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Effects of TrkB-related induced metaplasticity within the BLA on anxiety, extinction learning, and plasticity in BLA-modulated brain regions

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Abstract

Background Neuronal plasticity within the basolateral amygdala (BLA) is fundamental for fear learning. Metaplasticity, the regulation of plasticity states, has emerged as a key mechanism mediating the subsequent impact of emotional and stressful experiences. After mRNA knockdown of synaptic plasticity-related TrkB, we examined the impact of chronically altered activity in the rat BLA (induced metaplasticity) on anxiety-like behavior, fear memory-related behaviors, and neural plasticity in brain regions modulated by the BLA. These effects were investigated under both basal conditions and following exposure to acute trauma (UWT).

Results Under basal conditions, TrkB knockdown increased anxiety-like behavior and impaired extinction learning. TrkBKD also reduced LTP in the vSub-mPFC pathway but not in the dentate gyrus. Compared with those of control animals, acute trauma exposure led to increased anxiety-like behavior and impaired extinction learning in both the trauma-exposed group (CTR-UWT) and the trauma-exposed group on the background of TrkB knockdown (TrkBKD-UWT). However, the deficit in extinction learning was more pronounced in the TrkBKD-UWT group than in the CTR-UWT group. Accordingly, TrkBKD-UWT, but not CTR-UWT, resulted in impaired LTP in the vSub- mPFC pathway. Since LTP in this pathway is independent of BLA involvement, this result suggests that lasting intra-BLA-induced metaplasticity may also lead to transregional metaplasticity within the mPFC, as suggested previously.

Conclusions Taken together, these findings reveal the dissociative involvement of BLA function, on the one hand, in anxiety, which is affected by the knockdown of TrkB, and, on the other hand, in extinction learning, which is more significantly affected by the combination of intra-BLA-induced metaplasticity and exposure to emotional trauma.

Keywords BLA, TrkB, Metaplasticity, Transregional metaplasticity, mPFC, Fear extinction

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Background

Fear is a natural response developed through an evolutionary process to perceive environmental threats. The amygdala, particularly the basolateral amygdala (BLA), is a crucial structure connected with the hippocampus and prefrontal cortex as part of a fear learning and extinction memory circuit [31, 51, 59]. Under emotionally stressful situations, BLA activation is known to regulate synaptic plasticity and memory formation in other brain regions. This phenomenon, termed *emotional tagging*, can modify long-lasting plasticity and memory formation in these regions [12, 67]. Abraham and Bear [2] first coined the term "Metaplasticity" to address the incident of long-lasting modifications in long-term potentiation (LTP) or long-term depression (LTD) due to prior neural activation [2]. As their title indicates, metaplasticity is in fact a form of plasticity, which consequently affects a different form of plasticity ("plasticity of plasticity"). This term was later expanded to include persistent changes that may alter subsequent behavioral responses [3]. Various factors and events, such as behavioral or hormonal stress, environmental stimuli, learning, and memory, may produce changes in cellular or synaptic levels and subsequently induce LTP or LTD [1, 68, 79, 80, 97].

The BLA is a complex network of excitatory and inhibitory neurons that receive sensory information related to threatening stimuli from various brain regions. BLA principal neurons constitute the majority of approximately 80% of the BLA neuronal population (McDonald, [49]), whereas interneurons constitute only approximately 20% of the neurons [48, 50, 73]. GABAergic interneurons regulate activity and plasticity in the BLA. Therefore, modification of the activity of GABAergic interneurons may induce long-term metaplasticity within the BLA [75], as well as changes in activity and plasticity in other connected brain regions, ultimately influencing memory and coping with stress [43, 77, 88].

Tropomyosin receptor kinase B (TrkB) is an abundant cell surface receptor that is distributed throughout the amygdala and is activated mainly by brain-derived neurotrophic factor (BDNF) [63, 94]. TrkB and BDNF are involved in modulating fear learning and maintaining synaptic plasticity in the amygdala [29, 53, 57]. In addition, BDNF/TrkB signaling is essential for consolidating fear memories and fear extinction learning [18, 53, 58, 64]. The activation of TrkB has been shown to facilitate synaptic potentiation and enhance plasticity in various brain regions, including the hippocampus, cortex, and amygdala [36, 42, 56]. Furthermore, TrkB signaling is involved in the induction and maintenance of metaplasticity, such as heterosynaptic potentiation and metaplasticity priming [34, 95]. By regulating the plasticity threshold, TrkB activation can modulate the range and magnitude of subsequent plastic changes, influencing the overall plasticity dynamics within neural circuits. The dual role of TrkB in synaptic plasticity and metaplasticity suggests its importance in shaping the functional properties of neuronal networks. Dysregulation of TrkB signaling has been implicated in various neurological and psychiatric disorders, including Alzheimer's disease, depression, and anxiety disorders [11, 60, 98].

Notably, the TrkB receptor is found on both glutamatergic and GABAergic synapses [22]. The organization and function of glutamatergic synapses become regulated by TrkB-dependent signaling ([55, 96], 24744726). Moreover, a previous study revealed that transmembrane receptors, including TrkB, are involved in the stabilization of gephyrin clusters [93]. Gephyrin is a component of a submembranous scaffold that recruits GABAA receptors into the postsynaptic membrane [86]. Downregulation of these receptors destabilizes preformed gephyrin clusters and the clustering of GABAA receptors in postsynaptic compartments in vitro and in adult animals in vivo. Impaired gephyrin clustering caused by the knockdown of neurofascin, FGFR1, EphA7 or TrkB resulted in the selective loss of presynaptic inhibitory terminals. In these long-term knockdown experiments, the density of excitatory synaptic markers remained unaffected [15, 38, 75, 93]. The consequences for the microcircuitry are highly interesting since, depending on the investigated membrane receptor, compartment-specific elimination of GABAergic synapses on principal neurons was observed. The microcircuitry comprises different classes of interneurons that can be discriminated by several criteria, including the target compartment on principal neurons [30]. Neurofascin knockdown in principal neurons removes GABAergic synapses specifically on axon initial segments, whereas EphA7 knockdown removes GABAergic synapses on somata and proximal dendritic segments [15, 38]. Indirect evidence has shown that, like EphA7 signaling, BDNF–TrkB signaling also accounts for the stabilization of GABAergic synapses on somata [16].

Thus, reducing the expression of TrkB could, in principle, have a dual effect: affecting principal cell plasticity (inducing metaplasticity) and selectively altering BLA local microcircuit activity by destabilizing GABAergic synapses on somata (another form of metaplasticity).

The present study aimed to investigate the specific effects of inducing metaplasticity through TrkB modulation within the BLA on anxiety-like behavior and fear-extinction memory and on neural plasticity in BLA-modulated brain regions. To test this hypothesis, we applied viral vectors for TrkB knockdown in the rat BLA and assessed the effects of this manipulation on anxiety-related behaviors in the open field and elevated plus maze

tests. Furthermore, we tested fear extinction memory in the auditory cued fear conditioning paradigm, both under basal conditions and following acute underwater trauma (UWT) exposure. While cued fear memory acquisition was intact, TrkB-knockdown animals presented impaired extinction memory and increased anxiety-like behavior under both conditions. We further examined the effect of this manipulation on LTP in the vSub-mPFC pathway to determine whether this intra-BLA manipulation resulted in transregional metaplasticity [77]. Indeed, LTP in this pathway, which does not involve the BLA, was altered by intra-BLA TrkB knockdown. Similarly, we investigated the impact of reducing TrkB expression within the BLA on the induction of LTP in another brain region modulated by the BLA, the dentate gyrus, and found that plasticity in this region remained unaltered.

Methods

Lentivirus production and validation

The design and construction of pLenti04C_miCTR/SEW (miCTR) and pLenti04c_mi TrkB/SEW (miTrkB162 and miTrkB1973) were conducted as described in the Supplementary *Information. The* in vitro TrkB knockdown efficiency was evaluated via quantitative transduction of primary cortical neurons and subsequent quantitative RT–PCR as described previously [37]. In vivo validation was performed via a Western blot analysis following TrkB knockdown in the BLA.

(For a detailed description, see the Supplementary details.)

Animals

Male Sprague–Dawley rats (Envigo, Jerusalem, Israel) were procured at postnatal day (PND) 55 and weighed 200–224 g for all the experiments. Upon delivery, the animals were housed in groups of four per cage and acclimatized for 5 days (22 ± 2 °C; 12 h light–dark cycle) in a vivarium with regular food pellets and water ad libitum.

