

Differences in Stress-Induced Changes in Extinction and Prefrontal Plasticity in Postweanling and Adult Animals

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ABSTRACT

BACKGROUND: Postweaning is a critical developmental stage during which the medial prefrontal cortex (mPFC) undergoes major changes and the brain is vulnerable to the effects of stress. Surprisingly, the engagement of the mPFC in extinction of fear was reported to be identical in postweanling (PW) and adult animals. Here, we examined whether the effect of stress on extinction and mPFC plasticity would be similar in PW and adult animals.

METHODS: PW and adult animals were fear conditioned and exposed to the elevated platform stress paradigm, and extinction and long-term potentiation were examined. The dependency of stress-induced modulation of extinction and plasticity on *N*-methyl-D-aspartate receptors was examined as well.

RESULTS: We show that exposure to stress is associated with reduction of fear and enhanced induction of long-term potentiation (LTP) in PW pups, in contrast to its effects in adult animals. Furthermore, we report opposite effects in the occlusion of LTP following the enhanced or impaired extinction in the two age groups and that the reversal of the effects of stress is independent of *N*-methyl-D-aspartate receptor activation in PW animals.

CONCLUSIONS: Our results show that qualitatively different mechanisms control the modulatory effects of stress on extinction and plasticity in postweanling pups compared with adult rats. Our results point to significant differences between young and adult brains, which may have potential implications for the treatment of anxiety and stress disorders across development.

Keywords: Amygdala, Extinction, LTP, Metaplasticity, Postweanling, Prefrontal cortex, Stress

<http://dx.doi.org/10.1016/j.biopsycho.2014.10.004>

The interaction between the basolateral amygdala (BLA) and the medial prefrontal cortex (mPFC) is crucial for extinction of fear (1–5).

Fear associations can be learned by animals throughout development (6). However, developmental differences among different brain regions determine the age at which different subtypes of learned fear associations can be acquired. For example, whereas amygdala-dependent auditory fear conditioning emerges by postnatal day (PND) 16 to 18, hippocampal-dependent contextual fear conditioning was reported around PND 23 and has been attributed to the ongoing maturation of the hippocampus (7–12).

However, the mPFC and the BLA are late maturing structures and undergo major changes during postweaning (childhood in humans) in both rats and humans (13–18). In the mPFC, both interneurons (19,20) and *N*-methyl-D-aspartate receptors (NMDARs) (21,22) undergo dramatic changes during cortical development at postweaning (23). This may suggest that the inhibitory function of the mPFC on the BLA in early life may be dissimilar across development (24–27). Similarly, differences were reported between adults and postweanlings (PWs) in the hypothalamic-pituitary-adrenal axis response (28,29). Furthermore, the regulation of the hypothalamic-

pituitary-adrenal axis undergoes extensive morphologic and functional remodeling during this period (23,30–32).

Together, these observations raise the question of whether stress would similarly affect mPFC-dependent functions in the PW and adult animals.

In the adult animal, we and others have reported that exposure to behavioral stressors is associated with impairments in high-frequency stimulation (HFS)-induced potentiation (long-term potentiation [LTP]) in the BLA-mPFC pathway (33–35) and with impairments in extinction of fear (36–38). We have also shown that stress induces an NMDAR-dependent type of metaplasticity in the adult mPFC (34). In this study, we sought to examine 1) the effects of exposure to behavioral stressors on extinction and HFS-induced LTP in the BLA-mPFC pathway in the PW pup compared with the adult animal; 2) whether prior exposure to stress and extinction training would differentially affect the ability for further induction of HFS-induced LTP in both age groups; and 3) the role of NMDA receptors in occlusion of LTP in both age groups.

Our results clearly establish qualitative differences between the two age groups and show that stress differentially modulates extinction and plasticity in PW and adult animals.

METHODS AND MATERIALS

Adult (~60 days old) and postweanling (24–27 days old) male Sprague Dawley rats from the local animal colony at the Haifa University were used (for details, see Supplement 1).

Surgery and electrophysiological recordings were previously reported in our studies (39,40) (Supplement 1).

Stress procedure was detailed elsewhere (39,40) (Supplement 1). Corticosterone assessment is detailed in Supplement 1.

