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Safety signals mitigate the consequences of uncontrollable stress via a circuit involving the sensory insular cortex and bed nucleus of the stria terminalis

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Abstract

BACKGROUND—Safety signals exert a powerful buffering effect when provided during exposure to uncontrollable stressors. We evaluated the role of the sensory insular cortex (Si) and the extend amygdala in this “safety signal effect.”

METHODS—Rats were implanted with microinjection cannula, exposed to inescapable tailshocks either with or without a safety signal and later tested for anxiety-like behavior or neuronal Fos expression.

RESULTS—Exposure to the uncontrollable stressor reduced later social exploration, but not when safety signals were present. Temporary inhibition of Si during stressor exposure, but not during later behavioral testing, blocked the safety signal effect on social exploration. The stressor induced Fos in all regions of the amygdala, but safety signals significantly reduced the number of Fos immunoreactive cells in the basolateral amygdala and ventrolateral region of the bed nucleus of the stria terminalis (BNSTlv). Inhibition of BNSTlv neuronal activity during uncontrollable stressor exposure prevented the later reduction in social exploration. Finally, safety signals reduced the time spent freezing during uncontrollable stress.

CONCLUSIONS—These data suggest that safety signals inhibit the neural fear or anxiety response that normally occurs during uncontrollable stressors and that inhibition of the BNSTlv is sufficient to prevent later anxiety. These data lend support to a growing body of evidence that chronic fear is mediated in the basolateral amygdala and BNSTlv and that environmental factors that modulate fear during stress will alter the long-term consequences of the stressor.

Keywords

rat; fear; anxiety; conditioned inhibition; learned helplessness; amygdala

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INTRODUCTION

Exposure to acute traumatic stressor is a predisposing factor for a number of psychiatric conditions including posttraumatic stress disorder (PTSD) (1). A major symptom of PTSD is a state of fear or anxiety that persists even when trauma-related environmental cues are absent (2). The leading hypothesis regarding the etiology of PTSD posits that intense fear that occurred during trauma changes the way that fearful emotions are expressed following trauma and that the trauma-related fear generalizes to numerous other cues (3). Consequently, reducing the expression of such anxiety is a goal of PTSD treatment.

As with humans, traumatic stress exaggerates later fear (4–6) and anxiety (7) in the rat. Importantly, environmental factors that reduce the experience of fear during traumatic stress also prevent many of the long-term consequences of stress. For example, providing safety signals, stimuli that predict stressor-free periods, reduces fear conditioned to the stress environment (8) and prevents numerous sequelae of uncontrollable stress (9–13). Unlike danger signals that elicit conditioned stress responses, safety signals inhibit conditioned stress responses. Pretraining lesions to a region of the posterior insular cortex, termed “sensory insula (Si)” for its unique sensory characteristics (9, 14), completely prevented the stress-mitigating effect of a safety signal on later anxiety-like behavior (9).

In the present experiments Si activity was inhibited by local microinjection of the GABA_A agonist muscimol, either before stressor exposure with safety signals or 24 h later before a test of anxiety-like behavior. Because safety signals may reduce the fear conditioned during stress neuronal activity was assessed by Fos, the protein product of the immediate early gene, *c-fos* in the amygdala. Quantification of Fos revealed a reduction in neuronal activity in the posterior basolateral amygdala (BLAp) and lateral ventral bed nucleus of the stria terminalis (BNSTlv), structures involved in uncontrollable stress effects (15–17) and sustained fear behavior (18), suggesting that inhibition of this pathway is involved in stress mitigation. To determine if BNSTlv inhibition was sufficient to prevent stressor-induced anxiety, BNSTlv was inhibited during stress by tetrodotoxin (TTX) and anxiety-like behavior was assessed 24 h later. Freezing, a behavioral index of fear, was quantified during exposure to uncontrollable stress with or without a safety signal. Consistent with the anatomical data, safety signals led to a significant reduction of freezing during stress.

MATERIALS AND METHODS

Rats

Adult (65–75 days old at time of testing) male Sprague-Dawley rats (Harlan Sprague Dawley; Indianapolis, IN) and juvenile (28–32 day old at testing time) male rats of the same strain and source were used in each experiment. Adult rats were housed in groups of 2 per cage and juveniles were housed in groups of 4 on a 12-h light/dark cycle (lights on at 7:00 A.M. and off at 7:00 P.M.). Juvenile rats were used only as stimuli in social exploration tests. Rats were allowed to acclimate to colony housing for 7–10 days and had free access to standard lab food and water at all times. The University of Colorado Institutional Animal Care and Use Committee approved all procedures that involved animals.