Stereotaxic surgery

At PND60, the rats received bilateral microinjections of lentiviral vectors expressing miRNA against TrkB (miTrkB162) or a control miRNA (miCTR) into the basolateral amygdala (BLA) (coordinates according to Paxinos and Watson 2006 with AP: − 2.8 mm, ML: ± 4.7 mm from bregma; DV: − 7.7 mm from the brain surface). Two microliters of viral vector suspensions were injected (0.15 µl/min) in each hemisphere through a 10 µl Hamilton syringe (30G) connected to a motorized nanoinjector (UMP3 microsyringe pump and Micro4 controller, World Precision Instruments, USA) [26, 77, 85].

Underwater trauma (UWT)

Animals injected with miCTR/miTrkB virus were exposed to the UWT protocol adapted from [10, 26, 72].

Behavioral tests

We conducted two complementary experiments (Fig. 1a). In the first experiment, two weeks after recovery from stereotaxic surgery, the animals in both cohorts (miCTR or miTRKB) were subjected to a behavioral battery (PND 74–81), which included an open field test (OF), an elevated plus maze (EPM), and cued fear conditioning and extinction. The animals were subsequently subjected to in vivo electrophysiological recordings (from PND 82).

In the second experiment, the animals were divided into three groups: the CTR group, which was exposed to underwater trauma (CTR-UWT), and the TrkBKD group, which was exposed to UWT (TrkBKD-UWT). All three groups were subjected to the same behavioral tests as in the first experiment (PND 88–95) and were subsequently subjected to in vivo electrophysiological recordings (from PND 96).

The behavior of the animals in the OF and EPM tests was recorded and then analyzed via the EthoVision XT8 video tracking system (Noldus, Wageningen, Netherlands).

Open field (OF) test

Locomotor activity and anxiety-like behavior were assessed as described previously [9, 26, 85]. The anxiety index was expressed as a percentage.

$$Anxiety\ index = \frac{Distance\ covered\ or\ Time\ explored\ in\ the\ centre}{Total\ distance\ or\ Total\ time} \times 100$$

Elevated plus maze (EPM) test

Anxiety-like behavior was assessed via the EPM test 24 h after the OF test [9, 26, 85]. The anxiety index was expressed as a percentage.

$$Anxiety\ index = \frac{Distance\ covered\ or\ Time\ explored\ in\ the\ open\ arm}{Total\ distance\ or\ Total\ time\ on\ open\ and\ closed\ arm} \times 100$$

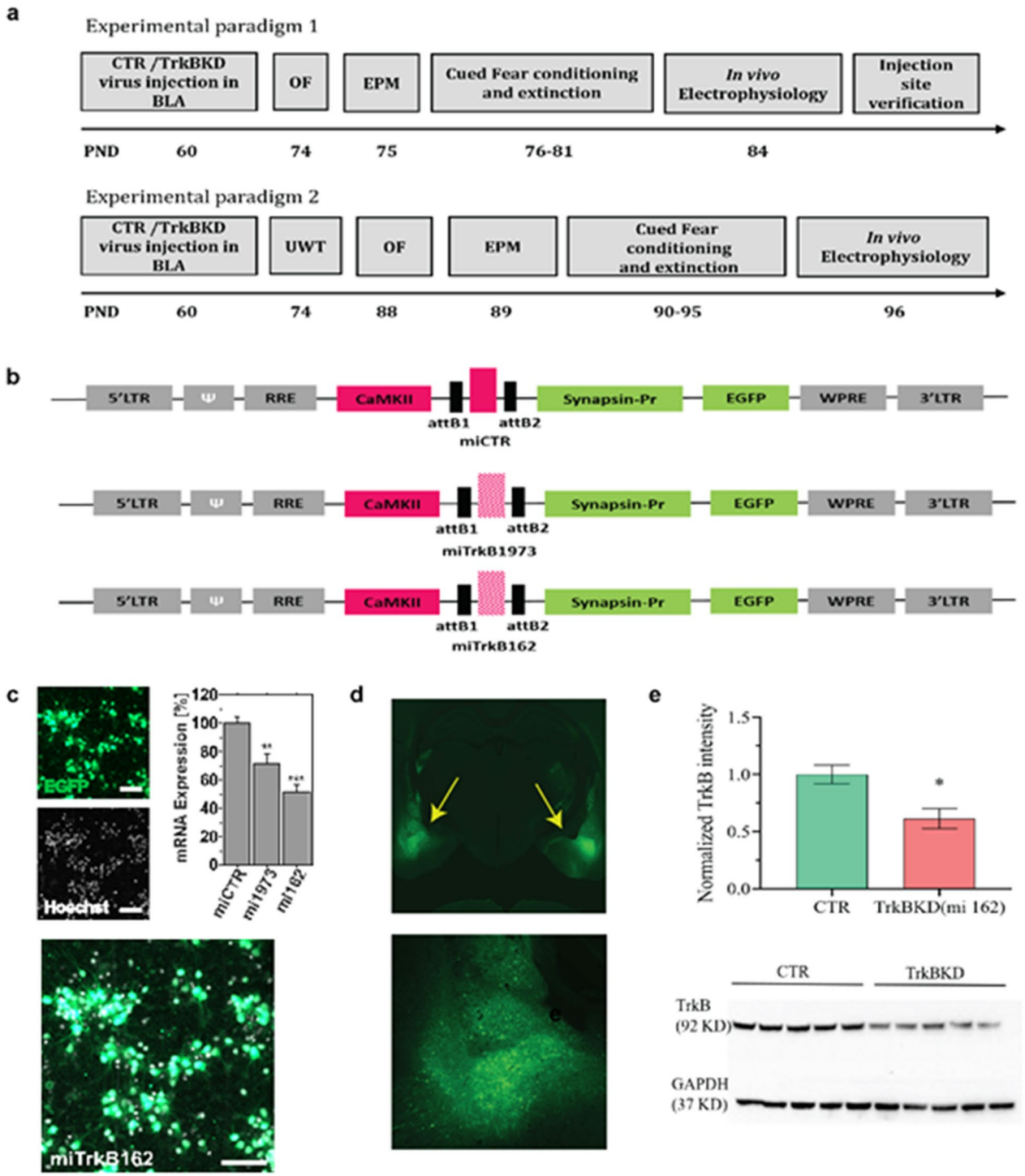


Fig. 1 Validation of TrkB knockdown virus in vitro and in vivo. **a** Schematic illustration of the experimental design. **b** Structure of viral vectors (pLenti04C_miRNA/SEW) used for RNAi induced knockdown in vitro and in vivo. Respective miRNA-oligos are expressed under control of a shortened mouse CaMKII promoter, and EGFP under the control of a rat synapsin promoter to visualize transduced neurons. Ψ, HIV-1 psi packaging signal; *RRE, Rev Response Element; WPRE, Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element*. **c** Evaluation of knockdown efficacy in vitro and EGFP expression in rat primary cortical neurons after viral transduction. Scale bars: 100 µm. **d** In vivo validation: viral vectors (CTR and miTrkB162) were bilaterally injected to the basolateral amygdala. Representative photomicrograph showing confirmation of injection site according to the expression of GFP; scale bar: 500 µm. **e** Western Blot revealed significant reduction of TrkB in the BLA with TrkB162 (n = 5) compared to CTR (n = 5)

Cued fear conditioning and extinction test

Cued fear conditioning and extinction were carried out 24 h after the EPM test in a sound debilitating chamber (Panlab, Harvard Apparatus, Barcelona, Spain), which contained either Context A or Context B [77, 89]. The animals were allowed to habituate for 2 consecutive days in context B for 10 min each day. Fear conditioning took place on Day 3 in Context A, and extinction training was carried out 24 h after the conditioning test for 3 consecutive days in Context B. Freezing, i.e., the absence of all movement (except respiration), was measured offline via Packwin Software (Panlab, Harvard Apparatus, Barcelona, Spain) during the CS presentation. Extinction analysis considers the average of a response during the presentation of two CSs as a 'Block'.

