a 3 mM dose of CGP 36216. Here, we confirmed that pharmacological blockade of $GABA_{B(1a)}$ receptors before training does not result in impaired contextual memory precision up to 6 h after training; vehicle-treated ($t(6) = 3.55$, $p < 0.01$), and drug-treated animals, ($t(6) = 6.63$, $p < 0.001$), displayed significant contextual memory precision (Figure 2c). Therefore, $GABA_{B(1a)}$ receptors are necessary for the maintenance of long term contextual memory precision beyond 6 h after training. This also rules out any differences in performance, acquisition, or perceptual abilities that may have occurred during training; mice given CGP 36216 were able to discriminate between the training and neutral contexts at 6 h.

Finally, to make sure effects of CGP 36216 on memory precision was not due to effects on locomotor activity, animals were given ICV infusions and tested 30 min later in an open field (Figure 2d). Distance traveled during a 10 min test revealed no significant differences in locomotor activity

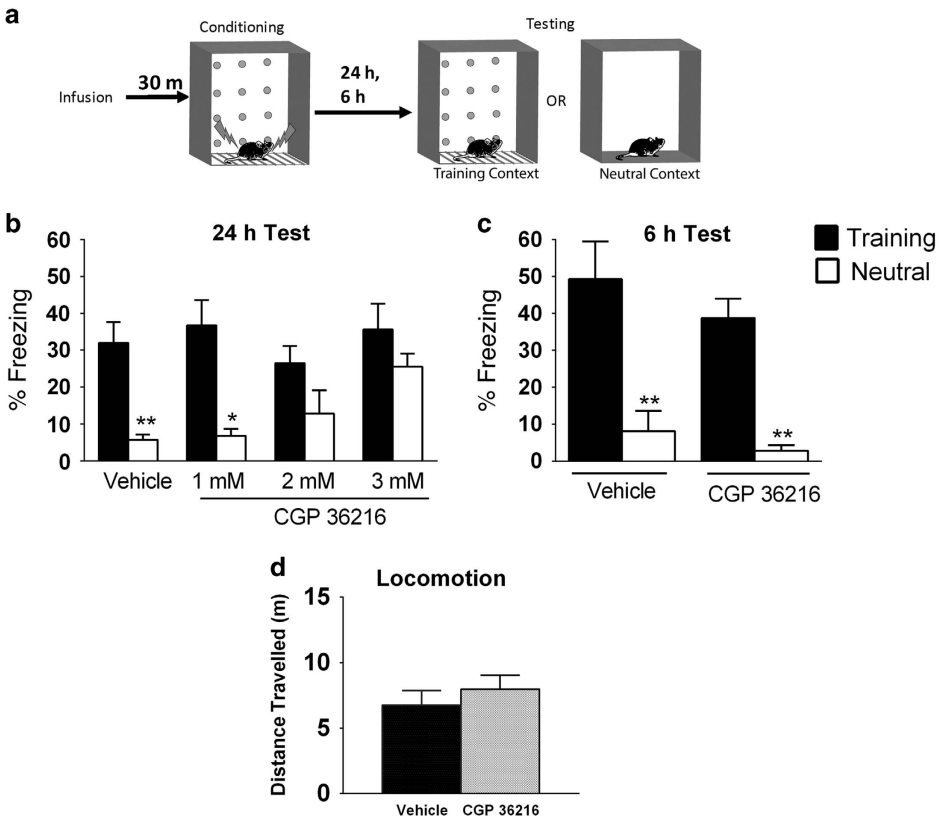

Figure 2 Central inhibition of $GABA_{B(1a)}$ receptors promotes generalized contextual fear. Animals received infusions into the lateral ventricle before training and were tested at different retention intervals. (a) Schematic representation of the experimental paradigm. Animals received infusions of vehicle or CGP 36216 into the lateral ventricle 30 min before context fear training and then were tested at distinct retention intervals in either the training or a neutral context. (b) Animals given infusions of CGP 36216 at 3 mM dose displayed impaired memory precision to the neutral context compared with vehicle-treated animals when tested 24 h after training. (c) When testing occurred 6 h after training, 3 mM CGP 36216 was not sufficient to impair memory precision. (d) Generalization was not due to differences in locomotor activity between vehicle and CGP 36216-treated mice. No differences were observed in distance traveled within an open field between vehicle and CGP 36216-treated mice. Values are displayed as mean ($\pm$SEM). Significance values were set at $p<0.05$. ($*=p<0.05$, $**=p<0.01$.)

