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Dendritic structural plasticity in the basolateral amygdala after fear conditioning and its extinction in mice

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Abstract

Previous research suggests that morphology and arborization of dendritic spines change as a result of fear conditioning in cortical and subcortical brain regions. This study uniquely aims to delineate these structural changes in the basolateral amygdala (BLA) after both fear conditioning and fear extinction. C57BL/6 mice acquired robust conditioned fear responses (70–80% cued freezing behavior) after six pairings with a tone cue associated with footshock in comparison to unshocked controls. During fear acquisition, freezing behavior was significantly affected by both shock exposure and trial number. For fear extinction, mice were exposed to the conditioned stimulus tone in the absence of shock administration and behavioral responses significantly varied by shock treatment. In the retention tests over 3 weeks, the percentage time spent freezing varied with the factor of extinction training. In all treatment groups, alterations in dendritic plasticity were analyzed using Golgi–Cox staining of dendrites in the BLA. Spine density differed between the fear conditioned group and both the fear extinction and control groups on third order dendrites. Spine density was significantly increased in the fear conditioned group compared to the fear extinction group and controls. Similarly in Sholl analyses, fear conditioning significantly increased BLA spine numbers and dendritic intersections while subsequent extinction training reversed these effects. In summary, fear extinction produced enduring behavioral plasticity that is associated with a reversal of alterations in BLA dendritic plasticity produced by fear conditioning. These neuroplasticity findings can inform our understanding of structural mechanisms underlying stress-related pathology can inform treatment research into these disorders.

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Keywords

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Post-traumatic stress disorder (PTSD) can be categorized as a disorder of dysregulated fear processing [1]. Aberrant fear learning is one of the central features of this disorder as demonstrated by cue-induced re-experiencing responses (e.g. flashbacks) that are slow to extinguish in humans [2]. Exposure therapy, a prominent treatment for PTSD, is a form of extinction training that has proven to be somewhat effective in improving the symptoms of PTSD [3]. However, the exact mechanism by which exposure therapy produces its therapeutic effects is unknown. It is well known that the amygdala, prefrontal cortex, and hippocampus are key sites of synaptic plasticity that mediate aspects of fear learning and memory [4]. It is believed that dysregulated fear conditioning in PTSD patients may be due to a hypoactive prefrontal cortex and/or a hyperactive amygdala [5–8]. Numerous animal studies have suggested that molecular mechanisms of synaptic plasticity in the amygdala may play a key role in fear extinction, and ultimately PTSD symptomatology [8].

Recent studies have found that the morphology and arborization of dendritic spines, small protrusions that form the majority of excitatory synapses, change as a result of fear conditioning and extinction in the cortical areas of the brain that are central to these learning processes [9–13]. Based on these findings, it is hypothesized that structural modifications may occur in connected subcortical regions such as the basolateral amygdala (BLA). To determine the changes in BLA neuroplasticity associated with fear conditioning and extinction, we quantified the development, expression, and extinction of conditioned fear responses behaviorally in C57BL/6 mice and then analyzed dendritic morphology during fear retention testing. Mice were trained in a fear conditioning paradigm, and either underwent fear extinction or sham fear extinction conditions and this was followed by fear retention trials. Immediately following the termination of all behavioral testing, mouse brains were prepared for Golgi–Cox impregnation, which allowed for dendritic morphology analysis in the BLA using brightfield microscopy [14,15].

Male C57BL/6, aged 6–8 weeks upon arrival (Charles River Laboratories, Raleigh, NC) were group-housed (4 per cage) in a temperature-controlled and light-controlled animal facility with ad libitum access to food and water. They were allowed to acclimate to a 12 h light/dark cycle for 7 days before testing. Mice had a mean body weight of 26 g at the time of behavioral testing. All animal testing took place in a facility that is approved by the Association for the Assessment and Accreditation of Laboratory Animal Care using protocols approved by the Institutional Animal Care and Use Committee of the VA Boston Healthcare System.