Behavioral profiling

Profiling of the animals as "affected" or "unaffected" was carried out according to the Behavioral Profiling approach [9, 26, 65, 85]. Behavioral profiling was performed on the basis of the behavioral parameters from the OF and EPM tests (Supplementary Table 1) for CTR and TrkBKD animals in both the UWT-exposed and nonexposed groups. Animals were characterized as "affected" if they exhibited behavioral deviations in at least 60% of all behavior parameters; animals that did not fall into this category were described as "unaffected" or "stress resilient.".

In vivo electrophysiology and long-term potentiation (LTP)

Recording and induction of LTP in the medial prefrontal cortex-ventral subiculum pathway (mPFC-vSub) or the dentate gyrus-perforant pathway (DG-PP) were carried out as described previously [26, 77, 85].

Brain removal and histology

Brain samples were collected either 3 weeks after virus injection or immediately after the completion of the behavioral experiments (for a detailed description, see the supplementary information).

Western blot analysis

For a detailed description, see the supplementary information.

Statistical analysis

The data were analyzed via IBM SPSS (21) statistical software (IBM, Armonk, NY, USA). All behavioral, electrophysiological, and immunoblot results were analyzed via independent sample t tests, one-way ANOVA, or repeated-measures ANOVA followed by post hoc Bonferroni correction, as indicated. For behavioral profiling, Pearson's chi-square test was used. Appropriate Greenhouse–Geisser or Huynh–Feldt corrections for sphericity issues were applied when necessary. All the results are presented as the means ± SEMs.

Results

In vitro validation of TrkB knockdown

To precisely remove TrkB from projection neurons in the BLA, we designed lentiviral vectors harboring a truncated CAMKII promoter to express miRNAs directed against *Ntrk2* transcripts or a control miRNA. Lentiviral vectors coexpressed green fluorescent protein (EGFP) controlled by the synapsin promoter to enable the identification of injection sites in the brain (Fig. 1b). To determine the efficacy of the lentiviral construct used for the miRNA-mediated knockdown of TrkB, we quantitatively transduced primary cortical rat neurons with the viral suspensions (Fig. 1c). Compared with those of the control miRNA (CTR-transduced culture), the expression levels of the corresponding TrkB mRNAs were markedly lower, as measured by quantitative RT–PCR, suggesting highly efficient suppression of TrkB mRNA expression (Fig. 1c). The TrkB162 miRNA (miTrkB162) appeared to be more effective than the TrkB1973 miRNA (miTrkB1973). Off-target effects were ruled out after the expression of miTRKB1973 and miTRKB162 in infected cells in vivo [14]. Thus, for the in vivo study, we further used the miTrkB162 construct only.

In vivo validation of TrkB knockdown

To validate the reduction in protein levels caused by TrkB knockdown, we performed Western blot analysis two weeks after viral injection. Only animals with correct injection sites on both hemispheres were included in the analysis, as ascertained by the selective expression of GFP in the BLA (Fig. 1d). Local injection of TrkB miRNA into the BLA resulted in 49% knockdown of endogenous TrkB protein expression compared with that in miCTR animals (Fig. 1e $t=3.257$, $df=8$; $p=0.012$).

Effect of selective TrkB knockdown in the BLA under basal conditions

BLA TrkB knockdown increased anxiety-like behavior without affecting locomotor activity

Two weeks after recovery from surgery, the locomotor activity of the CTR (n = 16) and TrkBKD (n = 22) groups was tested via an open field test (OF). TrkBKD did not affect locomotor activity, as analyzed by the total distance moved (Fig. 2a, $t(36)=0.467$, $p>0.05$). Anxiety-like behavior measured by the anxiety index of the distance between the periphery and center also remained unchanged (Fig. 2a, $t(36)=1.458$, $p>0.05$).

Next, anxiety-like behavior in both groups, CTR (n = 16) and TrkBKD (n = 22), was tested in the elevated

plus maze (EPM). Compared with the CTR group, the TrkBKD group presented a significant reduction in the total distance traveled in the arena (Fig. 2b, t (36) = 3.014, p < 0.01). Additionally, anxiety-like behavior increased, which was represented by a reduction in the anxiety index of the distance between the open and closed arms (Fig. 2b, t (36) = 5.028, p < 0.001). Taken together, these data suggest that viral knockdown of TrkB in the BLA resulted in increased anxiety-like behavior without affecting locomotor activity.

TrkB knockdown in the BLA increased the prevalence of "affected" individuals

After analyzing the behavioral group averages in the described tests (OF and EPM) and with the aim of examining the impact on the behavioral responses of individual animals, we applied the behavior profiling approach to identify "affected" and "unaffected" individuals, as previously reported [9, 65]. Seventeen key parameters from the OF and EPM tests were examined to identify stress-vulnerable and stress-resilient animals (Supplementary Table 1). For each behavioral parameter, a standard range was calculated as within the cutoff threshold values of either the 20th or 80th percentile of the average performance of the CTR group [6, 9]. Animals that deviated in at least 60% of all behavior parameters from that standard range were characterized as "affected" or "stress vulnerable". Comparisons between the CTR and TrkBKD groups via Pearson χ^2 analysis revealed a significantly greater proportion of "affected" animals in the TrkBKD group (64%) than in the CTR group (6%) ($X_2 = 12.77$, p < 0.001). (Fig. 2c).

Selective TrkB knockdown within the BLA did not alter the acquisition of conditioned fear but impaired fear extinction learning

After the basal anxiety level was assessed, the CTR (n = 16) and TrkBKD (n = 22) groups underwent auditory-cued fear conditioning. First, the effects of TrkB knockdown on the acquisition of conditioned fear were tested. All the animals in both the CTR and TrkBKD groups presented low preconditioned basal freezing levels in context A (Supplementary Figure S2n, t (36) = 0.160, p > 0.05). During the training phase, freezing increased over the three CS–US pairings in both groups, as analyzed by repeated-measures ANOVA (Fig. 2d, effect of tones: F (1.337, 48.126) = 485.001, p = 0.001; no group × tone interaction F (1.337, 48.126) = 0.787, p = 0.414; no group effect F (1,36) = 9.17, p = 0.122 Huynh–Feldt correction). Moreover, acquired fear was retained by all the animals and showed a similar level of freezing when tested after 24 h during the first 5 CS presentations at the beginning of the extinction training (Supplementary figure S2o, t (36) = 2.009, p > 0.05). Collectively, these data showed that intra-BLA TrkB knockdown did not affect fear learning or memory.

The following day, and every three consecutive days, a three-day extinction training paradigm was performed in context B. Each day, all the animals were exposed to 5 blocks of two CSs (Fig. 2e). Repeated-measures ANOVA revealed no significant difference between the groups on the first day of extinction (main group effect: F(1,36) = 2.501, p = 0.123; no block×group interaction: F(1.602,57.686) = 0.724, p = 0.46 Huynh-Feldt correction). Starting from day 2, a significant difference between the groups was observed (main group effect: F(1,36) = 8.888, p = 0.01; no block×group interaction: F(3.669, 132.088) = 1.575, p = 0.189 Huynh-Feldt correction), which increased on the 3rd day of extinction (main group effect: F(1,36) = 19.788, p < 0.001; block×group interaction: F(3.826, 137.725) = 9.497, p < 0.001 Huynh-Feldt correction). The intersession analysis (comparing the last CS block of a day with the first CS block of the session on the following day) did not reveal any group differences from day 1 to day 2 (main group effect: F(1,36) = 0.001, p= 0.989; block×group interaction: F(1, 36) = 0.151, p = 0.699 Huynh-Feldt correction). However, a significant group difference was found in the transition from day 2 to day 3 (main group effect: F(1,36) = 6.162, p = 0.018; block×group interaction: F(1, 36) = 0.784, p = 0.382 Huynh-Feldt correction). Further analysis of the total freezing percentage from day 1 to day 3 revealed reduced extinction in TrkBKD animals compared with CTR animals (Fig. 2f–h; day 1, t (36) = 1.923, p > 0.05;

(See figure on next page.)

