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Original research article

Brain-derived neurotrophic factor expression in serotonergic neurons improves stress resilience and promotes adult hippocampal neurogenesis

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ABSTRACT

The neurotrophin brain-derived neurotrophic factor (BDNF) stimulates adult neurogenesis, but also influences structural plasticity and function of serotonergic neurons. Both, BDNF/TrkB signaling and the serotonergic system modulate behavioral responses to stress and can lead to pathological states when dysregulated. The two systems have been shown to mediate the therapeutic effect of antidepressant drugs and to regulate hippocampal neurogenesis. To elucidate the interplay of both systems at cellular and behavioral levels, we generated a transgenic mouse line that overexpresses BDNF in serotonergic neurons in an inducible manner. Besides displaying enhanced hippocampus-dependent contextual learning, transgenic mice were less affected by chronic social defeat stress (CSDS) compared to wild-type animals. In parallel, we observed enhanced serotonergic axonal sprouting in the dentate gyrus and increased neural stem/progenitor cell proliferation, which was uniformly distributed along the dorsoventral axis of the hippocampus. In the forced swim test, BDNF-overexpressing mice behaved similarly as wild-type mice treated with the antidepressant fluoxetine. Our data suggest that BDNF released from serotonergic projections exerts this effect partly by enhancing adult neurogenesis. Furthermore, independently of the genotype, enhanced neurogenesis positively correlated with the social interaction time after the CSDS, a measure for stress resilience.

Abbreviations: BDNF, brain-derived neurotrophic factor; 5-HT, 5-hydroxytryptamin, serotonin; CSDS, chronic social defeat stress; mRN, midbrain raphe nuclei; TPH2, tryptophan hydroxylase 2; DG, dentate gyrus; TAM, tamoxifen; SERT, serotonin transporter.

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1. Introduction

Brain-derived neurotrophic factor (BDNF) and serotonin (5-hydroxytryptamin, 5-HT) are two distinct signaling systems, which are involved in the development and plasticity of neuronal networks (Edelmann et al., 2014; Gaspar et al., 2003; Park and Poo, 2013; Sasi et al., 2017), and are reported to be dysregulated in psychiatric diseases such as depression and anxiety disorders. Changes in BDNF and 5-HT signaling have been described as key triggers of e.g. enhanced anxiety, reduced stress coping ability or depressive-like behavior (Kraus et al., 2017; Martinowich and Lu, 2008). Both signaling systems influence each other. BDNF influences differentiation, structural plasticity and function of serotonergic neurons (Rumajogee et al., 2004). In addition, it increases 5-HT synthesis and activity of serotonergic neurons (Siuciak et al., 1998, 1996). On the other hand, 5-HT acts in an auto-paracrine loop to regulate BDNF mRNA levels in embryonic serotonergic cells of the raphe nuclei during development (Galter and Unsicker, 2000). Furthermore, the antidepressant action of selective serotonin-reuptake inhibitors (SSRIs) restores BDNF expression downregulated following stress, an important factor contributing to the emergence of depression (Numakawa et al., 2018; Warner-Schmidt and Duman, 2006). The neurotrophin hypothesis of depression presumes that reduced BDNF levels, particularly in the hippocampus, are causal to the disease and that a treatment option with antidepressants is to augment BDNF signaling and thereby to stimulate neurogenesis to normal physiological levels (Castrén, 2014). Growing evidence suggests that adult hippocampal neurogenesis plays an important role in hippocampal processing of emotional and cognitive information linked to stress resilience and hence psychiatric disorders (Leschik et al., 2021). Adult hippocampal cell proliferation has been shown to be sensitive to stress (Malberg and Duman, 2003; Tanti and Belzung, 2013), leading to reduced neurogenesis in depressed individuals (Berger et al., 2020; Eisch and Petrik, 2012). For this reason, it has been proposed that functional adult neurogenesis serves as a resilience mechanism (Anacker et al., 2018; Levone et al., 2015; Zimmermann et al., 2018; Leschik et al., 2021). Despite the fact that several studies implicate predominantly ventral hippocampal neurogenesis in emotional behavior, SSRIs act in a more uniform manner by augmenting ventral and dorsal adult neurogenesis (Tanti and Belzung, 2013). However, while BDNF and 5-HT both regulate adult neurogenesis (Alenina and Klempin, 2015), the relationship between BDNF, 5-HT and antidepressant action is rather complex and brain region-dependent. Apart from being reduced in human plasma of depressed patients (Cunha et al., 2009; Engelmann et al., 2019; Lieb et al., 2018; Wagner et al., 2018), BDNF levels are decreased in the hippocampus and prefrontal cortex, while BDNF is upregulated in the amygdala and nucleus accumbens (Autry and Monteggia, 2012; Wook Koo et al., 2016). Thus, besides their robust effect on hippocampal BDNF levels, antidepressants may exert opposite effects in other brain regions (Berton, 2006).