Mice were randomly assigned to three different treatment groups (5–7 mice per treatment group): unconditioned/extinction (control condition), conditioned fear/extinction, conditioned fear/sham extinction. Fig. 1A illustrates the study design used for the examination of fear acquisition, extinction, and then fear retention. The fear conditioning apparatus (Med Associates, St Albans, VT) consisted of electrified grid floors within sound

attenuated boxes equipped with infrared cameras for automated quantification of freezing accomplished by scanning the visual field for bodily movement of the mouse. On the first training day (27 min total duration), mice were exposed to three variable interval-3-min habituation trials which sounded a 10 kHz, 75 dB tone continuously for the last 30 s of each trial. This was followed by six variable interval-3-min fear acquisition trials co-terminating with the 30 s tone and a 2 s, 0.7 mA electric footshock. This training procedure results in cessation of motor behaviors (freezing) in response to subsequent exposure to the tone in the absence of footshock (conditioned fear). Control mice (unconditioned/extinction group) were exposed to the same procedures but without the footshock on the training day. For extinction training, mice underwent five daily variable interval-3-min extinction trials (15 min total duration) and the extinction groups were exposed to the conditioned stimulus tone for 60 trials in the absence of shock administration over 19 days of training (see Fig. 1A). A conditioned/sham extinction group was fear conditioned and placed for 15 min in a neutral environment during this extinction phase of the study. All treatment groups completed 15 fear retention trials on 3 separate days one week apart at days 23, 30 and 37 of the study following the completion of extinction/sham extinction training.

One hour following the final retention trial, mice were deeply anesthetized using pentobarbital. Golgi–Cox staining was performed using the FD Rapid Golgi Stain Kit (FD NeuroTechnologies, Inc., Ellicott City, MD). Brains were removed, rinsed with water, and placed directly in a mixture of 1 part Solution A and 1 part Solution B from the kit (see manufacturer's instructions for solution contents) for 2 weeks at room temperature in the dark. The solution was replaced and brains were then moved to Solution C, a cryoprotectant, for 3–5 days at 4 °C in the dark. Brains were snap frozen in isopentane cooled to –60 °C and embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC). Brains were coronally sectioned in a cryostat at –25 °C at a thickness of 100 µm and placed on chrome alum gel-coated slides. Sections were dried in the dark for 3–5 days and then underwent a stain development procedure. Following dehydration with ethanol, the slides were cleared with xylene, coverslipped, and allowed to dry. Sections were visualized using an Olympus BX51 bright field microscope at 60× magnification, manually traced and reconstructed using NeuroLucida 10.3 (MBF Bioscience, MicroBrightField, Inc., Williston, VT). Fifteen neurons per treatment group were analyzed. For the Sholl analyses, concentric circles (10 µm apart) were overlaid on the reconstruction from the cell body and outward (summarized in Fig. 2). The parameters analyzed were: total dendritic length, dendritic branch points, spine density, number of intersections by Sholl analysis, and number of spines by Sholl analysis.

For the behavioral data, percentage of time spent freezing was separately analyzed in the habituation, acquisition, extinction, and retention trials (Fig. 1A). There were no group differences evident in the habituation trial (data not shown). For the acquisition data, a two-way analysis of variance (ANOVA) of percentage of time spent freezing with shock exposure and experimental trial number as factors revealed significant effects of shock exposure [$F_{(2, 15)} = 27.6, p < 0.0001$], experimental trial number [$F_{(8, 120)} = 72.3, p < 0.0001$], and an interaction of the two factors [$F_{(16, 120)} = 15.4, p < 0.0001$] (Fig. 1B). For the extinction data, a two-way ANOVA examined percentage of time spent freezing with

shock treatment and experimental trial number as factors. Results revealed that there was a significant effect of shock exposure [$F_{(1, 10)} = 12.2, p < 0.01$] and a significant interaction of the two factors [$F_{(11, 110)} = 15.6, p < 0.0001$] without a significant effect of experimental trial number alone [$F_{(11, 110)} = 1.2, \text{n.s.}$]. In the retention tests, a three-way ANOVA of percentage time spent freezing, with shock exposure, extinction treatment, and experimental trial number as factors revealed a significant [$F_{(1, 15)} = 18.4, p < 0.0001$] overall effect of extinction training. This retention effect was produced in part by the increase in freezing behavior due to fear conditioning in the fear conditioned/sham extinction group compared to the fear conditioned/extinction group. There was no evidence of extinction over time in the conditioned/sham extinction group (see Fig. 1B in retention trial), which was not re-exposed to the conditioning tone cues on extinction training days and supports the use of our sham extinction paradigm.