Fig. 2 TrkB knockdown within BLA showed higher proportion of affected individuals and impaired fear extinction memory while cued fear conditioning remained unaltered. (**a**) Total distance moved in the open field arena and anxiety index of distance did not differ between the CTR (n = 16) and TrkBKD (n = 22) groups. **b** Total traveled distance and anxiety index of distance in the elevated plus maze significantly different between the CTR and TrkBKD groups. **c** Behavioral profiling based on anxiety-like behavior in OF and EPM revealed an increased proportion of 'affected' rats in the TrkBKD group compared to the CTR group. Values are the % 'unaffected' and 'affected' animals in each group. **d** Acquisition of fear to the auditory conditioned stimulus (CS) paired with shock, measured as % freezing to the CS, did not differ between CTR (n = 16) and TrkBKD (n = 22) groups. **e** Difference in freezing behaviorduring the extinction training showed reduced levels for freezing in CTR compared to TrkBKD group. **f**–**h** Total freezing (%) was gradually decreased from day 1 to day 3 in CTR animals compared to TrkB knockdown animals. Data displayed as means ± SEMs, *p < 0.05, **p < 0.01, ***p < 0.001

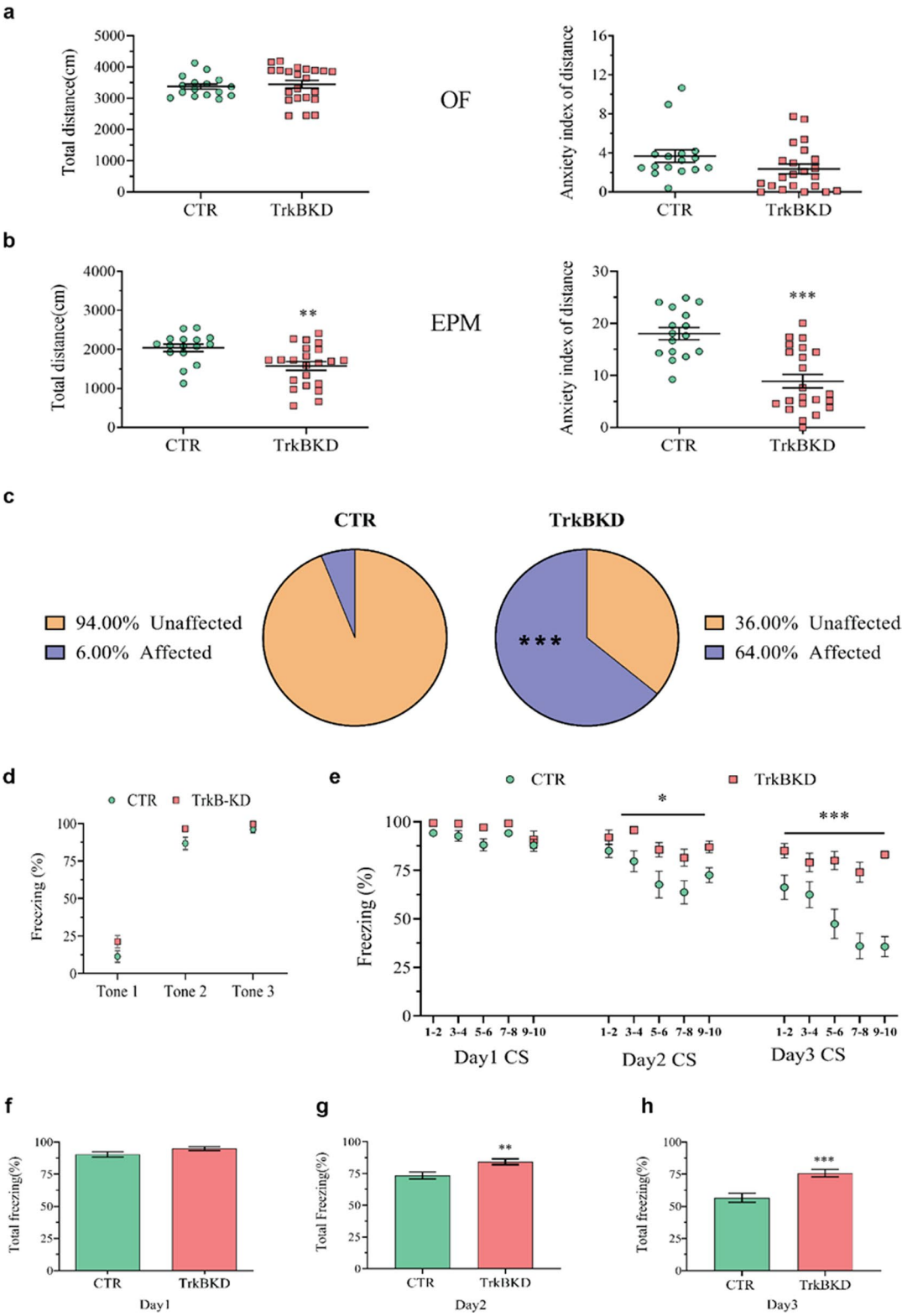


Fig. 2 (See legend on previous page.)

day 2, t (36) = 3.044, p < 0.01; and day 3, t (36) = 4.330, p < 0.001). Taken together, these data suggest that the knockdown of TrkB affects cued fear extinction learning.

Selective knockdown of TrkB in the BLA impairs LTP in the vSub-mPFC pathway

Twenty-four hours after the last behavioral test, LTP induction in the vSub- mPFC pathway was assessed. The vSub-mPFC pathway is a key circuit for fear extinction. Importantly, it is a BLA-independent, monosynaptic pathway [47, 68]. There was no significant difference in the input–output curve between the CTR and TrkBKD groups, indicating that there was no baseline difference in excitability. Additionally, no significant difference in the baseline EPSP slope was found prior to theta-burst stimulation (TBS) (Fig. 3a). TBS was applied to the vSub, and evoked field potentials were recorded in the mPFC. Sixty minutes after TBS, LTP was observed in the CTR animals, whereas no potentiation was evident in the TrkBKD group (Fig. 3b). Mixed model repeated-measures ANOVA revealed a significant difference in the EPSP slope between the two groups (group effect $F_{(1, 12)} = 20.59$, $p < 0.001$). Significant differences were also found in the time × group effect ($F_{(2.734, 32.805)} = 7.086$, $p = 0.001$ Greenhouse–Geisser correction). Furthermore, the average potentiation across time was significantly lower in TrkBKD animals than in CTR animals ($t = 4.537$, $p < 0.001$) (Fig. 3b).

To differentiate whether the preceding behavioral tests affected LTP or whether LTP in the vSub-mPFC pathway was influenced irrespective of the behavioral tests, we assessed the ability to induce LTP in this pathway without prior behavioral tests in another set of animals. Three weeks after BLA modulation with TrkBKD, the animals were subjected to electrophysiology experiments. TBS stimulation in the vSub resulted in LTP in the CTR group but was impaired in the TrkBKD animals, similar to the groups that were previously subjected to behavioral tests (Fig. 3c). Mixed model repeated-measures ANOVA revealed a significant difference in the EPSP slope between the CTR and TrkBKD groups (group effect $F_{(1, 6)} = 30.214$, $p = 0.002$) but no effect on the time × group interaction ($F_{(1.985, 11.908)} = 1.495$, $p = 0.263$ Greenhouse–Geisser correction). Furthermore, the average potentiation across time was significantly lower in the TrkBKD group than in the CTR group ($t = 5.497$, $p < 0.01$) (Fig. 3c), indicating that the impairment of LTP in the mPFC was a result of the modulation of TrkB in the BLA and not due to the interaction with the behavioral tests.

Selective knockdown of intra-BLA TrkB did not alter plasticity in the hippocampus

Another brain region in which plasticity is modulated by the BLA is the hippocampus [90, 91]. We thus also examined the impact of intra-BLA knockdown on LTP via the perforant path-dorsal dentate gyrus (PP-DG) pathway. The experiments started 24 h after the last behavioral test. There were no significant differences in the input–output curves or baseline recordings before TBS between the CTR and TrkBKD groups. Upon TBS, evoked field potentials in the DG were effectively potentiated in both the CTR and TrkBKD groups (Fig. 3e). Mixed model repeated-measures ANOVA revealed no significant differences between groups (group effect $F_{(1, 5)} = 0.162$, $p > 0.05$) and no time × group effect ($F_{(1.936, 9.679)} = 1.251$, $p > 0.05$ Greenhouse–Geisser correction). An independent t test revealed no significant differences in average potentiation across time after induction or between the groups ($t = 5.497$, $p > 0.05$) (Fig. 3e).

Coping with acute trauma exposure following selective TrkB knockdown within the BLA

Two weeks after surgery, we divided all the animals into three groups: one CTR group was not subjected to acute trauma, whereas the other two groups (CTR-UWT and TrkBKD-UWT) were subjected to acute underwater trauma. All the animals underwent a battery of behavioral tests two weeks later (Fig. 1a, experimental paradigm 2).