Detailed description of the procedure of fear conditioning and extinction was previously detailed in our work [e.g., (41)] (Supplement 1).

Details of the drugs that were used appears in our previous work (40) (Supplement 1).

The locations of the stimulating electrodes in the BLA were verified histologically (Figure S1 in Supplement 1; see Supplement 1 for details).

Data were analyzed using SPSS 19 Statistics software (IBM, Chicago, Illinois) (Supplement 1).

RESULTS

Effects of Exposure to Stress on Extinction in Adult and Postweanling Rats

Adult animals (adults, 60 days old) and postweanling pups (PW, 24–27 days old) were trained to associate a tone with an electrical footshock on the conditioning day. Twenty-four hours after conditioning, the animals were exposed to three tones for fear memory retrieval and immediately thereafter were either exposed to the elevated platform stressor (EP; adults-EP; $n = 8$; PW-EP; $n = 7$) or placed back in their home cage (adults-control; $n = 8$; PW-control; $n = 8$).

Two-way analysis of variance (ANOVA) (2×2 : group [stress (EP), control]; age [adult, PW]) of freezing levels during the retrieval test showed no significant main effect for any of the variables or for the interaction (group: $F_{1,28} = .3$, not significant (ns); age: $F_{1,28} = .09$, ns; age \times group: $F_{1,28} = .11$, ns; Figure 1), which suggests comparable freezing levels during the retrieval test.

Twenty-four hours after the retrieval session, the four groups of animals underwent extinction training during which

10 tones were presented in the absence of footshock. Repeated measures on the five extinction blocks showed no significant effect of group ($F_{1,28} = .15$, ns) or of age ($F_{1,28} = .3$, ns). However, there was a significant effect for the interaction between age and group ($F_{1,28} = 24.9$; $p < .0001$), suggesting that the two age groups behaved differently. Furthermore, there was a significant effect of block ($F_{1,28} = 24.3$, $p < .001$) in the absence of any significant interaction with the other variables ($F_{1,28} = .8$, ns), which suggests that the different age groups extinguished fear memory similarly over the extinction blocks.

Follow-up analysis of the observed interactions of age \times group using one-way ANOVA with repeated measures on the extinction trials showed differences between the adults-control and adults-stress groups ($F_{1,15} = 11.08$, $p < .005$, adults-control: $52.29 \pm 5.05\%$; adults-EP: $76.78 \pm 5.3\%$). The results show that stress impaired extinction of fear in adults.

In the postweanling animals, one-way ANOVA also revealed significant differences between the groups, with the PW-stress group showing lower freezing levels than the control group ($F_{1,13} = 52.2$, $p < .0001$, PW-control: $81.3 \pm 2.6\%$; PW-EP: $55.4 \pm 2.44\%$). This result suggests that stress facilitated extinction of fear in postweanling pups.

Similarly, two-way ANOVA on the retrieval of extinction 24 hours after extinction training showed a significant interaction effect (group \times age: $F_{1,28} = 41.3$; $p < .0001$) without any significant effect of group ($F_{1,28} = .6$, ns) or of age ($F_{1,28} = .85$, ns). A follow-up t test on the observed interactions showed that exposure to stress was associated with impaired extinction in adult rats ($t_{15} = 5.6$, $p < .001$, adults-control: $37.7 \pm 4.9\%$; adults-EP: $75.3 \pm 4.15\%$). In postweanling pups, the differences between the PW-control and PW-EP were also maintained during extinction retrieval, with the PW-EP group showing better fear extinction than control pups ($t_{13} = 4.5$, $p < .005$; PW-control: $68.6 \pm 4.1\%$; PW-EP: $42.9 \pm 3.8\%$).

Thus, stress exerts opposite effects on fear extinction in postweanling pups compared with adult rats.