between vehicle-treated and CGP-treated animals, ($t_{(12)}=0.79$, ns), suggesting that the effects of CGP on memory precision was not due to alterations in activity levels.

We next investigated if administering CGP 36216 would result in generalized contextual fear if given during the memory consolidation period. Animals received ICV infusions of vehicle or CGP 36216 immediately following context fear training and were tested 24 h later (Figure 3a). Independent *t*-test analyses reveal that vehicle-treated animals displayed intact memory precision, ($t(16)=4.56$, $p<0.001$), displaying significantly more fear in the training context as compared with the neutral context (Figure 3b). Drug-treated animals, however, displayed equivalent freezing in the training context and neutral context, suggesting impaired memory precision ($t(15)=1.36$, ns). Moreover, drug-treated animals displayed significantly more fear in the neutral context compared with vehicle-treated animals, ($t(17)=2.59$, $p<0.05$). Thus, post-training blockade of $GABA_{B(1a)}$ receptors disrupts the appropriate consolidation of a precise contextual memory and induces generalized contextual fear.

Having established that $GABA_{B(1a)}$ receptors are necessary for the consolidation of precise contextual fear memories and limit fear generalization, we next wanted to see if $GABA_{B(1a)}$ receptors were necessary for the retrieval of a precise contextual memory. In other words, would infusions of CGP 36216 before test produce retrieval discrepancies and induce contextual fear generalization? Therefore, animals underwent context fear training and received an ICV infusion of CGP 36216 or vehicle 30 min before a retrieval test that occurred 24 h after training in either the training context or the neutral context (Figure 3c). Independent *t*-test analyses reveal that both vehicle-treated, ($t(12)=3.73$, $p<0.01$), and drug-treated animals displayed significant memory precision ($t(21)=2.68$, $p<0.01$) (Figure 3d). However, CGP-treated and vehicle-treated animals did differ when tested in the neutral context, ($t(20)=2.408$, $p<0.05$). Despite the higher levels of freezing in the neutral context compared with vehicle-treated animals, animals given CGP before testing are not considered to have sufficient generalization as these animals do not meet both criteria for generalization. Therefore, these results suggest that $GABA_{B(1a)}$ transmission is not necessary for precise memory recall, but is required during memory consolidation in order to maintain a precise memory.

