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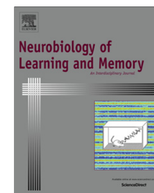

Posterior insular cortex is necessary for conditioned inhibition of fear

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ABSTRACT

Veridical detection of safety versus danger is critical to survival. Learned signals for safety inhibit fear, and so when presented, reduce fear responses produced by danger signals. This phenomenon is termed conditioned inhibition of fear. Here, we report that CS+/CS fear discrimination conditioning over 5 days in rats leads the CS to become a conditioned inhibitor of fear, as measured by the classic tests of conditioned inhibition: summation and retardation of subsequent fear acquisition. We then show that NMDA-receptor antagonist AP5 injected to posterior insular cortex (IC) before training completely prevented the acquisition of a conditioned fear inhibitor, while intra-AP5 to anterior and medial IC had no effect. To determine if the IC contributes to the recall of learned fear inhibition, injections of the $GABA_A$ agonist muscimol were made to posterior IC before a summation test. This resulted in fear inhibition *per se*, which obscured inference to the effect of IC inactivation with recall of the safety cue. Control experiments sought to determine if the role of the IC in conditioned inhibition learning could be reduced to simpler fear discrimination function, but fear discrimination and recall were unaffected by AP5 or muscimol, respectively, in the posterior IC. These data implicate a role of posterior IC in the learning of conditioned fear inhibitors.

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1. Introduction

Associative learning processes permit an organism to remember environmental cues that predict danger or safety. Danger learning has been extensively studied using Pavlovian fear conditioning procedures (McNally & Westbrook, 2006) and its neural mechanisms are very well understood (Johansen, Cain, Ostroff, & LeDoux, 2011; Kim & Jung, 2006; LeDoux, 2014; Maren, 2001; Tronson, Corcoran, Jovasevic, & Radulovic, 2012). By contrast, the mechanisms by which explicit environmental cues come to predict safety are largely unknown (Christianson et al., 2012; Kong, Monje, Hirsch, & Pollak, 2014). Learned safety signals are potent modulators of behavior and have the ability to inhibit fear responses, such as behavioral freezing, and promote exploration or foraging when presented in compound with learned danger cues (Chen, Foilb, & Christianson, 2015; Christianson et al., 2011; Konorski, 1967; Myers & Davis, 2004; Pollak et al., 2008; Rogan, Leon, Perez, & Kandel, 2005; Sangha, Robinson, Greba, Davies, & Howland, 2014). The phenomenon that underlies the blunting of fear by safety cues is termed conditioned inhibition and it can be investigated by providing discrete, unreinforced conditioned stimuli (CSs) in the midst of Pavlovian fear conditioning procedures (Christianson et al., 2012; Konorski, 1948; Rescorla, 1969). According to Rescorla (1969), two critical tests are necessary to assess conditioned inhibition. First, the strength of a conditioned inhibitor is assessed in a summation test in which the putative conditioned fear inhibitor (i.e. the safety signal) is presented in compound with a danger signal. In this test, well-learned safety signals reduce the conditioned freezing response typically evoked by the danger signal. Second, excitatory fear conditioning should be delayed if a conditioned inhibitor is paired with an aversive US. To test this, a learned safety signal and a novel CS are separately paired with an aversive US. If the safety cue is a true inhibitor, there will be reduced fear associated to it than to the novel CS.

The neuroanatomical loci that mediate learning and recall of conditioned fear inhibitors are currently unknown, but there have been a number of important investigations of conditioned inhibition in the context of appetitive learning. While a thorough review of this literature is not focal to the present study, these studies identify a number of neuroanatomical loci for conditioned inhibition that might contribute to conditioned inhibition as a general behavioral phenomenon seen across learning modalities, and so could be involved in the conditioned inhibition of fear. Noteworthy examples include the ventromedial prefrontal cortex (MacLeod & Bucci, 2010; Meyer & Bucci, 2014; Rhodes & Killcross, 2007),

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retrosplenial cortex (Robinson, Keene, Iaccarino, Duan, & Bucci, 2011), central nucleus of the amygdala (Holland, 2012), the perirhinal and postrhinal cortex (Campolattaro & Freeman, 2006; Gastelum, Guilhardi, & Burwell, 2012) and the serotonin system (Lister, Pearce, Butcher, Collard, & Foster, 1996; Watkins et al., 1998). As reviewed below, however, there are discrepancies between the appetitive and fear literatures with many more null results reported with regard to mechanisms for conditioned fear inhibition.

Numerous descriptive reports identify electrophysiological signatures and molecular correlates of safety signals in nuclei of the amygdala (Campeau et al., 1997; Likhtik, Stujenske, Topiwala, Harris, & Gordon, 2014; Pollak et al., 2008; Rogan et al., 2005; Sangha, Chadick, & Janak, 2013). However, the studies designed to determine the necessity of the amgydala, or any other structures for that matter, to conditioned inhibition of fear (i.e. inactivation or lesion) report null results, including the central amygdala (Falls & Davis, 1995), medial prefrontal cortex (Gewirtz, Falls, & Davis, 1997), perirhinal cortex (Falls, Bakken, & Heldt, 1997), auditory thalamus (Heldt & Falls, 1998), and nucleus accumbens (Falls & Davis, 1995; Falls et al., 1997; Gewirtz et al., 1997; Heldt & Falls, 1998, 2006; Josselyn, Falls, Gewirtz, Pistell, & Davis, 2005). Generalized fear is a key symptom of PTSD and is resistant to therapy (Rauch, Shin, & Phelps, 2006) and modulation of fear by safety signals is impaired in individuals with PTSD (Jovanovic et al., 2010). Thus, the basic need to identify the neuroanatomical mediators of conditioned inhibition will translate to a better understanding of fear related psychopathologies.

In the present study, we considered the posterior insular cortex (IC) as a novel participant in the learning and recall of conditioned fear inhibition and conditioned fear discrimination. The posterior IC has a number of features that position it to contribute to identifying environmental safety cues. These include access to auditory, visual, and somatosensory information (Benison, Rector, & Barth, 2007; Flynn, 1999; Gogolla, Takesian, Feng, Fagiolini, & Hensch, 2014; Mufson & Mesulam, 1982; Remple, Henry, & Catania, 2003; Robinson & Burton, 1980a, 1980b, 1980c; Shi & Cassell, 1998a; Sudakov, MacLean, Reeves, & Marino, 1971), a somatotopically organized body representation (Benison et al., 2007), multisensory integration (Rodgers, Benison, Klein, & Barth, 2008), afferent intracortical and thalamocortical connectivity (Shi & Cassell, 1998b) and efferent amygdala projections (McDonald, Shammah-Lagnado, Shi, & Davis, 1999; Shi & Cassell, 1998a). Extant data suggesting a role for posterior IC in processing safety signals were obtained using a backwards conditioned safety signal in the context of an unpredictable traumatic stressor. In the midst of the traumatic stressor, safety signals prevented the development of numerous stressor sequelae; these safety signal effects were blocked by both lesion and pharmacological inactivation of the posterior IC (Christianson et al., 2008, 2011). Whether the posterior IC contributes specifically to the learning or recall of safety signals, however, remains an unanswered question. The present study aimed to address this issue and extend these findings in a Pavlovian conditioned fear discrimination paradigm.