(See figure on next page.)

Fig. 3 TrkB knockdown results impaired LTP in the medial prefrontal cortex without affecting the synaptic plasticity in the hippocampus **a** Representative evoked field potential responses recorded in the mPFC of rats injected with CTR (n = 6) and TrkBKD (n = 8) groups before (black) and after theta burst stimulation (TBS) (gray dotted) of the vSub to induce LTP in the mPFC. **b** TBS to the vSub pathway induced a significant potentiation of mPFC field potentials in CTR animals, which was reduced in TrkBKD group. A significant difference in average percentage of LTP after TBS was found in the mPFC between CTR and TrkBKD groups. **c** Without prior behavioral tests, TBS of the vSub pathway induced a significant potentiation of mPFC field potentials in CTR (n = 4) animals, which was reduced in TrkBKD (n = 4) group (left); changes in average percentage of LTP after TBS in CTR and TrkBKD groups. **d** Representative evoked field potential responses recorded in the dentate gyrus of rats injected with CTR (n = 3) and TrkBKD (n = 5) group before (black) and after theta burst stimulation (TBS) (gray dotted) in the perforant pathway to induce LTP in the DG **e** After TBS to the perforant path both groups displayed a similar level of potentiation in the DG; percentage of average LTP in DG remained unaltered after TBS in CTR and TrkBKD groups. (Data are shown as means ± SEMs. ***p < 0.001)

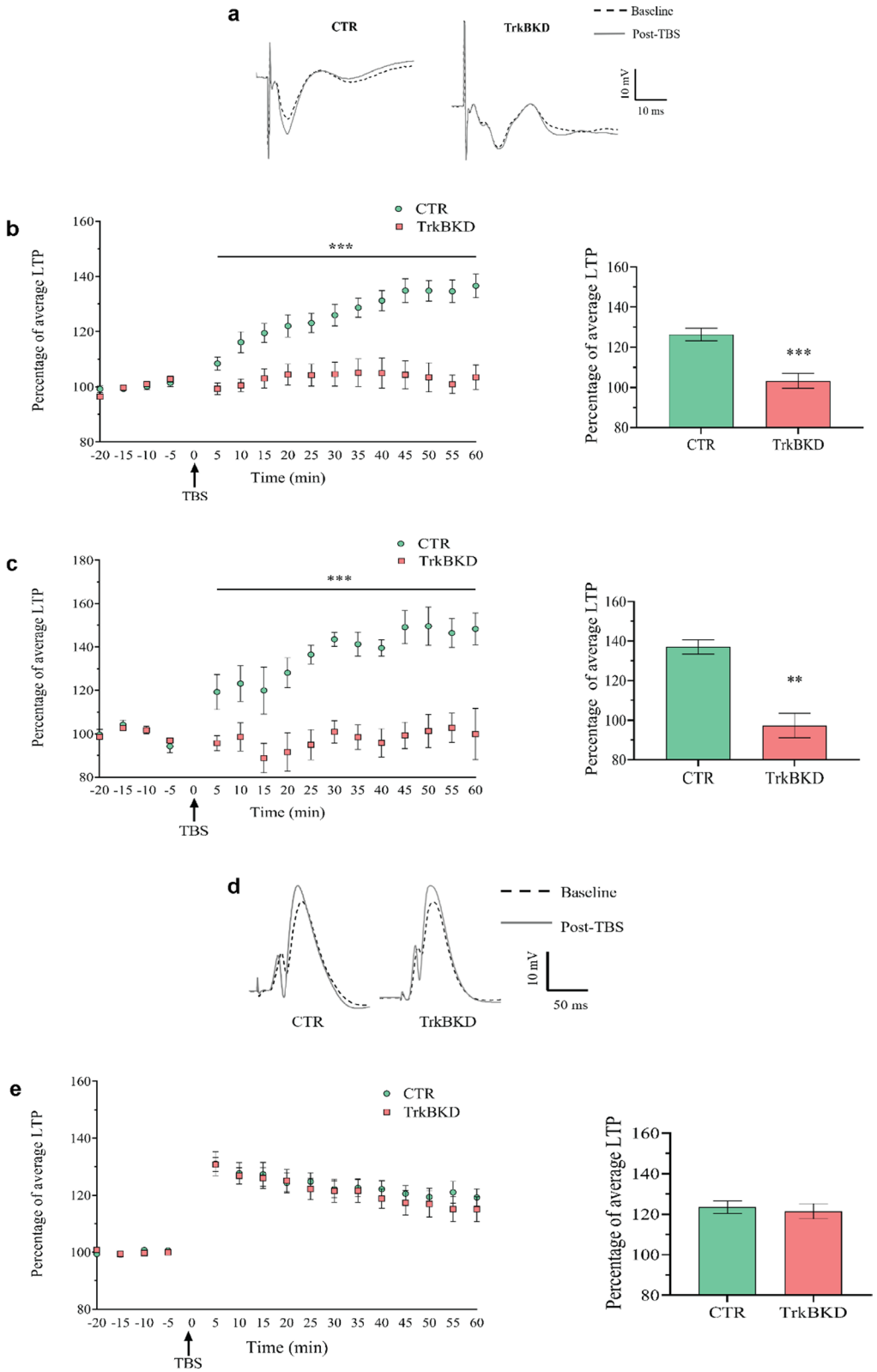


Fig. 3 (See legend on previous page.)

TrkB knockdown in the BLA did not contribute to the effect of acute trauma exposure on anxiety-like behavior.

To assess locomotor activity, we assessed the behavior of the CTR (n = 11), CTR-UWT (n = 15), and TrkBKD-UWT (n = 15) groups in the OF. One-way ANOVA revealed no significant effect of group on the total distance traveled in the OF test (Fig. 4a, $F_{(2,\ 38)} = 1.293$, $p = 0.286$). Furthermore, the OF anxiety index (for distance) revealed no significant effect between groups (Fig. 4a, $F_{(2,\ 38)} = 1.390$, $p = 0.261$).

All the groups were subsequently subjected to the EPM test. Traumatic stress exposure significantly reduced the total distance traveled in the arena (Fig. 4b, $F_{(2,\ 38)} = 10.13$, $p < 0.001$) in both the CTR-UWT (post hoc Bonferroni correction, $p < 0.01$) and TrkBKD-UWT ($p < 0.001$) groups compared with the CTR group, with no significant difference between the CTR-UWT and TrkBKD-UWT groups ($p > 0.05$). One-way ANOVA revealed a reduced anxiety index (for distance), suggesting enhanced anxiety-like behavior in trauma-exposed animals (Fig. 4b, $F_{(2,\ 38)} = 13.99$, $p < 0.001$). A post hoc Bonferroni correction revealed highly significant increases in anxiety levels in the CTR-UWT ($p < 0.001$) and TrkBKD-UWT ($p < 0.001$) groups compared with the CTR group, with no significant difference between the CTR-UWT and TrkBKD-UWT groups ($p > 0.05$). Overall, these findings indicate that anxiety-like behavior, as assessed via the EPM test, did not increase following both UWT and selective intra-BLA-mediated TrkB knockdown compared with the anxiety levels observed following UWT exposure alone.

TrkB knockdown in the BLA did not contribute to the effect of acute trauma exposure, as expressed by the proportion of affected individuals

The performance in the OF and EPM tests was then utilized for behavioral profiling analysis to distinguish affected and unaffected individuals, as described previously [9].

UWT significantly decreased resilience in the CTR-UWT group, and behavioral profiling revealed a significantly greater proportion of affected animals in that group (80%) than in the control group (0%) (Pearson $\chi 2$ analysis, $X_2 = 16.34$, $p < 0.001$). Similarly, the targeted knockdown of TrkB in the BLA combined with UWT significantly increased the proportion of affected animals in the TrkBKD-UWT group (87%) compared with that in the control group ($X_2 = 19.07$, $p < 0.001$) (Fig. 4c). However, the combination of targeted knockdown of BLA TrkB and UWT did not significantly differ from that of the CTR-UWT group. The percentages of the unaffected populations were 20% for the CTR-UWT group and 13% for the TrkBKD-UWT group.

