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Impaired fear extinction learning in adult heterozygous BDNF knock-out mice

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a crucial regulator of neuroplasticity, which underlies learning and memory processes in different brain areas. To investigate the role of BDNF in the extinction of amygdala-dependent cued fear memories, we analyzed fear extinction learning in heterozygous BDNF knock-out mice, which possess a reduction of endogenous BDNF protein levels to ~50% of wild-type animals. Since BDNF expression has been shown to decline with aging of animals, we tested the performance in extinction learning of these mice at 2 months (young adults) and 7 months (older adults) of age. The present study shows that older adult heterozygous BDNF knock-out mice, which have a chronic 50% lack of BDNF, also possess a deficit in the acquisition of extinction memory, while extinction learning remains unaffected in young adult heterozygous BDNF knock-out mice. This deficit in extinction learning is accompanied by a reduction of BDNF protein in the hippocampus, amygdala and the prefrontal cortex.

Studying the processes underlying acquisition and modulation of fear memories is probably one of the most important research areas in the field of behavioral neuroscience. Anxiety disorders such as phobias and posttraumatic stress disorders (PTSDs), are among the most commonly diagnosed mental health problems (Breslau, Peterson, Poisson, Schultz, & Lucia, 2004), and there is overwhelming evidence that patients suffering these diseases are impaired either in the learning and storage of fear memory or in fear extinction learning (Jovanovic & Ressler, 2010; Mahan & Ressler, 2012).

The classical Pavlovian fear conditioning paradigm is an adequate model to analyze the mechanisms of fear memory. Pairing an unconditioned stimulus (US), e.g. an aversive electric foot shock, with an initially neutral, ineffectual stimulus, e.g. a tone, results in a learning process that the neutral stimulus is a predictor for the occurrence of the US and thus the previously neutral stimulus is turned into a conditioned stimulus (CS). However, if the CS is presented repeatedly without the US, the conditioned response diminishes again until almost no remaining fear response can be observed. This phenomenon is called extinction of fear memory and is supposed to be a form of new learning that results in an inhibition of the original fear memory trace, since the original fear behavior can return after a delay of time (spontaneous recovery), or if the CS is presented in a different context than the extinction context (i.e., renewal; for reviews see e.g. Maren, 2011; Quirk & Mueller, 2008).

Brain-derived neurotrophic factor (BDNF) is a protein which is known to play a critical role in neuronal differentiation and survival (Davey & Davies, 1998; Lewin & Barde, 1996). Furthermore, BDNF is also involved in the induction and expression of activity-dependent synaptic plasticity (like e.g. long-term potentiation) thus regulating processes involved in learning and memory formation (see e.g. Bekinschtein et al., 2008; Cowansage, LeDoux, & Monfils, 2010; Gottmann, Mittmann, & Lessmann, 2009). BDNF also plays a critical role in the acquisition and consolidation of amygdala dependent fear memory (Endres & Lessmann, 2012; Ou & Gean, 2006; Ou, Yeh, & Gean, 2010; Rattiner, Davis, French, & Ressler, 2004; Rattiner, Davis, & Ressler, 2005) and very recently, it has been shown that BDNF is also involved in the extinction of fear memories (for review see: Andero & Ressler, 2012). Fear extinction training induces a strong increase in BDNF mRNA in the infralimbic medial prefrontal cortex (IL; Bredy et al., 2007) and BDNF-infusion in the IL facilitated extinction learning even without extinction training (Peters, Dieppa-Perea, Melendez, & Quirk, 2010). Furthermore, a region-specific genetic knock-down of BDNF in the hippocampus impairs fear extinction (Heldt, Stanek, Chhatwal, & Ressler, 2007). Chhatwal and colleagues showed that extinction training leads to an increase of BDNF mRNA within the basolateral amygdala (BLA), and that interfering with BDNF signaling by overexpressing non-functional TrkB receptors (tTrkB) in the amygdala, prevents the retention of extinction memory, but not the within-session extinction, suggesting a role of BDNF in the consolidation of extinction memory (Chhatwal, Stanek-Rattiner, Davis, & Ressler, 2006). Furthermore, Soliman et al. (2010) showed impaired fear extinction learning in mice that carry a heterozygous or homozygous mutation in the BDNF gene (Val/

