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Stressed rats fail to exhibit avoidance reactions to innately aversive social calls

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Disruptions in amygdalar function, a brain area involved in encoding emotionally salient information, has been implicated in stress-related affective disorders. Earlier animal studies on the behavioral consequences of stress-induced abnormalities in the amygdala focused on learned behaviors using fear conditioning paradigms. If and how stress affects unconditioned, innate fear responses to ethologically natural aversive stimuli remains unexplored. Hence, we subjected rats to aversive ultrasonic vocalization calls emitted on one end of a linear track. Unstressed control rats exhibited a robust avoidance response by spending more time away from the source of the playback calls. Unexpectedly, prior exposure to chronic immobilization stress prevented this avoidance reaction, rather than enhancing it. Further, this stress-induced impairment extended to other innately aversive stimuli, such as white noise and electric shock in an inhibitory avoidance task. However, conditioned fear responses were enhanced by the same stress. Inactivation of the basolateral amygdala (BLA) in control rats prevented this avoidance reaction evoked by the playback. Consistent with this, analysis of the immediate early gene cFos revealed higher activity in the BLA of control, but not stressed rats, after exposure to the playback. Further, in vivo recordings in freely behaving control rats exposed to playback showed enhanced theta activity in the BLA, which also was absent in stressed rats. These findings offer a new framework for studying stress-induced alterations in amygdala-dependent maladaptive responses to more naturally threatening and emotionally relevant social stimuli. The divergent impact of stress on defensive responses––impaired avoidance responses together with increased conditioned fear––also has important implications for models of learned helplessness and depression.

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INTRODUCTION

Stress-related psychiatric disorders are associated with a range of debilitating emotional symptoms, as well as structural and functional alterations in the amygdala [1, 2]. Rodent models have offered insights into how stress affects the amygdala across multiple levels of neural organization, including behavioral analyses of fear memories using Pavlovian conditioning [3–13]. As useful as these behavioral studies have been in exploring the functional consequences of stress-induced plasticity in the amygdala, they relied largely on stimuli that were not ethologically natural, e.g. exposure to foot shocks. Little is known about the impact of stress on unconditioned fear reactions to innately aversive stimuli that are ethologically relevant to rodents.

Accumulating evidence from studies of social interactions in rodents offer a useful framework for addressing this gap in knowledge [14]. For instance, rodents communicate their affective states through ultrasonic vocalizations (USVs), which constitute a key component of their social interactions [15–18]. Broadly, rats emit two distinct types of USV calls – 22-kHz alarm calls conveying negative emotional states triggered by aversive experiences or threats such as predators and painful stimuli, and 50-kHz appetitive calls elicited during mating, play behavior, direct social contact etc. [19, 20]. However, previous studies of defensive responses triggered by playback of aversive social calls, and other innately aversive auditory stimuli, yielded mixed results. While a few studies observed stimulus-induced defensive responses, others did not [21, 22]. Moreover, earlier analyses of playback-induced defensive responses focused on hypermotility as a primary behavioral readout, without taking the animals' direction of motion into consideration. Further, experience and environment also influence whether mice preferentially exhibit flight or freezing responses [23–26]. What kind of defensive reactions would playback of innately aversive 22-kHz alarm calls evoke in rats? Would prior exposure to chronic stress affect these defensive responses? Would stressed rats exhibit higher fear by responding with enhanced flight or avoidance reactions? Previous studies reported that chronic or repeated stress enhanced the recall of conditioned fear in rodents, manifested as higher levels of freezing to an auditory tone used as the conditioned stimulus [11, 27]. Neurons in the basolateral amygdala (BLA) are essential for the acquisition of the tone-shock association in auditory fear conditioning [10]. Would responses to innately aversive USV calls also depend on neural activity in the BLA? If so, how would stress affect this? Here we combine behavioral, pharmacological, immunohistochemical and in vivo electrophysiological analyses to address these questions using a well-established rat model of chronic immobilization stress [28, 29].

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MATERIALS AND METHODS

Details are provided in Supplementary information

Animals

Animal experiments were approved by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), and Institutional Animal Ethics Committee (IAEC) of NCBS, Bangalore.

Experimental procedures

Supplemental information contains all protocols for behavioral experiments, BLA inactivation, cFos immunohistochemistry and in vivo recordings.

Statistical analyses

All values are expressed as mean ± s.e.m., unless stated otherwise. GraphPad Prism (La Jolla, CA) was used for statistical tests; specific details are described in figure legends.

RESULTS

Playback of 22-kHz ultrasonic vocalizations elicits avoidance behavior in control rats

First, we set out to characterize innate behavioral responses of adult male rats to the playback of aversive 22-kHz vocalization (USV) calls. To this end, rats were habituated to a linear track (Supplementary Fig. 1A) for 5 minutes without any playback, during which control rats spent comparable durations of time in the proximal and distal halves of the track, exhibiting no preference for one or the other half (Fig. 1A, 1B–D, *left*). Following habituation, the rats were subjected to two 3-minute episodes of playback of 22-kHz USV calls, 5 minutes apart, on the same track. These USV calls caused them to spend significantly more time in the distal half of the track, away from the source of the playback calls (Fig. 1B right, 1D, left). This avoidance response is not habituated in these rats even after exposure to a prolonged aversive call playback episode (Supplementary Fig. 8). Next, a separate group of rats were subjected to a well-characterized model chronic immobilization stress (2 h/day for 10 days), the efficacy of which was verified using two separate measures. First, this chronic stress paradigm caused a significant increase in anxiety-like behavior in the open-field test (Supplementary Fig. 1B–G) [30]. Second, this chronic stress also led to a significant reduction in body weight gain [31]. Stressed rats were subjected to the same sequence of habituation followed by USV playback. Stressed rats also spent comparable amounts of time in the two halves of the track during habituation, similar to control rats (Fig. 1C right, 1E, left). Surprisingly, stressed rats continued to exhibit this lack of preference even when the aversive USV was played back. The aversive USV failed to elicit avoidance reactions in stressed rats as they spent similar amounts of time in either halves of the track (Fig. 1C right, 1E, left). The distance traveled by both control and stressed rats, in response to the aversive call playback, was also higher relative to habituation (Fig. 1D, 1E, right).

To ensure that the avoidance behavior and its impairment seen in control and stressed rats is specific to aversive calls, and not a generic response to auditory stimuli or USV calls, we subjected a separate group of rats to USV calls conveying a positive emotional valence [17, 32–47]. To this end, control and stressed rats were exposed to a playback of 50-kHz appetitive USV calls using the same protocol as the aversive calls (Supplementary Fig. 2A). During habituation, control rats spent equal time in the proximal and distal halves of the track. Control rats showed approach behavior during the 1st minute of the 1st playback episode of the appetitive call as they spent significantly more time in the proximal half of the track (Supplementary Fig. 2B, middle, 2D, left). Stressed rats also exhibited approach behavior (Supplementary Fig. 2C, middle, 2D, left) during 1st minute of the 1st playback. In striking contrast to control rats, however, stressed rats also showed avoidance behavior in response to the 2nd playback of the appetitive calls (Supplementary Fig. 2C, 2E, right), suggesting a switch in the perception of the emotional valence of the call from positive to negative. This suggests that social call playback-induced behavioral differences are not limited to aversive call playbacks but extend to appetitive call playback as well. Thus, the behavioral responses were specific and distinct between the appetitive and aversive call playback in the stressed animals.