To examine the role of IC in conditioned fear inhibition, animals were exposed to two CSs: a safe CS that was never paired with footshock (CS) and a danger CS that was always paired with shock (CS+) as previously (Chen et al., 2015; Foilb & Christianson, 2015). Because the IC connectivity to the amygdala varies across its length (McDonald et al., 1999; Shi & Cassell, 1998a, 1998b) and others have reported roles for anterior divisions of rodent insula in fear (Bermudez-Rattoni, 2014; Casanova et al., 2016), we targeted three points along the rostro-caudal axis. To examine the role of these regions in the acquisition of conditioned inhibition of fear, we blocked N-methyl-D-aspartate receptors (NMDAr), which are critical to synaptic plasticity and numerous mnemonic functions (Morris, 2013). NMDAr blockade prevented conditioned inhibition learning, but only when injected to the posterior IC. We next tested whether this region contributes to (1) the recall of the safety signal in a summation test, (2) fear discrimination learning and (3) fear discrimination recall. The results clearly implicate the posterior IC in conditioned inhibition learning.

2. Materials and methods

2.1. Rats

The conditioned inhibition studies shown in Fig. 1A and B were conducted at the University of Colorado and the remaining studies were conducted at Boston College. Adult (250300 g upon arrival) male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IA) for use at University of Colorado and from Charles River Laboratories (Wilmington, MA) for use at Boston College. Husbandry conditions at the different locations were nearly identical; rats were housed in pairs in plastic tub cages with free access to food and water at all times. Rats that underwent surgery were single housed. A piece of autoclaved manzanita wood was provided for enrichment (Rosenzweig & Bennett, 1996) in accordance with recommendations by the Boston College Institutional Animal Care and Use Committee (IACUC). All animals were given 710 days to acclimate to vivarium. Rats were kept on a 12-hour light/dark cycle with lights on at 0700 h and all testing occurred between 0800 and 1400 h. Procedures were reviewed and approved the University of Colorado Boulder and Boston College IACUCs and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Apparatus

Behavioral conditioning at Boston College was performed in 10 æ 11 æ 6 in (L æ W æ H) cages made of black plastic with wire mesh lids and a floor of stainless bars attached to a shocking grid (Model H10-11R-TC-SF, Coulbourn Instruments, Whitehall, PA). A 15 æ 12 æ 27 in (L æ W æ H) light and sound-attenuating enclosure chamber housed each conditioning cage. Ventilation and masking noise of 55 dB was provided by a fan. Conditioned stimuli were delivered via a white LED array (Model LPL620WTHD, Hampton Bay) and a speaker mounted at the top of the chamber. The chamber was illuminated with 2 infrared LEDs arrays (CMVision Model IR30) mounted to the ceiling of the enclosure and overhead cameras (Model VX-5000, Microsoft, Redmond, VA) with the infrared blocking filters replaced with infrared passing filters were used to recorded behavior. Freezing was detected using ANY-Maze software (version 4.99, Stoelting, Wood Dale, IL) with the manufacturers recommended settings (as previously, (Christianson et al., 2011). The apparatus used at the University of Colorado was nearly identical, as described (Christianson et al., 2011). In two experiments (Fig. 1B and C) a distinct context was made by dividing the conditioning chamber on the diagonal, removing the shock floor and replacing it with shaved wood bedding.

2.3. CS+/CS conditioning

Adapted from (Myers & Davis, 2004) and used previously (Chen et al., 2015; Foilb & Christianson, 2015), conditioning sessions involved 15 presentations each of shock-paired (CS+) or unpaired (CS) cues, for a total of 45 min per session. Each trial was signaled by a common element (X), a 5 s, 1 kHz tone (75 dB) immediately followed by a 15 s discrete auditory (white noise pips, duration = 10 ms, rate = 3 Hz, 75 dB) or visible (flashing LED light, 264.0 Lux, 20 ms on/off) CS. The aversive unconditioned stimulus

(US) was a 500 ms footshock (1.2 mA) that co-terminated with the CS+, such that each animal received 15 shocks per conditioning session. These parameters were adopted based on the results of a pilot experiment in which the conditioned inhibition of freezing was assessed after conditioning with either serial or compound transfer stimuli (as in the AX+/BX protocol of Myers & Davis, 2004); the serial conditioning protocol resulted in robust and reproducible conditioned inhibition of freezing, while the compound cues did not (Foilb & Christianson, 2015). As in the relative validity AX+/BX protocol (Wagner, Logan, Haberlandt, & Pricel, 1968) used by Myers and Davis (2004), the variation used here retains common element X in conditioning, which serves as a transfer stimulus on CS trials. Trials were presented in a quasi-random order, so that no cue occurred more than twice in succession. There was a fixed 70 s inter-trial-interval. Assignment of the light or pip as CS+ or CS was counterbalanced in each experiment, and equally represented in each treatment condition.