Stress-inducing stimuli lead to the activation of serotonergic neurons located in the midbrain raphe nuclei (mRN) (Hale et al., 2012), consisting of the dorsal (DRN) and median raphe nucleus (MRN). Both nuclei contain the majority of forebrain-projecting serotonergic neurons expressing the rate limiting 5-HT synthesizing enzyme tryptophan hydroxylase 2 (TPH2). The DRN and MRN are differentially responsive to stress-inducing stimuli (Hale and Lowry, 2010). For instance, acute optogenetic activation of the DRN was shown to increase active stress-coping (Nishitani et al., 2018), whereas lesion experiments revealed that the MRN is important for tolerance to repeated stressors (Pereira et al., 2019; Silva et al., 2016). The mRN highly innervate key limbic structures important for stress regulation including the dentate gyrus (DG), and hence, stress-induced serotonergic transmission could strongly influence adult neurogenesis. Whereas BDNF-mediated response of antidepressants was mostly attributed to BDNF expression in the hippocampus, specifically to the DG (Adachi et al., 2008), recent studies suggest that exclusively serotonergic neurons in the mRN mediate BDNF-induced antidepressant response. Treatment with SSRIs was shown to activate the BDNF-inducing transcription factor cAMP response element binding protein (CREB) in serotonergic but not in hippocampal neurons (Manners et al., 2019; Rafa-Zabłocka et al., 2018). Additionally, a drug-resistant phenotype was observed when selectively knocking out CREB in serotonergic neurons (Rafa, Zabłocka et al., 2017). This is in accordance with a study demonstrating that chronic social defeat stress (CSDS) leads to BDNF and CREB downregulation in mRN neurons besides reduced expression of serotonergic system related genes (Boyarskikh et al., 2013).

Recently, Meng et al. (2020) demonstrated for the first time BDNF expression in adult 5-HT neurons of the mRN and a decrease of total dorsal raphe BDNF by subchronic unpredictable stress. When specifically deleting BDNF from 5-HT neurons in mice, increased stress susceptibility to depression-related behavior was observed (Meng et al., 2020). Taken together, these results suggest an activity-dependent BDNF release from serotonergic neurons in the hippocampus by stress-inducing stimuli and consequent modulation of stress response. In the current study, our aim was to study whether increased levels of BDNF in mRN serotonergic neurons modulate the response to chronic stress. Here, we show that selective overexpression of BDNF in adult serotonergic neurons is sufficient to improve resilience to CSDS. In parallel, we observed enhanced 5-HT axonal sprouting in the DG and augmented neurogenesis in the ventral and dorsal hippocampal regions. Furthermore, we show that resilient/susceptible behavior after CSDS directly correlates with the individual degree of neural stem/progenitor cell proliferation in the hippocampus.