Having established that control rats exhibit avoidance behavior that is specific to the 22-kHz USV playback, we focused on the paradoxical finding that prior exposure to stress impairs, rather than enhance, the avoidance reaction to aversive calls. We tested if this stress-induced impairment generalizes to other forms of aversive auditory stimuli. Playback of auditory white noise has been reported to be an innately aversive stimulus that elicits avoidance/flight responses in rodents [24–26, 48]. In fact, it is aversive enough to be used as an unconditioned stimulus in Pavlovian fear conditioning paradigms [49]. Thus, a different group of control and stressed rats were presented with the same sequence of habituation and playback of white noise in the linear track (Supplementary Fig. 3A). Playback of white noise also elicited a robust avoidance reaction in control (Supplementary Fig. 3B, 3D), but not stressed rats (Supplementary Fig. 3C, 3E). Together, these results demonstrate that playback of aversive calls and white noise both elicited a robust avoidance behavior in control rats. However, this was absent in stressed rats, which explored the half of the track that was closer to the source of the aversive auditory stimuli to the same extent as the safer distal half that was preferred by the control rats.

Stress impairs inhibitory avoidance behavior

In an effort to further examine the robustness of these paradoxical findings, we adapted the inhibitory avoidance paradigm to our experimental design. The linear track was modified to include a small shock-grid at one end of the linear track (Supplementary Fig. 4, Supplementary Materials and Methods). Thus, in this experiment, one end of the track still contained an aversive stimulus (i.e. the "proximal" half), but now the USV call or white noise was replaced by a strong noxious stimulus in the form of a foot shock. First, control rats were habituated to the track for 10 minutes (habituation, Supplementary Fig. 4A), wherein they spent equal time in both halves (Supplementary Fig. 4B, 4D, left). Next, the shock-grid was turned on for 90 s such that rats received a DC foot-shock (0.4 mA) whenever they visited the end of the track containing the shock-grid (shock, Supplementary Fig. 4A). Once the shock-grid was turned off, the rat's behavior was monitored for another 10 minutes (post-shock, Supplementary Fig. 4A). Control rats spent significantly more time in the distal half of the track, away from the shock-zone (Supplementary Fig. 4B, 4D, right). Thus, exposure to the shock enhanced avoidance behavior in control rats. In contrast, stressed rats spent comparable time in both halves of the track despite exposure to the shock, similar to that exhibited during habituation in the absence of shock (Supplementary Fig. 4C, 4E). Further, while control rats avoided the shock zone following the cessation of shock, the stressed rats did not.

Despite the overall similarity in the findings on stress-induced suppression of avoidance behavior, the actual nature of the aversive stimuli across these paradigms were quite different. The playback of USV calls and white noise, although emanating from one end of the track, spread across the entire track. But, the shock grid was spatially restricted to a specific location on the track. This raises the possibility that despite spending comparable amounts of time post-shock in both halves overall, the stressed rats may still have successfully avoided the shock-zone itself. To test this, we first analyzed the time spent by control and stressed rats in the shock-zone. After receiving the shock, control rats showed a significant reduction in time spent in the shock-zone compared to habituation. However, stressed rats spent equal time in the