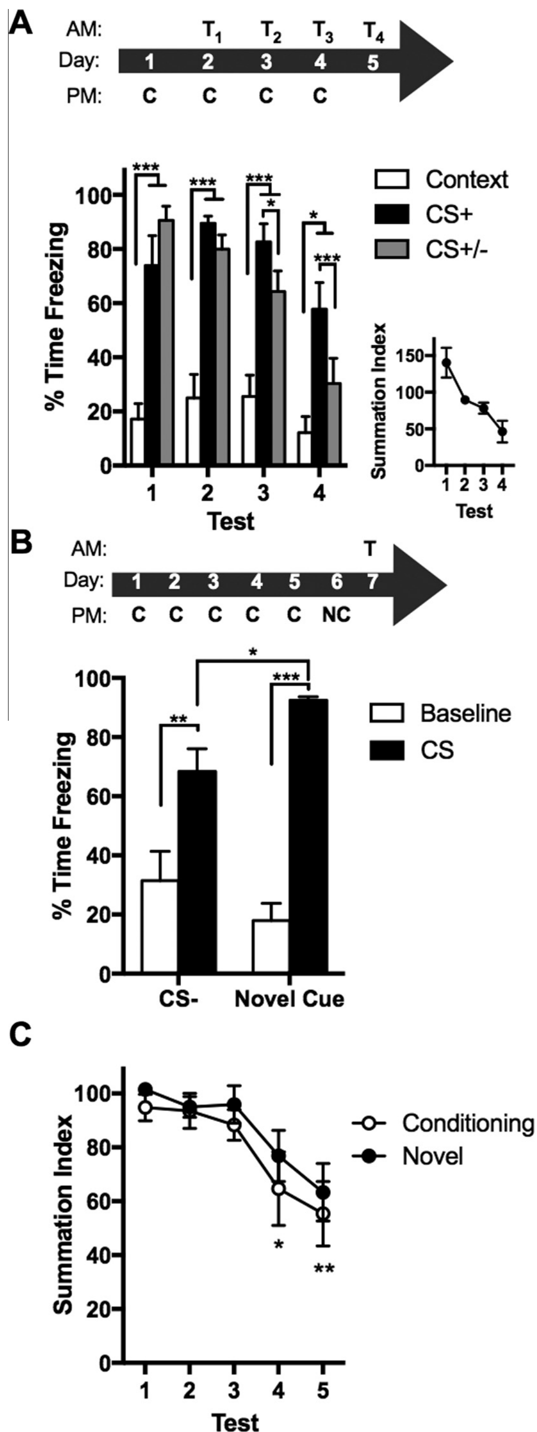

Fig. 1. Characterizing conditioned inhibition of fear. (A) *Above*: Timeline of CS+/CS conditioning sessions (C) and summation tests (T). *Below*: Mean (+SEM) percent time freezing to the training context at baseline and to presentations of the CS+ and the compound CS+/ cue in summation tests after each session of CS+/CS conditioning. Inhibitory summation to the CS+/ was evident as significantly less freezing during the CS+/ compound compared to the CS+ in tests 3 and 4. Inset displays the same data as mean summation index (˜S.E.M). Summation index scores were computed by dividing the time spent freezing to the CS+/ compound by the time spent freezing to the CS+ multiplied by 100. (B) *Above*: Timeline of conditioning for test of retardation of fear learning: 5 days of CS+/CS conditioning (C), new conditioning (NC) on day 6 with the CS or a novel cue paired with 2 footshocks, and recall test of fear to the CS or novel cue (T). *Below*: *Mean* (+SEM) percent time freezing to the CS and a novel cue after being paired with footshock. Presentation of the CS and novel cue caused significant increases in freezing compared to baseline but more freezing occurred to the novel cue than the CS. (C) Mean (˜SEM) summation index during tests for animals tested in either the conditioning chamber or a novel context following the same conditioning and test order as in A. Testing context did not appear to influence the emergence of conditioned inhibition as summation scores were significantly lower in both groups in tests 4 and 5 compared to tests 1, 2 and 3. Overhead brackets and asterisks indicate significant differences as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2.4. Summation tests

The efficacy of the CS safety signal to inhibit behavioral freezing was assessed in summation tests; the tests used at the University of Colorado began with 2 min of baseline context exposure, followed by a minute of the CS+ cue and a minute of CS+ and CS cues presented in compound (CS+/). At Boston college, an additional minute of the CS cue was included to permit simultaneous study of fear discrimination. At Boston College the cues were repeated for the following serial sequence: Baseline, CS+, CS+/, CS, CS+, CS +/, CS. Tests took place between 0800 and 1000 h each day. For figures and analysis, the two presentations of each cue were averaged.

2.5. Discrimination tests

In the experiments focused on fear discrimination, discrimination was assessed by presenting the CS+ and CS 6 times each in a quasi-random order, as previously (Chen et al., 2015); no summation trials occurred. Like the summation tests, these tests began with 2 min of baseline context exposure, followed by 60 s presentations of each cue in a quasi-random order. For figures and analysis, the six presentations of each cue were averaged.

2.6. Retardation test

After conditioning rats were divided into two groups: the first received 2 footshocks paired with the CS while the second group received 2 footshocks paired with a novel CS. The CS-shock pairings began 2 min after placement in the conditioning chamber. CS presentations were 30 s and co-terminated with a 5 s, 1 mA shock with a 60 s inter-trial-interval. Rats were removed from the chamber immediately after the second shock. Shock intensity was reduced relative to condition due to the longer US duration and to avoid a ceiling effect in later fear expression that would obscure any retarding effects of the putative conditioned inhibitor. On the next day, all rats were placed in a novel context (shaved bedding floor, red ambient light, triangular shape) and freezing was observed for 3 min of baseline and then during 3 min of CS

or novel CS presentation. The purpose of this experiment was to assess retardation of subsequent excitatory conditioning to the putative conditioned inhibitor (CS).

2.7. Cannula placement and microinjections

Surgical procedures and cannula placements were conducted as previously described (Chen et al., 2015; Christianson et al., 2011). Rats were anesthetized with isoflurane (3% in oxygen; Isothesia, Henry Schein, Dublin, OH) and placed in a stereotaxic apparatus. Stainless steel guide cannula (22 g; Plastics One, Roanoke, VA) were implanted bilaterally to target anterior (AP +2.7, ML˜3.9, DV 5.2), medial (AP +0.5, ML˜4.9, DV 6.2), or posterior (AP 1.8, ML 6.5, DV 6.2) insular cortex. All coordinate measures (mm) were taken from the skull surface at bregma. The IC can be subdivided into granular, dysgranular and agranular regions along its dorsal-ventral axis. Here, cannula were targeted for the central agranular region and the injection volume (0.5 L) was selected to permit diffusion throughout the three regions. Cannula tips found within any of the three subdivisions were included, thus conclusions from these were not intended to be specific to any of the IC subregions. Cannula were fixed to the skull with stainless steel screws and acrylic cement and a stylet extending 1 mm below the tip of the guide was placed in the cannula. Rats were given 1 mg/kg Loxicom (Eloxiject, Henry Schein) and penicillin (15,000 Units, Combi-Pen-48, Henry Schein) after surgery. A minimum of 7 days of recovery were allotted before behavioral testing, during which time rats were periodically handled and stylets were checked to ensure the cannula remained unobstructed. Microinjections were made by gently restraining the rat in a cloth towel and replacing the stylet with a microinjector connected with PE-50 tubing to a 25 L syringe (Hamilton, Reno, NV) in a micromanipulator (Model 5000, Kopf Instruments, Tujunga, CA). The injector protruded 1 mm beyond the cannula tip (33 g; Plastics One, Roanoke, VA). NMDArs were blocked with receptor antagonist D-()-2-Amino-5-phosphonopen tanoic acid (AP5). AP5 (Tocris, Minneapolis, MN) was dissolved in sterile saline at 6 g/L (as in (Amat et al., 2014; Bast, da Silva, & Morris, 2005; Christianson et al., 2014). The $GABA_A$ agonist muscimol was used to temporarily inactivate IC. Muscimol (Sigma, St. Louis, MO) was dissolved in sterile saline at 100 ng/L (Moscarello & LeDoux, 2013). Each drug was administered bilaterally at 0.5 L per side at a rate of 1 L/min, with an additional minute allowed for diffusion. Vehicle treated animals received saline injections at the same volume and rate as the drug infusions. AP5 injections were completed 15 min before conditioning (Bast et al., 2005) and muscimol injections were completed an hour before testing (Amat et al., 2005). At the end of each experiment, rats were overdosed with tribromoethanol (Sigma, St. Louis, MO). Brains were removed and flash-frozen in 2-methylbutane on dry ice, and stored at 80 C until they were sliced at 40 m on a freezing cryostat (20 C). Slices were stained with cresyl violet, coverslipped, and allowed to dry overnight before cannula placement was determined by comparison with the Rat Brain Atlas in Stereotaxic Coordinates (Paxinos & Watson, 2007). Data from rats for which cannulas were not found or were located outside of the targeted areas of IC were excluded in statistical analysis (see Fig. 2).