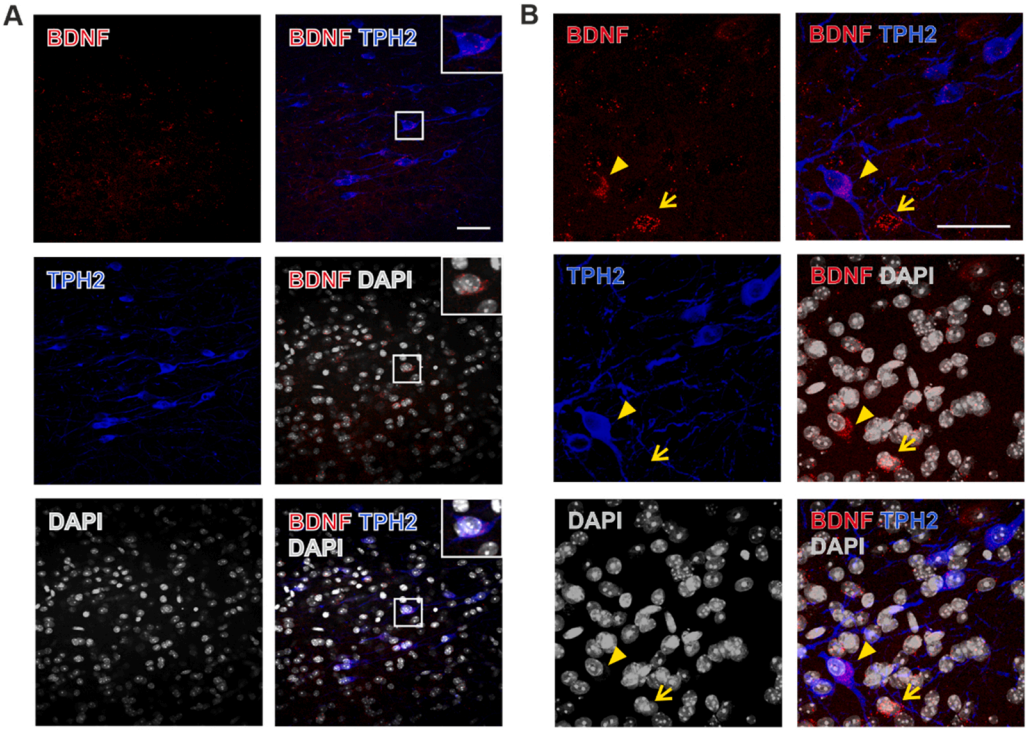
2. Results

2.1. Endogenous BDNF expression in serotonergic neurons of the raphe nuclei

First, we addressed whether adult serotonergic neurons of the mRN endogenously express BDNF. To determine physiological BDNF expression in adult serotonergic neurons, we combined single-molecule fluorescent in situ hybridisation (RNAscope ISH) with immunohistochemistry against the serotonergic neuron marker TPH2 on wild-type mouse brain sections (Fig. 1). Confocal microscopy revealed the presence of BDNF mRNA in TPH2+ cells of the mRN (overview in Fig. 1A). Fig. 1B depicts also other cell types with BDNF mRNA expression in the mRN, which could be e.g. glutamatergic. Semi-quantitative microscopic analysis revealed that the vast majority (80.43 ± 2.23 %) of TPH2+ cells express BDNF (Fig. 1C).

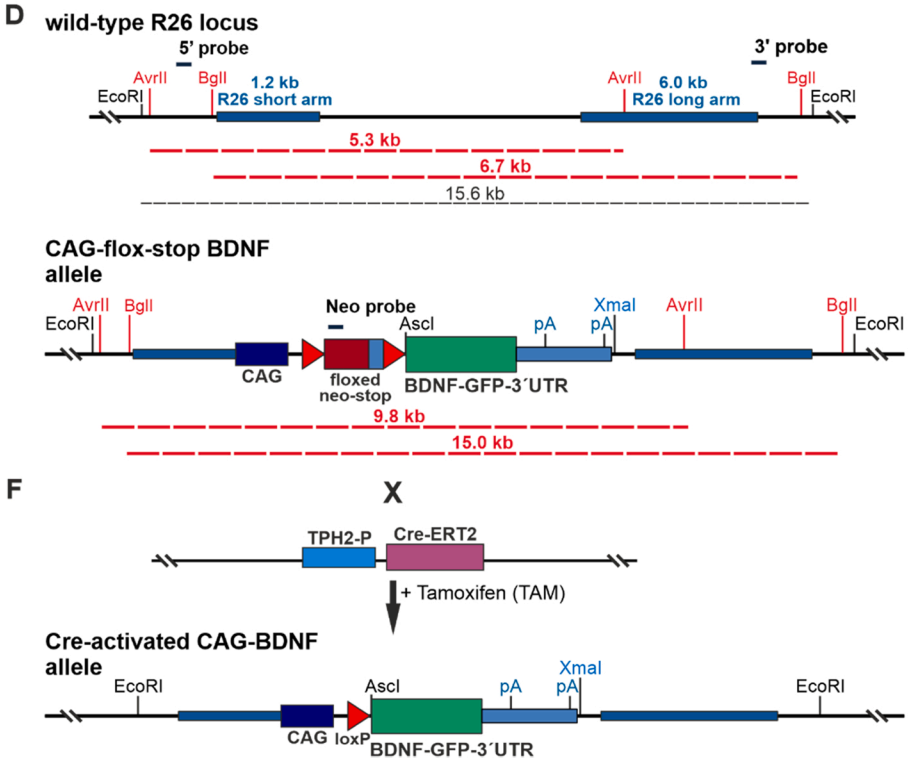
2.2. Generation of a knock-in mouse model of increased BDNF expression in serotonergic neurons

In order to generate a mouse line allowing inducible BDNF (over) expression in distinct neuronal populations, a knock-in targeting strategy into the Rosa26 (R26) locus was chosen (Fig. 1D). To enable analysis and visualization of transgenic BDNF, the coding sequence of mouse BDNF was C-terminally fused in-frame with GFP, which maintains physiological BDNF secretion and function (Brigadski et al., 2005; Kolarow et al., 2007; Leschik et al., 2019). Additionally, the sequence of the long 3′UTR, including the proximal and distal polyadenylation sites, of the endogenous mouse *Bdnf* gene was retained, allowing correct processing and subcellular targeting of BDNF–GFP-encoding mRNA (Tongiorgi and Baj, 2008). Upstream to the BDNF-GFP-3'UTR sequence, a transcriptional stop cassette (floxed-neo-stop) was introduced to suppress transcription but allowing inducible transcription when Cre recombinase is present. Induced expression is under the control of the ubiquitous CAG promoter (early cytomegalovirus enhancer element and the chicken β-actin promoter). After successful germline transmission, genomic tail DNA of founder animals was tested by Southern blot analysis, verifying the correct integration of the mutant allele into the



C

microscopic field, 40x	TPH2+	TPH2+/BDNF+	% (to all TPH2+)
1	18	15	83.33
2	39	29	74.36
3	25	20	80.00
4	19	16	84.21
		mean ± SEM	80.43 ± 2.23%