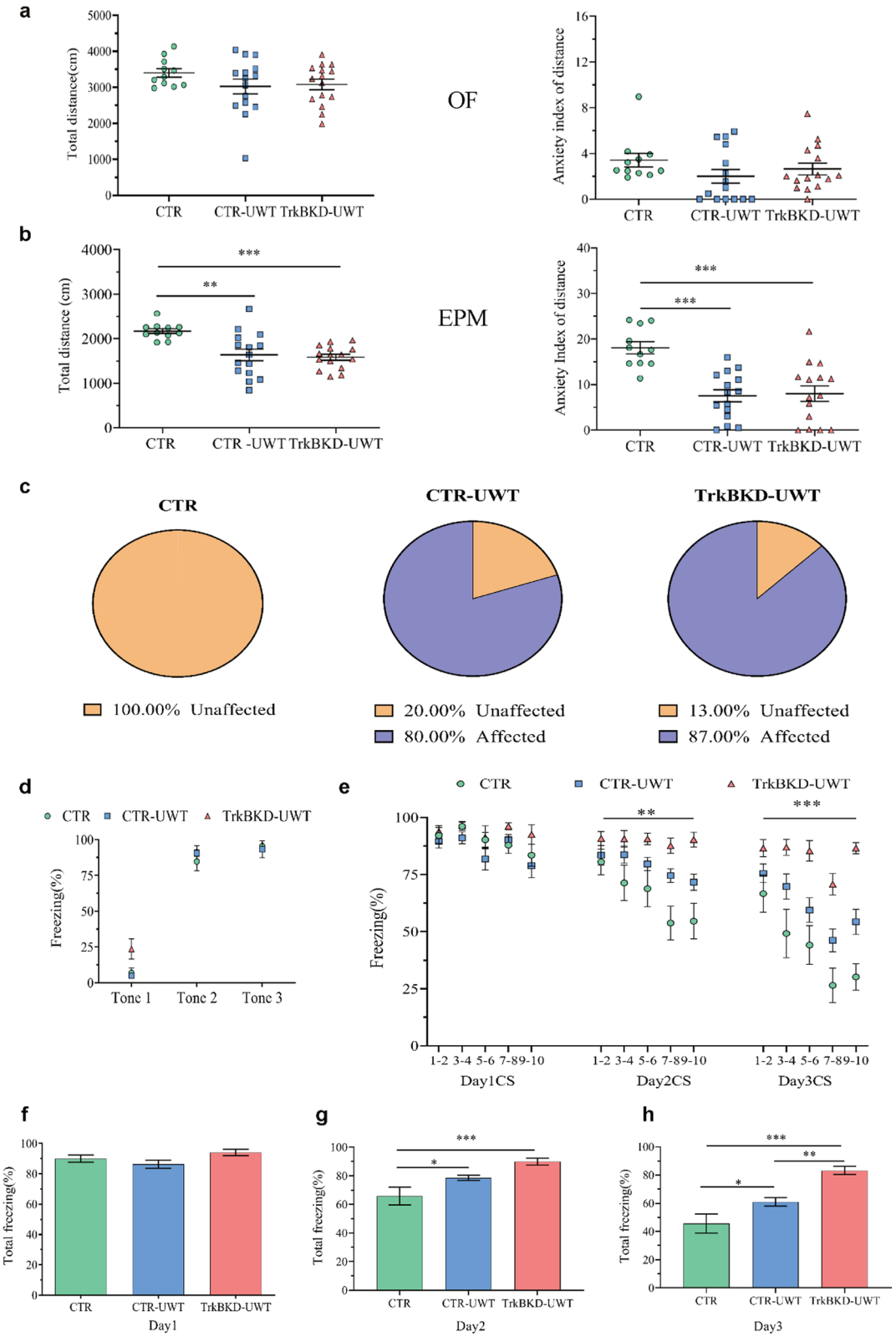
Combining the UWT with BLA TrkB knockdown amplifies fear extinction impairment.

During the fear conditioning training day, animals in the CTR (n = 11), CTR-UWT (n = 15), and TrkBKD-UWT (n = 15) groups presented similar low preconditioning freezing levels in context A (Supplementary Figure S4n, one-way ANOVA $F_{(2,\ 38)} = 1.281$, $p = 0.289$). During the training phase, all groups displayed increasing levels of freezing upon delivery of the three CS–US pairings. A repeated-measures ANOVA revealed a significant group effect ($F(2,38) = 3.355$, $p = 0.046$) and effect of tones ($F(2,76) = 344.056$, $p = 0.001$) but no group × tone interaction ($F(4,76) = 1.656$, $p = 0.169$ Huynh–Feldt correction). Post hoc comparisons revealed no significant differences between the groups (Fig. 4d). Additionally, all groups retained fear even after 24 h, as examined during the first 5 CS presentations at the beginning of extinction training (Supplementary figure S4o, $F_{(2,\ 38)} = 1.029$, $p = 0.367$).

Extinction training spanning three days started the next day with context B (Fig. 4e). Repeated-measures ANOVA revealed no significant difference between the groups on the first day (main group effect $F(2,38) = 2.839$, $p = 0.071$; no block × group interaction $F(6.403,\ 121.656) = 0.858$, $p = 0.534$; Huynh–Feldt correction). On day 2, the freezing percentage was significantly lower in CTR animals than in both trauma-exposed groups (main group effect $F(2,38) = 11.294$, $p = 0.001$; block × group interaction $F(6.250,\ 118.751) = 2.625$, $p = 0.019$); post hoc: CTR vs.

(See figure on next page.)

Fig. 4 Exposure to underwater trauma following knockdown of TrkB in the BLA significantly increased prevalence of affected individuals and impaired fear extinction memory without affecting the conditioning of fear. **a** Total distance traveled in the open field arena and anxiety index of distance did not differ between the CTR (n = 8), CTR-UWT(n = 15) and TrkBKD-UWT (n = 15) groups. **b** Total distance moved and anxiety index of distance in the elevated plus maze significantly differ between the CTR, CTR-UWT and TrkBKD-UWT groups. **c** Behavioral profiling based on anxiety-like behavior in OF and EPM revealed an increased proportion of 'affected' rats in the trauma-exposed groups (CTR-UWT & TrkBKD-UWT) compared to the CTR group. Values are the % 'unaffected' and 'affected' animals in each group. **d** Acquisition of fear to the auditory conditioned stimulus (CS) paired with shock, measured as % freezing to the CS, did not differ between CTR (n = 8), CTR-UWT (n = 15) and TrkBKD-UWT (n = 15) groups three groups (**e**) Freezing difference during the extinction training showed reduced level for freezing in CTR compared to trauma exposed (CTR-UWT & TrkBKD-UWT) groups (**f**–**h**) Total freezing (%) was gradually decreased from day 1 to day 3 in CTR animals compared to trauma-exposed animals. Data are shown as means ± SEMs, *p < 0.05, **p < 0.01, ***p < 0.001