2.8. Experimental approach

The central goal of this study was to gain understanding of the neural mechanisms underlying fear inhibition by discrete safety signals. Thus, the critical first step was to establish a behavioral paradigm that met the criteria of conditioned inhibition. A priority was to utilize behavioral freezing as the endpoint of fear behavior as this behavior is central to the majority of research into the neural mechanisms of danger learning. A series of pilot studies culminated in the protocol described above which is fashioned from the procedure originally reported by (Myers & Davis, 2004) who used fear potentiated startle to observe summation and retardation effects to the unpaired CS cue. The experiments are divided into three sets. In set 1, we demonstrate that the current CS+/CS conditioning procedure led to a significant reduction in fear in summation tests and that excitatory fear conditioning to the CS was reduced when later paired with the US, and that the CS exhibits inhibitory effects when tested within the familiar conditioning context or in a novel context. Next, in set 2 we tested whether IC is necessary for learning and expression of conditioned fear inhibition. Because insula NMDAr blockade may have prevented either the recall or consolidation of the CSs by interfering with the more elementary process of fear discrimination, experiment set 3 sought to test whether IC contributes to fear discrimination or recall.

2.8.1. Experiment Set 1: A paradigm for the study of conditioned inhibition of fear

In the first experiment, rats were exposed to 4 consecutive days of fear discrimination conditioning and daily summation tests. Discrimination conditioning involved reinforcing the CS+ with a footshock on every trial and never reinforcing the CS. The morning after each conditioning session, all rats received a summation test. Because summation alone is insufficient evidence of conditioned inhibition (Rescorla, 1969), the second experiment tested whether the putative conditioned inhibitor would display retarded excitatory conditioning. Rats received CS+/CS conditioning on 4 consecutive days without summation tests. At this point, rats received retardation testing. Animals were divided into two groups, and received footshock paired with either the CS or a novel CS. The next day, fear to the CS and novel CS was tested. At this point, the laboratory moved from the University of Colorado to Boston College. The last experiment of this set sought to determine whether expression of summation was dependent upon testing within the conditioning apparatus. It also provided an opportunity to replicate the fundamental conditioned inhibition phenomenon in the new laboratory. The issue of context is important for two reasons. First, the context in which an excitatory CS is extinguished can become a conditioned inhibitor (Polack, Laborda, & Miller, 2012) which could augment the apparent fear inhibition in summation tests and make resolving the neural mechanisms of the discrete versus contextual conditioned inhibitors difficult. Second, the inhibition of fear that occurs when presented with an extinguished CS+ is tied to the context in which extinction occurred (Bouton, Westbrook, Corcoran, & Maren, 2006). Thus, it was conceivable that the inhibitory conditioning that occurred to the CS in the current preparation could be tied to the conditioning apparatus. It is worth noting that conditioned inhibition learning occurred at a slightly slower rate at Boston College, possibly due to differences in the breeding source or local nuances in the vivarium. Thus in experiments at Boston College, rats received an additional day of conditioning for a total of 5 consecutive days of conditioning. The morning after each day of conditioning rats received a summation test in either the conditioning context or the distinct testing context.

2.8.2. Experiment Set 2: Role of insular cortex in the acquisition and expression of conditioned inhibition of fear

To test the necessity of IC for the acquisition of conditioned inhibition, cannulas were implanted in three regions of ICanterior, medial, and posterior. Rats received conditioning for 5 days, with summation tests in the conditioning chamber each subsequent morning. In pilot experiments, rats that received injections of vehicle on the first day of conditioning never expressed conditioned inhibition, possibly due to tissue damage caused by repeated microinjections (5 consecutive days). Since conditioned inhibition is not evident before day 3 (as shown in Fig. 1 and in (Foilb &

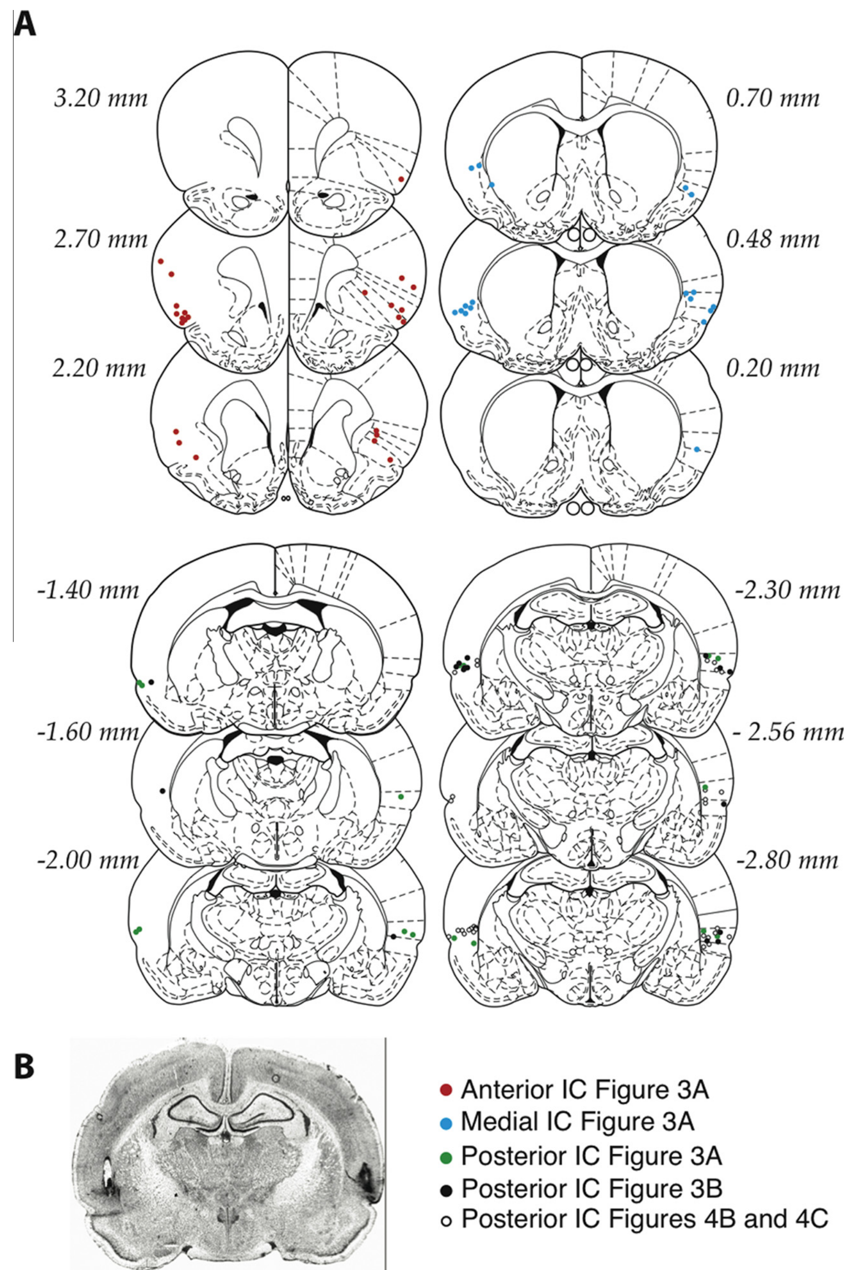

Fig. 2. Cannula placements. (A) Sites of microinjections for all cannula experiments. (B) Representative cannula tract in posterior IC. Images were reconstructed from the atlas of Paxinos and Watson (2007).