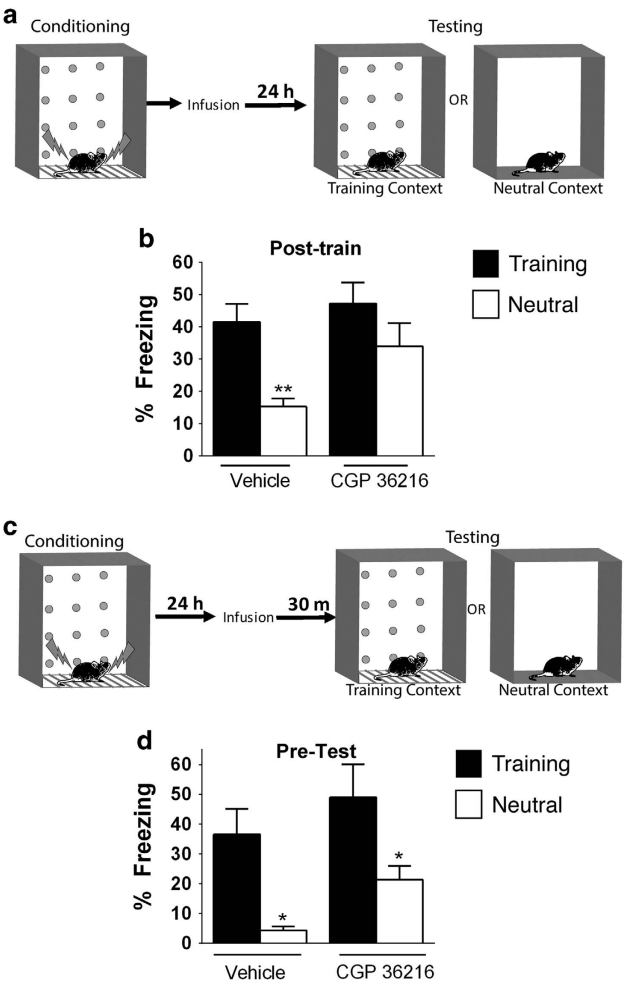

Figure 3 Pre-testing or post-training inhibition of $GABA_{B(1a)}$ receptors promotes generalized contextual fear. Animals received infusions into the lateral ventricle immediately following training or 30 min before testing. (a) Schematic representation of the experimental paradigm. Animals received infusions into the lateral ventricle immediately following training and were tested at 24 h. (b) Animals receiving infusions of CGP 36216 immediately following fear training, displayed impaired memory precision when tested 24 h later. (c) Schematic representation of the experimental paradigm. Animals received infusions into the lateral ventricle 30 min before memory testing. (d) When animals received infusions 30 min before testing, CGP 36216 did not impair memory precision. Values are displayed as mean (±SEM). Significance values were set at $p<0.05$. (* = $p<0.05$, ** = $p<0.01$.)

Hippocampal $GABA_{B(1a)}$ Receptors Constrain Contextual Fear Generalization

The dorsal CA1 hippocampus is implicated in the processing of contextual information (Bannerman *et al*, 2002; Bannerman *et al*, 1999; Kjelstrup *et al*, 2002; Moser *et al*, 1993; Moser and Moser, 1998). Therefore, to evaluate if $GABA_{B(1a)}$ receptors within the dorsal CA1 region of the hippocampus are required for precise contextual memory and limiting generalized fear, animals received infusions of CGP 36216 or vehicle into the dorsal CA1 immediately after context fear training (Figure 4a) or 5 min before testing (Figure 4d). For animals receiving immediate post-training infusions, independent *t*-test analyses revealed that vehicle-treated animals displayed significantly more fear in the training context as compared with the neutral context, indicating intact memory precision, ($t(16)=3.78$, $p<0.001$). However, drug-treated animals displayed equivalent fear in the training context and neutral context ($t(16)=0.93$, ns), and significantly more fear in the neutral context compared with vehicle-treated animals, ($t(16)=2.29$, $p<0.05$) (Figure 4b). When infusions were directed towards the dorsal CA1 before context memory retrieval, vehicle-treated, ($t(23)=2.94$, $p<0.01$), and drug-treated animals displayed significant memory precision, ($t(14)=3.22$, $p<0.01$) (Figure 4e), indicating little generalized fear.