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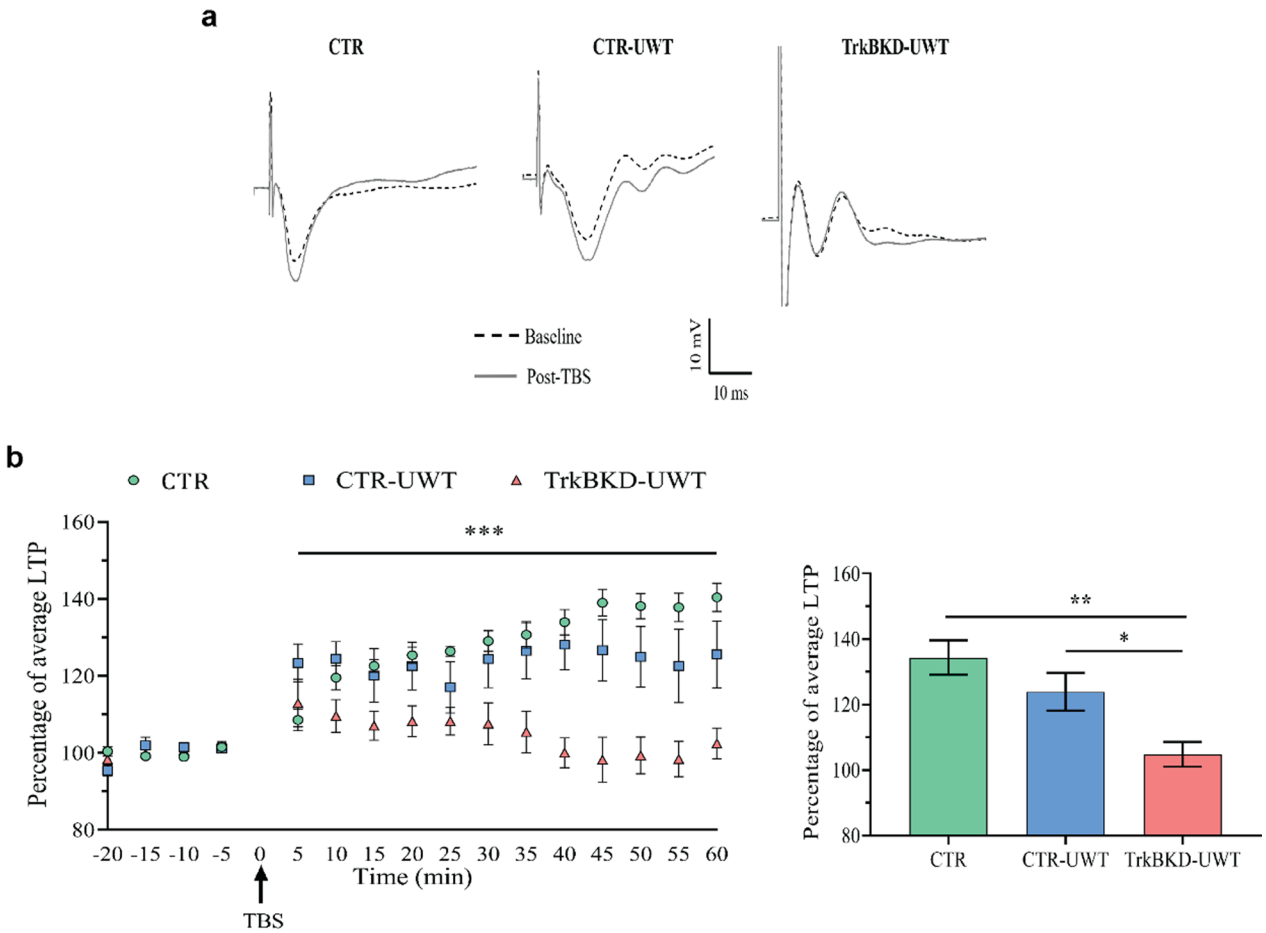

Fig. 5 TrkB knockdown in combination with underwater trauma resulted in impaired synaptic plasticity in the vSub–mPFC pathway. **a** Representative evoked field potential responses recorded in the mPFC of rats injected with CTR (n = 5), CTR-UWT(n = 5) and TrkBKD-UWT (n = 6) groups before (black) and after theta burst stimulation (TBS) (gray dotted) in the vSub to induce LTP in the mPFC **b** TBS to the vSub pathway induced a significant potentiation of mPFC field potentials in CTR animals, which was reduced in CTR-UWT and TrkBKD-UWT groups; changes in average percentage of LTP after TBS in CTR and trauma-exposed (CTR-UWT and TrkBKD-UWT) groups. Data are shown as means ± SEMs, *p < 0.05, **p < 0.01, ***p < 0.001

CTR-UWT $p < 0.05$; CTR vs. TrkBKD-UWT $p < 0.001$), an effect that was still observable on the third day (main group effect $F(2,38) = 20.299$, $p < 0.001$; block × group interaction $F(7.720, 146.686) = 3.362$, $p = 0.002$); post hoc: CTR vs. CTR-UWT $p < 0.05$; CTR vs. TrkBKD-UWT $p < 0.001$). One-way ANOVA of the total freezing percentage from day 1 to day 3 revealed reduced extinction in the trauma-exposed groups compared with the CTR groups (Fig. 4f–h; day 1, $F(2,38) = 2.840$, $p > 0.05$; day 2, $F(2,38) = 11.20$, $p < 0.001$; and day 3, $F(2,38) = 20.30$, $p < 0.001$). Post hoc comparisons revealed significant differences on days 2 and 3 between the CTR group and both the CTR-UWT and TrkBKD-UWT groups. Notably, post hoc comparison on day 3 revealed a significant difference between the CTR-UWT and TrkBKD-UWT groups, indicating that the knockdown of TrkB exacerbated the effect of UWT on fear extinction learning.

Combining the UWT with BLA TrkB knockdown impaired (transregional) LTP in the mPFC

There were no significant differences in the input–output curves or baseline recordings between the groups. While applying TBS stimulation to the vSub induced LTP in the CTR group (Fig. 5a), no significant changes were observed between the CTR and CTR-UWT groups, indicating that exposure to UWT alone did not result in any significant effect on LTP. However, in the TrkBKD-UWT group after TBS stimulation, no EPSP slope potentiation was evident, and after 60 min, the EPSP slope did not differ from that at baseline (Fig. 5b). Repeated-measures ANOVA revealed a significant group effect ($F_{(2,14)} = 10.148$, $p = 0.002$; time × group effect $F_{(6.558,42.627)} = 5.055$, $p < 0.001$ Greenhouse–Geisser correction). Post hoc analysis revealed a significantly impaired EPSP slope in the TrkBKD-UWT group

compared with the CTR ($p < 0.01$) and CTR-UWT groups ($p < 0.05$). Moreover, the comparison of average potentiation across time after TBS induction also revealed a significant difference among the three groups (one-way ANOVA, $F_{(2,14)} = 9.672$, $p = 0.002$): post hoc Bonferroni CTR vs. TrkBKD-UWT ($p < 0.01$) and CTR-UWT vs. TrkBKD-UWT ($p < 0.05$) (Fig. 5b).

This impairment of LTP in the BLA-independent vSub-mPFC pathway provides additional evidence supporting the idea that targeted TrkB knockdown in the BLA induces transregional alterations within the mPFC, which affects the ability to induce synaptic plasticity in a direct monosynaptic pathway in that region, independent of the BLA.

Discussion

In the present study, we investigated the impact of intra-BLA metaplasticity induced by selective TrkB knockdown on anxiety-like behavior, fear memory, and neural plasticity in brain regions that are involved in emotional processing, in which activity and plasticity are known to be modulated by the BLA.

In our study, we have chosen to drive miRNA expression targeting TrkB through a shortened CamKII promoter which was previously shown to account for specific transgene expression in glutamatergic neurons (e.g. [78]). This view was recently challenged by another report showing that the CamKII promoter is also active in inhibitory neurons (Veres et al. [87]). While we cannot formally rule out an impact of TrkB knockdown in inhibitory neurons, a demonstration of functional relevance remains elusive.

TrkB, a member of the neurotrophin receptor tyrosine kinase family, has been recognized for its crucial role in the regulation of amygdala fear circuitry and plasticity [44]. The activation of TrkB by its ligand BDNF has been implicated in various aspects of fear-related processes, including fear acquisition, consolidation, and extinction [41, 53], as well as in the modulation of synaptic plasticity [23, 32]. Studies have shown that TrkB signaling within the amygdala is involved in fear learning and memory formation, while the inhibition of TrkB activity impairs fear conditioning and attenuates fear-related behaviors [18, 64], highlighting the importance of TrkB in mediating fear responses. Moreover, conditional knockout of TrkB in specific amygdala subregions, such as the basolateral amygdala (BLA), has revealed deficits in fear memory extinction [41]. We have not observed effects of TrkB KD on fear learning and memory, but did observe significant effects on fear extinction. The differences may reflect differences in protocols and manipulations, but taken together, these findings support a role of BDNF and TrkB within the amygdala in regulating fear learning, memory and in particular, fear extinction.

Furthermore, TrkB signaling contributes to the modulation of synaptic plasticity and metaplasticity, influencing the plasticity states of synapses within the amygdala. Metaplasticity serves as a gating mechanism that regulates the threshold and extent of subsequent synaptic plasticity. The activation of TrkB has been shown to induce metaplasticity in the amygdala, resulting in increased synaptic potentiation and plasticity [92]. Conversely, inhibition of TrkB activity leads to reduced plasticity and altered neuronal function [8]. These findings highlight the dual role of TrkB in both synaptic plasticity and metaplasticity within the amygdala.