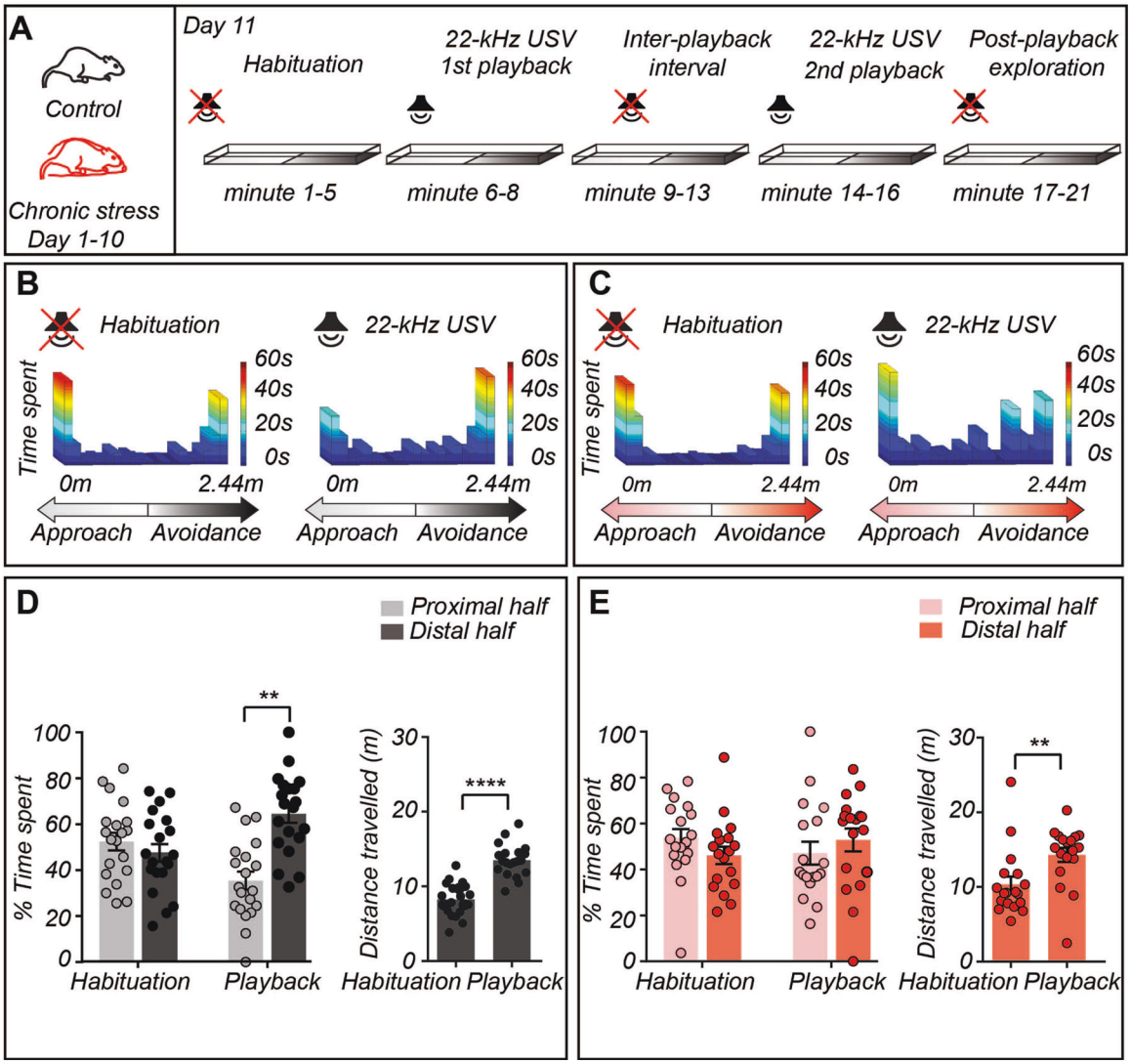

Fig. 1 Effects of stress on avoidance behavior elicited by playback of aversive 22-kHz USV calls. A Experimental design. **B**, **C** Time spent by a representative control (**B**) and stressed (**C**) rat along the track during habituation (left) and playback (right). **D**, **E** Time spent in proximal and distal halves and distance traveled. **D** Control: Left: Two-way RM ANOVA, Sidak's multiple comparisons test, location: $F_{1,\ 19} = 5.03$, $p < 0.05$, playback: $F_{1,\ 19} = 1.00$, $p > 0.05$, location X playback: $F_{1,\ 19} = 9.28$, $p < 0.01$, $N = 20$. Right: Paired t-test, $t_{19} = 12.58$, $p < 0.0001$. **E** Stress: Left: Two-way RM ANOVA, Sidak's multiple comparisons test, location: $F_{1,\ 17} = 0.02$, $p > 0.05$, playback: $F_{1,\ 17} = 1.00$, $p > 0.05$, location X playback: $F_{1,\ 17} = 1.97$, $p > 0.05$, $N = 18$. Right: Paired t-test, $t_{17} = 3.36$, $p < 0.01$.

shock-zone both during habituation and post-shock exploration (Supplementary Fig. 4F). The impairment of avoidance behavior in stressed rats may also arise due to control and stressed rats receiving different extents of foot shocks during the shock period. We quantified the time spent on, and visits to, the shock-grid *during* the shock period. This analysis revealed no difference in these two measures between control and stressed rats (Supplementary Fig. 4G). Thus, stress-induced suppression of unconditioned avoidance behavior is not limited to innately aversive auditory stimuli, but also extends to a noxious somatosensory stimulus, thereby showing that impairment in the avoidance response to aversive stimuli in stressed rats to be a robust phenomenon that generalizes across stimulus modalities.

Stress impairs avoidance from, but increases freezing to, the conditioned stimulus in an auditory fear conditioning paradigm

Having demonstrated that exposure to chronic stress causes a deficit in the avoidance response to innately aversive unconditioned stimuli, we next asked if stress also impairs a conditioned avoidance response. To this end, we used a Pavlovian auditory fear conditioning paradigm, but with an additional behavioral readout (Fig. 2). In addition to testing for the recall of conditioned fear manifested as a freezing response in the usual testing context, we also assessed their conditioned avoidance response in the linear track (Fig. 2A, right). Rats were first habituated to the conditioning context for 20 minutes for two days. 24 h later, they were subjected to five presentations of the conditioned stimulus (CS) alone (tone habituation). This was immediately followed by auditory fear conditioning using seven pairings of the CS co-terminating with an unconditioned stimulus (US, 0.7 mA foot shock; Fig. 2A, conditioning). After the end of conditioning, control rats showed robust acquisition of fear memory, as evidenced by significantly higher freezing relative to tone habituation (Fig. 2B). A day later, these rats were divided into two groups to assess their behavioral responses to the tone CS either in their home cage or in the linear track (Fig. 2A, right). During fear recall in their home cage, control rats exhibited significantly higher freezing to the CS (Fig. 2C). The other group of control rats were first allowed to get habituated to the track for 10 minutes without the CS