Christianson, 2015), drug manipulations began prior to conditioning session 3. On days 3, 4, and 5, rats received intra-IC (anterior, medial, or posterior) injections of AP5 or vehicle 15 min before conditioning. Only posterior IC NMDAr blockade interfered with acquisition of conditioned inhibition. To further understand the role of posterior IC in conditioned inhibition of fear, a separate set of rats were implanted with cannula in the posterior IC and received conditioning on consecutive days until conditioned inhibition was evident in summation tests given each subsequent morning. Importantly, animals were handled 1 h prior to each summation test to habituate to the microinjection procedure. Handling prior to summation tests slowed the expression of conditioned inhibition; therefore rats were only included in the inactivation phase of the experiment if they reached a summation index (see Section 2.9) of 80 or less. IC neuronal activity was pharmacologically inhibited by intra-IC injections of the $GABA_A$ receptor agonist muscimol or saline 1 h prior to a final summation test.

2.8.3. Experiment Set 3: Insular cortex and fear discrimination

A simple account of the IC contribution to conditioned inhibition would be a role in learning the prerequisite CS+/CS discrimination. To determine if the IC contributes to basic fear discrimination we first established a paradigm to test discrimination of fear and safety, which could then be used to determine the necessity of IC in this process. To this end, rats received one session of CS+/CS conditioning, and were tested in a fear discrimination recall test the following morning. To examine the involvement of IC in acquisition fear discrimination, a second group of rats received cannula implants within the posterior IC. Intra-IC injections of AP5 or saline were made 15 min before CS+/CS conditioning. The following

morning, animals were tested for fear discrimination. No effect of pre-training AP5 was evident on any aspect of later fear discrimination. We next sought to determine if posterior IC neuronal activity was required for fear discrimination recall. Using the same set of animals, all received a second, drug-free conditioning session. Prior to a second fear discrimination test, muscimol or saline microinjections (exactly as above) were made to posterior IC and fear discrimination was tested 1 h later. To increase the experimental power, rats were given a third fear discrimination test and received the opposite muscimol or vehicle treatment 2 days later, for a within subjects comparison.

2.9. Data analysis

Freezing was analyzed as percent time freezing during the relative cue condition. In summation tests, a summation index was calculated as freezing to CS+/ divided by freezing to CS+ times 100. Thus values greater than 100 would reflect excitatory summation whereas values less than 100 would reflect conditioned inhibition. Group differences in behavioral freezing data were then evaluated by analyses of variance (ANOVA) with drug treatment treated as a between-subjects factor, and cue, day or test treated as within-subjects factors, except where noted. Main effects and interactions were deemed significant with $p < 0.05$ and between-subjects post hoc comparisons were made with Tukeys HSD correction while within subjects comparisons were made using Sidaks correction. All analyses were made using GraphPad Prism 6.0 with experiment-wise error set to = 0.05.

3. Results

3.1. Experiment Set 1: Characterizing conditioned inhibition

In the first experiment, rats received a CS+/CS conditioning protocol in which the CS+ was always reinforced with a footshock (n = 7). In all reported experiments freezing was assessed during the conditioning sessions as previously (Foilb & Christianson, 2015). There were never any stimulus group differences in freezing during the conditioning sessions (data not shown; please refer to Foilb & Christianson, 2015) for a detailed account of freezing during conditioning using the same procedure). Freezing to the context, CS+, and compound CS+/ are shown in Fig. 1A. Presentation of the CS+/ initially led to excitatory summation, i.e. greater freezing in the presence of both CSs, but over the course of training, rats displayed significant conditioned inhibition as less freezing during the CS+/ trial than to the CS+. These observations are supported by significant main effects of test day, $F(3,18) = 9.96$, $p < 0.001$, of summation cue type, $F(2,12) = 93.34$, $p < 0.001$ and a day by cue interaction, $F(6,36) = 7.023$ $p < 0.001$. Post hoc comparisons between cue for each test day revealed greater freezing during the CS+ and the CS+/ trials on all days compared to the context (ps < 0.05). Conditioned inhibition was evident as less freezing to the CS+/ trial compared to the CS+ in tests 3 and 4 (ps < 0.05).

In the next experiment, all rats were given 5 days of CS+/CS conditioning without any summation tests. On the 6th day, rats received two footshocks paired with either the CS or a novel CS (ns = 8/condition). Excitatory fear conditioning to either CS was assessed on the next day. While presentation of each CS evoked freezing, more freezing was evoked by presentation of the novel CS compared to the CS (Fig. 1B). This observation was supported by a significant effect of CS presentation (baseline vs. CS presentation), $F(1,14) = 94.33$, $p < 0.001$, and significant CS presentation by CS type (CS vs. Novel CS) interaction, $F(1,14) = 5.497$, $p = 0.034$. Post hoc comparisons revealed significantly greater freezing to the CS compared to the pre-CS baseline (ps < 0.01) and significantly more freezing during the novel CS compared to the CS ($p = 0.05$). These results provide evidence of retardation of excitatory conditioning to the CS.

To determine the importance of testing context in conditioned inhibition, animals were conditioned in rectangular chambers and tested each following morning in either the same (n = 8) rectangular conditioning chambers or in different (n = 8) triangular chambers with shaved wood bedding. As above, conditioned inhibition appeared to get stronger with each test day, which was apparent in the summation index scores regardless of testing context (Fig. 1C). ANOVA revealed a main effect of test day, $F(4,56) = 12.00$, $p < 0.001$, but no main effect of test context, $F(1,14) = 0.8254$, $p = 0.379$, and no context by day interaction, $F(4,56) = 0.1528$, $p = 0.961$. Post hoc comparisons showed a simple effect of test, where summation index was significantly decreased in test 4 compared to tests 1, 2 and 3 (*ps* < 0.05) and test 5 showed further improvements with significantly lower summation scores compared to tests 1, 2 and 3 (*ps* < 0.01). Freezing to each cue was analyzed by context group by each test. There was no evidence of the context as an inhibitor or context specificity of conditioned inhibition because freezing to the context and presentations of CS+, CS and CS+/ compound never differed significantly between groups in any of the 5 tests (data not shown, *ps* > 0.05).

