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Basolateral amygdala Thy1-expressing neurons facilitate the inhibition of contextual fear during consolidation, reconsolidation, and extinction

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ABSTRACT

Disrupted fear inhibition is a characteristic of many anxiety disorders. Investigations into the neural mechanisms responsible for inhibiting fear will improve understanding of the essential circuits involved, and facilitate development of treatments that promote their activity. Within the basolateral amygdala (BLA), Thy1-expressing neuron activity has been characterized by us and others as promoting fear inhibition to discrete fear cues by influencing consolidation of cued fear learning or cued fear extinction. Here, we evaluated how activating BLA Thy1-expressing neurons using DREADDs affected the consolidation, expression, reconsolidation, and extinction of contextual fear. Using an inhibitory avoidance paradigm, our present findings indicate a similar involvement of BLA Thy1-expressing neuron activity in the consolidation and extinction, but not expression, of fear. Importantly, our data also provide the first evidence for involvement of these neurons in inhibiting fear reconsolidation. Therefore, these data enhance our understanding of the roles that Thy1-expressing neurons within the BLA play in inhibiting fear when examining avoidance, in addition to the already established role in Pavlovian fear paradigms. Future investigations should further explore the circuits responsible for these contextual effects modulated by BLA Thy1 neuron activation, and could promulgate development of therapies targeting these neurons and their downstream effectors.

1. Introduction

As essential as fear is to an organism's survival, so too is an organism's ability to inhibit fear. Insufficient inhibition of fear can impair the execution of behaviors supporting survival in animals (e.g., foraging, reproduction), as well as interrupt daily human activities including eating, working, and socializing. Indeed, an inability to inhibit fearful responses to stimuli in humans can result in neuropsychiatric disorders such as specific phobias or more generalized fear disorders, including post-traumatic stress disorder, social anxiety disorder, and generalized anxiety disorder (Christianson et al., 2012; Graham & Milad, 2011; Grupe & Nitschke, 2013; Lopresto, Schipper, & Homberg, 2016; Quirk & Gehlert, 2003). Understanding how the brain can inhibit fear will not only advance comprehension of the complex circuits responsible for emotion regulation, but also facilitate identification of novel pathways and mechanisms integral to the development of more efficacious therapeutic strategies.

One of the subnuclei within the amygdala, the basolateral amygdala (BLA), is central to a fear circuit that regulates responses to discrete as well as ambiguous fear cues (Davis, 1997; Rosen & Schulkin, 1998). Indeed, the BLA possesses a critical role in the regulation of fear and anxiety behaviors (see Tovote, Fadok, & Luthi, 2015). We previously hypothesized that a cell population in the BLA identified by Lüthi and colleagues as "extinction neurons" (Herry et al., 2008), neurons that become more active during extinction learning, might overlap with glutamatergic Thy1-expressing neurons. In agreement with this hypothesis, we found that fear inhibition to auditory fear cues was facilitated by optogenetic activation of Thy1-expressing neurons (Jasnow et al., 2013). This was the first characterization of the behavioral effects of activating Thy1 neurons, as most researchers primarily employ mice with Thy1 promoter-driven transgenes as reporter lines to drive expression in a fraction of forebrain glutamatergic projection neurons (Dana et al., 2014; Feng et al., 2000; Heldt et al., 2014; Oakley et al., 2006; Porrero, Rubio-Garrido, Avendaño, & Clascá, 2010; Zagoraiou et al., 2009).

Further supporting a fear-inhibiting role of Thy1 neurons in the

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BLA, Ressler and colleagues have recently demonstrated that optogenetic inhibition of Thy1-expressing neurons enhances fear responses to auditory fear cues, whereas chemogenetic activation of these neurons reduces cued fear responding (McCullough et al., 2016). In sum, Thy1-expressing neuron activity in the BLA appears critical for the inhibition of fear to discrete cues and may therefore inhibit fear more generally. However, whether Thy1-expressing neurons also attenuate contextual fear, avoidance, or are involved in fear reconsolidation, remains unclear.

Here we employed a chemogenetic method for activation of Thy1 neurons by using designer receptors exclusively activated by designer drugs (DREADDs). In order to selectively activate Thy1-expressing neurons only within the BLA, we infused a Cre-dependent virus (from the UNC Vector Core) that codes for the activational G_s-coupled DREADD rM_3D (Guettier et al., 2009) specifically into the BLA of Thy1-Cre mice. With the second and third intracellular loops of the rat muscarinic acetylcholine M3 receptor replaced by those from turkey β_1 adrenergic receptor, this genetically engineered GPCR is only activated by the otherwise biologically inert clozapine *N*-oxide (CNO). For investigation of contextual fear, we employed an inhibitory avoidance paradigm (Lynch, Cullen, Jasnow, & Riccio, 2013) and examined the influence of activating BLA Thy1 neurons at different stages during fear processing (i.e., consolidation, reconsolidation, extinction, and expression). Thus, the findings presented here demonstrate the effect of GPCR-mediated selective activation of BLA Thy1 neurons on the learning and expression of fear behaviors in response to contextual stimuli.

2. Materials and methods

2.1. Animals

All mice were bred in-house in a breeding colony at the Department of Psychological Sciences at Kent State University. Adult male Thy1-Cre mice (FVB/N-Tg(Thy1-cre)1Vln/J; Jax stock no. 006143) on an FVB/N (Friend virus B NIH) background strain were used as experimental mice and were housed 2–5 mice per cage. Thy1-driven Cre expression and molecular characterization of Thy1-expressing neurons in the BLA has been demonstrated previously in this mouse strain using the same AAV-based rM_3D DREADD viral vector (see below) (McCullough et al., 2016). All mice were maintained on a 12:12 light:dark cycle with *ad libitum* access to food and water. All experiments were approved by the Kent State Institutional Animal Care and Use Committee, conducted in a facility accredited by the Association for Assessment and Accreditation and Laboratory Animal Care (AAALAC), and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, 8th Ed.

2.2. Genotyping

Ear punches were taken for DNA extraction following the procedures of Truett et al. (2000) and genotyped for the presence (Cre+) or absence (Cre−) of the Cre gene, as previously described (Gafford, Jasnow, & Ressler, 2014; Gilman, DaMert, Meduri, & Jasnow, 2015).