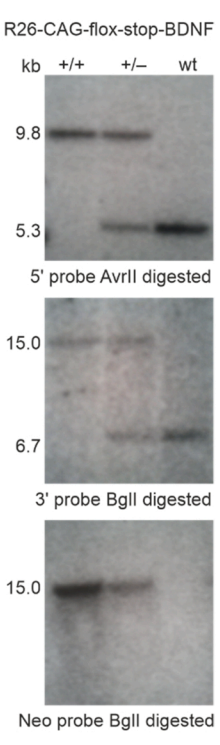


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Fig. 1. BDNF expression in serotonergic cells and generation of R26-CAG-flox-stop-BDNF knock-in mice. (A) BDNF-RNAscope in situ hybridization combined with anti-TPH2 immunohistochemistry in the adult mouse brain. Overview single plane LSM micrographs show BDNF mRNA expression in the raphe nuclei. TPH2+ neurons express BDNF mRNA. White box depicts the magnified area in the right corner, clearly demonstrating the occurrence of BDNF mRNA around the nucleus (DAPI staining) of TPH2+ neurons. (B) Cropped maximum intensity projection of 15 μm z-stack, acquired in the same area on the slide as in A. BDNF mRNA expression is not only present in serotonergic TPH2+ neurons (arrowhead), but also in other cells of the mRN (arrow). Scale bar, 50 μm. (C) Semiquantitative microscopic analysis of BDNF RNA-Scope with subsequent TPH2 staining by cell counting demonstrates that the vast majority of TPH2+ cells in the raphe nuclei of C57BL/6 N wild-type mice express BDNF. (D) Generation of R26-CAG-flox-stop-BDNF knock-in mice. Upper scheme: R26 genomic locus with depiction of short and long arms to mediate homologous recombination, length of the generated fragments by *Avr*II and *Bgl*I restriction, and the Southern blot 5′ and 3′ probes positioned outside of the short and long arms. Lower scheme: Representation of R26 locus after homologous recombination with the targeting construct containing the following features: an ubiquitous promoter (CAG), a loxP-flanked neomycin resistance gene-transcriptional stop cassette (floxed neo-stop), the coding sequence of BDNF–GFP fused to the long 3′UTR with proximal and distal polyadenylation signals (pA) from the *Bdnf* gene (BDNF-GFP-3′UTR). The position of the Neo probe, used for Southern blot analysis, is also shown. (E) Southern blot analyses of *Avr*II digested genomic DNA, hybridized to 5' probe, or *Bgl*I digested genomic DNA, hybridized to 3' or Neo probe. DNA of homozygous (+/+) and heterozygous (+/-) mice showed the expected mutant bands at 9.8 kb and 15.0 kb. (F) Cre-activated CAG-BDNF allele leading to transcription of BDNF-GFP in serotonergic cells after crossbreeding with TPH2–CreER^{T2} mice (P, promoter) and tamoxifen treatment.

R26 locus (Fig. 1E). Results of Southern blot analysis of homozygous (+/+), heterozygous (+/-) R26-CAG-flox-stop-BDNF animals and wild-type (WT) littermates demonstrated the correct integrity of the knock-in allele when probing for 5', 3' and the neomycin resistance gene (neo). After crossbreeding mice with the inducible Cre line TPH2-CreER^{T2} (double transgenic termed *tph2*-BDNF) and tamoxifen (TAM) treatment, Cre-mediated recombination of the knock-in allele is expected to lead to the expression of BDNF-GFP exclusively in serotonergic neurons of the mRN (Fig. 1F).

2.3. Characterization of mouse line and BDNF-GFP expression analysis in *tph2*-BDNF mice

Brain tissue clearing of TAM-treated *tph2*-BDNF mice and subsequent light-sheet microscopy demonstrated BDNF-GFP expression throughout the whole DRN (Fig. 2A and Video 1). Furthermore, immunofluorescent staining analysis on tissue sections with anti-GFP and anti-Cre antibodies showed vast co-localization of BDNF-GFP and Cre recombinase in raphe cells of *tph2*-BDNF animals treated with TAM, which was absent without TAM-treatment (Fig. 2B, upper panel). Furthermore, when quenching autofluorescence of perfused tissue with sudan black, GFP fluorescence without signal amplification by anti-GFP-antibody staining was detectable. GFP signal co-localized with anti-TPH2 staining, thereby validating BDNF-GFP expression in serotonergic neurons (Fig. 2B, lower panel) with a recombination efficiency of 81.72 ± 5.66 % (Fig. S1A).

When comparing overexpression of BDNF-GFP to endogenous BDNF expression by quantitative PCR (qPCR), an approximately 12-fold overexpression in the mRN of TAM-treated *tph2*-BDNF animals was detected (Fig. 2 C, and further qPCR control experiments in Fig. S1B). Correct molecular weight and processing of pro-BDNF-GFP in the mRN of TAM-treated *tph2*-BDNF animals was analyzed by Western blot after anti-GFP immunoprecipitation (IP) (Fig. 2D). With supersensitive ECL detection system, we were able to confirm protein expression of the precursor protein pro-BDNF-GFP in *tph2*-BDNF, but not WT animals with an apparent molecular weight of 58 kDa and the cleaved mature form of BDNF-GFP with 43 kDa. Hippocampal lysates of mice heterozygous for BDNF-GFP homologously recombined into the endogenous BDNF gene (kiBE mouse line) (Leschik et al., 2019) served as Western blot positive control. In the mRN, both BDNF-GFP species were detectable in *tph2*-BDNF animals when probing with an anti-BDNF antibody. In contrast, anti-GFP IP in *tph2*-BDNF hippocampal lysates pulled down only mature BDNF-GFP, which suggests transport and release of mainly mature BDNF-GFP from serotonergic synapses to the hippocampus. This is strengthend by immunohistochemical GFP positivity co-localizing with or being in proximity to the SERT signal of serotonergic fibers in the DG (Fig. S2). Even if the IP experiments demonstrated that the detection limit is reached, altogether the data validate correct molecular weight and processing of pro-BDNF-GFP by inducible transgene expression in the mRN.