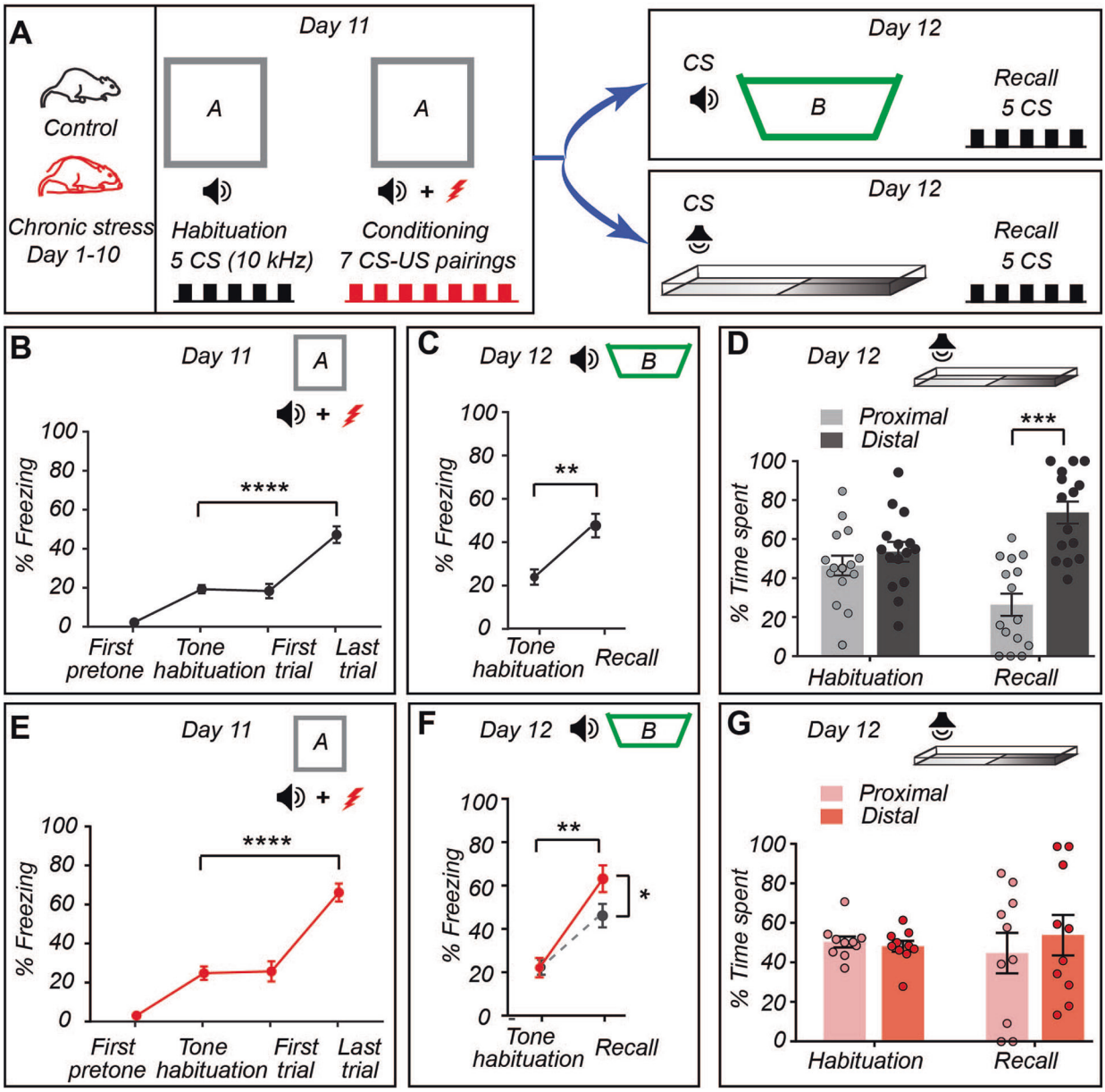

Fig. 2 Effects of stress on defensive responses triggered by the conditioned stimulus following auditory fear conditioning. **A** Experimental design. (**B**, **E**) Freezing response during first pretone, tone habituation, first and last trials of conditioning. **B** Control: One-way RM ANOVA, Tukey's multiple comparisons test, $F_{3,\ 84} = 50.22$ $p < 0.0001$, $N = 29$; (**E**) Stress: One-way RM ANOVA, Tukey's multiple comparisons test, $F_{3,\ 60} = 50.1$ $p < 0.0001$, $N = 21$. **C**, **F** Freezing response to CS before (in conditioning context) and 24 h after (during fear recall in home cage) fear conditioning: (**C**) Control: Paired t-test, $t_{13} = 3.74$, $p < 0.01$, $N = 14$; (**F**) Stress: Paired t-test, $t_{10} = 4.29$, $p < 0.01$, $N = 11$. Fear recall: Control vs. Stress: Unpaired t-test, $t_{26} = 2.37$, $p < 0.05$. **D**, **G** Time spent in proximal and distal halves during fear recall in linear track. **D** Control: Two-way RM ANOVA, Sidak's multiple comparisons test, location: $F_{1,\ 14} = 10.29$, $p < 0.01$, *CS*: $F_{1,\ 14} = 3.50$, $p > 0.05$, location X CS: $F_{1,\ 14} = 8.93$, $p < 0.01$, $N = 15$; (**G**) Stress: Two-way RM ANOVA, Sidak's multiple comparisons test, location: $F_{1,\ 9} = 0.53$, $p > 0.05$, *CS*: $F_{1,\ 9} = 0.01$, $p > 0.05$, location X CS: $F_{1,\ 9} = 1.00$, $p > 0.05$, $N = 10$.

(habituation), wherein they spent comparable amounts of time in both halves (Fig. 2D, left). This was followed by five presentations of the same tone CS through a speaker at one end of the track (Fig. 2A), identical to the earlier USV playback experiments. During this test in the linear track, CS presentations triggered a strong avoidance reaction in control rats (Fig. 2D, right). When stressed animals were subjected to the same sequence of training and tests, their behavioral response to the CS was similar to their control counterparts except for in the linear track. Stressed rats also exhibited robust acquisition of fear memory (Fig. 2E). Stressed rats exhibited significantly higher levels of freezing than their control counterparts during fear acquisition (Supplementary Fig. 11). 24 h later, when tested for recall of conditioned fear in their home cages, one group of stressed rats also showed CS-induced freezing that was significantly higher than that shown by control rats (Fig. 2F). However, in the linear track, the same CS failed to elicit avoidance behavior in the other group of stressed rats as they spent comparable amounts of time in both the proximal and distal halves (Fig. 2G, right). Further, we confirmed that the CS by itself was not innately aversive because it did not elicit avoidance behavior in experimentally naive rats (Supplementary Fig. 5A). Also, while motility, measured as the overall distance traveled along the track, of control and naive rats during habituation and CS presentation was comparable, stressed rats