Cannula Placement and Microinjection Procedure

Bilateral guide cannulae were implanted under isoflurane anesthesia (3% in O₂). Stainless steel dual cannula guides (26 gauge, 1 mm center to center, Plastics One, Roanoke, VA) were placed in the Si (−1.75, −2.75 AP, ± 6.5 ML, −6.5 DV, see Figure 1) or bilateral single guides (26 gauge, 7.5 mm, Plastics One) were aimed at the BNSTlv at a 22° angle relative to midline at: AP −0.4 mm, ML ± 4.4 mm, DV −6.3 mm and fixed to skull with screws and acrylic cement. All coordinates were relative to Bregma and obtained from the atlas of

Paxinos and Watson (19). A stylet that projected 1 mm below the guide cannula was inserted with a dust cap secured over the top to insure patency. Each rat received 0.25ml of penicillin (s.c., Twin-Pen; AgriLabs, St. Joseph, MO, USA) per kg body weight and allowed 2 weeks of postoperative recovery. Intracerebral microinjections were made while gently restraining the rat and replacing the stylet with a microinjector that extended 1 mm beyond the cannula tip (33 ga, Plastics One) into the Si or BNSTlv region. For Si injections, each rat received either 500 ng of muscimol in 0.9% saline (Sigma, St. Louis, MO) or 0.9% saline alone at a rate of 1 μ l/min through PE-50 tubing by a 25 μ l Hamilton syringe and Kopf micromanipulator. For BNSTlv injections, each rat received either 160 ng of TTX (Sigma) in artificial cerebrospinal fluid (aCSF) or aCSF alone. All injection volumes were 0.5 μ l. Following injection, the injectors were left in place for an additional 2 min to prevent backflow and permit diffusion. Electrophysiological verification, using established methods (Christianson et al., 2008) confirmed that muscimol sufficiently inhibited evoked neuronal activity in Si without influencing evoked responses in the surrounding cortex (data not shown). At the end of the experiment, rats were deeply anesthetized with sodium pentobarbital (65 mg/kg i.p.) and the brains were removed and frozen. 40 μ m sections were mounted on gelatin-coated slides, air-dried, stained with cresyl violet, and cover slipped. Rats with misplaced cannula that received muscimol or TTX injections were excluded from analysis; saline injected rats were included regardless of cannula placement to reduce animal usage.

Safety Signal and Inescapable Shock Procedure

Inescapable tail shock (IS) was administered in clear acrylic restraining tubes that were placed in sound-attenuating chambers with a houselight (60 lux). The tail was taped to a Plexiglas rod that extended from the back of the chamber and 2 copper electrodes were attached to the tail with electrolyte paste. Tailshock was delivered by a Precision Regulated Animal Shocker operated by a LabLinc computer interface and Graphic State 3.0 software (Coulbourn Instruments, Allentown, PA). Each rat received 100 trials of tailshock (10 trials $\times$ 1.0 mA, 10 trials $\times$ 1.3 mA, and 80 trials $\times$ 1.6 mA) on a variable interval schedule (average 60 seconds; range = 20–140 s). Shock length varied at random within a range of 1 to 9 s with an average length equal to 5 s. In the safety signal group (SAFE-IS), the houselight was turned off for 5 s immediately after the termination of each shock. The random shock group (RANDOM-IS) was exposed to 5 s light-off periods distributed at random on an average of 60 seconds with no specific relationship to shock onset or offset. When only IS was used shock duration was fixed at 5 s with a constant houselight. These procedures are identical to our prior work (9).

In the final experiment rats were placed in chambers modified for the assessment of freezing. Rats were placed on a grid of steel rods (1cm center-center; Coulbourn Instruments) within a black acrylic enclosure (27cm $\times$ 25 cm $\times$ 12 cm) with a transparent mesh lid. The chamber was placed inside a sound-attenuating chamber fitted with a houselight (20 Lux), 2 infrared illuminators (850nm wavelength; IR 30, www.yytrade.net) and webcams (VX-6000, www.microsoft.com) mounted (32cm) directly above the black enclosure. A 850nm infrared passing filter was added to the webcam to visualize only the infrared spectrum. Footshocks (scrambled 0.8mA) and light-off periods were administered as above to rats in either SAFE-IS or RANDOM-IS treatments. ANY-Maze software (version 4.75, www.anymaze.com) was used to quantify freezing.