2.3. Surgeries

At 7–9 weeks of age, Cre+ mice underwent stereotaxic surgeries for infusion of the Cre-dependent control mCherry recombinant virus (rAAV5-hSyn-DIO-mCherry; UNC Vector Core) or rM_3D recombinant virus (rAAV5-hSyn-DIO-$rM_3D(G_s)$-mCherry; UNC Vector Core). This rM_3D construct and viral vector has been previously characterized to increase Gs signaling *in vitro* and *in vivo* (Guettier et al., 2009; Marchant et al., 2016; McCullough et al., 2016; Nakajima et al., 2016). Virus titers were 5.2×10^{12}–6.5×10^{12} vg/mL as analyzed by qPCR. Sham mice (expression and consolidation experiments only) were infused with sterile saline. Animals were anesthetized by subcutaneous (sc.) injection of a ketamine (75 mg/kg) and dexmedetomidine (1 mg/kg) mixture prepared in sterile saline. After completion of surgery, atipamezole hydrochloride (1 mg/kg) was administered to counteract the effects of dexmedetomidine. Perioperative analgesia was provided by sc. administration of ketoprofen (5 mg/kg). Intracranial infusion was performed bilaterally with 33–34 G NanoFil needles (World Precision Instruments, Sarasota, FL) connected to Hamilton syringes (Model 85; Hamilton, Reno, NV) by polyethylene tubing (0.015″ ID × 0.043″ OD; BD Medical, Franklin Lakes, NJ). Rate of infusion was 0.1 μL/min for a final volume of 1 μL, controlled by a PHD 2000 syringe pump (Harvard Apparatus, Holliston, MA). Needles were positioned at −1.6 AP, ± 3.4 ML, −4.9 DV relative to bregma to target the BLA. Following completion of each infusion, needles were left in place for 10 min before removal to facilitate diffusion of the infusion solution. Scalp incisions were closed using 5 μL of Vetbond (3 M, Maplewood, MN) and animals were allowed to recover for a minimum of 3 weeks before commencement of behavior, a time span previously employed to permit sufficient protein expression following rAAV transduction of neurons (Aschauer, Kreuz, & Rumpel, 2013).

2.4. Histological verification of viral expression

Recombinant virus (rAAV5-hSyn-DIO-$rM_3D(G_s)$-mCherry or rAAV5-hSyn-DIO-mCherry) expression in Cre+ virus-infused mice was confirmed histologically. Within one week after testing, mice were deeply anesthetized with sodium pentobarbital (Fatal-Plus; Vortech Pharmaceuticals, Dearborn, MI), then transcardially perfused with ice-cold saline followed by 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer, pH 7.4. Brains were immediately extracted, then post-fixed in PFA overnight, followed by 48–72 h cryoprotection in 30% sucrose in 0.1 M sodium phosphate buffer. Brains were then cut into 40 μm sections on a freezing microtome, rinsed in water to remove salts, then mounted onto slides (Superfrost Plus; VWR, Arlington Heights, IL) and coverslipped with Mowiol mounting medium containing 2.5% DABCO. Sections from each brain were sampled approximately every 240 μm coronally in a rostro-caudal manner from the prefrontal cortex through the dorsal raphe to detect any off-target expression of the transgene fluorophore mCherry. Sections were viewed on a Nikon Eclipse Ti-S light microscope using a Cy3 filter to confirm BLA-restricted expression of mCherry. Cre+ virus-infused mice lacking bilateral, BLA-restricted expression due to missed targets were omitted from analyses. See Fig. 1 for representative images of mCherry expression in BLA and mapping of transgene expression. No off-target expression was observed in regions projecting to the BLA or in regions outside of the immediate infusion area.

2.5. Clozapine N-oxide administration

Clozapine *N*-oxide (CNO) was obtained from Cayman Chemicals (cat. no. 16882; Ann Arbor, Michigan). All mice (regardless of whether stereotaxically infused with mCherry or rM_3D) were injected intraperitoneally (ip., 10 mL/kg) with 2 mg/kg CNO (Zhong et al., 2014) dissolved in sterile saline (vehicle). A subset of sham mice in expression and consolidation experiments were injected with vehicle only. When testing for the effect of BLA Thy1 neuron activation on inhibitory avoidance consolidation, mice were injected with CNO immediately after inhibitory avoidance training. Those mice undergoing reactivation or extinction training received CNO injections immediately after completion of the reactivation exposure or extinction training session, respectively. For the no reactivation group, CNO injections occurred in the home colony, 48 h after training and 48 h prior to testing, in the absence of any exposure to the training environment. To examine the effects of BLA Thy1 neuron activation on expression of fear, mice were injected with CNO 30 min prior to testing in inhibitory avoidance.

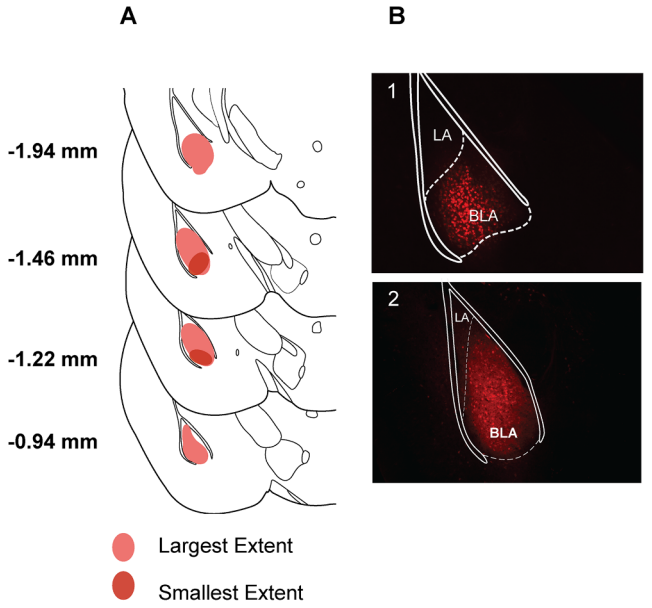

Fig. 1. Viral-induced transgene expression in the basolateral amygdala (BLA). Brains of all experimental animals were perfused and examined to verify BLA-restricted mCherry or rM_3D-mCherry expression for inclusion in data analyses. (A) Atlas of the mouse brain displaying the largest extent allowed (light red), and smallest extent required (dark red), of mCherry-tagged rM_3D expression for animals to be included in analyses. Numbers indicate posterior distance from bregma. (B) Representative sections from Thy1-Cre+ mice infused with the DIO-mCherry control virus (B1) or (B2) DIO-rM_3D-mCherry DREADD virus. Robust, BLA-restricted expression was present in both posterior and anterior sections from Thy1-Cre+ mice. Abbreviations: lateral amygdalar nucleus (LA); basolateral amygdala (BLA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Inhibitory avoidance

Also known as passive avoidance, inhibitory avoidance involves use of a step-through apparatus (52 cm L × 30 cm W × 35 cm H, Model 7551; Ugo Basile, Varese, Italy) containing a white, brightly lit side and a black, unilluminated side connected by an automated door. A grid floor is present on both sides, and a single lid has one half opaque black that rests over the black side, with the other half clear acrylic containing an illuminated light bulb oriented to shine down on the white side. One apparatus was located in the training context, whereas another was located in a neutral context; these contexts have been previously described (Lynch, Winiecki, Vanderhoof, Riccio, & Jasnow, 2016). The training context was a 1.6 × 2.33 m room with house fluorescent lights and contained bare white walls, no artificial scents or sounds, and was cleaned with 70% ethanol. The neutral context was a 1.83 × 2.74 m room lit by a 25 W red light bulb and the room had colorful posters on the walls. The neutral context had white noise (70 dB) and was cleaned with 50% quatricide. In each context, the experimenter wore different gloves (latex dishwashing glove in training; nitrile lab glove in neutral) to handle mice, and white lab coats were only worn when handling animals within the training context.