2.4. Enhanced serotonergic fiber outgrowth in the hippocampus of *tph2*-BDNF mice

Next, we investigated whether overexpression of BDNF in serotonergic neurons affects plastic remodeling of axonal fibers innervating the hippocampus (Fig. 3). Five week old *tph2*-BDNF and WT mice were treated with TAM and immunohistochemically analyzed 6 weeks later. Serotonergic fiber density in the dorsal and ventral DG was measured by 3D reconstruction using the semi-automatic filament tracing tool of Imaris software (Fig. 3A and B). Immunohistochemical staining against the marker for serotonergic axons SERT (serotonin transporter) revealed increased serotonergic fiber density in the DG of *tph2*-BDNF mice compared to WT (Fig. 3B). Quantification revealed that *tph2*-BDNF mice displayed a significantly increased serotonergic fiber length ($p = 0.0334$) and volume ($p = 0.007$) in the dorsal and ventral part of the DG compared to WT littermates (analyzed by 2-way ANOVA without interaction for DG region) (Fig. 3C). No differences in fiber diameter were detected, which accounts for increased axonal sprouting, which is one mechanism of enhanced neuroplasticity. Remarkably, the data suggest remodeling by BDNF in the adult hippocampus after the basic layout of serotonergic fibers is already established.

2.5. Contextual fear memory and adult neurogenesis are increased in animals with BDNF overexpression in serotonergic neurons

Animals were treated with TAM at an age of 5 weeks. In order to reveal possible neurogenesis-dependent effects, behavioral analysis and neurogenesis analysis by BrdU application was performed 6 weeks after the last TAM injection (experimental timeline in Fig. 4A, left panel). After TAM-mediated recombination resulting in BDNF overexpression in serotonergic neurons, mice were not affected regarding body weight gain (Fig. S3A), explorative (Open field, OF) or motor (Rotarod, RR) behavior (Fig. S3B and C). In addition, basic anxiety measures as time spent in the center of the open field arena (Fig. S3B) and performance in the light/dark test (LDT) (Fig. S3D) were unchanged compared to their WT littermates. In accordance, stress-induced anxiety by the novelty-suppressed feeding (NSF) paradigm did not result in differences between experimental groups (Fig. S3E). Significant differences ($p = 0.0493$) were found in hippocampus-dependent contextual fear learning (Fig. 4B). *Tph2*-BDNF animals showed increased memory for the fearful context 6 weeks after induction of BDNF overexpression. In contrast, amygdala-dependent cued fear learning was unchanged between experimental groups. Prior work has shown that spatial memory in contextual (fear) learning is increased by upregulated adult neurogenesis (Akers et al., 2014) and reduced by blocking neurogenesis, whereas cued (fear) learning is neurogenesis-independent (Saxe et al., 2006). Therefore, we asked whether the spatial memory enhancement in *tph2*-BDNF animals is associated with an increase in adult neurogenesis induced by BDNF during 6 weeks of overexpression. When pulsing animals for five days with BrdU, addressing proliferation/survival of neural stem and progenitor cells, we found a significant upregulation