We previously demonstrated that activity of the ventral CA1 of the hippocampus and the ACC were critically involved in time-dependent contextual fear generalization (Cullen *et al*, 2015). Inactivation of the ventral CA1 or the ACC during a remote retrieval test (21 days after training) attenuated generalized contextual fear (Cullen *et al*, 2015). We therefore wanted to assess if blockade of presynaptic $GABA_{B(1a)}$ receptors within these regions would promote generalized fear on a shorter time scale during the consolidation process or during retrieval. Animals received local infusions of vehicle or CGP 36216 into the ventral CA1 or the ACC immediately after context fear training or 5 min before testing. Immediate post-training infusions of CGP 36216 into the ventral CA1 induced contextual fear generalization (Figure 4c). Vehicle-treated animals displayed significantly more freezing in the training context as compared with the neutral context, indicating intact memory precision, ($t(13)=5.13$, $p<0.001$). Drug-treated animals, however, displayed equivalent fear to both contexts, ($t(13)=1.70$, ns), and significantly more fear in the neutral context compared with vehicle-treated animals, ($t(15)=2.25$, $p<0.05$). When infusions were given into the ventral CA1 before context memory retrieval test, vehicle-treated, ($t(9)=2.29$, $p<0.05$), and drug-treated animals displayed significant memory precision, ($t(10)=3.80$, $p<0.01$) (Figure 4f). Finally, infusions of CGP 36216 into the dorsal hippocampus 5 min before exposure to the open field did not affect locomotor activity, ($t_{(10)}=1.37$, ns), similar to what was found with ICV infusions, indicating that impaired memory precision via hippocampal infusions is not due to alterations in locomotor activity (Figure 4g). Infusions of CGP 36216 into the ACC had no effect on fear generalization during the consolidation process (Figure 5a and b). Vehicle-treated, ($t(11)=2.635$, $p<0.05$), and drug-treated animals displayed significant memory precision, ($t(11)=4.71$, $p<0.001$). In addition, infusions of CGP 36216 into the ACC during the context retrieval test did not affect fear generalization (Figure 5c and d). Vehicle-treated, ($t(13)=3.49$, $p<0.001$), and drug-treated animals displayed significant memory precision, ($t(14)=3.10$, $p<0.001$). Taken together, these results suggest that presynaptic $GABA_{B(1a)}$ receptors within the dorsal and ventral CA1 are necessary to constrain contextual fear generalization during memory consolidation, but not necessary for retrieval of precise contextual fear memories. Presynaptic $GABA_{B(1a)}$ receptors in the ACC are not necessary for precise fear memory consolidation or retrieval, and do not appear to play a role in early forms of generalized contextual fear, despite the importance of this region in time-dependent fear generalization (Cullen *et al*, 2015).