These results show that the similar kinetics of extinction in all groups suggest a reduction in expression of fear rather than facilitated extinction. To better dissociate between the two, we

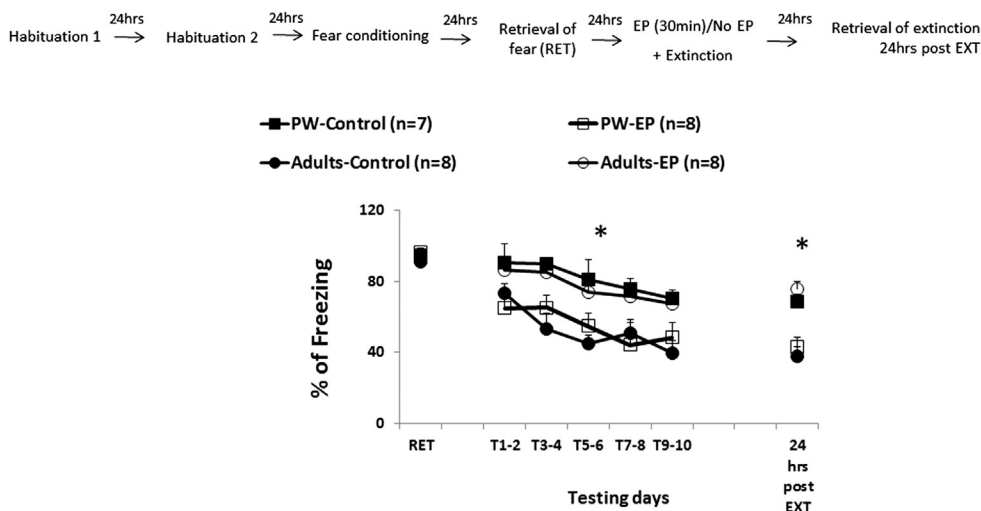


Figure 1. Schematic representation of the experimental protocol. A total of four groups were tested: postweanling (PW) pups and adult rats were conditioned on the first day. The next day they underwent retrieval testing (RET) and 24 hours later, the four groups of animals underwent extinction training (EXT). The results indicate that stress increased the expression of fear in adults and reduced it in PW pups ($p < .001$ for significant effect between adults-control and adults-elevated platform stressor [EP] and PW-control and PW-EP). These differences were maintained 24 hours later (24 hrs post-EXT; $p < .005$).

carried out an experiment in which animals were exposed to the platform immediately after the retrieval of fear memory, and 24 hours later they started with extinction training. Results show significant interactions between group \times age ($F_{1,27} = 11.3$; $p < .005$) and block \times group \times age ($F_{1,27} = 8.02$; $p < .001$), suggesting that the groups in both ages extinguished fear memory differently (Figure S2 in Supplement 1).

Exposure to the Elevated Platform Stressor Facilitates LTP in the BLA-mPFC Pathway Only in Postweanling Pups

Adult animals and postweanling pups were assigned to two groups (stress and control). The stress group was exposed to the platform stressor for 30 minutes before commencement of the electrophysiological experiment (adults-EP, $n = 6$; PW-EP, $n = 6$). Control animals were taken from the home cage and immediately anesthetized (adults-control, $n = 7$; PW-control, $n = 7$).

Two-way ANOVA [group (control, stress) \times age (adults, PW)] on the amplitude values of the baseline evoked field potentials before the application of theta-burst stimulation (TBS) did not show significant differences for any of the variables or for the interaction ($F_{1,22} < 1$). Thus, baseline values (in mV) were similar across all groups and ages (adults-control: $5.5 \pm .63$; adults-EP: $5.0 \pm .68$; PW-control: $4.72 \pm .61$, PW-EP: $5.13 \pm .4$ mV). Furthermore, there was no significant difference in the stimulation intensities required to achieve baseline values ($F_{1,22} = .9$, ns).

Repeated measures on the percentage of change following TBS (group [control, stress (EP)] \times age [adults, PW] \times 12 time points) revealed a significant effect for group ($F_{1,22} = 5.15$, $p < .05$), a significant age \times group interaction ($F_{1,22} = 28.13$; $p < .001$), but no significant effect for age ($F_{1,22} = .05$, ns).