For the dendritic data, statistical analyses were performed to determine the effects of fear conditioning and extinction on dendritic branch points, total dendritic length, spine density, and Sholl analyses of dendritic arborization. A one-way ANOVA for branch points and total dendritic length with treatment group as the sole factor revealed that there were no significant differences between treatment groups (data not shown). Spine density was analyzed overall (Fig. 3A) and by dendritic order (Fig. 3B). A one-way ANOVA revealed a significant effect of treatment on overall spine density [$F_{(2, 42)} = 4.2, p < 0.05$]. The Tukey–Kramer method revealed that overall spine density differed between the unconditioned/extinction mouse controls and the fear conditioned/sham extinction mice ($p < 0.05$). A two-way ANOVA revealed a significant effect of dendritic order [$F_{(4, 207)} = 50.9, p < 0.0001$] and treatment [$F_{(2, 207)} = 13.5, p < 0.0001$] on spine density (Fig. 3B). Bonferroni post hoc tests revealed significant differences in spine density between the unconditioned/extinction controls and fear conditioned/sham extinction treatment groups for second order ($p < 0.05$), third order ($p < 0.05$), and fourth order dendrites ($p < 0.01$). Tukey–Kramer testing revealed that spine density differed between the fear conditioned/sham extinction group and both the unconditioned/extinction and the fear conditioned/extinction groups on third order dendrites ($p < 0.05$), a key finding.

Dendritic complexity was also analyzed using Sholl analyses of intersections (Fig. 3C) and number of spines (Fig. 3D). The two-way ANOVA of intersections revealed a significant effect of Sholl radius distance [$F_{(11, 504)} = 58.5, p < 0.001$], which signified that dendritic complexity was affected by the distance from the soma. Also, there was a significant effect of treatment group on intersections [$F_{(2, 504)} = 14.5, p < 0.0001$]. In Fig. 3C, the Tukey–Kramer test revealed a significant difference in the overall number of intersections between the fear conditioned/sham extinction group and the unconditioned/extinction and the fear conditioned/extinction treatment groups ($p < 0.05$). There was no difference in the overall number of intersections between the unconditioned/extinction controls and the fear conditioned/extinction group. In Fig. 3D, a two-way ANOVA of spine number revealed a significant effect of radial distance on spine number [$F_{(11, 504)} = 58.5, p < 0.001$], which signified that spine number was affected by the distance from the soma. There was also a significant effect of treatment group on spine number [$F_{(2, 504)} = 34.3, p < 0.0001$]. Bonferroni post hoc tests revealed significant differences between the fear conditioned/sham

extinction and both the unconditioned/extinction ($p < 0.05$) and fear conditioned/extinction ($p < 0.05$) treatment groups in spine number at 50, 60, and 70 μm radii from the soma (Fig. 3D).

In the present study, brief exposures to an aversive stimulus paired with a distinctive tone cue produced behavioral plasticity in the form of robust conditioned freezing and also synaptic plasticity in the form of alterations in BLA dendritic morphology. Additionally, the gradual extinction of conditioned freezing behavior following initial fear learning resulted in changes in the dendritic spine profile in treatment groups. Key findings in dendritic morphology revealed that the number of intersections differed between the fear conditioned group and the fear extinction treatment groups. Fear conditioning appeared to increase BLA structural plasticity measures while subsequent extinction training appeared to reverse these effects. Our findings extend neurophysiological studies which demonstrate that fear memories produce BLA dendritic changes and that subsequent extinction training produces changes in neural networks responsible for informational transfer to the BLA [16].

The overall profile of spine number in the present study is in good agreement with other quantitative studies which report [17] about 5 spines per 10 μm of dendritic length (Fig. 3) and [18] that the majority of spines are located about 150 μm from the soma (Fig. 3). The novel findings in the present study show BLA dendritic spine changes that persisted for more than one month following initial fear learning, suggesting long-term and enduring effects on dendritic plasticity. While exposure to late-occurring retention testing procedures following conditioning and extinction training may well have altered the absolute amount of BLA spine plasticity, retention testing was applied uniformly across all treatment groups and is thus not likely to have biased the present sham extinction versus active extinction group differences in BLA spine plasticity. Although Golgi–Cox analysis of structural plasticity may reveal changes in spine density, branch arborization, and ring intersections in some studies [19], the present alterations in spine number and density were selective and not accompanied by a significant change in any other aspect of dendritic morphology, such as dendritic length and dendritic branching.