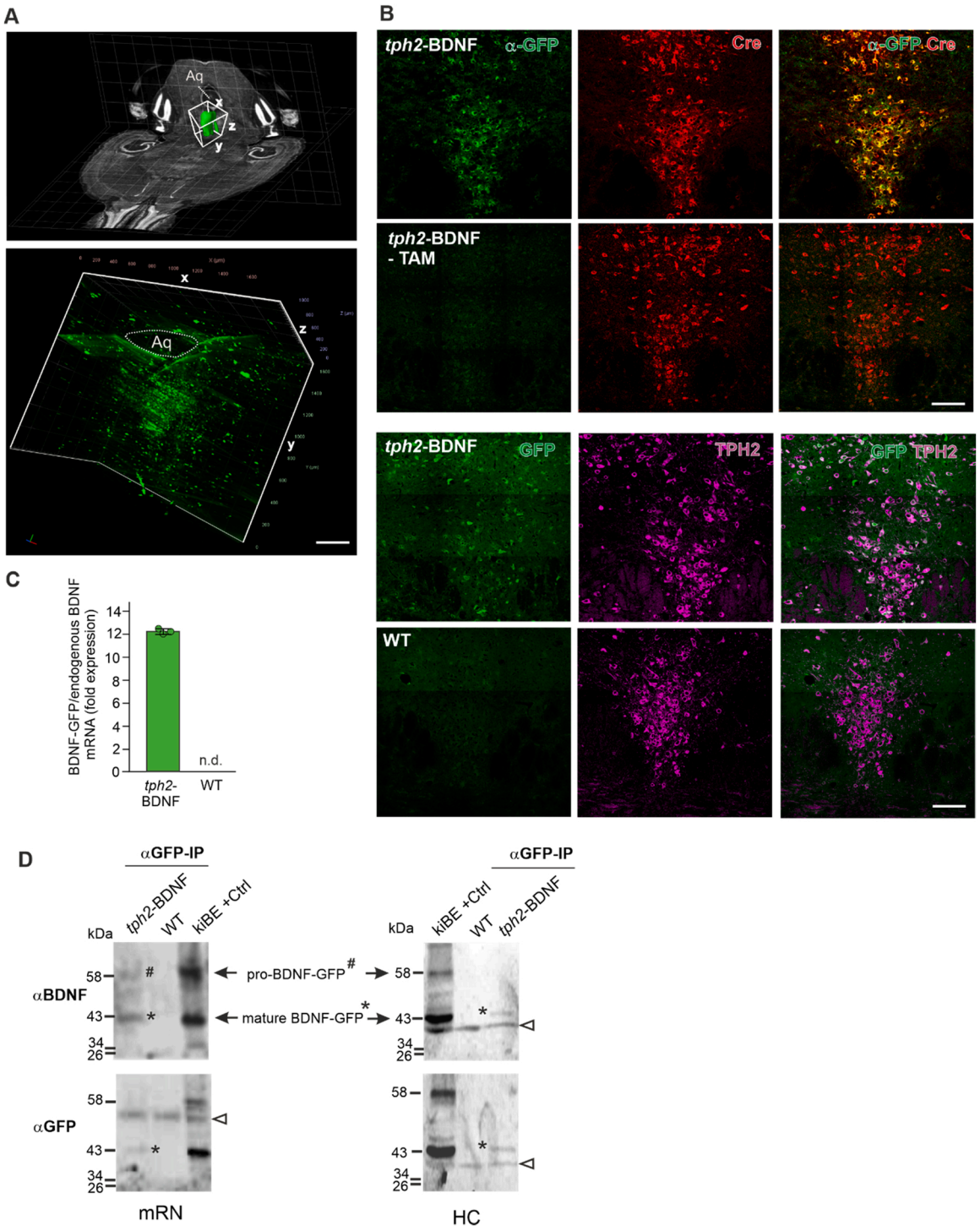

Fig. 2. Analysis of BDNF-GFP overexpression in serotonergic cells. (A) Schematic 3D representation of DRN (green) location in the mouse brain by the use of Allen Brain (Mouse) Atlas-Brain Explorer ® 2 with coronal and horizontal planes indicated for orientation; x,y, and z axes define acquisition window (white box) for light-sheet microscopy. In lower panel, 3D micrograph of light-sheet microscopy of TAM-treated *tph2*-BDNF mice depicting cellular BDNF-GFP signal throughout the DRN. Scale bar, 300 μm. Aq: aquaeduct. (B) Confocal micrographs of raphe *tph2*-BDNF sections showing anti-GFP and anti-Cre immunostaining, demonstrating BDNF-GFP expression only after TAM-treatment in serotonergic neurons expressing Cre recombinase (upper panel). GFP fluorescence co-localizes with anti-TPH2 signal of serotonergic cells in *tph2*-BDNF but not in WT littermates after TAM treatment (lower panel). Scale bar, 100 μm. (C) Expression analysis of BDNF-GFP relative to endogenous BDNF by qPCR in the mRN of TAM-treated *tph2*-BDNF mice, n = 3; n.d., not defined. (D) Western blot probed against BDNF and GFP after immunoprecipitation (IP) with anti-GFP antibody, demonstrating correct translation and processing of pro-BDNF-GFP ($^{\#}$) protein in the mRN (pooled n = 8 animals for one IP). In HC lysates, only the mature form of BDNF-GFP (*) is detected. Unspecific bands marked by open arrowheads. Knock-in BDNF-GFP (kiBE) hippocampal lysates were used for positive control (+Ctrl). mRN: midbrain raphe nucleus, HC: hippocampus.