To better understand the source of the interaction and since we were interested in how stress modulates LTP in each age group, we performed repeated ANOVA test for each age group. In the adults, exposure to stress was associated with impaired LTP, as the levels of potentiation were significantly higher in the control group ($F_{1,11} = 17.8$, $p < .001$; adults-control: $134.4 \pm 4.7\%$; adults-EP: $103.3 \pm 5.1\%$; Figure 2B). By contrast, in PWs, the stress-exposed rats showed higher levels of potentiation compared with the control rats, which showed moderate levels of potentiation ($F_{1,11} = 11.7$, $p < .005$; PW-control: $109.3 \pm 2.3\%$; PW-EP: $126.5 \pm 2.5\%$; Figure 2B). Thus, stress is associated with impairment of LTP in the BLA-mPFC pathway in the adult animal and with facilitation of LTP in the postweanling pup.

Enhanced Extinction Following Exposure to the EP Occludes the Induction of LTP in PW Pups

It has been previously shown that HFS-induced LTP is occluded following learning. This phenomenon is thought to occur owing to natural LTP-like mechanisms, which take place during the behavioral task to enable the learning process, resulting in inability of electrical stimulation to induce further LTP (39–41). Furthermore, we have previously shown that exposure to stress induces NMDAR-dependent metaplasticity that affects further ability to induce LTP in the mPFC of the

adult animal (34,42). To examine the ability of TBS to induce further LTP following stress, we used the elevated platform.

Similar to the data described in Figure 1, exposure to stress resulted in reduced fear expression in the postweanling pups and increased fear in the adult animal (interaction: $F_{3,44} = 15.3$; $p < .001$; data not shown).

Following the termination of behavioral testing, the animals were anesthetized for electrophysiological recording. To assess the dependency of learning and stress-induced metaplasticity on NMDAR activity, two mirror experiments were planned. As stress and extinction in the adult animal resulted in enhanced LTP, we injected the NMDAR antagonist before TBS. In the PW, EP and extinction resulted in occlusion of LTP, and thus the NMDAR agonist D-cycloserine (DCS) was injected to assess whether it could rescue the occlusion of LTP. Three groups of adult rats were tested: extinction group without exposure to the EP (EXT): $n = 9$; extinction with EP (EXT + EP): $n = 10$; extinction + EP + MK801 before TBS (EXT + EP + MK801): $n = 6$. Repeated measures ANOVA revealed a significant effect of group ($F_{2,22} = 13.2$; $p < .001$; Figure 3B) without significant effect of time following TBS or interaction ($F_{1,22} = .4$, ns for both).

Post hoc analysis showed that EXT + EP significantly differed from both groups in the levels of potentiation following TBS (EXT + EP: $117.8 \pm 2.8\%$; EXT: $95.4 \pm 10.05\%$; EXT + EP + MK801: $102.4 \pm 3.9\%$; $p < .005$ for significant difference from the other groups). These results show that in the adult animal, impaired extinction of fear following exposure to stress resulted in induction of LTP and that extinction training alone was associated with occlusion of LTP. Injection of MK801 blocked the LTP induced by the combined extinction and exposure to stress (EXT + EP). There were no significant differences in baseline amplitudes or stimulation necessary to elicit baseline response ($F_{2,22} = .53$ for both).

In PW animals, three groups were tested: extinction group without exposure to the EP (EXT): $n = 8$; extinction with EP (EXT + EP): $n = 10$; extinction + EP + DCS before TBS (EXT + EP + DCS): $n = 7$. Repeated measures ANOVA showed significant differences between the three groups ($F_{2,22} = 6.37$; $p < .005$; Figure 3C), without significant effect of recording time or interaction ($F_{2,22} = .87$). Follow-up analysis using post hoc comparison showed that the EXT group significantly differed from the other groups, while the other two groups did not differ from each other (EXT: $117.25 \pm 3.4\%$; EXT + EP: $105.6 \pm 3.2\%$; EXT + EP + DCS: $99.47 \pm 3.7\%$; $p < .05$ for significant difference of the EXT group from the two groups).

These results show that reduced fear following exposure to stress results in occlusion of LTP in PW pups. DCS failed to promote the induction of LTP.

In contrast to the effects on responses following TBS, there were no significant differences in baseline responses ($F_{2,22} = 1.6$; ns) or the stimulation intensity ($F_{2,22} = .68$).