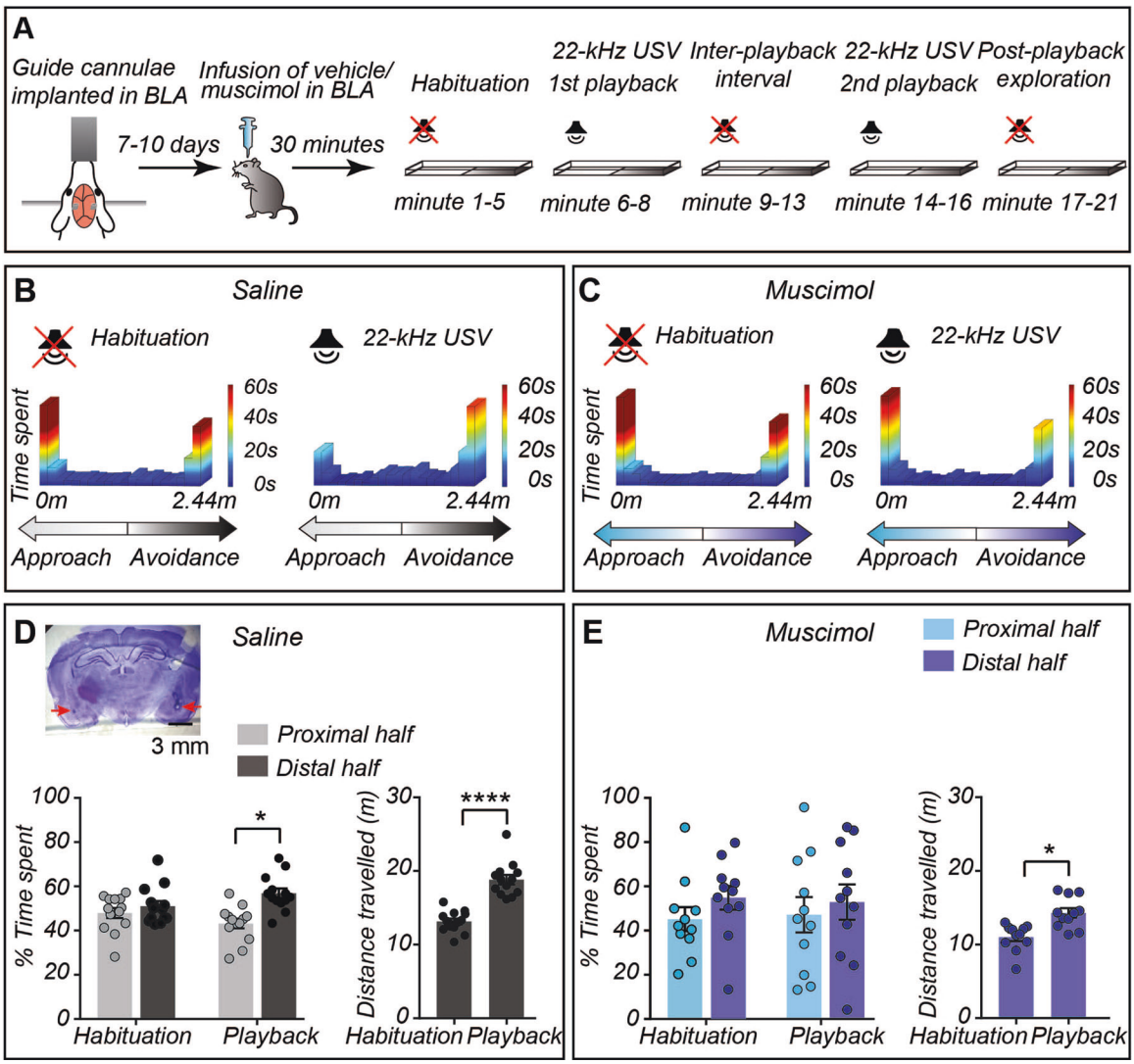

Fig. 3 Effects of pharmacological inhibition of BLA activity on avoidance response to aversive USV call playback. A Experimental design. **B**, **C** Time spent by an exemplar vehicle-infused (**B**) and muscimol-infused rat (**C**) along the track during habituation (left) and playback (right). **D**, **E** Time spent in proximal and distal halves and distance traveled. **D** Top: Representative photomicrograph showing infusion sites in the BLA (red arrows). Bottom: Left: Vehicle: Two-way RM ANOVA, Sidak's multiple comparisons test, location: $F_{1,\ 12} = 4.82$, $p < 0.05$, playback: $F_{1,\ 12} = 1.00$, $p > 0.05$, location X playback: $F_{1,\ 12} = 5.13$, $p < 0.05$, $N = 13$; Bottom: Right: Paired t-test, $t_{12} = 10.87$, $p < 0.0001$. **E** Muscimol: Left: Two-way RM ANOVA, Sidak's multiple comparisons test, location: $F_{1,\ 10} = 0.43$, $p > 0.05$, playback: $F_{1,\ 10} = 1.00$, $p > 0.05$, location X playback: $F_{1,\ 10} = 0.08$, $p > 0.05$, $N = 11$. Right: Paired t-test, $t_{10} = 3.10$, $p < 0.05$.

showed lower motility (Supplementary Fig. 5B). More detailed trial-by-trial analyses of time spent by the rats along the track revealed that control rats spent significantly greater time in the distal half of the track from the 2nd trial onwards. In contrast, stressed and naive rats spent comparable time in either halves of the track in all trials (Supplementary Fig. 5C).

Together, these results reveal that stress selectively suppresses avoidance behavior in response to the CS in the linear track, while enhancing conditioned freezing to the same CS in the home cage.

Targeted inactivation of the basolateral amygdala in control rats blocks avoidance behavior elicited by playback of aversive USV calls

Since aversive social calls are used by rodents to warn conspecifics about potential threats and the amygdala plays a role in defensive responses to threatening stimuli, we hypothesized that amygdalar activity might be necessary for mediating the avoidance behavior seen in the present study. Hence, we carried out bilateral in vivo infusions of the $GABA_A$-receptor agonist muscimol directly into the BLA of control rats to test its impact on avoidance behavior triggered by the playback of 22-kHz USV calls (Fig. 3A, Supplementary Fig. 7). Rats infused with vehicle spent equal time in both halves of the track during habituation, but spent significantly more time in the distal half of the track during USV playback (Fig. 3B, 3D). Thus, vehicle infusion into the BLA did not interfere with these rats' ability to exhibit avoidance behavior during USV playback. On the other hand, rats infused with muscimol spent equal time in the proximal and distal halves of the track during both habituation and playback (Fig. 3C, 3E). Thus, activity in the BLA is necessary for the expression of avoidance behavior evoked by aversive USV playback.

Playback of aversive calls increases cFos expression in the basolateral amygdala of control but not stressed rats

Results presented so far show that inactivation of the BLA prevents the avoidance reactions (Fig. 3) to 22-kHz USV playbacks. Interestingly, chronic stress has the same effect on avoidance behavior. Does this mean that stress blocks avoidance behavior by