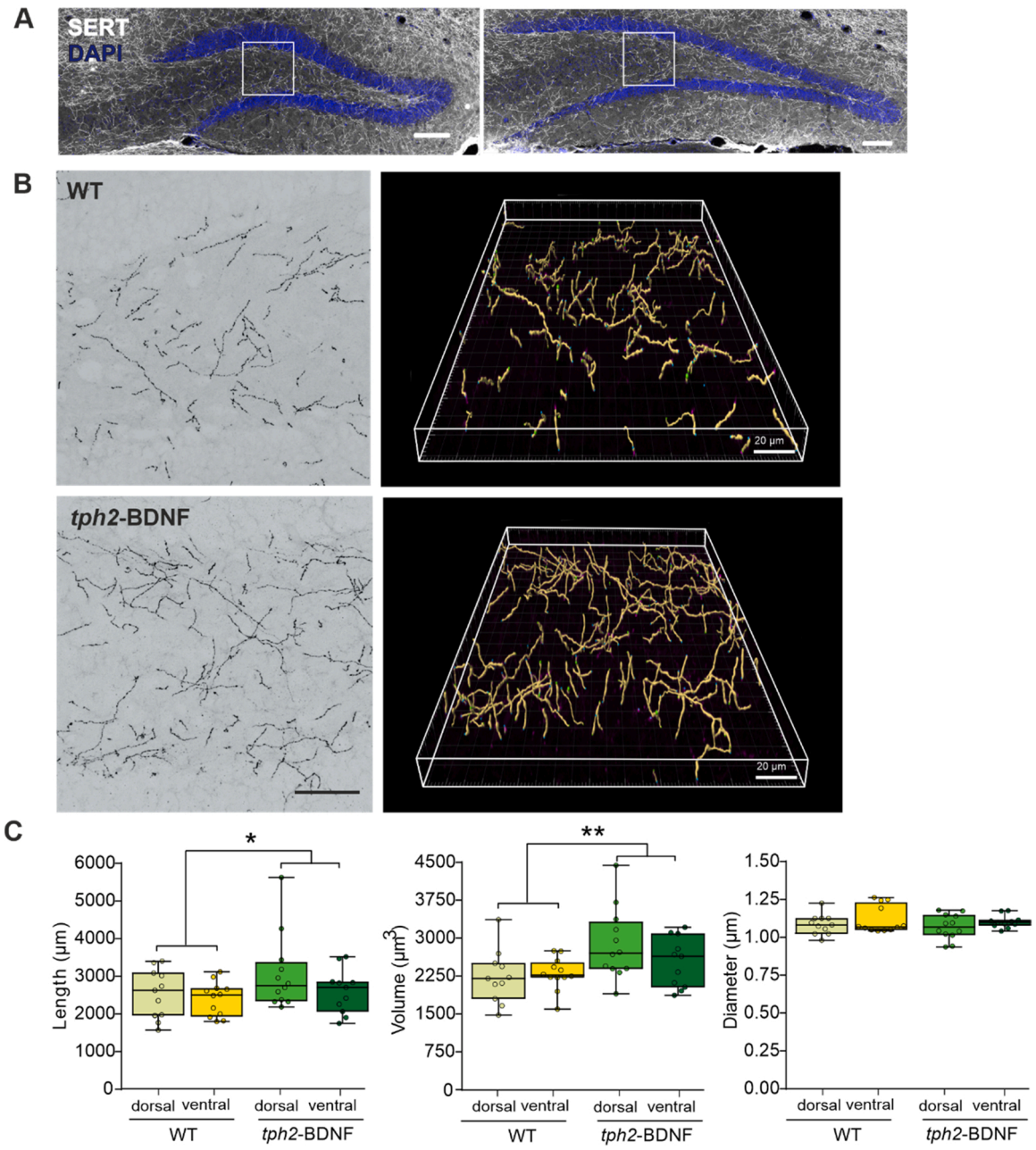

Fig. 3. Three-dimensional quantitative analysis of serotonergic axonal fibers in the DG of *tph2*-BDNF mice. (A) Representative overview confocal micrographs serving as scheme to illustrate dorsal (left) and ventral (right) DG localization of image acquisition (white squares). Scale bar, 100 µm. (B) Representative micrographs of SERT-stained serotonergic fibers in the dorsal DG of WT and *tph2*-BDNF mice taken for quantification (scale bar, 50 µm) and adjacent the same images illustrating traced SERT-positive fibers by Imaris software in 3D format. (C) *Tph2*-BDNF mice display an increased fiber length and volume of serotonergic fibers in the dorsal and ventral DG compared to WT animals, but no differences in fiber diameter. n = 12 DG sections for each genotype and hippocampal region (3 mice, 4 DG/animal). 2-way ANOVA, with p = 0.0334 (length) and p = 0.007 (volume) for genotype, no interaction with region.

(p = 0.0329) of proliferating cells in *tph2*-BDNF mice, which displayed 2571 ± 198 BrdU+ cells compared to 2002 ± 64 BrdU+ cells in WT littermates (Fig. 4C). Furthermore, enhanced neuronal differentiation was seen by co-staining of BrdU+ cells with the neuronal differentiation marker doublecortin (DCX) (DCX+ BrdU+ / total BrdU+ 12.97 ± 1.15 % *tph2*-BDNF; 10.27 ± 0.71 % WT; p = 0.0335) (Fig. 4D and E).

2.6 Serotonergic neuron BDNF overexpression protects against CSDS in an antidepressive-like manner

Depressive-like behavior, which was analyzed by the forced swim test (FST), was unchanged between WT and *tph2*-BDNF animals (Fig. 5A) and both genotypes responded to the antidepressant fluoxetine (FLX) (Fig. S4). However, when animals were exposed to chronic social defeat stress (CSDS) (experimental timeline in Fig. 5B), *tph2*-BDNF animals did not show depressive-like behavior after CSDS, but displayed a similar degree of immobility as WT animals treated with the antidepressant fluoxetine (FLX) (Fig. 5C). The antidepressant effect of FLX was not observed in *tph2*-BDNF treated with FLX, suggesting that BDNF overexpression in chronically stressed mice occludes further antidepressant effects of FLX.