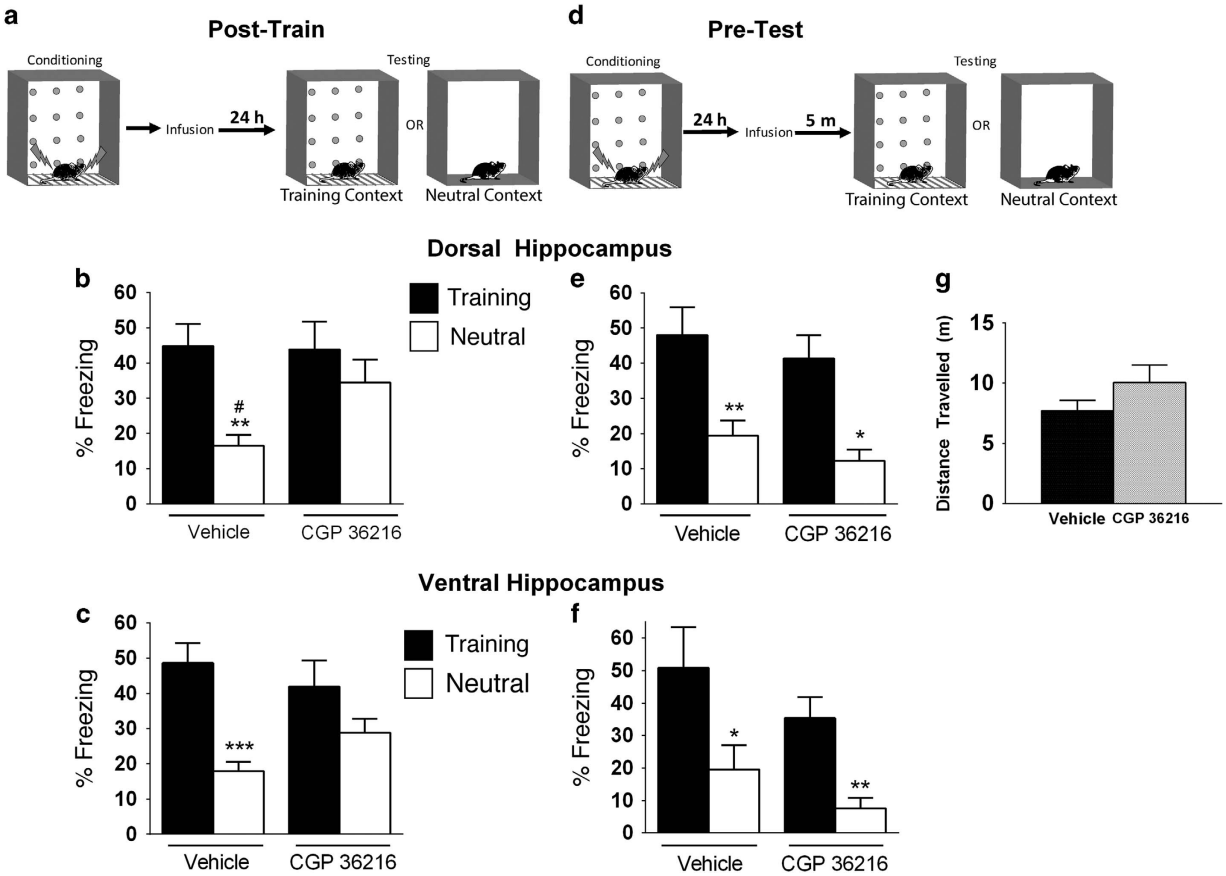

Figure 4 Hippocampal $GABA_{B(1a)}$ receptors constrain contextual fear generalization. Animals received infusions into the dorsal or ventral CA1 region of the hippocampus immediately after training or 5 min before testing. (a) Schematic representation of the experimental paradigm. Animals received infusions immediately following fear training and were tested 24 h later. (b) When infusions were given into the dorsal hippocampus, CGP impaired memory precision. (c) Infusions of CGP into the ventral hippocampus also impaired memory precision. (d) Schematic representation of the experimental paradigm. Animals received infusions 5 min before testing. (e) Infusions of CGP into the dorsal hippocampus before testing was not sufficient to impair memory precision. (f) Infusions of CGP into the ventral hippocampus before testing was not sufficient to impair memory precision. Values are displayed as mean (±SEM). Significance values were set at $p<0.05$. (*/# = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.) (g) Generalization was not due to differences in locomotor activity between vehicle and CGP 36216-treated mice. No differences were observed in distance traveled within an open field between vehicle and CGP 36216-treated mice.

DISCUSSION

The current results significantly improve our understanding of how GABA-mediated presynaptic inhibition contributes to memory specificity and constrains generalized fear. Here, we expand upon the findings of global $GABA_{B(1a)}$ KO mice displaying generalized contextual fear 24 h after context fear training (Cullen *et al*, 2014) by showing that GABA-mediated presynaptic inhibition is required during the consolidation of memory within the dorsal and ventral hippocampus in order to maintain memory precision. Utilizing a $GABA_{B(1a)}$-specific receptor antagonist, we found that inactivation of $GABA_{B(1a)}$ receptors during memory formation or consolidation results in generalized contextual fear 24 h later, although no loss of memory precision is seen if testing occurs up to 6 h later. Additionally, we demonstrated that site-specific infusions of the $GABA_{B(1a)}$ receptor antagonist promoted generalized contextual fear when infused into the dorsal or ventral CA1 region of the hippocampus during memory consolidation, but not during memory retrieval. Finally, infusions of the $GABA_{B(1a)}$ receptor antagonist into the ACC did not promote generalized fear when infused at either time. Our current results demonstrate a loss of fear memory precision for contextual information; animals display heightened levels of fear in a neutral context when $GABA_{B(1a)}$ receptors are blocked during consolidation. These results are unlikely the result of overall increases in anxiety-like behavior as $GABA_{B(1a)}$ KO do not demonstrate enhanced anxiety-like behavior across several different commonly used mouse behavioral procedures (Jacobson *et al*, 2007a), which may or may not represent anxiety in humans. Fear generalization, however, is a common finding in people with anxiety disorders and may contribute significantly to the spreading of fear cues across contexts leading to sustained anxiety symptoms (Lissek, 2012; Lissek *et al*, 2005). Thus, the current data are relevant to the understanding of human anxiety disorders.