For all training procedures, mice are held for 30 s by an experimenter wearing a white lab coat and textured latex dishwashing glove, then gently placed in the white side with the automated door closed. The lid to the apparatus is immediately closed, and following a 20 s delay, the door opens and animals may cross into the black side. Once an animal crosses to the black side, the door automatically shuts and, following a 2 s delay, the mouse receives a single, 2 s foot shock (0.8 mA for all experiments except optimization and reconsolidation; see Figs. 2 and 4). The time span between the door's first opening and the animal crossing from the white side to the black side with all 4 paws is measured in seconds and recorded as the initial latency to cross. Ten seconds after the end of the shock, mice are removed from the black side and returned to their home cage. We, and others, have demonstrated

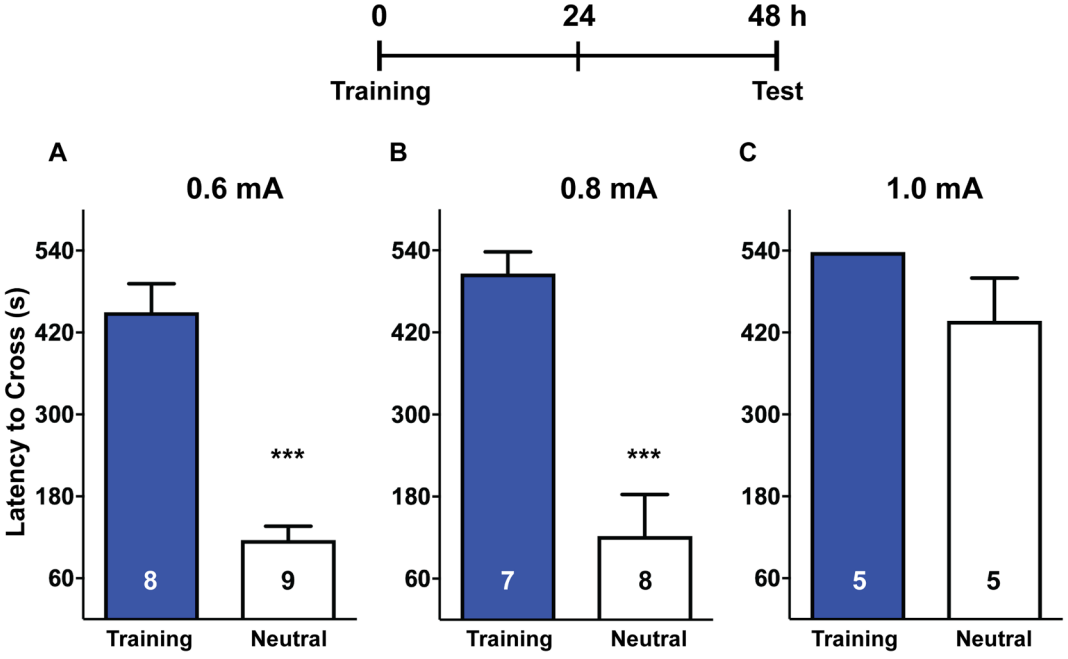

Fig. 2. Optimizing inhibitory avoidance parameters in FVB/N mice. To optimize inhibitory avoidance conditions in mice, three different shock levels were employed for training: (A) 0.6 mA; (B) 0.8 mA; (C) 1.0 mA. All mice were trained in the training context at the corresponding shock level, then 48 h later mice were tested (see timeline in figure) in either the training context or a neutral context for their latency to cross from the white side of the apparatus to the black side. $^{***}p < 0.001$ vs. animals tested in training context at same training shock level. No mice trained at 1.0 mA crossed when tested in the training context (C), so there is no error bar for this group. Data are presented as mean ± S.E.M. Numbers within each bar represent Ns.

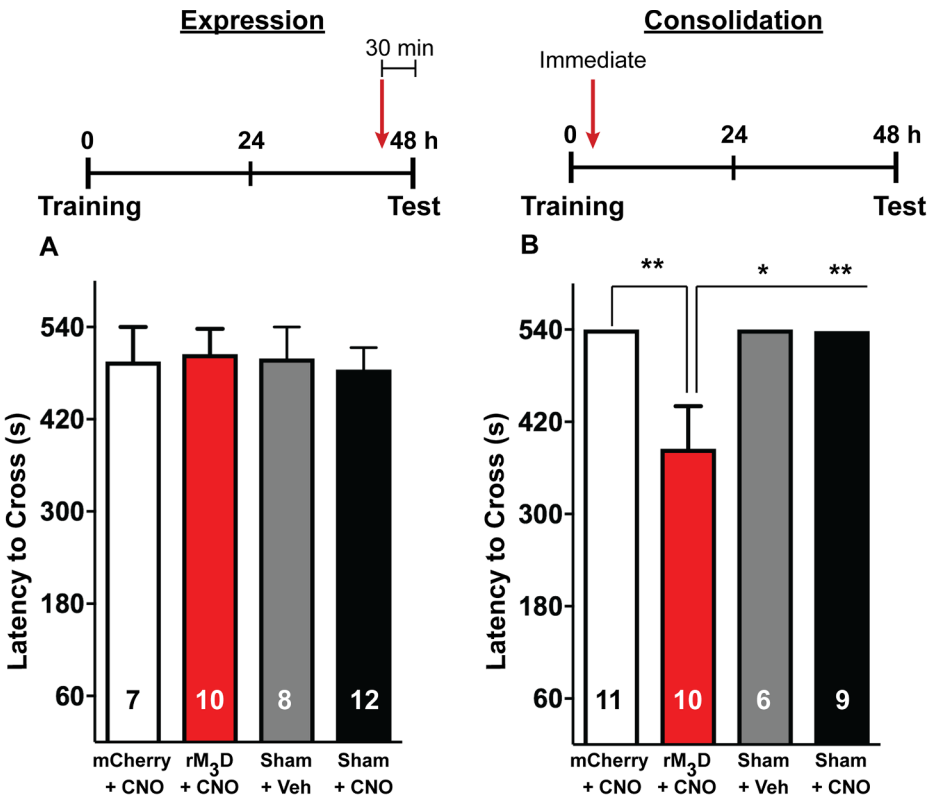

Fig. 3. BLA Thy1 neuron activation on contextual fear expression and consolidation. Mice underwent inhibitory avoidance training (0.8 mA) and then BLA Thy1 neurons were activated by administration of 2 mg/kg CNO, ip. (red arrow on timelines), at 30 min before test (Expression, A) or immediately after training (Consolidation, B). Mice were tested 48 h after training in the training context for their latency to cross. $^{**}p < 0.01$; $^{*}p < 0.05$ (none of the control mice used to investigate consolidation (B) crossed during testing, so there are no error bars for those data). In addition, CNO in sham mice (no virus) had no effect on latency to cross compared to sham mice administered vehicle (saline) or to mice expressing the control virus and administered CNO in either experiment. These data demonstrate that in the absence of rM_3D expression, CNO does not alter fear memory. Data are presented as mean ± S.E.M. Numbers within each bar represent Ns. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