Depression and anxiety-like behaviors are reported to develop due to reduced BDNF and TrkB expression in various brain regions [7]. In mice, the absence of active full-length TrkB receptors in newly synthesized neurons increases anxiety-like behavior [13]. TrkB-BDNF pathway inhibition in early life may give rise to depressive and anxiety-like symptoms in adulthood [62]. The administration of a TrkB agonist, 7,8-dihydroxyflavone (7,8-DHF), has been shown to reduce anxiety-like behaviors in animal models [17, 61]. Conversely, inhibition of TrkB signaling, either through genetic manipulation or pharmacological blockade, has been associated with increased anxiety-like behaviors [71]. In line with these findings, our results also indicate that a reduced level of TrkB in the BLA leads to increased anxiety-like behavior.

Because of the high individual variability in response to stress and trauma, searching for neural mechanisms associated with stress-related psychopathology or resilience requires identifying and differentiating between 'affected' and 'unaffected' individuals [69]. Toward that end, the behavioral profiling analysis approach was developed, enabling the separation of affected from unaffected animals and the identification of mechanisms that would have otherwise been masked by the analysis of the averaged exposed group responses [6, 9, 19, 65]. In the present study, we assessed the effects of TrkB knockdown in the BLA on the proportion of animals falling into the category of being 'affected', despite not being exposed to a particular trauma, by profiling analysis [9]. Overall, compared with the proportion of animals injected with the control virus, the behavioral profile revealed an increased percentage of affected individuals following TrkB knockdown in the BLA.

Sex-related differences significantly contribute differences is response to stress and trauma in both humans and animals [52, 70, 82]. In that respect, a limitation of the current findings is that only male rats were examined, and it would be important in future studies to examine also females. However, in this context, it is pertinent to

indicate that there are also relevant similarities. Both males and females are sensitive to pre-trauma factors such as childhood adversities, which may impact resilience later in life [33, 35], though the specific neural mechanisms underlying these effects could differ [39, 52, 82].

The amygdala acts as a critical structure in the acquisition, storage, and expression of conditioned fear memory [40, 74], and the activity of the BLA is considered to contribute to the initial acquisition of fear extinction memory [31]. TrkB knockdown in the BLA did not alter cued fear memory acquisition or consolidation. However, it did lead to impaired fear extinction memory, a result that aligns with previous research demonstrating the involvement of BDNF–TrkB signaling in the plasticity processes underlying fear extinction [18, 28]. Furthermore, it was previously demonstrated that animals with reduced TrkB expression display normal fear responses during fear conditioning, but they exhibit impaired fear extinction [8]. These effects could result from the impact of reduced TrkB on excitatory or inhibitory aspects of BLA activity. In vivo knockdown of GAD67 in the amygdala impaired fear extinction [27]. Previous findings in our laboratory also showed that modification of specific BLA GABAergic synapses altered fear extinction memory without affecting fear acquisition and consolidation [75].

The neural network for fear and extinction memory formation includes a close association between the amygdala, hippocampus, and prefrontal cortex [21, 31, 45, 46]. Numerous studies have established that the mPFC acts as a key region in fear extinction [25, 54]. BLA fear neurons project specifically to the prelimbic cortex (PL), but extinction neurons are connected to the infralimbic cortex (IL). This network activity of the BLA to the PL or IL is responsible for the expression of fear and extinction memories [84]. Synaptic potentiation within the BLA-mPFC pathway is essential for normal extinction of fear [89]. We previously showed that manipulating BLA-specific GABAergic synapses can affect fear extinction memory and alter synaptic plasticity in the BLA-mPFC pathway [77]. The BLA is reciprocally connected with both the mPFC and the ventral hippocampus. BLA priming can block the induction of LTP in the vSub-mPFC pathway [68]. However, chronic modulation of GABAergic synapses within the BLA can impair LTP induction in the vSub-mPFC pathway, which is BLA independent [77]. This result implies that long-term modulation within the BLA may induce transregional metaplasticity in related regions, such as the mPFC, further leading to reduced plasticity in the mPFC input from the vSub [77]. The current results further support the existence of this novel form of metaplasticity—transregional metaplasticity—affecting mPFC plasticity and related behaviors.

To verify whether the impact of intra-BLA TrkB knockdown on plasticity within the mPFC was the outcome of an interaction with exposure to the behavioral tests required for behavioral profiling, we repeated the same experiment of examining mPFC LTP induction, but this time without prior exposure to any behavioral tests. The results were similar to those of prior behavioral testing, indicating that the altered LTP in the vSub-mPFC pathway was exclusively a result of intra-BLA TrkB knockdown.

BLA modulation was previously found to also affect the induction of DG-LTP [4, 5, 88, 90, 91]. However, while previous findings demonstrated intraamygdala modification-induced transregional metaplasticity in the mPFC, the hippocampal pathway remained unaltered [76]. In the present study, we also examined whether intra-BLA TrkB knockdown had an impact on neural plasticity in the PP-DG pathway, but similar to previous results regarding intra-BLA neurofascin expression modulation [76], TrkB knockdown in the BLA did not affect neural plasticity in the dentate gyrus. This finding suggests that the BLA-mPFC pathway might be more amenable to BLA-induced transregional metaplasticity.

Until that point, we examined the basic impact of TrkB knockdown. We subsequently aimed to examine the impact of such intra-BLA-induced metaplasticity on the ability of the animals to cope with exposure to trauma.

Traumatic stress has been shown to modulate the activity of GABAergic and glutamatergic neurons in the BLA [24], and acute trauma has been found to affect anxiety-like behavior in rodents, with effects that persist for prolonged periods [20, 83]. UWT alone was previously shown to increase anxiety-like behavior acutely and enduringly [9, 10, 66, 81]. In the present study, behavioral profiling revealed a significant increase in the proportion of affected individuals among both the trauma-exposed CTR-UWT (80%) and TrkBKD-UWT (87%) groups in a similar way. Therefore, TrkB knockdown did not seem to augment the impact of the UWT. The lack of added contribution could be due to a ceiling effect, since the percentage of affected individuals was already very high for the CTR-UWT group.

A different outcome was found regarding the impact of intra-BLA TrkBKD on extinction learning. The UWT alone had a mild effect on extinction learning, whereas the combination of the UWT and TrkBKD revealed a clearly impaired extinction learning. Similarly, while UWT had only a mild effect on synaptic plasticity in the mPFC, the combination of UWT and TrkBKD significantly impaired mPFC LTP. These results demonstrate an impact of TrkB knockdown manipulation on the

consequences of exposure to trauma, which could have a long-lasting effect on the severity and duration of anxiety-like symptoms in these individuals.

Conclusions

These results indicate that induced intra-BLA metaplasticity may affect emotional behavior, including basal levels of anxiety. Furthermore, a natural difference in the levels or functionality of the intra-BLA TrkB-related circuits could contribute to individual differences in response to trauma. A more profound understanding of the underlying mechanisms could provide valuable insights and may help characterize signs of vulnerability, which in turn may serve to develop preventive interventions.

Abbreviations

BLA	Baso-lateral amygdala
DG	Dentate gyrus
mPFC	Medial prefrontal cortex
BDNF	Brain-derived neurotrophic factor
TrkB	Tropomyosin receptor kinase B
EPM	Elevated plus maze
OF	Open field
UWT	Under-water trauma
LTP	Long-term potentiation
LTD	Long-term depression
TBS	Theta-burst stimulation
CTR	Control group
CTR-UWT	A group exposed to UWT
TrkBKD	A group injected with the TrkB knockdown virus into the BLA
TrkBKD-UWT	A TrkB group exposed to UWT

Supplementary Information

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Additional file 1.

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Author contributions

Authors' contributions—Joyeeta Dutta Hazra: Investigation, Validation, Formal analysis, Writing—Original Draft, Visualization. Kuldeep Srivastava: Investigation, Validation, Formal analysis. Lisa-Sophie Wüstner: Review & Editing, Visualization. Rachel Anunu: Investigation, Validation, Project administration. Erez Chervinsky: Investigation. Somoday Hazra: Investigation. Simone Beuter: Visualization. Martin Kriebel: Methodology, Validation, Writing—Review & Editing, Visualization, Supervision. Mouna Maroun: Supervision. Hansjuergen Volkmer: Conceptualization, Methodology, Resources, Writing—Original Draft, Writing—Review & Editing, Visualization, Supervision, Project administration. Gal Richter-Levin: Conceptualization, Methodology, Resources, Writing—Original Draft, Writing—Review & Editing, Supervision, Project administration.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments, and were approved by the University of Haifa Animal Care and Use Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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