Increase in Plasma Corticosterone Following Exposure to the Elevated Platform Stressor

Four groups were tested: adults-control ($n = 8$), adults-EP ($n = 8$), PW-control ($n = 6$), and PW-EP ($n = 6$). Two-factor ANOVA (2 \times 2: group [stress, control]; age [adult, PW]) of plasma corticosterone showed significant main effects

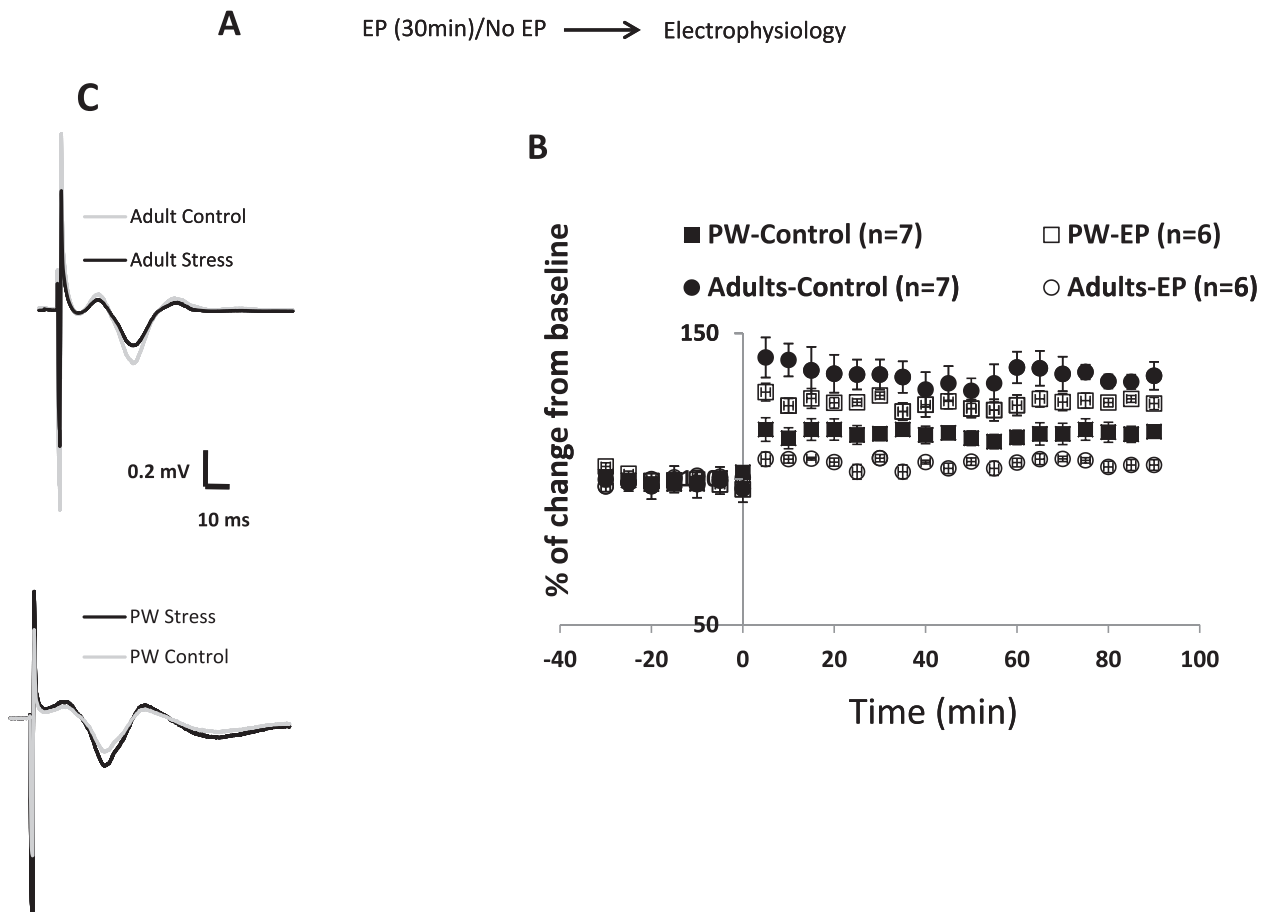


Figure 2. Four groups were tested: postweanling (PW) pups and adult animals that were either assigned to the control group or exposed to 30 minutes stress on an elevated platform (EP). **(A)** Schematic representation of the experimental protocol. **(B)** Repeated measures showed a significant effect of group ($p < .05$) and a significant interaction of age \times group ($p < .001$), without a significant effect of age. Follow-up repeated analysis of variance for each age showed significant differences between the control and stress subgroups within each age group ($p < .005$), with stress inhibiting the induction of long-term potentiation in the adults and facilitating potentiation in the postweanling pup. Time zero indicates the application of high-frequency stimulation. **(C)** Representative average waveforms. Horizontal bar = 10 msec; vertical bar = .2 mV.