using this procedure that rats and mice can distinguish between the contextual cues of the training context and neutral context in which the apparatus is located. Rats and mice show long latencies to cross in the training context, indicating a strong fear memory, whereas they show short latencies to cross in the neutral context, suggesting that their fear memory is, at least in part, contextually-dependent (Lynch et al., 2013, 2014; Lynch, Vanderhoof, et al., 2016; Lynch, Winiecki, et al., 2016; Riccio & Joynes, 2007; Zhou & Riccio, 1994, 1996).

For testing, mice were brought to the training or neutral context, held for 30 s, and gently placed in the apparatus. After the 20 s door delay, mice were allowed 9 min to cross to the black side. If mice crossed from the white to the black side (defined as a 4 paw entry into the black side) the testing latency to cross was recorded. If mice did not cross, they were assigned a latency to cross of 9 min (540 s). Once mice crossed to the black side, or after the allowed 9 min passed, mice were removed from the apparatus and returned to their home cage.

Neutral context testing, 48 h after initial training, was only performed for optimization experiments. Testing in the training context took place 48 h after initial training for optimization, consolidation, and expression experiments (see Figs. 2 and 3). Testing for reconsolidation or extinction experiments occurred in the training context 48 h after the experimental manipulation (see Figs. 4 and 5). No foot shocks were administered during testing. Experimenters performing neutral context testing did not wear lab coats, and held mice with non-textured nitrile gloves; experimenters performing training context testing were dressed as described for the training procedure.

To first optimize inhibitory avoidance conditions for FVB/N male mice, we evaluated three different foot shock amplitudes (0.6, 0.8, and 1.0 mA) and assessed the ability of the mice to discriminate between the training context and a neutral context. For this optimization, adult naïve male mice of both Cre+ and Cre− genotypes that had not undergone surgery were used. Mice were only tested in the neutral or in the training context – no mouse was tested in both contexts.

Mice in reactivation groups were trained at either 0.6 or 0.8 mA, the former to allow for detection of potentiation of the fear memory following reactivation (Inda, Muravieva, & Alberini, 2011). Reactivation consisted of mice being brought to the training context by a researcher not wearing a lab coat and held in a hand covered with a non-textured nitrile glove. Mice were immediately placed into the white side of the apparatus and the lid was closed. Mice remained in the white side for 30 s, then were removed, immediately injected with CNO, and then returned to their home cage.

To rule out a confounding role of CNO-mediated Thy1-expressing neuron activation on fear memory stability in the absence of discrete consolidation processes, a separate no reactivation cohort was tested. Mice were trained at 0.6 mA to facilitate detection of any memory deterioration, then injected with CNO in the home colony (in the absence of any memory reactivation) at the same 48 h time point as the reactivation groups (see Fig. 4). These mice were then tested 48 h after injection, as with the reactivation groups (Fig. 4B).

Extinction took place 48 h after the initial training in the training context. To extinguish inhibitory avoidance, mice were brought to, and placed in, the apparatus just as with training. After the door opened, mice were given 90 s to cross to the black side. If the mice did not cross, they were gently guided by hand through the door to the black side, at which point the door closed. No shock was administered during extinction training. Mice stayed on the black side for the remainder of 10 min from the time the door initially opened. Then, mice were removed from the black side, injected with CNO, returned to their home cage, and tested 48h after injection.