Social Exploration Test

Social exploration tests were conducted in a quiet testing room as described previously (20). Each experimental subject was assigned a separate plastic tub cage (18 $\times$ 24 $\times$ 18 cm) with 1cm of shaved wood bedding and a wire lid. Before testing, experimental subjects were

placed in the test cage and were allowed 1 h to acclimate in the testing room. A 28 ($\pm$ 2) day old juvenile was introduced to the adult's test cage for 3 min and exploratory behaviors, including sniffing, pinning, grooming and sparring, initiated by the adult rat, were timed by an observer blind to treatment. A social exploration baseline was obtained to identify rats with unusual baseline social exploration. Healthy naïve rats on average explore for approximately 80 s; rats with baseline social exploration times less than 50 s were precluded as outliers. The tests were performed 24 h before stress, and 24 h after stress. Social interaction tests are widely used to assess anxiety-like behaviors (21). These procedures are sensitive to anxiolytic and anxiogenic drugs (7) and reliably detect the effect of safety signals on stressor induced anxiety-like behavior (9).

Fos immunohistochemistry

Tissue preparation—Fos expression peaks between 1 and 3 h after stress (22, 23).

Therefore, 2 h after the last tailshock, rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and perfused with 200 ml of heparinized saline and then 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were collected, postfixed in paraformaldehyde for 24 h and transferred to 30% sucrose. 30 μ m sections were collected on a freezing cryostat (-20°C) and stored in cryoprotectant at 4°C until sectioning.

Immunohistochemistry—Immunohistochemical staining in sections containing the LA, BLAp and CeA were processed for CamK-II alpha immunoreactivity following the procedure of Reznikov et al. (24) who used this procedure to identify pyramidal neurons in the BLAp. Although the CamK-II alpha stain appears to be robust in the photomicrograph, pyramidal staining was quite variable between subjects and so the data are not presented. Since the goal of the study was to assess neuronal activity, with cellular specificity a secondary aim, this tissue and BNST tissue was then processed for Fos as follows. First, endogenous peroxidase, avidin and biotin were blocked with hydrogen peroxide and avidin/biotin blocking Kit (Vector Laboratories # SP-2001), respectively. Tissue was then incubated with Fos primary antibody (rabbit anti-Fos 1:15,000 Santa Cruz Biotechnology, Lot #D012) in a blocking solution containing 1% bovine serum albumin, 0.3% Triton-X, and 0.1% sodium azide. After incubation, sections were washed in PBS and then incubated for 60 min in biotinylated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) diluted 1:200 in blocking solution. Following another series of washes in PBS, sections were exposed to 1:200 dilution of avidin-biotin horseradish peroxidase (ABC) solution, Vectastain ABC Kit (Vector Laboratories #PK-4000) for 60 min. Sections were then washed with 0.1 M phosphate buffer (PB) and visualized with 3'3 diaminobenzidine enhanced with colbalt and nickel.

Fos Quantification—Using bright-field microscopy, an observer, blind to treatment, quantified the number of Fos immunoreactive cells in 4 representative sections of the central nucleus of the amygdala (CeA; Bregma -2.56 mm to -2.8 mm), 2 representative sections containing the lateral amygdala (LA) and BLAp (Bregma -3 mm to -3.3 mm), and 1 representative section containing the BNSTlv (Bregma -0.8 mm). The average count per section, per subject was analyzed. Anatomical location was verified by comparing adjacent Nissl stained sections with a stereotaxic atlas. Fos cells were identified by blue/black ovoid nuclei. Although the CamK-II alpha stain was variable, Fos counts were normally distributed in each treatment condition.

Experimental Procedures

Inhibition of Si prior to stressor exposure—After 2 weeks of postoperative recovery, rats were given a social exploration pretest on Day 1 and were then randomly assigned to one of 4 groups in a 2 (muscimol or vehicle) $\times$ 2 (SAFE-IS or RANDOM-IS) between

subjects design ($n_s = 8/\text{group}$). On Day 2 rats received either muscimol or saline 1 h before shock with safety signals or random signals. On the following day, all rats were given the social exploration test. Since post-stress social exploration could be compared to baseline behavior, an unstressed group was not necessary in this design and data were analyzed as percent of baseline social exploration.

Inhibition of Si before social exploration testing—Rats were given a social exploration pretest on Day 1. Rats were randomly assigned to one of 6 groups in a 2 (muscimol or vehicle) $\times$ 3 (SAFE-IS, RANDOM-IS or no stress home cage control: HC) between subjects factorial design ($n_s = 7-9/\text{group}$) and on Day 2 received stress treatment and then returned to the vivarium. On Day 3 rats received intra-Si muscimol or saline 1 h before the social exploration test.