(group: $F_{1,24} = 11.8$, $p < .001$; age: $F_{1,24} = 16.5$, $p < .001$) but without significant interaction (age \times group $F_{1,24} = 1.8$, ns) (Figure S3 in Supplement 1).

DISCUSSION

Using behavior, electrophysiology, and pharmacology, the major finding of this study is that following stress, the response profile of postweanling pups differs from that of the adult animal. However, it should be noted that baseline differences between the two age groups also exist, pointing to developmental differences that will be discussed in the next sections.

Stress, Extinction, and LTP

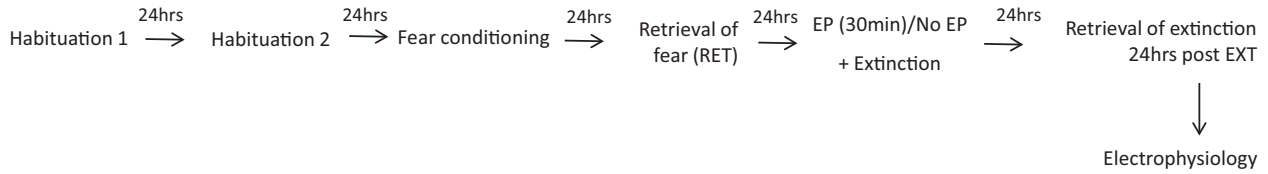
The mechanisms mediating fear and its extinction, both of which depend on the interaction between the BLA and the mPFC, were reported to be similar in the PW pup and the adult animal (43–45). The present data show that the ability of the PW pup to form fear memory and to extinguish it is not

different from that of the adult animal. However, a differential pattern emerged when the animals were challenged by stress. Namely, exposure of the PW pup to stress weakened fear expression and this effect is in contrast to the detrimental effects of exposure to stress on extinction, which have been reported in the adult animal here and elsewhere with the same stressor (4,37) or with other stressors (36,46–50).

In addition, we showed that stress before extinction training only weakened the expression of fear in the PW pups without affecting extinction per se, as there were no differences in the kinetics of extinction between the two age groups (Figure 2). However, targeting the consolidation phase by exposing the animals to the EP stressor after the retrieval of fear resulted in differential patterns of extinction, i.e., enhanced extinction in the PW pups and impaired extinction in the adult animals (Figure S2 in Supplement 1). Together, the data show that not only fear expression was weakened but also extinction was affected (36–38), further confirming opposite effects of stress on extinction in PW and adult animals.

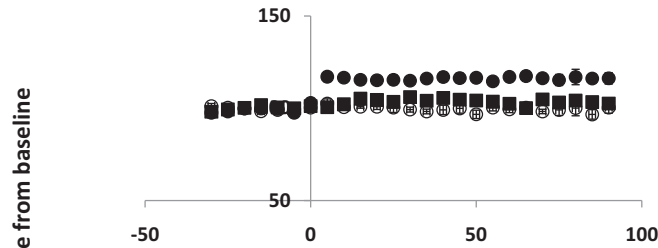
Stress Effects on Extinction and Long-Term Potentiation

A



○ Adults-EXT (n=9) ● Adults-EXT+EP (n=10) ■ Adults-EXT+EP+MK801 (n=6)