These findings are consistent with other recent studies, which have found that significant remodeling occurs as a result of fear conditioning and that these modifications may be reversed by extinction in a site-specific nature [10,13]. All of these findings suggest that fear extinction may partially eliminate the original fear memory in addition to creating a new type of memory, as previously demonstrated [12]. Although the exact functional correlate of increased dendritic complexity is unclear, studies have shown that a mature dendritic spine has a greater density of receptors associated with synaptic plasticity, and more ribosomes for local protein synthesis, thereby suggesting a stronger post-synaptic connection within a synapse than an immature spine [20]. Therefore, techniques that assess the branching and morphology of dendritic spines have long been useful in revealing functionally relevant neural plasticity due to their activity-dependent modifications [21,22]. Our structural dendritic findings are novel but fit with functional plasticity findings in which fear conditioning-induced synaptic potentiation on lateral amygdala neurons [22,23]. In this last study, this effect was reversed by extinction and reinstated by subsequent re-conditioning. A limitation of our current study is that structural plasticity was measured at one post-retention

testing time point. Future studies can sample structural plasticity at multiple post-acquisition and pre-extinction time points. This will enable a more complete argument linking the effects conditioned freezing and extinction in mice with associated dendritic morphology [19].

Anatomical convergence and association of the conditioned stimulus and unconditioned stimulus occurs in the amygdala and contextual information processed by the hippocampus can also enter into association with the unconditioned stimulus in the amygdala. Conditioned and unconditioned fear responses are mediated by projections from the amygdala to an array of brain areas involved in autonomic, somatic, and defensive responses. Similarly, the persistence of conditioned fear expression following successful completion of fear extinction training, such as in the case of fear renewal, suggests the existence of a distributed network model of structural plasticity. In this network, BLA spine density changes may be decoupled from fear expression so that other brain circuits subserve fear recovery and renewal phenomena following extinction. The retention of fear memories may also involve a hippocampal–prefrontal cortical network regulating balance of excitation and inhibition in the BLA that either enhances or reduces, respectively, fear to an extinguished conditioned stimulus [24].

In conclusion, the current study presents novel findings regarding long-lasting morphological alterations in the BLA in fear conditioned and fear extinguished mice. These findings are consistent with hypotheses that fear extinction reverses alterations in synaptic plasticity produced by fear conditioning. In addition, the present animal model of conditioned fear and extinction is analogous to acute trauma exposure and recovery in patients. The clinical implications of these findings are that changes underlying stress-related pathology, such as in PTSD, can be partially reversed by extinction learning and such findings can inform treatment research into these disorders. A greater understanding of the structural and molecular mechanisms underlying fear extinction will better enable translational efforts toward the design of more effective and enduring new treatments for PTSD.

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Abbreviations

BLA	basolateral amygdala
PTSD	post-traumatic stress disorder

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highlights

- C57BL/6 mice acquired conditioned fear and subsequent fear extinction that were retained weeks later.
- During retention testing, dendritic spines and intersections were measured in basolateral amygdala neurons.
- Fear conditioning increased spines and intersections and fear extinction reversed these effects.

Fig. 1.

Acquisition and extinction of cue conditioned fear. (A) A representation of the behavioral paradigm. (B) The figure exhibits percentage of time spent freezing (mean $\pm$ SEM) in the fear acquisition, extinction, and retention phases of the study. $*p < 0.05$, $***p < 0.001$ simple main effects of shock exposure relative to unconditioned, and $\diamond p < 0.05$ conditioned/sham extinction relative to conditioned/extinction.

Fig. 2.

Stages of Golgi–Cox analysis of dendritic complexity reflecting neuron tracing, reconstruction, and Sholl analysis. (A) A photomicrograph (60×) of a Golgi–Cox-stained neuron in the basolateral amygdala with complete dendritic arborization. (B) The neuronal reconstruction created by tracing the neuron in Neurolucida (Microbrightfield), including the Sholl analysis using concentric circles of increasing radii to measure dendritic complexity.

Fig. 3.

Parameters of structural plasticity change as a result of fear conditioning and extinction in the BLA. (A and B) exhibit overall spine density (A) and spine density by order (B) spine density, represented as the mean number of spines per 10 μm . Spine density significantly differed between the unconditioned/extinction mice and the conditioned/sham extinction treatment group (A). There were significant effects of dendritic order and treatment group on spine density (Fig. 3B) and post hoc tests revealed significant differences in spine density between treatment groups for second, third, and fourth order dendrites. (C and D) exhibit differences between treatment groups as measured by dendritic intersections and number of dendritic spines, respectively, as analyzed by Sholl analysis with radii 10 μm apart (0 μm is the center of the cell body). * $p < 0.05$, ** $p < 0.01$ for differences between the unconditioned/extinction control group and the fear conditioned/sham extinction group and # $p < 0.05$ for differences between the fear conditioned/sham extinction group and the fear conditioned/extinction group ($n = 15$ neurons/treatment group).