Effect of safety signals on stress induced neuronal activity in the amygdala—Rats were randomly assigned to one of 3 groups: SAFE-IS, RANDOM-IS or HC, $n_s = 8/\text{group}$. Brains were collected and processed for Fos immunoreactivity and quantified as described above.

BNSTlv inhibition during stressor exposure—BNSTlv inhibition was examined to determine whether it would prevent IS-induced anxiety. Rats were assigned to one of 4 groups in a 2 (IS or HC) by 2 (TTX or vehicle) design, $n_s = 10/\text{group}$. TTX, a sodium channel antagonist, was used to inhibit BNSTlv neuronal output. Although TTX would also inhibit fibers of passage, BNSTlv outputs are regulated by local GABAergic neurons. Thus, alternatives such as muscimol could release the output neurons from inhibition confounding interpretation. On Day 1, all rats were given a social exploration baseline test. On Day 2, rats received intra-BNSTlv injection of TTX or vehicle 30 min before IS or HC treatment. No safe or random signals were presented during IS. After stress rats were returned to the vivarium. On Day 3, social exploration tests were given as on day 1.

Assessment of freezing during unpredictable stress—Prior work (8) and the current data suggest that safety signals inhibit fear during stress exposure. Behavioral freezing is a commonly used assay of fear in rat (25) but cannot be assessed in the tailshock restraint tubes used in the previous experiments. Thus, rats were exposed to the same shock and signal protocol in a footshock environment in which freezing could be assessed by a video tracking system. Rats were randomly assigned to either SAFE-IS or RANDOM-IS in the footshock apparatus.

Results

Inhibition of Si before stressor exposure prevented the stress-mitigating effect of safety signals

Rats that had baseline social exploration times less than 50 sec and/or had missing or damaged cannula were excluded from the behavioral analysis, yielding the following group sizes: SAFE-IS/Saline, $n = 8$, SAFE-IS/Muscimol, $n = 8$, RANDOM-IS/Saline, $n = 11$, RANDOM-IS/Muscimol, $n = 8$. Locations of cannula tips are illustrated in Figure 1, Panel C. Each rat served as its own control and social exploration is expressed as the percentage of baseline. Baseline social exploration did not differ between treatment groups ($p > 0.05$). Social exploration in the SAFE-IS/Saline group was near 100% of baseline while the RANDOM-IS groups showed marked reductions. Thus, safety signals mitigated the impact of IS on later social exploration. However, inhibition of the Si by muscimol before stress eliminated the effects of the safety signal (Figure 1, Panel A). A two-way analysis of variance (ANOVA) with stress and drug treated as between-groups factors revealed a main

effect of Stress, $F(1, 28) = 6.65$, $p = 0.016$, a main effect of Drug, $F(1, 28) = 16.83$, $p < 0.001$ and a Stress by Drug interaction, $F(1, 28) = 12.40$, $p = 0.001$. Post-hoc comparisons (Fisher's Protected Least Significant Difference test) indicated that mean percent of baseline in the SAFE-IS/Muscimol group was significantly less than SAFE-IS/Saline, $p < 0.001$. Pair-wise comparisons found that all groups were significantly lower than the SAFE-IS/Saline group, $p < 0.05$.

Inhibition of Si before social exploration testing did not influence behavior

Rats that had baselines below 50 seconds or misplaced cannula were excluded resulting in $n = 8$ in all groups; cannula tips are illustrated in Figure 1, Panel C. Again, each rat served as its own control and social exploration is expressed as the percentage of baseline. An unstressed group was included in order to demonstrate that muscimol injections into the Si region did not affect the social exploration time of stress-naïve rats. The safety signal reduced the impact of tailshock on later social exploration as above but inhibition of the Si with muscimol 1 h before the social exploration test had no effect (Figure 1, Panel B). A two-way ANOVA identified a main effect of Stress, $F(2, 42) = 46.92$, $p < 0.001$, but the main effect of Drug and the Stress by Drug interactions did not reach significance, $ps > 0.5$. Post hoc comparisons revealed that random shock significantly reduced social exploration compared with both safety signal and HC groups, $p < 0.001$, which did not differ, $p > 0.05$.