B



C

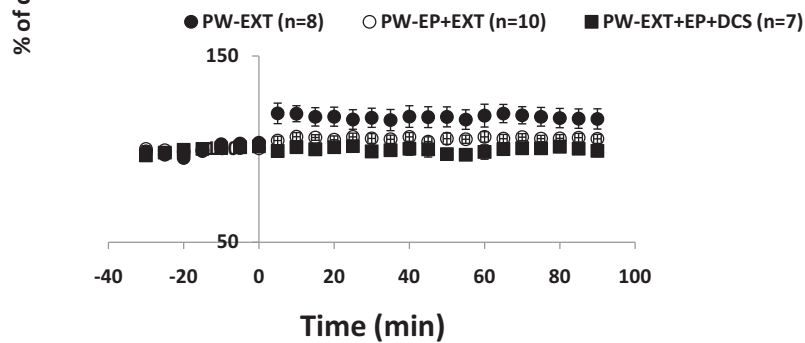


Figure 3. (A) Schematic representation of the experimental protocol. (B) In the adult animal, repeated measures on the potentiation levels post theta-burst stimulation revealed a significant difference between the three groups ($F_{2,22} = 13.2; p < .001$). Post hoc analysis showed that extinction training (EXT) + stress on an elevated platform (EP) had significantly higher levels of potentiation compared with the group that underwent extinction only (Adults + EXT) and with the group that underwent extinction, were exposed to the EP, and received the *N*-methyl-D-aspartate antagonist (Adults + EXT + EP + MK801). These two groups did not show potentiation of postsynaptic potentials. (C) In postweanling (PW) animals, repeated measures showed significant differences between the three groups ($F_{2,22} = 6.37; p < .005$). Post hoc comparison showed that the EXT group significantly differed from the other groups, expressing high levels of potentiation while the other groups did not show any level of potentiation. Time zero indicates the application of high-frequency stimulation. DCS, D-cycloserine; EXT, extinction training.

Differences in the behavioral profile following exposure to stress were previously reported in adult and young animals. It was specifically shown that the same stress resulted in hyperactivity in juvenile animals (same age used in this study) and in hypoactivity in adult animal (51). This may raise the question that reduced freezing in PW animals is due to hyperactivity; however, this could be excluded, as the groups show similar freezing levels during the first two blocks on the extinction training (Figure S2 in Supplement 1). However, previous reports have shown that animals can display high anxiety but decreased cued fear expression (52), showing that fear may be manifested by low freezing. Future studies should, however, combine different methods to assess fear.

The differences between the two age groups could not be explained by differences in stress-induced changes in plasma corticosterone levels, as both age groups showed an increase in corticosterone levels following exposure to the platform.

Prior exposure to the stressor impaired LTP in the adult mPFC, consistent with previous reports (33–35). However, exposure to the same stressor resulted in enhanced mPFC-LTP in the PW animals, in line with reports that showed exposure to stress was associated with a significant potentiation of glutamatergic transmission in slices from the prefrontal cortex (53,54). Interestingly, the authors do not address the possibility that their data could be ascribed to the age of the animals (25–28 days), which is the same age used in the present study. Furthermore, they have shown that acute stress at 25 to 28 days enhanced prefrontal cortex-dependent memory in the delayed alternation task (53–55). All in all, these findings raise the question of whether all mPFC-dependent cognitive processes are enhanced following exposure to stress in PW animals; this question remains to be elucidated.

Our results were further confirmed by the ability to induce LTP in the experiment combining behavior and electrophysiology in

the same animal, in which we addressed the outcome of enhanced/impaired extinction in the PW and adult animals, respectively. The enhanced extinction in the PW pup resulted in occlusion of LTP. Similarly, in the adult animal, the group that underwent extinction training did not show LTP following TBS, and in contrast, the EXT + EP group that showed impaired extinction had high levels of LTP. Studies have shown an overlap of mechanisms mediating HFS-LTP and training-induced synaptic potentiation in relevant brain structures mediating these types of learning [e.g., (39–41,56,57)]. Importantly, potentiation induced by the behavioral training occluded the subsequent induction of LTP by electrical HFS (39–41). All in all, our data join previously reported data to indicate that the occlusion of LTP induced by electrical stimulation suggests that an LTP-like mechanism was activated during the learning task.

In the PW animal, only the animals that underwent extinction expressed LTP, hinting that potentiation does not occur during extinction training, unlike our previous report on adult animals (57). These results also support that stress-induced extinction alterations (and possibly other forms of extinction as well) are mediated by different mechanisms in the two age groups.