To summarize the results of Experiment Set 1, inhibitory summation was evident as reduced freezing to the CS+/ compound than to the CS+ alone. Importantly, this effect was not present in the first summation test but only after 3 or more conditioning sessions which indicates the inhibitory feature is a result of the CS conditioning history. Retardation was evident after pairing the CS with footshock resulted in less freezing than observed to a novel CS. Together these data show that CS+/CS conditioning results in a CS that meets the requirements of a conditioned inhibitor set forth by Rescorla (1969). Furthermore, the expression of conditioned inhibition was equal when tested in either the conditioning or a distinct context. Based on these results, a minimum of 5 days of conditioning was used and all other testing occurred in the conditioning chamber.

3.2. Experiment Set 2: Role of insular cortex in conditioned inhibition

Microinjections of AP5 were made to anterior (n = 12), medial (n = 9) and posterior (n = 8) IC before conditioning on days 3, 4, and 5, the days that precede evidence of conditioned inhibition in the summation test. Vehicles received microinjections of saline and included subjects with cannula placements in each of the three insular regions (n = 12 vehicles total). Reconstructions of the cannula tip locations for all rats included in analysis receiving drug injections are depicted in Fig. 2. To determine if there were any effects of AP5 on behavior during the conditioning sessions, all conditioning data were analyzed with 3 (cue) by 4 (region) ANOVAs. In no case was a main effect or interaction found for drug suggesting that AP5 did not influence freezing *per se*. The only significant effect of AP5 was found on Day 5 when comparing freezing to vehicles on the CS trials. Thus, during conditioning on Day 5, animals with posterior IC AP5 injections froze significantly more than vehicle ($p < 0.001$) or medial IC AP5 animals ($p < 0.05$). This is consistent with the failure to inhibit fear in the presence of the CS in summation tests in this group and does not reflect a general effect of AP5 on fear expression *per se*.

Performance in the summation tests for each drug and region group are shown in Fig. 3A. A 4 (region) by 5 (test) ANOVA showed a significant main effect of test, $F(4,148) = 21.63$, $p < 0.001$, and a significant main effect of region, $F(3,37) = 5.238$, $p = 0.004$. Post hoc comparisons showed that summation in vehicle animals was significantly reduced in tests 3 ($p < 0.01$), 4 and 5 (*ps* < 0.001) compared to test 1, in tests 4 and 5 compared to test 2 (*ps* < 0.001), and

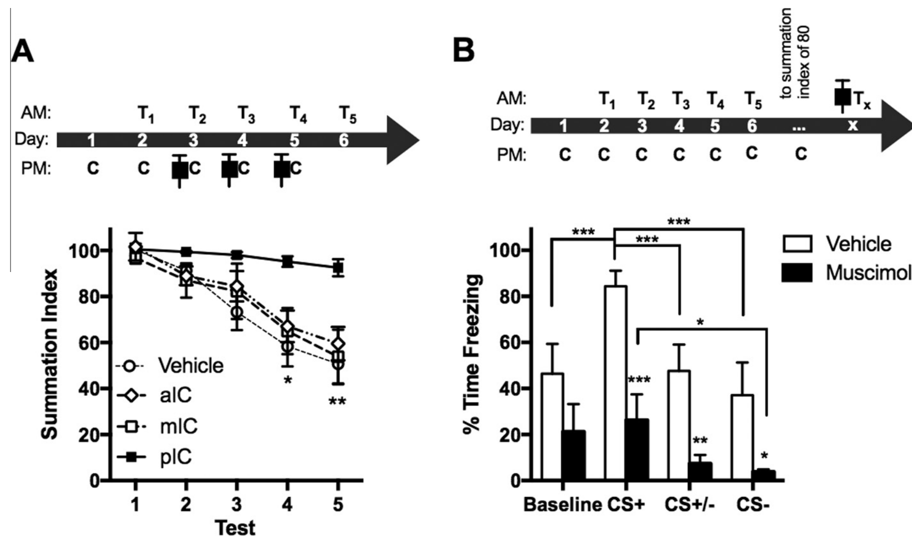

Fig. 3. Role of IC in conditioned inhibition of fear. (A) *Above*: Timeline of conditioning (C), summation tests (T), and intra-IC AP5 injections (syringe icon) before conditioning. *Below*: Mean ˘(SEM) summation index in tests given the morning after pre-conditioning with vehicle or AP5 injections to anterior (aIC), medial (mIC) or posterior IC (pIC) on days 3, 4, and 5. Greater conditioned inhibition was evident as lower summation scores in all groups compared to the pIC in tests 4 and 5. All groups except the pIC showed significant improvement in summation over the course of conditioning with significantly lower summation indices in tests 4 and 5 compared to their respective tests 1, 2 and 3 (ps < 0.05). B) *Above*: Timeline of conditioning, summation tests, and muscimol injections to the pIC before a summation test. Animals received repeated conditioning and recall testing until they reached a summation index less than 80. *Below*: *Mean* (+SEM) freezing to baseline context, CS+, CS+/ and CS 1 h after muscimol injections. There were main effects of both drug and cue where animals froze significantly more to the CS+ than to the CS+/, or CS and there was significantly reduced freezing to presentations of CS + and CS+/ in the muscimol-treated animals compared to vehicles. Overhead brackets and asterisks indicate significant differences as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

in test 5 ($p < 0.05$) compared to test 3. This gradual decrease in summation index was comparable to the animals without cannula or injections (Fig. 1C). Similarly, in animals with medial and anterior IC injections of AP5, summation scores were significantly lower in tests 4 ($ps < 0.01$) and 5 ($ps < 0.001$) compared to test 1, and further improved in test 5, which was significantly reduced compared to tests 2 ($ps < 0.01$) and 3 ($ps < 0.05$). Conversely, AP5 injections to the posterior IC resulted in significantly greater summation scores compared to anterior and medial AP5 injections and vehicle injections on tests 4 (ps < 0.05) and 5 ($ps < 0.01$). Thus, only posterior IC NMDAr appear to be critical to the acquisition of conditioned inhibition as measured by summation.

Because the CS appears to gain strength as an inhibitor after each conditioning session, it is possible that recall and reconsolidation processes occur within the posterior IC during the conditioning sessions. Thus, the preceding results could be attributed to a role of posterior IC in either the recall the CS or the subsequent consolidation of new learning to the CS during conditioning. To investigate this possibility that IC contributes to the recall of either the CS+ or CS and to test whether IC contributes to the expression of summation, animals received daily conditioning and summation tests for 78 days at which point the majority of test subjects had summation scores less than 80%; subjects not meeting this criterion were excluded from analysis (as in Likhtik et al., 2014). Before the final summation test, microinjections of muscimol (n = 8) or vehicle (n = 9) were made to posterior IC, the region identified as critical above. Despite observing an increase in freezing to the CS +/ compound when NMDAr were blocked in posterior IC during acquisition, pharmacological inactivation of posterior IC before recall surprisingly reduced freezing to all cues (Fig. 3B). A 2 (drug) by 4 (cue) ANOVA revealed significant main effects of drug, $F(1,15) = 10.76$, $p = 0.005$, and cue, $F(3,45) = 9.135$, $p < 0.001$. Because there was no significant drug by cue interaction, post hoc analyses included both groups. Although freezing was reduced, all rats appeared to discriminate between the different CSs in the summation test. Conditioned inhibition remained intact with significantly reduced freezing to context ($p < 0.05$), CS+/ ($p < 0.01$) and CS ($p < 0.001$) compared to CS+. The main effect of drug was evident as a significant reduction in fear in the muscimol condition compared to vehicles, where muscimol-treated animals froze significantly less to presentations of CS+ ($p < 0.001$) and CS+/ ($p < 0.05$).