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Met and Met/Met) which affects BDNF secretion (Egan et al., 2003). In addition, systemic application of the TrkB-receptor agonist 7,8-Dihydroxyflavone was able to foster fear extinction learning (Andero et al., 2010). In conclusion, the above mentioned studies suggest that BDNF is an important regulator of fear extinction learning of amygdala-dependent cued fear memories. Given the known physiological decline of endogenous BDNF protein expression with aging (Boger et al., 2011; Carreton et al., 2012; Silhol, Bonnichon, Rage, & Tapia-Arancibia, 2005) we here focused on the role that diminished endogenous BDNF levels exert on fear extinction during early aging.

In a recent study we could show, that heterozygous BDNF knock-out mice ($BDNF^{+/-}$ mice), which retain roughly 50% of BDNF protein levels compared to their wild-type littermates, display an age-dependent deficit in fear learning (Endres & Lessmann, 2012): if the mice were 3 months of age or beyond, the ability to consolidate fear memories was impaired. Based on these results we hypothesized that these mice might exhibit also an age-dependent deficit in fear extinction learning. Several studies have shown that the protein levels of BDNF in the brain decreases with increasing age (see e.g. Boger et al., 2011; von Bohlen und Halbach, 2010). Thus, in young $BDNF^{+/-}$ mice the level of BDNF might still be adequate to form new extinction memory traces, whereas during aging the quantity of BDNF might fall below a critical threshold, so that no extinction memory can be formed. In order to test this, we analyzed fear extinction learning in young adult (2 months) and older adult (7 months) $BDNF^{+/-}$ mice and their wild-type littermates. In addition, we quantified BDNF protein levels in brain areas which are well known to be essential for fear extinction learning, i.e. basolateral amygdala, hippocampus and the mPFC, using an enzyme-linked immunosorbent assay (ELISA).

To test our hypothesis, we used 2 or 7 months old male $BDNF^{+/-}$ mice (Korte et al., 1995), which were backcrossed to a C57BL/6J genetic background (Charles River, Sulzfeld, Germany) for more than 10 generations. Wild-type littermates (WT) served as controls. The experimental group sizes were between eight and twelve mice per genotype. Mice were group-housed (2–4 per cage) in a temperature controlled room (22 °C) with a 12 h light–dark cycle (lights on at 7 a.m.) with food and water access *ad libitum*. All experimental procedures were performed in accordance with the ethical guidelines for the use of animals in experiments and were approved by the local animal care committee (Landesverwaltungsamt Sachsen-Anhalt, IPHY/G/01-872/08). For the fear conditioning experiments we used an automated setup that assessed the activity of the animals via an array of infra-red light beams (Fear conditioning system, TSE systems GmbH, Bad Homburg, Germany) which was located in a separate testing room. For the 2 months old mice we used an 8 kHz sine tone (30 s, 75 dB, SPL) as CS, that was paired three times with a co-terminating scrambled foot shock (1 s, 0.7 mA). Since we knew from our previous experiments that this protocol was not able to induce fear in $BDNF^{+/-}$ mice, which are older than 3 months of age (Endres & Lessmann, 2012), we first established a fear conditioning protocol that could compensate this learning deficit in older $BDNF^{+/-}$ mice. It turned out that a protocol applying five CS–US pairings with a shock intensity of 0.4 mA was able to induce a comparable amount of conditioned fear in older $BDNF^{+/-}$ mice and their wild-type littermates, as observed with three pairings in the younger animals. In addition, we verified that the two conditioning protocols induced a comparable amount of conditioned fear and a similar extinction of fear in young wild-type mice ($F_{6,133} = 1.6$, $p > 0.05$, data not shown). On the first day of the experiment, animals were fear conditioned according to the above mentioned protocols and 24 h later the animals underwent extinction training. The extinction training was identical for both age groups and was performed in a different context than the fear conditioning. It consisted of 28 CS presentations with a consistent inter-stimulus interval of 5 s. On day three we tested the animals for extinction memory by exposing them to the extinction context where they received three CS presentations after a habituation phase of 180 s.