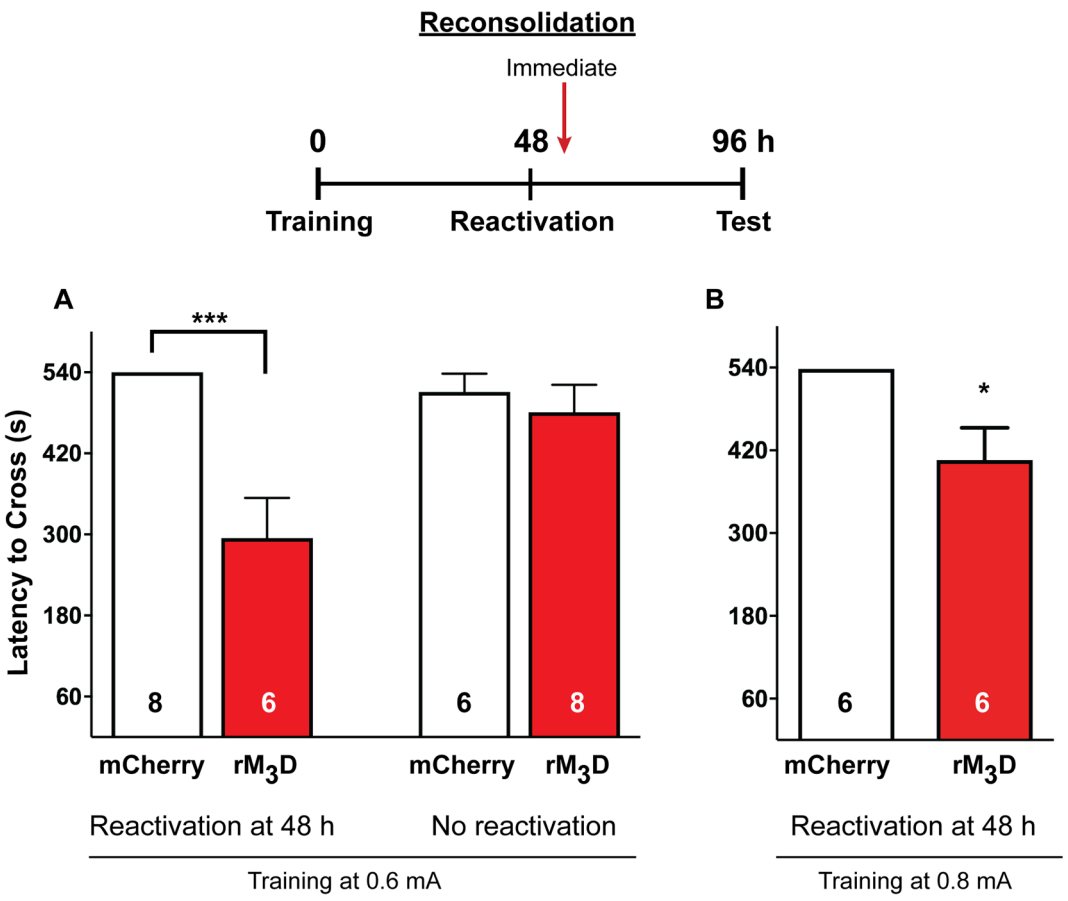

Fig. 4. BLA Thy1 neuron activation on reconsolidation of contextual fear. Mice underwent inhibitory avoidance training (0.6 (A) or 0.8 mA (B)) and then BLA Thy1 neurons were activated by administration of 2 mg/kg CNO, ip. (red arrow on timeline) at 48 h after training. This injection occurred either immediately following a 30 s reexposure to the white side, in the absence of any foot shock (A, left; B) to test the effects of BLA Thy1 neuron activation on contextual fear reconsolidation, or in the home colony with no exposure to the training context (A, right) to assess whether BLA Thy1 neuron activation in the absence of a contextual fear learning event affects the fear memory. Regardless of reactivation status, all mice were tested 48 h after CNO injection in the training context for their latency to cross (see timeline on figure). $^{***}p < 0.001$; $^{*}p < 0.05$ vs. respective mCherry group. In the absence of reactivation, Thy1 neuron activation did not impair reconsolidation (A, right). None of the mCherry mice after having been exposed to reactivation in (A) or (B) crossed during testing, so there are no error bars for these groups. Data are presented as mean ± S.E.M. Numbers within each bar represent Ns. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7. Statistics

Inhibitory avoidance data were analyzed using parametric unpaired *t*-tests or a one-way ANOVA for groups with variances. Non-parametric Mann-Whitney U or Kruskal-Wallis tests were used when there was no variance in one or more groups because they performed at ceiling. Significance was set *a priori* at $p < 0.05$. For the 0.6 mA reconsolidation experiment comparing reactivation to no-reactivation groups, data were analyzed using a Mann-Whitney U with Bonferroni correction. In this case, significance was set at $p < 0.025$. All data were graphed as mean ± S.E.M., and analyzed using GraphPad Prism 7.0d (GraphPad Software, La Jolla, CA).

3. Results

3.1. Inhibitory avoidance optimization

First we optimized inhibitory avoidance conditions for FVB/N mice based on our previous work in rats (Lynch et al., 2013, 2014). To do this, we piloted 3 different shock levels (0.6, 0.8, and 1.0 mA) and tested mice 48 h later in the training context or in a distinct, neutral context (Fig. 2). No main effects of genotype across training contexts were detected with a two-way ANOVA (0.6 mA: $F_{(1,\ 16)} = 1.00$, $p > 0.33$; 0.8 mA: $F_{(1,\ 11)} = 0.0582$, $p > 0.81$; 1.0 mA: $F_{(1,\ 6)} = 0.260$, $p > 0.62$), so within each foot shock amplitude and context (e.g., 0.6 mA neutral context), genotypes were combined. Initial latencies for each of the different shock levels were not significantly different between mice that were subsequently tested in the training or neutral contexts (Table 1). Mice trained at 0.6 mA ($t_{(15)} = 7.44$, $p < 0.01$; Fig. 2A) or 0.8 mA ($t_{(13)} = 5.363$, $p < 0.01$; Fig. 2B) successfully distinguished between the training and neutral contexts, as indicated by significantly greater latencies to cross in the training context compared with the neutral context. However, mice trained at 1.0 mA had similar latencies to cross irrespective of the testing context. All mice tested in the training context performed at ceiling (i.e., did not cross after 9 min), resulting in no variance. Therefore, these data were analyzed with a Mann-Whitney U ($U = 7.5$, $p = 0.44$; Fig. 2C). Subsequent experiments therefore primarily employed the 0.8 mA shock level, given the maximal latency to cross for those mice tested in the training context, and the significantly lower latency to cross in mice tested in the neutral context (Fig. 2B). The only exception is reactivation experiments, in which mice were trained at either 0.6 mA or 0.8 mA.

3.2. Activation of Thy1 neurons in the BLA does not affect the expression of inhibitory avoidance memory.

The influence of BLA Thy1 activation on expression of contextual fear was examined by injecting mice with CNO 30 min prior to testing

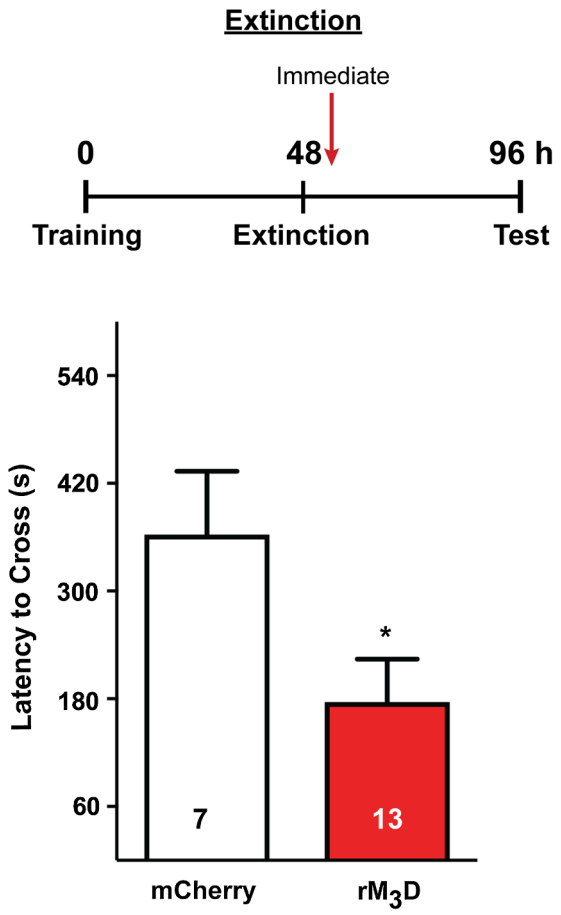

Fig. 5. BLA Thy1 neuron activation on contextual fear extinction. Mice underwent inhibitory avoidance training (0.8 mA) and then 48 h later underwent extinction training in the training context. This involved a 90 s exposure to the white side, followed by guidance through the door to the black side. Mice remained on the black side for the remainder of 10 min, then were removed and immediately injected with CNO (2 mg/kg, ip.; red arrow on timeline). Testing for latency to cross occurred in the training context 48 h after CNO injection (see figure timeline), $^{*}p < 0.05$ vs. mCherry group. Data are presented as mean ± S.E.M. Numbers within each bar represent Ns. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the training context. In this experiment, to control for any non-DREADD effects of CNO or its reverse metabolism to clozapine (Gomez et al., 2017; MacLaren et al., 2016), a separate group of mice underwent sham infusions and were administered CNO or vehicle (saline). Three mice were excluded from the analysis because they were identified as behavioral outliers. Initial (Table 1) and testing latencies ($F_{(3,\ 33)} = 0.0681$, $p = 0.977$) were not significantly different among all groups of mice (Fig. 3A). This indicates that activating BLA Thy1 neurons during context fear testing does not affect contextual fear expression. In addition, these data indicate that CNO alone, or its potential reverse metabolism to clozapine, has no effect on avoidance behavior.