3.3. Experiment Set 3: Posterior insular cortex and fear discrimination

To establish whether the role of the posterior IC in conditioned inhibition was the consequence of a simpler role in conditioned discrimination, we adapted the summation test to optimize detection of discrimination (see Section 2). One group of rats (n = 8) was given one day of CS+/CS conditioning and the next day received a discrimination test. Fear discrimination was evident as differential freezing to the CS+ and CS (Fig. 4A). This was supported by a significant main effect of cue, $F(2,14) = 13.39$, $p = 0.006$. Post hoc comparisons revealed significantly more freezing to CS+ compared to CS and baseline context ($ps < 0.01$). This procedure was then used to test the involvement of posterior IC in the learning and recall of fear, and fear discrimination.

In a new group of animals, microinjections of AP5 (n = 9) or saline (n = 10) were made to posterior IC prior to CS+/CS conditioning on day one. As above, there was no effect of AP5 on freezing during conditioning (data not shown). In the discrimination test on the following day, rats in both AP5 and saline groups appeared to have acquired equal fear to CS+ with discrimination evident to the CS (Fig. 4B). Accordingly, there was a main effect of cue, $F(2,34) = 71.01$, $p < 0.001$, but no main effect of drug, $F(1,17) = 0.43$, $p = 0.523$, or interaction of drug and cue, $F(2,34) = 0.93$, $p = 0.402$. Post hoc comparisons showed effective fear discrimination with significantly greater freezing to the CS+ compared to both the CS and context ($ps < 0.001$). Therefore, posterior IC NMDAr do not appear to be critical to the acquisition of a basic fear discrimination.

The same cohort of animals was subjected to an additional drug-free conditioning session and discrimination tests to determine the role of posterior IC on fear discrimination recall. All rats were trained again without drug on day 2. We used a within-

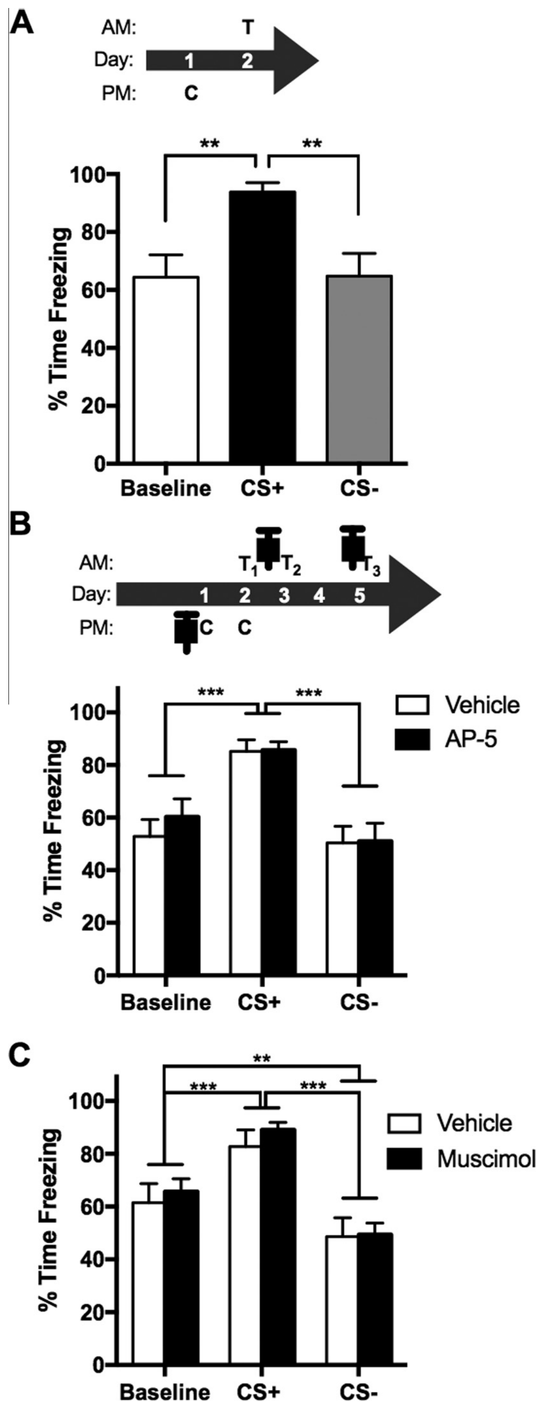

Fig. 4. Role of IC in fear discrimination. (A) *Above*: Timeline of conditioning (C) and discrimination tests (T). *Below*: Mean (+SEM) percent freezing to baseline context, CS+ and CS during discrimination test. Discrimination was evident as significantly greater freezing to the CS+ than to either the context (baseline) or the CS. (B) *Above*: Timeline of conditioning (C), recall (T) and intra-posterior IC AP5 injections (syringe icon) before conditioning and muscimol injections before recall Tests 2 and 3. *Below*: Mean (+SEM) percent freezing during recall Test 1 after intra-IC AP5 injections before conditioning. There was no effect of drug, but a significant effect of cue, with increased freezing to the CS+ compared to both the CS and context in both treatment conditions. (C) Mean (+SEM) percent freezing in discrimination test 1 h after intra-IC muscimol injections. There was no effect of drug, but a main effect of cue where freezing to each cue was significantly different from all other cues. Overhead brackets and asterisks indicate significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

subjects design in which all animals received 2 fear discrimination tests, one each on day 3 and 5. On day 3, one half of the subjects received muscimol prior to the test while the other received vehicle; on day 5 the treatments were reversed. Rats were left alone for 1 day between tests to ensure washout of muscimol. Data were first analyzed for effects of initial drug treatment (AP5 or vehicle); none were apparent (ps > 0.12) and so the data were pooled. A 3 (cue) by 2 (drug) within subject ANOVA was used (n = 12). Pretest muscimol did not appear to influence freezing to any cue or discrimination (Fig. 4C). Thus, a significant main effect of cue, $F(2,22) = 60.30$, $p < 0.001$, but no effect of drug, $F(1,11) = 0.2669$, $p = 0.616$, and no interaction of drug and cue, $F(2,22) = 0.7717$, $p = 0.474$, were found. Freezing to each cue was significantly different from all other cues: context vs. CS+, CS+ vs. CS, and context vs. CS (*ps* < 0.001). Together, these results show that the conditioning paradigm used for conditioned inhibition, reliably and effectively produces fear discrimination but neither pre-training AP5 nor pre-testing muscimol appeared to influence acquisition or expression of fear discrimination.