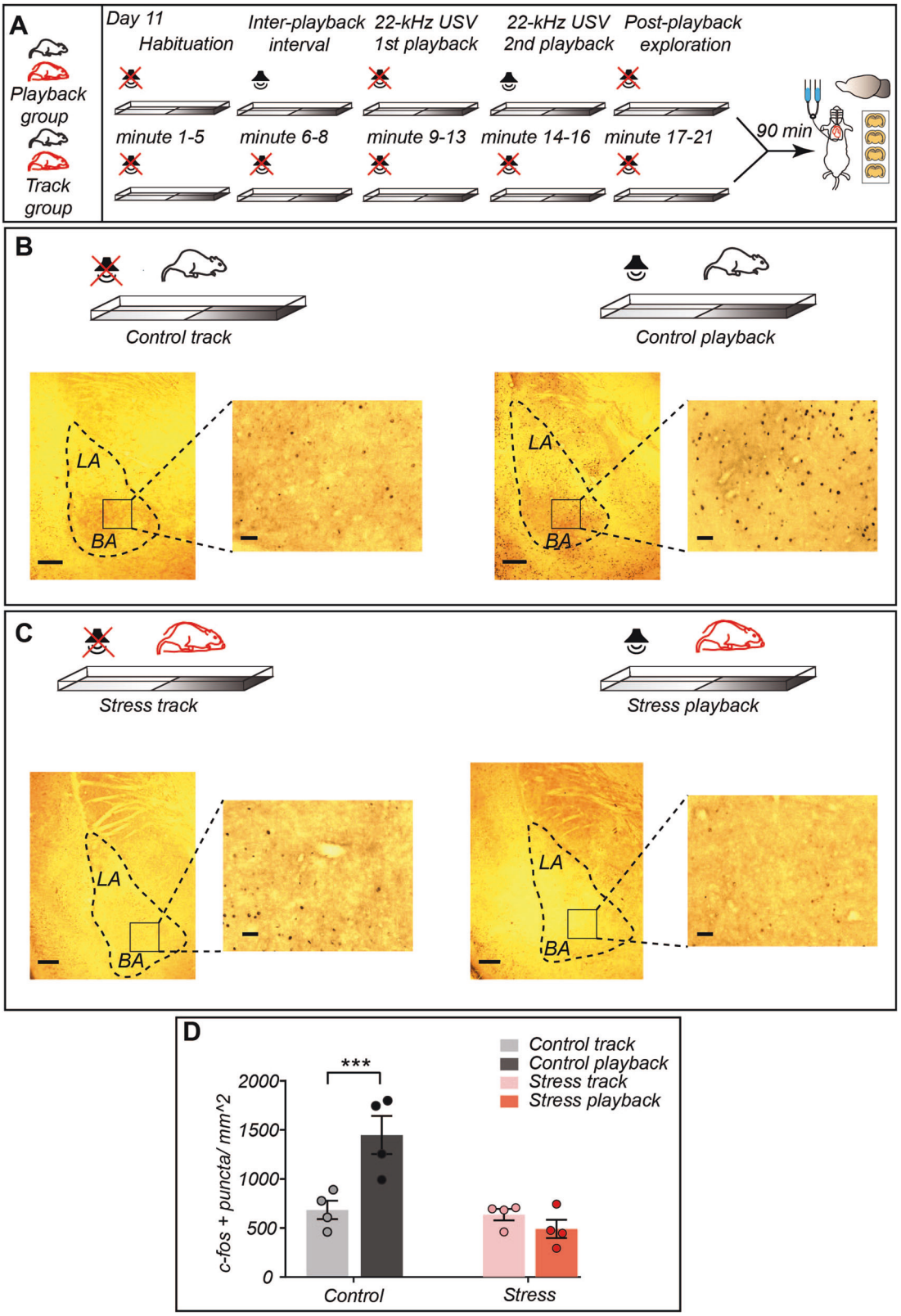

Fig. 4 Effects of stress on cFos expression in the BLA elicited by aversive USV call playback. A Experimental design. **B**, **C** Top: Sub-groups for estimating cFos expression in BLA. (**B**) Control; (**C**) Stress. Bottom: Representative images (4X and 20X magnified) showing cFos expression in BLA from different sub-groups. (**B**) Control; (**C**) Stress. Scale bar measures 500 μm and 50 μm for 4X and 20X magnified images. **D** cFos expression in BLA of control and stressed rats. Two-way ordinary ANOVA, Sidak's multiple comparisons test, playback: $F_{1,\ 12} = 6.44$, $p < 0.05$, stress: $F_{1,\ 12} = 17.10$, $p < 0.01$, playback X stress: $F_{1,\ 12} = 14.03$, $p < 0.01$, $N = 4$.

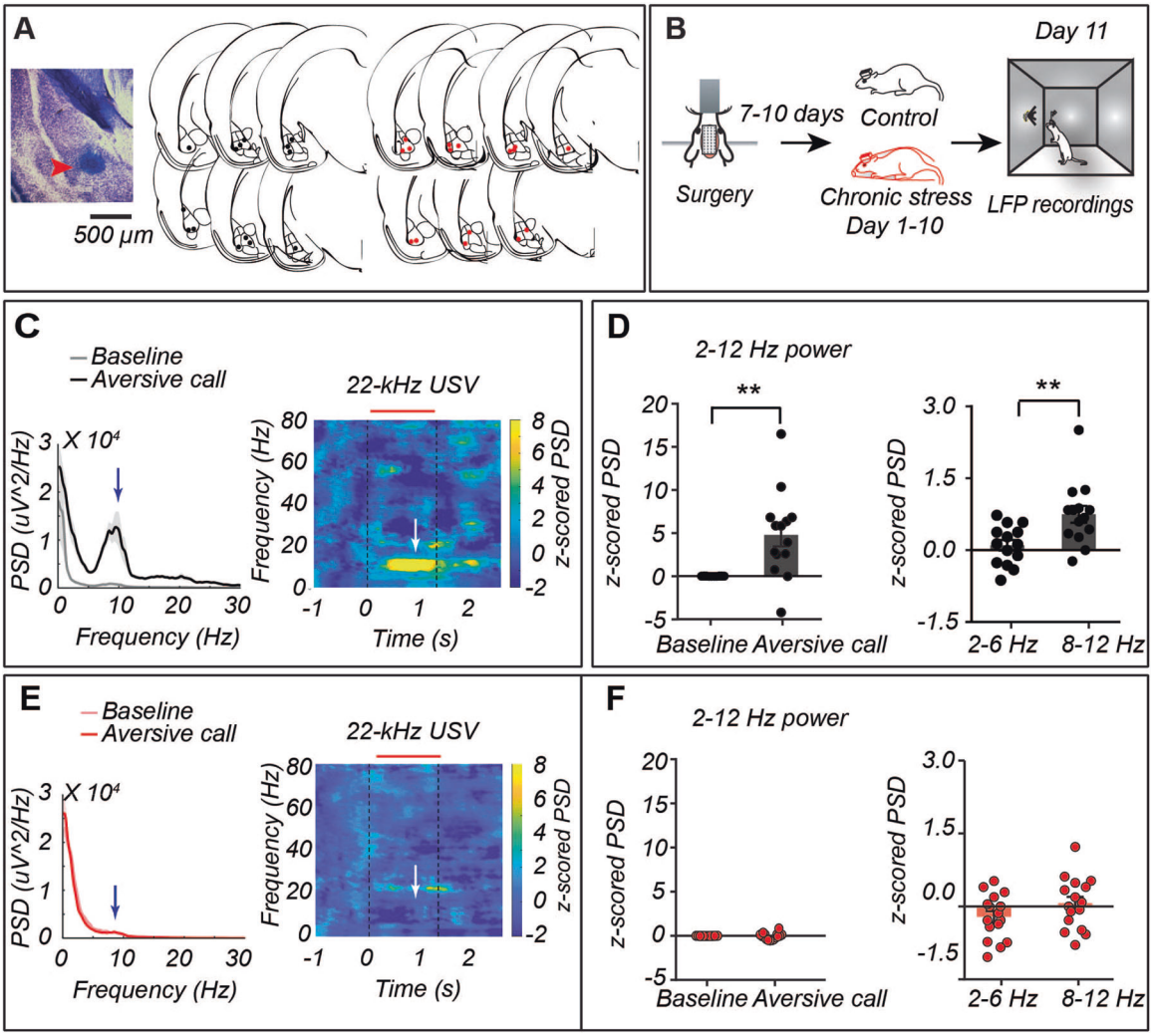

Fig. 5 Effects of stress on theta activity in the BLA evoked by aversive USV calls. A Recording sites in BLA. Left: Representative photomicrograph showing recording site (red arrow head). Right: Schematic BLA coronal sections showing recording sites. **B** Experimental design for recording LFPs in BLA. **C**, **E** Left: Trial-averaged raw power spectrum from an exemplar control (**C**) and stressed (**E**) rat showing changes in BLA theta band power (blue vertical arrow). Thick solid and shaded lines represent mean and ±s.e.m. respectively. Right: Spectrogram showing baseline-corrected trail-averaged power in BLA of a representative control (**A**) and stressed (**E**) rat. Stimulus onset and offset are marked by vertical dashed black lines. Stimulus duration is marked by a horizontal solid red line. Vertical white arrow points to changes in theta band power. **D**, **F** Left: Baseline-corrected trial-averaged BLA theta band power. **D** Control: Paired t-test, $t_{13} = 3.63$, $p < 0.01$, $N = 14$. **F** Stress: Paired t-test, $t_{15} = 0.52$, $p > 0.05$, $N = 16$. Right: Baseline-corrected trial-averaged power in theta sub-bands. **D** Control: Paired t-test, $t_{13} = 4.10$, $p < 0.01$. **F** Stress: Paired t-test, $t_{15} = 1.52$, $p > 0.05$.