3.3. Activation of Thy1 neurons in the BLA attenuates the consolidation of inhibitory avoidance learning.

To evaluate the influence of BLA Thy1 neurons on the initial consolidation of context fear, all mice were trained using inhibitory avoidance with a 0.8 mA shock, then immediately injected with 2 mg/kg CNO, ip. In this experiment, to control for any non-DREADD effects of CNO or its reverse metabolism to clozapine, a separate group of mice underwent sham infusions and were administered CNO or vehicle (saline). Initial latencies to cross were not significantly different among all groups (Table 1), and testing occurred 48 h later in the training context. Several groups of mice performed at ceiling (i.e., did not cross), therefore these data were analyzed using the Kruskal-Wallis test with Dunn's multiple comparisons. Two mice were removed from the analysis because they were identified as outliers. There was a significant effect of treatment $\chi^2_{(3,\ N = 36)} = 21.63$, $p < 0.01$). Mice expressing rM_3D that were administered CNO displayed significantly lower latencies to cross during testing compared to mCherry-expressing mice that were administered CNO ($p < 0.01$), sham mice that were administered vehicle ($p < 0.05$) and sham mice that were administered CNO ($p < 0.01$). There were no differences among the control groups ($p > 0.05$). (Fig. 3B). These data suggest BLA Thy1 neuron activation attenuates fear consolidation. Subsequent experiments characterized the influence of BLA Thy1 neuron activation on two different stages in the consolidation of fear processing: reconsolidation and extinction.

3.4. Activation of Thy1 neurons in the BLA attenuates the reconsolidation of inhibitory avoidance.

For reconsolidation experiments, mice were trained at either 0.6 or 0.8 mA. Training at 0.6 mA was intended to enable detection of contextual fear potentiation following reactivation (Inda et al., 2011), whereas training at 0.8 mA was for consistency with the other inhibitory avoidance conditions employed herein. To examine the impact of BLA Thy1 neuron activation on fear reconsolidation, the contextual fear memory was reactivated in mice by re-exposing them to the white side of the training apparatus for 30 s in the absence of any foot shock (Reactivation). Mice received CNO injections immediately after reactivation. An additional group of mice received CNO injections at the same time but were not re-exposed to the training apparatus (No Reactivation). None of the mice trained at 0.6 and 0.8 mA for reconsolidation experiments exhibited any differences in initial latency to cross with respect to infusion condition (see Table 1). One group of mice trained at 0.6 mA performed at ceiling, therefore the 0.6 mA data were analyzed using a Mann-Whitney U with Bonferroni correction for multiple comparisons. Across all reconsolidation experiments (0.6 mA and 0.8 mA training), 2 mice were removed from analysis as behavioral outliers and 9 mice were removed because they either had poor transgene expression, unilateral BLA transgene expression or a complete missed targeting of the BLA. Activating Thy1-expressing neurons by injecting CNO immediately following reactivation significantly reduced the cross latency in rM_3D-expressing mice compared to mCherry-expressing mice ($U = 0$, $p = 0.0003$; Fig. 4A, left). This effect was specific to animals experiencing reactivation, as the effect was lost if CNO was instead given in the absence of any reactivation exposure ($U = 22$, $p = 0.852$; Fig. 4A, right). After 0.8 mA training, activating BLA Thy1 neurons immediately after reactivation similarly attenuated the latency to cross in rM_3D-infused animals versus mCherry-infused mice tested 48 h after reactivation ($U = 6$, $p = 0.03$, 1-tailed sig); Fig. 4B). These data are the first to implicate BLA Thy1 neurons in attenuating fear during reconsolidation processes.

3.5. Activation of Thy1 neurons in the BLA facilitates the consolidation of inhibitory avoidance extinction.

Given the ability of chemogenetic BLA Thy1 neuron activation to attenuate fear consolidation and reconsolidation (Figs. 3B and 4), and our previous work demonstrating optogenetic activation of these neurons enhances the consolidation of cued fear extinction (Jasnow et al., 2013), we sought here to characterize the impact of BLA Thy1 neuron activity on the consolidation of contextual fear extinction. Initial latencies were not significantly different between mCherry controls and rM_3D groups (see Table 1). Extinction occurred 48 h after 0.8 mA training and consisted of a 10 min reexposure to the training context in the absence of any foot shock (see Section 2.6), followed immediately

Table 1
Inhibitory avoidance initial latencies.

Context:	Training			Neutral		
Optimization	Mean	SEM	N	Mean	SEM	N
0.6 mA $t_{(15)} = 1.078$, $p > 0.29$	41.1	9.2	8	28.7	7.14	9
0.8 mA $t_{(13)} = 0.068$, $p > 0.94$	44.6	16.9	7	43.4	8.98	8
1.0 mA $t_{(8)} = 0.706$, $p > 0.50$	26.6	9.23	5	38.7	14.5	5

Stereotaxic Infusion:	mCherry			rM3D			Sham-Vehicle			Sham-CNO		
BLA Thy1 Activation	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
Expression $F_{(3, 33)} = 0.767$, $p > 0.66$	17.6	5.54	7	35.8	10.38	10	30.68	13.18	8	23.8	4.514	12
Consolidation $F_{(3, 34)} = 1.329$, $p > 0.28$	41.5	10.0	11	21.9	5.29	10	49.88	13.67	8	38.09	10.92	9
0.6 mA Reactivation $t_{(12)} = 0.255$, $p > 0.80$	27.9	5.06	8	30.1	7.06	6						
0.6 mA No Reactivation $t_{(12)} = 0.185$, $p > 0.85$	30.9	6.43	6	33.1	9	8						
0.8 mA Reactivation $t_{(10)} = 0.840$, $p > 0.42$	19.8	7.96	6	28.3	6.41	6						
Extinction $t_{(18)} = 0.770$, $p > 0.44$	43.8	11.54	7	34.3	9.36	13						

by injection of CNO. Testing occurred 48 h after this extinction procedure. One mouse was removed from analyses as a behavioral outlier and 3 additional mice were removed because of poor transgene expression, unilateral BLA transgene expression or a complete missed targeting of the BLA. CNO administration following extinction resulted in a significant facilitation of extinction consolidation in rM_3D-expressing mice compared to mCherry-expressing mice ($t_{(18)} = 2.25$, $p = 0.037$; Fig. 5). These data are consistent with previous results (Jasnow et al., 2013; McCullough et al., 2016) suggesting that activation of Thy1-expressing neurons in the BLA specifically facilitates the consolidation of inhibitory, or extinction, learning.