Safety signals reduced Fos immunoreactivity in the BLAp and BNSTlv

The average number of Fos immunoreactive cells per region of interest was computed for each rat and used for the group analysis. Stress induced Fos in the LA, BLAp, CeA (Figure 2, Panel A), and BNSTlv (Figure 3, Panel A). Between group comparisons were analyzed with separate one-way ANOVAs in each region of interest; group sizes were $n = 6-8$. In the LA and CeA, Stress effects were found, $F(2, 21) = 10.15$ and 5.530 , respectively, $ps < 0.01$, and post hoc comparisons found that both SAFE-IS and RANDOM-IS were significantly greater than HC controls ($ps < 0.05$) but did not differ from each other. In the BLAp, a Stress effect was found, $F(2, 21) = 6.51$, $p = 0.007$. Post hoc comparisons found that both SAFE-IS and RANDOM-IS to be greater than HC controls and that RANDOM-IS was greater than SAFE-IS ($ps < 0.05$). Finally, in the BNSTlv a Stress effect was found, $F(2, 21) = 8.50$, $p = 0.002$. Post hoc tests found RANDOM-IS to be greater than both other groups and SAFE-IS to be greater than HC ($ps < 0.05$).

Inhibition of BNSTlv during inescapable stress

Exclusion of rats in the TTX groups with cannula tips outside of the BNSTlv or with failed injections yielded the following group sizes: IS-TTX, $n = 7$; IS-Vehicle, $n = 9$; HC-TTX, $n = 8$; HC-Vehicle, $n = 8$; cannula tips are illustrated in Figure 4, Panel B. IS reduced social exploration compared to unstressed HC controls but TTX in the BNSTlv completely prevented the development of this effect (Figure 4, Panel A). A two-way ANOVA revealed a significant main effect of Stress, $F(1, 29) = 17.98$, $p < 0.001$; Drug, $F(1, 29) = 7.28$, $p = 0.012$; and a Stress by Drug interaction, $F(1, 29) = 4.55$, $p = 0.041$. Post hoc comparisons between all groups found the mean social exploration in the IS-Vehicle group was significantly lower than all other groups ($ps < 0.05$), which did not differ ($ps > 0.05$). It is worth noting that the TTX injection volume, $0.5\mu\text{L}$, may have led to some diffusion of drug outside of the target nucleus. However, data obtained from rats that received IS with misplaced cannula indicated that TTX injections outside of the BNSTlv did not influence later social exploration ($n = 5$, mean = $46.1s$, S.E.M. = 5.8). Thus, the effect of TTX was specific to the region of interest.

Safety signals inhibited the expression of freezing during inescapable footshock stress

Freezing was quantified during SAFE-IS or RANDOM-IS treatment ($n = 8/\text{group}$) using the “freezing module” of the ANY-Maze software. Freezing was defined as complete immobility except for movement required for respiration. ANY-Maze computed a freezing “score” that correlated with the amount of movement made by the rat. The threshold freezing score was set at 30, which corresponded with freezing scores generated by a human observer. The percentage of time spent with a freezing score less than 30 during the inter-shock intervals was computed from all rats and pooled into blocks of 2 trials; group means are depicted in Figure 5. A 2 (SAFE-IS vs RANDOM-IS) $\times$ 50 (trial block) ANOVA revealed a significant effect of Stress, $F(1, 49) = 5.708, p = 0.032$, Block, $F(1, 49) = 3.416, p < 0.001$, and a Stress by Block interaction, $F(1, 49) = 3.346, p < 0.001$. Post hoc comparisons between Safe-IS and Random-IS groups revealed significant differences in blocks 1–14, 23, 24, 27, 49 and 50 ($ps < 0.05$, Sidak correction).

Discussion

The current studies examined the neural systems that underlie the mitigating properties of a safety signal on behavioral effects induced by an uncontrollable stressor. Exposure to unpredictable and inescapable tailshock led to a temporary anxiety-like state that was observed here as a reduction in exploration of a juvenile conspecific and chronic fear. Consistent with our previous report, provision of a 5 s chamber blackout at the beginning of the inter-trial-intervals completely prevented the later anxiety. Like pretraining lesions (9), temporary inhibition of Si during the stressor session eliminated the safety signal effect. Stress induced Fos in all regions studied, but the safety signal attenuated Fos expression in the BLAp and BNSTlv suggesting that inhibition in this pathway contributes to the prevention of anxiety. Temporary inhibition of the BNSTlv during stress completely prevented the stress-induced anxiety. Lastly, the safety signal significantly reduced fear during unpredictable shock exposures.

Fear-induced behaviors are the product of a relatively well-characterized neural circuit. Upon exposure to an aversive stimulus, such as a tailshock, environmental and sensory cues converge in the basolateral amygdala complex (including the LA and BLAp) where a Pavlovian association is made between stimuli (26). Upon subsequent presentation of the environmental cue, i.e. the shock context, outputs of the BLAp trigger specific fear behaviors. Recently, Davis and colleagues provided evidence that distinct circuits mediate “phasic” and “sustained” fear. The phasic circuit is activated upon brief exposures to the fear stimuli (on the order of seconds), but control transitions to the sustained circuit as the stimuli persist. The phasic circuit utilizes the CeA as the critical output structure, while the sustained circuit employs the BNSTlv. A wealth of data support this model in rat and human (for an extensive review see (18)).