That pre-training infusions of CGP 36216 had no effect on memory precision when retrieval occurred 6 h later suggests that $GABA_{B(1a)}$ receptors are not required for the acquisition or establishment of precise contextual memory. Rather, these data suggest that $GABA_{B(1a)}$ receptors, and the resultant

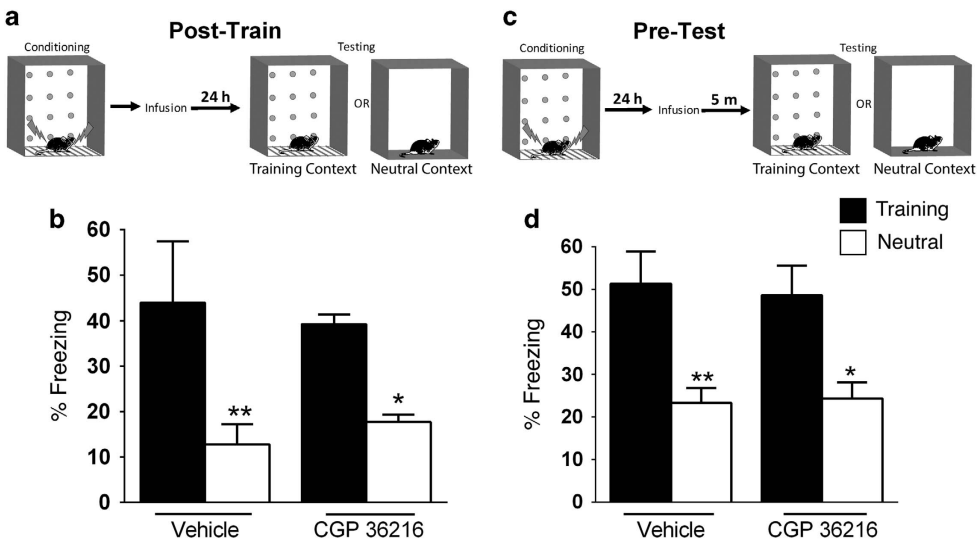

Figure 5 Inhibition of $GABA_{B(1a)}$ receptors in the ACC does not promote generalized contextual fear. Animals received infusions into the ACC immediately after training or 5 min before testing. (a) Schematic representation of the experimental paradigm. Animals received infusions immediately following fear training and were tested 24 h later (b). When infusions were given into the ACC following fear training, CGP did not impair memory precision. (c) Schematic representation of the experimental paradigm. Animals received infusions 5 min before testing. (d) Infusions of CGP into the ACC before testing was not sufficient to impair memory precision. Values are displayed as mean (±SEM). Significance values were set at $p<0.05$. (* = $p<0.05$, ** = $p<0.01$.)

presynaptic inhibition, are required for the long-term consolidation or maintenance of precise contextual memory and function to constrain contextual generalization. These findings are in line with what is seen in $GABA_{B(1a)}$ KO mice; they display precise memory recall for contextual fear or object recognition 2 h, but not 24 h after training (Cullen *et al*, 2014).