4. Discussion

Building upon previous findings (Jasnow et al., 2013; McCullough et al., 2016), here we extended characterization of the types of aversive learning events that BLA Thy1 neurons modulate. These results reveal novel roles of BLA Thy1 neuron activity in attenuating contextual fear responses and in fear memory reconsolidation. Together with previous data, we show consistent effects of BLA Thy1-expressing neuron activation during consolidation and extinction of fear memories (Jasnow et al., 2013; McCullough et al., 2016). Activation of BLA Thy1-expressing neurons during consolidation of an initial contextual fear learning event, or during reconsolidation following reactivation of a contextual fear memory, inhibits contextual fear expressed 48 h later. However, acute activation of BLA Thy1 neurons had no impact on fear expression. Importantly, these findings indicate that BLA Thy1 neuron activation does not interfere with learning, as consolidation of extinction learning is *enhanced* by activation of these neurons. Indeed, extinction learning is a process distinct from fear learning (Dunsmoor, Niv, Daw, & Phelps, 2015; Quirk et al., 2010). Thus, BLA Thy1-expressing neurons not only dampen fear responses to discrete fear cues (Jasnow et al., 2013; McCullough et al., 2016), but they also reduce fear to contextual stimuli in more complex behavioral tasks that are not produced by purely classical conditioning (present results).

Before exploring the impact of BLA Thy1 neuron activation on contextual fear processing, we first confirmed that male FVB/N mice could learn inhibitory avoidance successfully. Mice on an FVB/N background do not exhibit as high freezing responses to fearful stimuli as other strains, such as C57BL/6 or 129S1 (Farley, McKay, Disterhoft, & Weiss, 2011; Hefner et al., 2008; Keum et al., 2016), resulting in a floor effect that makes employment of freezing as an indicator of fear disadvantageous. Such a lack of freezing could also be mistakenly interpreted as an impaired ability of the strain to learn fear, but here we detected robust context-specific fear responses in inhibitory avoidance in FVB/N male mice. These were indicated by significantly increased latencies to cross in the training context, but not the neutral context, when training occurred 48 h prior at 0.6 or 0.8 mA, suggesting inhibitory avoidance is a useful tool to measure contextual fear in FVB/N mice. Future investigations could employ this paradigm to explore the role of BLA Thy1 neurons in contextual fear generalization using the 1.0 mA foot shock.

Having established the utility of inhibitory avoidance to assess context-specific fear in male FVB/N mice, we moved to evaluate how DREADD-mediated activation of Thy1-expressing neurons in the BLA affected different stages of contextual fear processing. The learning-specific involvement of BLA Thy1 neurons in fear processing that has been previously reported with cued fear (Jasnow et al., 2013; McCullough et al., 2016) was supported by our contextual fear observations. We noted that activation of these neurons was without effect on expression of contextual fear, and similarly did not alter learned contextual fear if activation occurred in the absence of any reexposure to the training context (No Reactivation group). Rather, the impact of activating BLA Thy1 neurons was most evident when it occurred immediately after training (consolidation), brief reexposure to the training context (reconsolidation), or prolonged reexposure to the training context (extinction), in accord with work by us and others demonstrating that these neurons affect fear learning but not fear expression. Importantly, and as described previously (Jasnow et al., 2013; McCullough et al., 2016), this is not to say that BLA Thy1 neuron activation impairs learning, as consolidation of cued (previous work) and contextual (present findings) extinction is *facilitated* after their activation.

We have previously determined that BLA Thy1 neuron activation promotes polysynaptic feed-forward inhibition of the medial portion of the central amygdala (CeM; Jasnow et al., 2013), the amygdalar subnucleus primarily responsible for initiating behavioral expressions of fear (see Duvarci & Pare, 2014). This polysynaptic feed-forward

inhibition onto CeM fear output neurons by BLA Thy1-expressing neurons could involve intermediary activation of neurons within the lateral portion of the central nucleus of the amygdala (CeL). For example, activation of CeL neurons classified as PKC_{δ}-expressing (Haubensak et al., 2010) or oxytocin receptor-expressing (Knobloch et al., 2012; Viviani et al., 2011) reduces fear expression by inhibiting CeM output. Therefore, it is quite possible that the attenuated contextual fear observed here is a consequence of this downstream inhibition of the CeM, potentially via CeM-inhibiting neurons located in the CeL.

One of the novel findings of the present manuscript is that activation of Thy1-expressing neurons in the BLA impaired fear reconsolidation. The traditional view of reconsolidation is that recently reactivated memories are initially labile and susceptible to disruption, but over time become stable through an activity- and/or protein-dependent process (Misanin, Miller, & Lewis, 1968; Nader, Schafe, & Le Doux, 2000; Przybyslawski & Sara, 1997; Sara, 2000a, 2000b). An assumption of the reconsolidation hypothesis is that memory impairments produced by administration of amnesic agents (e.g., anisomycin, cycloheximide, electroconvulsive shock) following a reactivation episode are caused by disruption of the "re-storage" of the reactivated memory. A more contemporary framework to explain reconsolidation has been termed trace updating (McKenzie & Eichenbaum, 2011), which suggests that the molecular processes surrounding reactivation serve to modify the memory trace and integrate new information (Dudai & Eisenberg, 2004; Gisquet-Verrier et al., 2015; McKenzie & Eichenbaum, 2011). Thus, it is generally thought that reconsolidation serves to either maintain or strengthen memories, and this has been supported by a number of studies (Inda et al., 2011; Lee, 2008; Tronson, Wiseman, Olausson, & Taylor, 2006). The current findings support the idea that reactivation serves to modify memory traces through integration of new information. Our data suggest that the memory trace was modified by activating neurons that selectively inhibit fear (BLA Thy1 neurons) after memory reactivation, which served to impair fear reconsolidation. It is unlikely that activation of Thy1-expressing neurons shortly after memory reactivation impaired "re-storage" of the memory, as would be predicted by the traditional view of reconsolidation. This is supported by our evidence that activation of Thy1-expressing neurons in the BLA reduced fear by inhibiting the consolidation of fear learning *and* by facilitating the consolidation of avoidance extinction (present study) and cued fear extinction (Jasnow et al., 2013; McCullough et al., 2016) - both of which require a consolidation period similar to reconsolidation. Taken together with our previous findings, these data add support for the idea that specific activation of a functional subpopulation of pyramidal neurons can selectively inhibit fear responses. Thy1-expressing neurons may inhibit fear through their inherent molecular properties (McCullough et al., 2016) or their functional connectivity to other brain regions involved in fear processing, such as the CeA (Jasnow et al., 2013), the nucleus accumbens (NAcc) (Porrero et al., 2010), bed nucleus of the stria terminalis (BNST), the infralimbic cortex (IL), and medial intercalated nuclei (mITC) (McCullough et al., 2016). These regions, in particular the NAcc, IL, and mITC, have all been implicated in suppressing fear (Correia, McGrath, Lee, Graybiel, & Goosens, 2016; Likhtik, Popa, Apergis-Schoute, Fidacaro, & Pare, 2008; Quirk, Garcia, & González-Lima, 2006). Molecular characterization of BLA Thy1-expressing neurons revealed that these neurons display enriched expression of the neurotensin receptor 2 (Ntsr2) among a number of other genes (McCullough et al., 2016). Direct pharmacological manipulation of Ntsr2 in the BLA using agonists and antagonists bidirectionally modified fear responses (McCullough et al., 2016), suggesting that increased signaling through Ntsr2, possibly within Thy1-expressing neurons, serves to suppress fear.