4. Discussion

The goal of this project was to advance understanding of the neural circuitry that mediates fear inhibition by learned safety signals. To this end, we first characterized a fear conditioning paradigm in which rats were exposed to danger cues paired with footshocks (CS+) and safety cues which were never paired with shock (CS). After several conditioning sessions, upon later presentation of the CS+ and CS in compound, the CS appeared to modulate the conditioned fear response and the expression of freezing was attenuated. Prior work had identified the posterior IC as a region capable of integrating the sensory information required to distinguish between safety and danger and modulate the output of fear circuits. Here, blockade of the posterior IC NMDAr completely prevented conditioned inhibition learning, which to our knowledge is the first evidence indicating any brain region as necessary for acquiring a conditioned fear inhibitor. We conducted a number of control experiments, which suggest that the posterior IC plays a unique role in learning about safety signals that could not be reduced to a more simplistic role in fear discrimination. These findings have important implications for understanding the neural regulation of fear and introduce a number of new questions about the functions of insula in cognition.

Although first described by Pavlov (1927), Rescorla (1969) later outlined the standard assessment of conditioned inhibition (1969). The ability of a conditioned inhibitor to reduce the conditioned response elicited by a conditioned excitor is the most direct method to measure conditioned inhibition. In experiment set 1 (and see (Foilb & Christianson, 2015)), we provide clear evidence of inhibitory summation after 3 or more conditioning sessions. Rescorla argued that new, excitatory conditioning to the putative conditioned inhibitor should occur more slowly than excitatory conditioning to a novel CS. Here, when pairing the learned CS with footshocks we observed significantly less CS evoked freezing in a later recall test than to a novel CS. Consistent with the prior work of (Myers & Davis, 2004) using a very similar protocol, the safety signal CS used here appears to meet the classic requirements of a conditioned inhibitor.

The blockade of posterior IC NMDAr prevented acquisition of conditioned inhibition of fear, whereas NMDAr blockade in anterior and medial insula did not alter acquisition. This marks an important step towards understanding the neural circuitry

underlying conditioned inhibition of fear. Prior mechanistic experiments have failed to find a critical roles for central nucleus of the amygdala (Falls & Davis, 1995), medial prefrontal cortex (Gewirtz et al., 1997), perirhinal cortex (Falls et al., 1997), auditory thalamus (Heldt & Falls, 1998), hippocampus (Heldt & Falls, 2006), or nucleus accumbens (Josselyn et al., 2005) in the acquisition of conditioned inhibition of fear. That neither NMDAr blockade or pharmacological inhibition of posterior IC with muscimol interfered with any aspect of fear discrimination suggests that the differential associations formed between the footshock, the CS+ and the CS that permit discrimination are mediated by subcortical, namely amygdala, circuits.

As noted, IC receives multisensory inputs and is positioned to interface with the amygdala fear circuit. The insula has access to auditory (Robinson & Burton, 1980a), visual (Guldin & Markowitsch, 1983; Miller & Vogt, 1984) and somatosensory information (Benison et al., 2007; Shi & Cassell, 1998a) and plays role in multisensory integration (Gogolla et al., 2014; Rodgers et al., 2008). Thus, the partial reduction in freezing observed after IC inactivation before summation test (Fig. 3B) may be the result of impairment in relaying sensory information to other neural circuits. However, this is not a complete account because IC inactivation did not alter fear recall when rats had received insufficient training for conditioned inhibition (Fig. 4C). Until now, only studies focusing on conditioned inhibition of other behaviors have found neuroanatomical bases as noted above. Importantly, other reports fail to find a necessary role of the IC in fear expression (Rosen et al., 1992; Shi & Davis, 1999); although see (Casanova et al., 2016) for exception). That expression of conditioned inhibition appeared intact when posterior IC was inactive suggests that this region is not critical to the recall of the safety cue.

Considerable electrophysiological evidence suggests that the expected outcome of a danger or safety signal is encoded within the amygdala: conditioned inhibitors evoke smaller local field potentials in the lateral amygdala (Rogan et al., 2005), single units in the basal amygdala encode both danger and safety (Genud-Gabai, Klavir, & Paz, 2013; Sangha et al., 2013), safety signals alter amygdala-prefrontal synchrony (Likhtik et al., 2014), and safety and danger signals differentially potentiate inputs (Ostroff, Cain, Bedont, Monfils, & Ledoux, 2010) and outputs (Amano, Unal, & ParÕ, 2010) of the lateral amygdala to the central amygdala. Our results suggest that conditioned inhibition arises after (or more slowly, but in parallel to) simple fear discrimination learning, which occurs within the amygdala and is independent of IC.

The reciprocal connectivity between posterior IC and the BLA (Shi & Cassell, 1998b) positions this structure as a critical intersection for incoming sensory cues to be compared with learned associations. With additional conditioning, an association between the safety signal and the nonoccurrence of shock gains strength because of negative prediction errors generated on no shock trials. That posterior IC inactivation by muscimol reduced the fear response to the CS+ (Fig. 3B) suggests that IC plays a role in danger expectation which could be relayed to the amygdala to provide a basis for a prediction error on no shock trials. Although the effect of muscimol on fear recall (Fig. 3B) contrasts with studies which found no critical role of IC in simple fear conditioning, our result is consistent with other findings that over time, a fear CS undergoes systems consolidation such that the conditioned response becomes dependent on the IC (Izquierdo et al., 1997). The systems consolidation view also accounts for the discrepancy that inactivation of the IC during recall of conditioned inhibition produced a reduction in fear to the CS+ after several days of conditioning, but had no effect on the recall of fear discrimination after one day of conditioning (Fig. 4C). Thus, the evidence suggests that NMDAr blockade during conditioning may have interfered the systems consolidation processes supporting the CS+ and the relay of fear expectancy from the IC to amygdala nuclei; these will be the focus of future investigation.

The present work has important implications for understanding fear related behavior and psychopathology in humans. Individuals with PTSD display hyperactivity in the insular cortex (Etkin & Wager, 2007), which may interfere with the integration of stimuli needed to compute whether or not a given environment is safe or dangerous. Although safety learning has received only limited attention in human neuroimaging studies, insular activity is positively correlated with expectations of danger (Phelps et al., 2001) and pain (Ploghaus et al., 1999). That we observed a reduction in fear with insular inactivation during a summation test is consistent with these findings (Fig. 3B) and suggests that hyperactivity of the insula in a relatively safe environment could shift appraisal to danger and manifest as fear in an individual with PTSD. Thus, developing treatments that normalize insular cortex activity may allow individuals with PTSD to better utilize environmental safety cues and achieve greater treatment outcomes.

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