The Role of the NMDA Receptor in Metaplasticity

Previous reports have established the role of the NMDA receptor in the induction of LTP in the mPFC (33,58). Acute stress was associated with an increase in glutamate release in the mPFC (59), which has a crucial role in stress-mediated effects on the mPFC (60).

We have previously shown that stress also induces a form of NMDAR-dependent metaplasticity (61,62) in the adult mPFC that can lead to further ability to induce LTP. This type of metaplasticity could be manipulated by either the NMDAR partial agonist DCS or by the NMDAR antagonist MK801 (34). Our present data confirm that metaplasticity induced by stress could be inhibited by the NMDAR antagonist MK801 at a dose that does not affect LTP per se (34,63). Likewise, DCS injection before TBS also rescued stress-induced impairment of LTP in the mPFC (34). These findings are also in line with reported data that showed the blockade of NMDA receptors during restraint stress prevented stress-induced apical dendritic retraction in the mPFC (60), which is associated with impairments in extinction of fear (36). All in all, activation of NMDA receptors during stress contributes to stress-induced alterations in mPFC functioning and confirms the protective nature of NMDA receptor blockade, at least at low doses.

By contrast, in the PW pup, the injection of DCS was ineffective in modifying the occlusion of LTP following the enhanced extinction and exposure to stress. These results may suggest that metaplasticity in the PW animal is not dependent on NMDA receptors and further suggest that metaplasticity in the two age groups is mediated by different mechanisms.

Developmental Differences

In rats, the mPFC undergoes a rapid period of growth from birth until around PND 20. Although the mPFC reaches adult-like thickness around PND 24, it continues to develop well into adulthood (64,65). Because the mPFC is a late-maturing structure in both humans and rodents (64,66,67), it was

reported that whereas extinction is mPFC-independent at the age of 12 to 17 days in pups, it becomes mPFC-dependent a few days later (68,69). Fear inhibition was suggested to rely on functional amygdala and periaqueductal gray structures, as they have been shown to mature earlier than the mPFC [e.g., (6,26,70)]. Gogolla *et al.* (44) investigated the mechanisms that mediate differences in extinction between the preweanling pup (extinction leads to memory erasure) as compared with postweanlings and adults (fear memories are actively protected after extinction). The authors showed that this protection is conferred by extracellular matrix chondroitin sulfate proteoglycans in the amygdala and that the organization of chondroitin sulfate proteoglycans into perineuronal nets (PNNs) coincided with the developmental switch in fear memory resilience, with adult and PW animals having significantly higher numbers of PNNs than preweanling pups. However, a careful inspection of their results clearly shows that the PW pups (23–28 days old) also have a significantly different number of PNNs compared with adult animals, suggesting that even at the level of the amygdala, there exist differences between PW and adult animals. This finding strengthens our claim that the circuit mediating fear and extinction is distinctive in PW pups compared with the adult animal.

It should also be noted that the levels of potentiation in the control group of PW animals were moderate. Marked differences were reported in intrinsic excitability and local circuit activity between PW and adult animals in the dentate gyrus of the hippocampus (71). Future studies, however, should address whether other high-frequency stimulation protocols can induce comparable potentiation in the two age groups.

Implications of the Research

Studies in humans and animal models have described age-related shifts in cellular and molecular brain architecture and disparities in the pharmacologic effects of various drugs on different age groups (23,51,72–75). Although PW pups exhibit similar fear and extinction behaviors to adults, the mechanisms through which stress modulates LTP and extinction are fundamentally different. These results provide novel evidence that a stressful experience is processed differently in the PW brain compared with the adult brain; this could be of crucial importance when considering how to treat juveniles suffering from psychiatric disorders.

ACKNOWLEDGMENTS AND DISCLOSURES

This project was supported by an Israel Science Foundation grant to MM (663/13).

We thank Hamutal Rosengarten and Nissrin Lahoud for valuable help.

The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

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Received May 22, 2014; revised Sep 10, 2014; accepted Oct 7, 2014.

Supplementary material cited in this article is available online at <http://dx.doi.org/10.1016/j.biopsych.2014.10.004>.

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