Several studies demonstrate the importance of the hippocampus in context memory precision (Ruediger *et al*, 2011; Wiltgen and Silva, 2007; Wiltgen *et al*, 2010; Winocur *et al*, 2007). According to the transformation hypothesis of memory function over time, detailed context-specific memories are initially dependent on the hippocampus, but are transformed into schematic (less specific) memories as they are stored in the neocortex, resulting in generalized memory recall (Frankland *et al*, 2006; Frankland *et al*, 2001; Kim and Fanselow, 1992; McGaugh, 1966; Vetere *et al*, 2011; Zola-Morgan and Squire, 1990). The gradual decline in memory precision over time (Jasnow *et al*, 2012) may be due to some modulation of memory storage in the neocortex or its potential interaction with the ventral hippocampus (Cullen *et al*, 2015). Given the results presented here and previous findings within $GABA_{B(1a)}$ KO mice that have normal hippocampal $GABA_{B(1a)}$ autoreceptors (Gassmann and Bettler, 2012), we conclude that the effect of CGP, and thus the role of $GABA_{B(1a)}$ receptors in fear generalization, occurs through presynaptic mechanisms on glutamatergic terminals. In isolation, however, the CGP experiments cannot rule out effects on presynaptic autoreceptors. These effects were not due to impairments in the perceptual abilities of the animals because they were able to discriminate between similar contexts up to 6 h after training. Thus, GABA-mediated presynaptic inhibition is necessary for the consolidation of precise long term contextual memories. These findings do not fit within the transformation hypothesis, which requires the passage of time in order for cortical and more generalized memories to become dominant. Therefore, the current results demonstrate a form of fear generalization that does not require the passage of time as indicated by the transformation hypothesis.

The dorsal, but not ventral, hippocampus is required for the acquisition of spatial information, (Bannerman *et al*, 2002; Bannerman *et al*, 1999; Kjelstrup *et al*, 2002; Moser *et al*, 1993; Moser and Moser, 1998), but see (de Hoz *et al*, 2003; Ferbinteanu and McDonald, 2000). One model of hippocampal interconnectivity in relation to fear training suggests that the spatial information encoded by the dorsal hippocampus is transferred to the ventral hippocampus, which via interconnections with the amygdala (Canteras and Swanson, 1992; Pitkänen *et al*, 2000) helps associate contextual information with representations of shock (US) (Maren, 2001). Integrating the current findings with this model would suggest that GABA-mediated presynaptic inhibition through $GABA_{B(1a)}$ receptors is required for processing specific spatial information within the dorsal hippocampus and for the association of the US with contextual information via ventral hippocampal connections, possibly to the amygdala.

Recent data suggest the involvement of the ventral hippocampus in memory precision. The ventral hippocampus is active during memory retrieval in a familiar and novel context at both early (1 day) and remote tests (21 days), as measured by Arc mRNA (Cullen *et al*, 2015). At the remote retrieval test, context memory has become generalized and animals exhibit fear to the training (familiar) and the novel contexts. However, temporary inactivation of the ventral hippocampus selectively reduces fear to the novel context—but not fear to the training context—only at the remote test. This suggests that although the ventral hippocampus remains active during remote context fear retrieval regardless of context, its activity is more tightly linked to generalized fear responses (Cullen *et al*, 2015). In