suppressing neural activity in the BLA? We addressed this question by testing whether differences in avoidance behavior are reflected in changes in BLA neuronal activity in control and stressed rats. The expression of the immediate early gene *c-fos*, and its protein product cFos, is a reliable marker for neuronal activation [50–53]. Thus, to assess how the playback of aversive calls affect cFos expression in the BLA (Fig. 4A), control and stressed rats were either exposed to the linear track alone (control and stress track, Fig. 4B, 4C, left), or subjected to the aversive call playback on the linear track (control and stress playback, Fig. 4B, 4C, right). These rats exhibited the same behavioral response as depicted in Fig. 1 (Supplementary Fig. 13). Approximately 90 minutes after the behavioral sessions on the track, rat brains were prepared for quantification of cFos-labeled cells in the BLA (Supplementary Materials and Methods; Fig. 4A). USV aversive calls elicited a significant increase in cFos expression in the BLA of control rats relative to those exposed only to the track (Fig. 4B, 4D). Strikingly, this increase in BLA cFos expression was not seen in stressed rats (Fig. 4C, 4D). Also, the density of cFos positive nuclei was similar in the control and stressed rats that were only exposed to the track, suggesting that basal activity in BLA neurons was not affected by stress (Fig. 4B, 4C, 4D). Additional analyses revealed cFos expression in the CA1 sub-region of the dorsal hippocampus to be similar across control and stressed animals subjected to the same playback of aversive USV calls (Supplementary Fig. 12). Overall, this analysis revealed that aversive call playback recruits lower numbers of BLA neurons in stressed rats compared to control rats. Hence, the increase in BLA cFos expression in control, but not stressed rats, mirrors their behavioral response to the aversive USV playback (Fig. 1B–1E).

Aversive USV calls increase theta power in the BLA of control, but not stressed rats

Our post-mortem analysis of cFos expression suggests that the same aversive USV calls that elicit robust activation of BLA neurons in control rats, fail to do so in stressed animals. Therefore, in the final set of experiments, we probed the neural correlates of this in the intact BLA of freely behaving rats. Relatively little is known about

neural activity in the amygdala in response to either playback of social calls [22, 54] or vocalizations of conspecifics in free social interactions [55], and the impact of stress on such processes remains unexplored. Hence, rats were unilaterally implanted with in vivo electrodes to record local field potentials (LFPs) from the BLA (Fig. 5A, Supplementary Fig. 6). Upon recovery from surgery, these implanted rats were randomly assigned to either control or stress groups. On day 11, LFPs were recorded while rats were subjected to 100 presentations of single 22-kHz USV calls (Fig. 5B). Relative to the baseline period, there was a significant increase in theta power in the BLA of control rats triggered by the 22-kHz USV (Figs. 5C, 5D). Notably, no such enhancement in BLA theta power was observed in stressed rats (Figs. 5E, 5F). Alterations in the power of distinct theta sub-bands in the amygdala have been correlated with distinct behavioral and internal states, and have been hypothesized to underlie distinct functions in a context-dependent manner [56–58]. Hence, we carried out a more detailed analysis of two different theta sub-bands––2–6 Hz and 8–12 Hz [56–59]. This revealed that while increased theta power in response to the aversive USV was specific to the 8–12 Hz frequency range in control rats (Fig. 5D, right), neither of the two sub-bands in stressed rats showed any significant change (Fig. 5F, right). In addition to the BLA, we recorded LFPs from the dorsal medial PFC (dmPFC), in the same rats, while presenting them with aversive USV calls. Similar to what was seen in the BLA, we observed a smaller but still significant increase in theta band activity in dmPFC of control rats (Supplementary Fig. 9A, 9B, left). But, this was not seen in stressed rats (Supplementary Fig. 9C, 9D, left). Unlike the changes in BLA, the increase in theta band activity in dmPFC during aversive call presentations was not exclusive to any frequency sub-band (Supplementary Fig. 9B, 9D, right). Also, during aversive call presentations, we found enhanced BLA-dmPFC theta synchrony (measured as magnitude-squared coherence) in control but not stressed rats (Supplementary Fig. 10). This finding is consistent with a potential role for BLA-dmPFC communication in mediating the appropriate avoidance responses in control rats that is impaired in stressed animals.

DISCUSSION

This study is one of the first attempts to examine the effects of repeated stress on avoidance behavior triggered by innately aversive stimuli, and a role for the basolateral amygdala (BLA) in such behavior. We found that playback of aversive USV social calls elicited avoidance reactions in rats, but prior exposure to chronic stress suppressed this. On the other hand, both control and stressed rats exhibited an initial approach behavior in response to playback of an appetitive USV calls. Unlike control rats, however, stressed rats also showed a late avoidance response to the appetitive call, suggesting a switch in the perception of the emotional valence of the calls from positive to negative. Notably, stress-induced impairment of avoidance also extended to other aversive stimuli – white noise and electric shock in an inhibitory avoidance task. During recall of conditioned fear, stressed rats exhibited higher conditioned freezing to the CS auditory tone compared to controls. However, avoidance reactions to the same CS tone was impaired in stressed but not control rats. This reveals that the same stress can have contrasting effects on the expression of defensive responses – impaired avoidance responses together with increased conditioned fear. This contrast led us to explore a role for the BLA because it not only plays a central role in conditioned fear, but is also affected by chronic stress. USV playback increased BLA neural activity, as evidenced by enhanced cFos expression and theta activity in control rats. Conversely, inactivation of the BLA prevented the avoidance response. Consistent with the stress-induced impairment in the avoidance behavior, both measures of enhanced USV-induced neural activity in the BLA were also suppressed by stress. Together, these findings add a new dimension to earlier work that focused primarily on how stress modulates learned behaviors, such as recall and extinction of conditioned fear, as well as appetitive conditioning tasks.

A role for amygdalar activity and its behavioral consequences

Our analyses identifying a role for the BLA in mediating the avoidance response adds to evidence on the presence of neural correlates for both appetitive and aversive USs in this brain area [60, 61]. This is also in agreement with a role for the BLA in aversive conditioning and avoidance learning [62–66]. Further, our electrophysiological data are in line with an earlier report that 22-kHz USV increased single-unit firing rates in the BLA [22]. Future studies will be needed to examine whether BLA activity alone is sufficient to trigger avoidance responses, as well as potential contributions from other areas like the central amygdala [67, 68]. In this context it is also worth noting that while previous studies assessing the facilitating effects of stress on conditioned fear reported stressed-induced potentiation of BLA activity [69, 70], we found attenuated amygdalar activity in stressed rats in response to innately aversive social call playbacks. Similarly, exposure to chronic stressors, such as maternal maltreatment or prenatal stress, was reported to impair behavioral responses to social stimuli and reduce neural activity in the BLA [71, 72]. Whether the blunted amygdalar responses seen in stressed rats are specific to innate fear cues or arise from a generalized attenuation in amygdalar responsiveness to social cues needs further investigation.