In the current procedure, 100 tailshocks caused a state of sustained fear quantified by high levels of behavioral freezing. Accordingly, neuronal activation in the nuclei of the sustained circuit, the LA, BLAp and BNSTlv, was high in rats receiving IS with random signals. However, adding the safety signals reduced both Fos immunoreactivity in the BLAp and BNSTlv and behavioral freezing. Interestingly, Fos in the CeA was insensitive to the safety signal, a pattern that suggests that stress drove CeA activity to a limit. The fact that pharmacological BNSTlv inhibition prevented the stressor-induced anxiety suggests that factors modulating BNSTlv activity can, in principle, influence the stress outcome.

In addition to demonstrations that safety signals reduce fear conditioned to a tailshock context (8, 12), safety signals presented in this manner become Pavlovian conditioned inhibitors of fear (11). A conditioned inhibitor is a stimulus that predicts the non-occurrence

of an unconditioned stimulus. When presented simultaneously with conditioned excitatory stimuli, such as the tailshock context, conditioned inhibitors prevent the expression of learned responses. This is precisely what occurred when the safety signal was provided during unpredictable tailshocks; fear was reduced. A growing body of research has identified mechanisms by which conditioned inhibitors reduce fear and amygdala neuronal activity. Rogan and colleagues identified a trend toward synaptic long-term depression in LA circuits after conditioned inhibition training, a pattern that is opposite of the long-term potentiation found after fear training (27, 28). Conditioned inhibition training reduced synaptic size in LA synapses, also indicating inhibition (29). Plasticity in inhibitory BLAp to CeA neurons, specifically the ones that terminate in the lateral division, has also been observed (30).

The current data suggest that when safety signals are present, the Si may contribute to fear inhibition. The anatomy supports this possibility because the insula receives multimodal sensory inputs (14), projects directly to the basolateral amygdala complex (31, 32), and cortical sensory information is conveyed to the amygdala via insular cortex (33). Indeed, insular cortex is critical to the anticipation of pain (34) and fear (35), but insula lesions in rat suggest that it is not necessary for basic fear learning (33, 36). We speculate that the insular cortex works in concert with the basolateral amygdala during safety signal learning leading to inhibition of the BLAp to BNSTlv circuit. Future studies will address whether Si activity is causally related to the inhibition of fear and amygdala neuronal activity when safety signals are present.

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References

1. Nutt, D.; Garcia de Miguel, B.; Davies, JC. Phenomenology of anxiety disorders. In: Blanchard, RJ.; Blanchard, DC.; Griebel, G.; Nutt, D., editors. *Handbook of Anxiety and Fear*. 1. Amsterdam: Associated Press; 2008. p. 365-393.
2. Jovanovic T, Norrholm SD, Fennell JE, Keyes M, Fiallos AM, Myers KM, et al. Posttraumatic stress disorder may be associated with impaired fear inhibition: relation to symptom severity. *Psychiatry Res*. 2009; 167:151–160. [PubMed: 19345420]
3. Hartley CA, Phelps EA. Changing fear: the neurocircuitry of emotion regulation. *Neuropsychopharmacology*. 2010; 35:136–146. [PubMed: 19710632]
4. Baratta MV, Christianson JP, Gomez DM, Zarza CM, Amat J, Masini CV, et al. Controllable versus uncontrollable stressors bi-directionally modulate conditioned but not innate fear. *Neuroscience*. 2007; 146:1495–1503. [PubMed: 17478046]
5. Rau V, DeCola JP, Fanselow MS. Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neurosci Biobehav Rev*. 2005; 29:1207–1223. [PubMed: 16095698]
6. Greenwood BN, Strong PV, Fleshner M. Lesions of the basolateral amygdala reverse the long-lasting interference with shuttle box escape produced by uncontrollable stress. *Behav Brain Res*. 2010; 211:71–76. [PubMed: 20226213]
7. Christianson JP, Paul ED, Irani M, Thompson BM, Kubala KH, Yirmiya R, et al. The role of prior stressor controllability and the dorsal raphe nucleus in sucrose preference and social exploration. *Behav Brain Res*. 2008; 193:87–93. [PubMed: 18554730]
8. Mineka S, Cook M, Miller S. Fear conditioned with escapable and inescapable shock: effects of a feedback stimulus. *J Exp Psychol Anim Behav Process*. 1984; 10:307–323.