Expression of Thy1 in the mouse brain occurs exclusively in glutamatergic projection neurons located in numerous brain areas, including the BLA, hippocampus, and multiple cortical regions (Jasnow et al., 2013; Sugino et al., 2006). Here, we specifically examined Thy1-expressing neurons in the BLA through the use of a Cre-dependent DREADD virus in Thy1-Cre mice, permitting selective study of this neuronal subpopulation. In support of this specificity, we did not observe any evidence of retrograde labeling of regions projecting to the BLA or viral transduction of non-Thy1 fibers passing close to the needle track. Further, bilateral BLA-restricted expression of mCherry in Cre + mice was required for the inclusion of animals in analyses; animals that had unilateral expression or had extra-BLA expression because of a missed target were excluded. Moreover, the results we observed here are in agreement with our previous report on the fear inhibitory effect of optogenetic BLA Thy1 neuron activation (Jasnow et al., 2013). Those findings used a different neuronal activation technique, different background strain of mice, and different fear training paradigm, yet here we observed parallel learning-specific effects on the inhibition of contextual fear. Further, complementary findings using cued fear conditioning were reported by Ressler and colleagues using the same mouse line and viral vector we used here (McCullough et al., 2016). Thus, we are confident in attributing these results to the specific activation of Thy1-expressing neurons within the BLA.

Initial characterization of rM_3D in pancreatic β cells suggested mild constitutive activity of this DREADD (Guettier et al., 2009), but this was not detected later in transgenic mice expressing rM_3D in striatal neurons (Farrel et al., 2013) nor in the recent study by McCullough et al. (2016). We observed no evidence for constitutive activation here either; if constitutive activation were present we would anticipate consistent discrepancies between the infusion conditions. Further, for the expression and consolidation experiments, we included animals that were sham-infused and either received CNO or vehicle to control for drug exposure, as well as for any potential reverse metabolism to clozapine producing non-DREADD mediated effects (Gomez et al., 2017). We observed effects of CNO solely when rM_3D was present. Sham-infused, CNO-injected mice behaved similarly to vehicle-treated mice and to CNO-treated mice that received control virus (Fig. 3A and B). In addition, we saw no effects of CNO when mice did not receive reactivation in the reconsolidation experiments (Fig. 4), an additional piece of evidence suggesting that our observed results were not due to off target effects of CNO or its reverse metabolism to clozapine. Further, initial latencies to cross during inhibitory avoidance training were not significantly different between infusion groups. Finally, testing latencies for the expression and no reactivation groups were not significantly different, further providing support for a lack of constitutive activity when rM_3D is expressed in BLA Thy1-expressing neurons.

The apparent differences in the proportion of animals exhibiting a maximum latency to cross (540 s) during testing after 0.6 mA training for the no reactivation group, as compared to those of animals trained at 0.6 mA for optimization of inhibitory avoidance in FVB/N mice, did not escape our notice. Our reconsolidation cohort timeline was twice the length of our initial optimization timeline, and this added time may be responsible for the enhanced latency to cross in the no reactivation group. We implemented this extended timeline to allow for direct comparison with the consolidation group (injected with CNO 48 h prior to test), while maintaining the undisturbed 48 h time window following inhibitory avoidance training employed for all groups tested. This phenomenon certainly warrants continued study, and indeed we are performing parametric studies to continue characterizing the time course of contextual fear processing with inhibitory avoidance in FVB/N mice of both sexes.

The experiments presented here confirm a fear suppressing role of BLA Thy1-expressing neurons, and further suggest that their activation during the consolidation or reconsolidation of fear learning, or during extinction learning, may inhibit fear in a mechanistically similar way, although this may involve anatomically distinct downstream brain regions (e.g., NAcc, BNST). Subsequent investigations aimed at mapping out the effector brain regions and molecular targets involved in this fear inhibition will enable the parsing apart of possible learning-specific effects. It should be noted that while our modified training procedure

enables us to make inferences about contextual learning, other than the optimization experiments we did not measure the animal's ability to distinguish between the training and neutral contexts. Inhibitory avoidance is a complex behavioral paradigm that involves aspects of both Pavlovian and instrumental conditioning. Thus, while we infer that the mice learn about where they were shocked (the context), the avoidance behavior is reinforced during testing when they do not receive a shock if they remain in the light compartment. Thus, in addition to reducing fear to contextual cues, activation of Thy1-expressing neurons likely also reduced the reinforcing properties of an instrumental avoidance response. This has implications for how Thy1-expressing neurons are functionally connected such that they can both reduce fear to purely Pavlovian associations (Jasnow et al., 2013; McCullough et al., 2016) and to more complex avoidance tasks (present results). Finally, exploration of the influence that BLA Thy1 neurons have on fear generalization processes (see Jasnow, Lynch, Gilman, & Riccio, 2016) will advance investigations into the potential therapeutic utility of the circuit in which these fear inhibiting neurons participate.

5. Conclusions

In sum, the data presented here demonstrate that activation of BLA Thy1-expressing neurons inhibits the consolidation and reconsolidation of fear to contextual stimuli, resulting in reduced fear expression at testing. Further, BLA Thy1 neuron activation facilitates consolidation of avoidance extinction learning. Future experiments examining the behavioral effects of selectively activating BLA Thy1 neuron afferents in regions such as the CeM, NAcc, and IL will enable extended characterization of this fear inhibitory circuit. Improved understanding of BLA Thy1-centered circuitry could have significant implications for improved treatment of fear and anxiety disorders stemming from varied etiologies, particularly those that involve avoidance as a key symptom.

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