contrast to our previous results (Cullen *et al*, 2015), the current study suggests that the ventral hippocampus is critical for the long-term consolidation of precise contextual fear memory, serving to constrain generalized fear. Here, we find increased generalized contextual fear when $GABA_{B(1a)}$ receptors are blocked within the ventral hippocampus after training. $GABA_{B(1a)}$ receptors are commonly localized to presynaptic terminals of glutamatergic neurons within the hippocampus (Gassmann and Bettler, 2012; Guetg *et al*, 2009; Vigot *et al*, 2006). Thus, blocking these receptors within the dorsal or ventral CA1 could result in augmented glutamate release from CA3 or possibly entorhinal inputs, resulting in saturated postsynaptic responses during memory consolidation (Gassmann and Bettler, 2012; Vigot *et al*, 2006). Saturated postsynaptic responses would likely impair LTP in CA3-CA1 synapses similar to what is observed in $GABA_{B(1a)}$ KO mice (Vigot *et al*, 2006), moving synaptic plasticity outside of a dynamic range that would support LTP (Gassmann and Bettler, 2012; Vigot *et al*, 2006). This effect could contribute to a lack of context specificity during consolidation. However, despite the lack of notable LTP, $GABA_{B(1a)}$ KO mice are still able to learn context and cued fear, as well as passive avoidance (Cullen *et al*, 2014; Jacobson *et al*, 2007b; Shaban *et al*, 2006). In the current study, animals treated with CGP 36216 can also learn context fear, but they are unable to maintain contextual precision. Alternatively, pharmacological blockade of $GABA_{B(1a)}$ receptors could produce a type of non-associative, NMDA-independent form of LTP in the CA1 similar to what has been observed in the lateral amygdala (Shaban *et al*, 2006). This could account for contextual generalization as has been demonstrated for cued fear generalization. It is important to note that a shift of the generalization threshold to lower shock intensities as hypothesized by Shaban *et al* (2006) cannot account for the contextual fear generalization observed here because $GABA_{B(1a)}$ KO mice are able to discriminate between contexts 2 h after training, and also show generalization of non-aversive stimuli. Similarly, here CGP-treated mice were able to discriminate contexts up to 6 h after training, but generalized 24 h later. If a shift of the generalization threshold to lower shock intensities accounted for this difference, we would have observed generalization at all testing points in both studies. A final explanation is that augmented glutamate release enhances contextual-US associations; animals have improved pattern completion of overlapping contextual features during retrieval tests in a distinct but similar context. This would result in fear expression in the neutral context. This possibility, however, is difficult to support given the animals' successful discrimination of contexts up to 6 h after training. Thus, although the specific LTP phenomenon that supports generalized contextual fear is currently unknown, it is apparent that $GABA_{B(1a)}$ receptors within the hippocampus support long-term consolidation or maintenance of contextually precise memories, but not their establishment.

Our previous data demonstrate a role of the prefrontal cortex, specifically the ACC, in contextual fear generalization. Inactivation of the ACC at a remote time point specifically reduced generalized fear, but did not affect fear to the training context, suggesting that imprecise contextual memory recall is supported by the ACC (Cullen *et al*, 2015). Similarly, inactivation of the ACC before training produced fear generalization, but only at remote time points (Xu *et al*, 2012; Xu and Sudhof, 2013). In the current study, blocking $GABA_{B(1a)}$ receptors within the ACC did not affect contextual fear generalization when the antagonist was given during memory consolidation or before memory retrieval when animals were tested 24 h after training. These findings are in agreement with previous findings demonstrating no ACC involvement in recent memory recall and extend the finding to show that $GABA_{B(1a)}$ receptor activity is not involved in recent memory recall within the ACC. Future studies will be needed to determine if presynaptic inhibition underlies the imprecise memory recall driven by the ACC at remote time points.

Overall, these experiments extend the previous findings with $GABA_{B(1a)}$ KO mice and demonstrate a specific role of $GABA_{B(1a)}$ receptors in the long term maintenance of contextual memory precision, specifically within the dorsal and ventral hippocampus. To date, theories of memory precision all share the idea that the passage of time is required in order for generalized responding to occur (Biedenkapp and Rudy, 2007; Jasnow *et al*, 2012; Lynch *et al*, 2013; Matynia *et al*, 2008; Wiltgen and Silva, 2007; Winocur *et al*, 2007). The current data suggest, in some cases, the loss of memory precision does not require a significant passage of time (ie, several or more days) and can be dependent upon the maintenance of the originally formed memory trace during memory consolidation. These findings demonstrate a need to gain a better, more comprehensive understanding of the mechanisms contributing to the establishment and maintenance of precise contextual memories and how they function to constrain generalized fear, which is particularly relevant to human anxiety disorders. Understanding how GABA-mediated presynaptic inhibition contributes to contextual fear memory specificity may lead to new targets for treatments of several anxiety disorders in which generalization of fear cues is a common symptom.

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