To rule out that older $BDNF^{+/-}$ animals show a reduced exploratory activity which might interfere with the analysis of freezing behavior as an indicator of fear behavior, we performed open field experiments with both genotypes. The animals were exposed to an open field arena (30 × 30 × 45 cm) under dim light conditions for 10 min. The distance traveled by the animals was recorded and analyzed by a video tracking system (Any-maze, Stoelting Co).

In order to compare the baseline BDNF protein levels in the extinction learning relevant brain areas between the two genotypes as well as between the different age groups, we used a second set of animals ($n = 4$–8 per age/genotype), which were sacrificed and the brains were removed and sliced using a brain matrix (AgnTho's AB, Lidingö, Sweden). Probes of the amygdala, the hippocampus and the mPFC were taken with a soft tissue biopsy puncher (Zivic instruments, Pittsburgh PA, USA) and stored at −80 °C. BDNF protein levels were measured with the BDNF Quantikine ELISA kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instruction.

During the first three presentations of the CS in the extinction session, young $BDNF^{+/-}$ mice as well as their wild-type littermates showed a robust and comparable amount of conditioned fear behavior in response to the CS. With ongoing CS presentations the freezing behavior declined (Fig. 1A, phase: $F_{5,84} = 5.21$, $p = 0.0003$) and there were no differences between the two genotypes (genotype: $F_{1,84} = 0.07$, $p = 0.79$; genotype × phase: $F_{5,84} = 0.62$, $p = 0.68$), indicating successful fear learning as well as successful within-session extinction regardless of the genotype (Fig. 1A). In the 7 months old animals we observed also a comparable extent of conditioned freezing in both genotypes during the first three CS presentations, indicating successful fear learning even in the adult $BDNF^{+/-}$ mice. However, with ongoing CS presentations the $BDNF^{+/-}$ mice did not show a reduction in freezing behavior contrary to their wild-type littermates, indicating no within-session extinction in these animals (Fig. 1C). An ANOVA revealed no significant general effect of the number of CS presentations (phase: $F_{5,126} = 1.59$, $p = 0.16$), indicating such an unsuccessful extinction learning. But we observed a significant effect of the factor genotype ($F_{1,126} = 18.64$, $p < 0.0001$), indicating a different extinction performance between wild-type and $BDNF^{+/-}$ mice. However, we did not see a significant interaction between these two factors (genotype x phase: $F_{5,126} = 1.26$, $p = 0.28$).

Twenty-four hours after the extinction training, we re-exposed the animals to the CS in the extinction context in order to test their extinction memory. Consistent with our results for the within-session extinction, we observed successful extinction memory in young animals for both genotypes, since $BDNF^{+/-}$ as well as the wild-type mice showed significantly less freezing in response to the CS compared to the beginning of the extinction training (paired t-test comparison, p's < 0.05) (Fig. 1B). In contrast to the results for young animals but in accordance with our observation regarding the within-session extinction, in the 7 months old animals we observed a successful extinction memory only in wild-type mice ($p < 0.05$) whereas the $BDNF^{+/-}$ mice still showed a high amount of freezing behavior in response to the CS, which was not different from the freezing behavior at the beginning of the extinction training ($p > 0.05$). This result further demonstrates the impairment in fear extinction learning in 7 months old $BDNF^{+/-}$ mice. Importantly, the increased freezing in response to the CS in the 7 months old $BDNF^{+/-}$ mice is not due to any generalization of fear in these animals since we observed a similar low amount of freezing during the habituation period of the extinction memory test as in their wild-type littermates (Fig. 1D, $p > 0.05$).