Exposure to aversive USV playback also increased cFos expression in the BLA of control rats, which is consistent with previous work showing enhanced cFos expression in the BLA and other brain areas induced by artificial and natural vocalizations [21, 23, 73–76]. On the other hand this increase in cFos expression was absent in stressed animals, which is similar to several earlier studies on stress-induced habituation of immediate early gene expression [77–83]. Further, while an acute bout of restraint stress was shown to increase expression of *c-fos* mRNA in multiple brain regions, repeated exposure to the same stress caused a habituation in *c-fos* mRNA expression [77]. This holds for audiogenic stress as well [80]. Finally, a novel acute stressor following a chronic exposure to homotypic stressor does not change *c-fos* expression in rodents [78, 79]. Since the absence of stress-induced cFos expression in the BLA mirrored the impairment in avoidance behavior, we probed this further using in vivo recordings in awake, behaving rats. This part of our analysis was guided by previous studies on the roles of neural oscillations in the amygdala in the context of consolidation, retrieval and extinction of fear memories [56, 84–91] and social behaviors [58, 92]. Our findings on enhanced BLA theta activity elicited by aversive USV playback is in agreement with growing evidence regarding changes in theta rhythms during states of arousal, especially while responding to a fearful stimulus [93]. The specific increase in theta power in the 8–12 Hz range in the BLA, caused by the aversive USV, is interesting in light of a previous report on two divergent forms of arousal in rats caused by fearful and social stimuli [58]. While the fearful stimulus evoked a theta rhythm in the 3–7 Hz range, the social stimulus induced a distinct theta rhythm in the 7–10 Hz range. Other studies have also shown 2–6 Hz oscillations to overlap with freezing episodes during fear recall in mice [59]. This raises the possibility that enhanced BLA theta power in the 8–12 Hz range seen here may signal a heightened state of arousal associated with a social stimulus. Further, theta-range communication between the PFC and the BLA is also known to play an important role in fear discrimination. Hence, we also examined changes in BLA-dmPFC communication in mediating avoidance responses to aversive call playbacks. We found aversive USV playbacks to increase theta band activity in the dmPFC, as well as synchrony between BLA and dmPFC in the theta frequency band in control but not in stressed rats. This is consistent with a potential role for BLA-dmPFC communication in mediating the

appropriate avoidance responses in control rats that is impaired in stressed animals.

While the use of innately aversive social calls in our study helped reveal stress-induced impairment in avoidance response, such ethologically natural stimuli also pose certain challenges. For instance, rats could be emitting aversive USVs during and after the 2-hour immobilization over the course of the chronic stress paradigm, thereby causing habituation to such USVs during subsequent behavioral tests in the linear track. Since the ability to vocalize is innate to the rats and a principal mode of communication for them, and given that rats are housed in the vivarium in colonies, it is quite challenging to control for this factor. Enhanced aversive vocalizations and reduced appetitive vocalizations have also been reported after exposure to chronic unpredictable stress or juvenile stress [94, 95]. However, these vocalizations were recorded not when the rats were being stressed, rather when they were subjected to a separate behavioral paradigm. To the best of our knowledge, no such data exist with the chronic immobilization stress paradigm. Further, there are some aspects of our experimental design that are likely to have helped reduce the impact of such factors. Notably, we did not rely only on the 22-kHz aversive USV call playback to establish the key finding of stress-induced impairment in avoidance behavior. We used two other, very different, aversive auditory stimuli (white noise and the tone CS used in fear conditioning) to confirm that the same chronic stress also impaired avoidance in those experiments. The stressed rats were not repeatedly exposed to those auditory stimuli (CS/white noise) over the course of the 10-day paradigm, thereby ruling out habituation to those stimuli; yet they too exhibited impaired avoidance.

Stress and learned helplessness

What are the potential implications of the surprising finding that stress impaired, rather than enhanced, avoidance behavior evoked by a range of innately aversive auditory and somatosensory stimuli in stressed rats? Interestingly, these results are reminiscent of several earlier behavioral observations. For instance, rats and mice experiencing chronic immobilization stress [96, 97] and inescapable foot shocks [98] exhibited impaired active defensive responses like avoidance in a conditioned avoidance response [96, 97] or to innately aversive looming stimulus [98]. Taken together, these results suggest that repeated encounters with an inescapable stressor might tip the balance in favor of passive defensive responses (e.g. freezing) over active ones (e.g. flight or avoidance). This would be consistent with previous observations that stressed rats show enhanced fear recall (i.e. higher freezing), yet impaired avoidance responses as reported here and elsewhere [96–98]. Moreover, the impaired avoidance behavior may also be indicative of "learned helplessness" [99] wherein an organism, when challenged repeatedly with inescapable stressors, eventually learns that avoidance reactions are fruitless [99–101]. In such a framework, chronic immobilization stress would serve as the inescapable stressor inducing a state similar to "learned helplessness" such that when they are subsequently faced with aversive/stressful experiences, they no longer exhibit avoidance behaviors. Hence, it would be interesting to further explore the utility of this behavioral paradigm as an animal model of learned helplessness. While our results were obtained using male rats, growing evidence highlights the importance of sex differences in the effects of stress on fear and anxiety-like behavior, and their neural underpinnings in the amygdala [29, 102–104]. However, the impact of sex difference in stress-induced modulation of innate fear and avoidance behavior remains unexplored and the findings presented here offer a framework to address this gap in knowledge.

Clinical implications for affective symptoms of stress disorders

In conclusion, the paradigm presented here combines an animal model of stress with natural, social calls to reveal amygdala-dependent behavioral changes akin to learned helplessness. These findings suggest future directions of enquiry that may be of clinical relevance. For instance, pioneering studies by Seligman and colleagues had explored the possibility of learned helplessness serving as a laboratory model of clinical depression [105, 106]. As depression-like symptoms are often precipitated by some form of stress, animal models of stress have been used to elucidate the neural mechanisms of depression. These studies underscored the importance of stress-induced plasticity in corticolimbic structures, such as the amygdala, that are thought to contribute to emotional symptoms of depression [107]. Moreover, neuroimaging studies in depression patients also implicate many of the same brain areas, thereby providing convergence between animal models and clinical observations. Interestingly, similar to the stress-induced suppression of avoidance behavior and BLA activity seen here, blunted amygdalar activity was associated with depression severity in treatment-resistant depression [108]. In another clinical study, while depressed children exhibited a blunted response in the amygdala to fearful faces, children with anxiety disorders showed an exaggerated amygdala response to fearful faces compared with healthy children [109]. In this context, it is worth noting that the chronic stress paradigm used here also enhanced anxiety-like behavior in earlier studies [30, 110] (Supplementary Figs. 1B–F). This suggests that assessing the impact of the same stressor with a diverse range of behavioral readouts, such as those involving learned versus innate behaviors, can help capture a wider constellation of amygdala-dependent changes that, in turn, can be mapped to distinct stress disorder symptoms in humans. Together such analyses may offer a more comprehensive understanding of how severe stress leads to symptoms of affective disorders and possible therapeutic interventions to reverse them.

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AUTHOR CONTRIBUTIONS

AS and SC designed the study. AS conducted the experiments and analyzed data. AS and SC interpreted the results and wrote the paper.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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