9. Christianson JP, Benison AM, Jennings J, Sandsmark EK, Amat J, Kaufman RD, et al. The sensory insular cortex mediates the stress-buffering effects of safety signals but not behavioral control. *J Neurosci*. 2008; 28:13703–13711. [PubMed: 19074043]
10. Maier SF, Keith JR. Shock signals and the development of stress-induced analgesia. *J Exp Psychol Anim Behav Process*. 1987; 13:226–238.
11. Maier SF, Rapaport P, Wheatley KL. Conditioned inhibition and the UCS-CS interval. *Anim Learn Behav*. 1976; 4:217–220. [PubMed: 964450]
12. Minor TR, Trauner MA, CL, Dess NK. Modeling signal features of escape response: effects of cessation conditioning in “learned helplessness” paradigm. *Journal of Experimental Psychology: Animal Behavior Processes*. 1990; 16:123–136. [PubMed: 2335768]
13. Weiss JM. Effects of coping behavior with and without a feedback signal on stress pathology in rats. *J Comp Physiol Psychol*. 1971; 77:22–30. [PubMed: 5166077]
14. Rodgers KM, Benison AM, Klein A, Barth DS. Auditory, Somatosensory, and Multisensory Insular Cortex in the Rat. *Cereb Cortex*. 2008
15. Christianson JP, Ragole T, Amat J, Greenwood BN, Strong PV, Paul ED, et al. 5-hydroxytryptamine 2C receptors in the basolateral amygdala are involved in the expression of anxiety after uncontrollable traumatic stress. *Biol Psychiatry*. 2010; 67:339–345. [PubMed: 19914601]
16. Hammack SE, Richey KJ, Watkins LR, Maier SF. Chemical lesion of the bed nucleus of the stria terminalis blocks the behavioral consequences of uncontrollable stress. *Behav Neurosci*. 2004; 118:443–448. [PubMed: 15113272]
17. Maier SF, Grahn RE, Kalman BA, Sutton LC, Wiertelak EP, Watkins LR. The role of the amygdala and dorsal raphe nucleus in mediating the behavioral consequences of inescapable shock. *Behav Neurosci*. 1993; 107:377–388. [PubMed: 8484901]
18. Davis M, Walker DL, Miles L, Grillon C. Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. *Neuropsychopharmacology*. 2010; 35:105–135. [PubMed: 19693004]
19. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. 4. New York: Academic Press; 1998.
20. Christianson JP, Thompson BM, Watkins LR, Maier SF. Medial prefrontal cortical activation modulates the impact of controllable and uncontrollable stressor exposure on a social exploration test of anxiety in the rat. *Stress*. 2009; 12:445–450. [PubMed: 19051125]
21. File SE, Seth P. A review of 25 years of the social interaction test. *Eur J Pharmacol*. 2003; 463:35–53. [PubMed: 12600701]
22. Kovacs KJ. c-Fos as a transcription factor: a stressful (re)view from a functional map. *Neurochem Int*. 1998; 33:287–297. [PubMed: 9840219]
23. Sonnenberg JL, Mitchelmore C, Macgregor-Leon PF, Hempstead J, Morgan JI, Curran T. Glutamate receptor agonists increase the expression of Fos, Fra, and AP-1 DNA binding activity in the mammalian brain. *J Neurosci Res*. 1989; 24:72–80. [PubMed: 2553994]
24. Reznikov LR, Reagan LP, Fadel JR. Activation of phenotypically distinct neuronal subpopulations in the anterior subdivision of the rat basolateral amygdala following acute and repeated stress. *J Comp Neurol*. 2008; 508:458–472. [PubMed: 18335544]
25. Fanselow MS. Conditioned and unconditional components of post-shock freezing. *Pavlov J Biol Sci*. 1980; 15:177–182. [PubMed: 7208128]
26. Kim JJ, Jung MW. Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. *Neurosci Biobehav Rev*. 2006; 30:188–202. [PubMed: 16120461]
27. Rogan MT, LeDoux JE. LTP is accompanied by commensurate enhancement of auditory-evoked responses in a fear conditioning circuit. *Neuron*. 1995; 15:127–136. [PubMed: 7619517]
28. Rogan MT, Leon KS, Perez DL, Kandel ER. Distinct neural signatures for safety and danger in the amygdala and striatum of the mouse. *Neuron*. 2005; 46:309–320. [PubMed: 15848808]
29. Ostroff LE, Cain CK, Bedont J, Monfils MH, Ledoux JE. Fear and safety learning differentially affect synapse size and dendritic translation in the lateral amygdala. *Proc Natl Acad Sci U S A*. 2010; 107:9418–9423. [PubMed: 20439732]

30. Amano T, Unal CT, Pare D. Synaptic correlates of fear extinction in the amygdala. *Nat Neurosci*. 2010; 13:489–494. [PubMed: 20208529]
31. Shi CJ, Cassell MD. Cascade projections from somatosensory cortex to the rat basolateral amygdala via the parietal insular cortex. *J Comp Neurol*. 1998; 399:469–491. [PubMed: 9741478]
32. McDonald AJ, Shammah-Lagnado SJ, Shi C, Davis M. Cortical afferents to the extended amygdala. *Ann N Y Acad Sci*. 1999; 877:309–338. [PubMed: 10415657]
33. Shi C, Davis M. Pain pathways involved in fear conditioning measured with fear-potentiated startle: lesion studies. *J Neurosci*. 1999; 19:420–430. [PubMed: 9870970]
34. Ploghaus A, Tracey I, Gati JS, Clare S, Menon RS, Matthews PM, et al. Dissociating pain from its anticipation in the human brain. *Science*. 1999; 284:1979–1981. [PubMed: 10373114]
35. Phelps EA, O'Connor KJ, Gatenby JC, Gore JC, Grillon C, Davis M. Activation of the left amygdala to a cognitive representation of fear. *Nat Neurosci*. 2001; 4:437–441. [PubMed: 11276236]
36. Romanski LM, LeDoux JE. Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning. *J Neurosci*. 1992; 12:4501–4509. [PubMed: 1331362]

Figure 1.

Mean (+S.E.M.) social exploration expressed as the percentage of baseline in a 3-minute test 24 h after stress treatment. (A) Inescapable shock with random signals (RANDOM) significantly reduced social exploration from baseline, but provision of a safety signal (SAFE) completely mitigated the effect. Temporary inhibition of the insular cortex by muscimol prevented the safety signal effect * $p < 0.05$ Safe-Saline compared to all other groups. (B) Inhibition of insular cortex just prior to social exploration had no effect on either stress with random signals or the safety signal effect. * $p < 0.05$ Random groups compared to homecage control and SAFE groups. (C) Illustration of the rostral canula tips of rats receiving muscimol during safety signals (black circles) or before social exploration (white circles). Illustrations adapted from *The Rat Brain Atlas in Stereotaxic Coordinates* (Paxinos & Watson, 1998) with permission.

Figure 2.

(A) Mean (+S.E.M.) number of Fos immunoreactive cells per rat in the LA, BLAp, and CeA. Stress treatment induced Fos in all regions of interest but provision of a safety signal inhibited neuronal Fos in the BLAp. Each region was analyzed separately and each treatment compared to the others. Treatments that were significantly different ($p < 0.05$) are marked with different letters (a, b, or c). Bars marked with the same letter did not differ. (B) Photomicrograph containing a representative coronal section including the LA, BLAp and CeA. (C) Magnification of the boxed area in B identifying typical Fos immunoreactive neurons (black arrows).

Figure 3.

(A) Mean (+ S.E.M.) number of Fos immunoreactive cells per rat in the BNSTlv. Stress increased Fos counts but the safety signal caused significant inhibition of Fos in the BNSTlv. Each treatment was compared to the others and treatments that were significantly different ($p < 0.05$) are marked with different letters (a, b, or c). (B) Cresyl Violet stained section of the BNST at Bregma -0.3mm with overlay of BNST compartments according to Paxinos & Watson (1998). (C) Adjacent section from the same rat treated with RANDOM-IS. (D) Comparable section from a rat treated with SAFE-IS. Fos was counted bilaterally in the BNSTlv, BNSTlm, and fusiform area (FU). (D) AC, anterior commissure; PS, parastria area; FU, fusiform region.

Figure 4. (A) Mean (+S.E.M.) social exploration in a 3 minute test given 24 h after inescapable stress (IS) or no stress homecage control (HC) with either tetrodotoxin or vehicle injections in the BNSTlv. Stress caused a reduction in social exploration that was prevented by BNSTlv inhibition, * $p < 0.05$ compared to all other groups. (B) Illustration of the cannula tips (grey circles) of rats receiving inescapable shock and tetrodotoxin. Adapted from *The Rat Brain Atlas in Stereotaxic Coordinates* with permission.

Figure 5. Mean ($\pm$ S.E.M.) percentage of time spent freezing during the inter-shock-interval in two consecutive trials with either safety signals (SAFE) or random signals (RANDOM). The provision of a safety signal led to a significant reduction of freezing in 22 of 50 trial blocks, $*p < 0.05$.