Protection against CSDS was also observed when testing *tph2*-BDNF animals in the social interaction test as a measure of stress resilience (Krishnan et al., 2007) (Fig. 5D). Stressed *tph2*-BDNF animals displayed

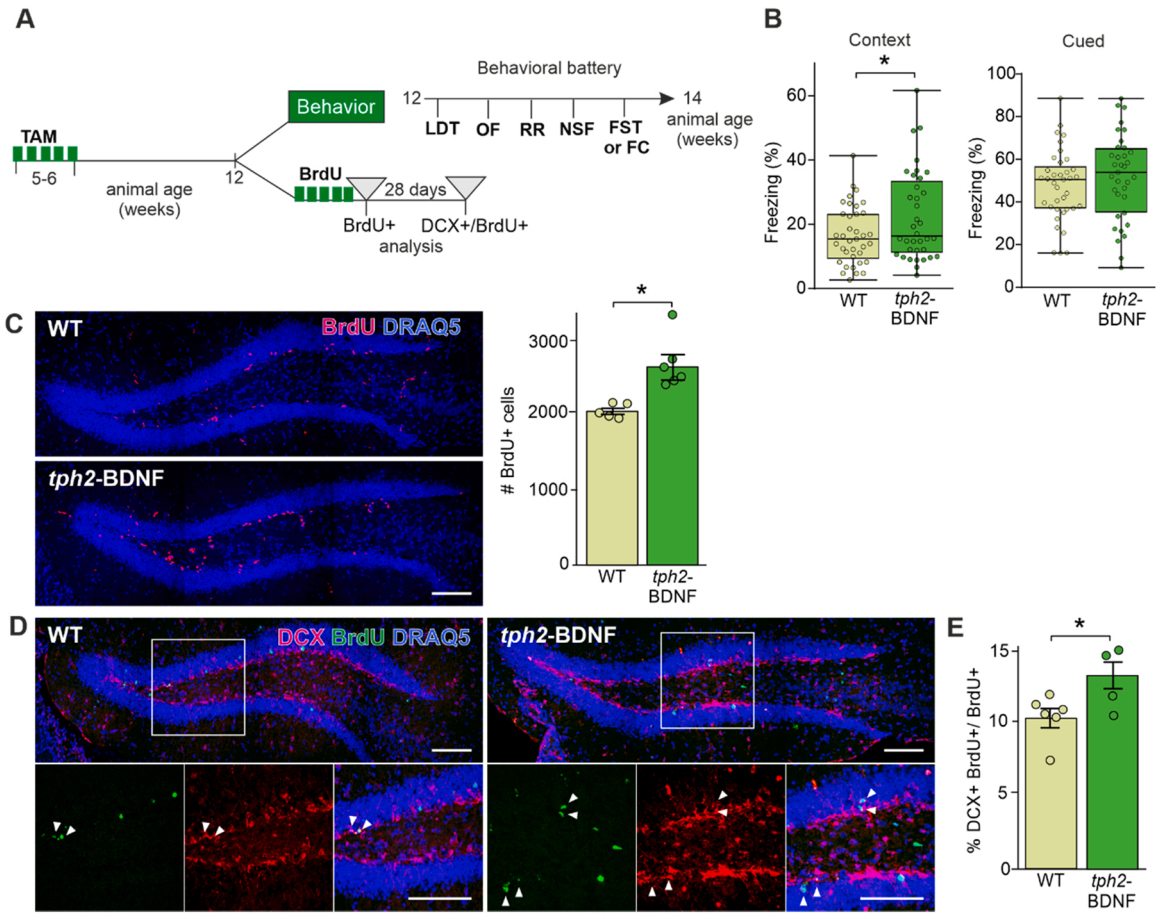

Fig. 4. Fear memory and adult neurogenesis in *tph2*-BDNF mice. (A) Scheme of animal experiment with TAM and BrdU injections, and subsequent behavioral analyses. At an age of 5 weeks animals were i.p. injected with TAM once per day for 5 days. At 12 weeks of age, mice were either i.p. injected with BrdU for five days or underwent behavioral assays. Immunohistochemical analysis took place on the day after the last BrdU injection or after 28 days. The behavioral battery (right scheme) consisted of the light/dark test (LDT), open field (OF), rotarod (RR), forced swim test (FST) or fear conditioning (FC). (B) *Tph2*-BDNF mice showed enhanced fear learning but only in contextual not in cued fear conditioning (n = 37 for WT and n = 36 for *tph2*-BDNF), 2-tailed, unpaired student's t-test. (C) Representative confocal micrographs showing BrdU-positive cells in the DG of WT and *tph2*-BDNF mice. Scale bar, 100 μm. Number (#) of BrdU+ cells in the DG is significantly increased in *tph2*-BDNF mice as compared to WT mice. n = 5 for WT and n = 6 for *tph2*-BDNF. 2-tailed, unpaired student's t-test. (D) Representative confocal micrographs showing double immunohistochemistry for DCX and BrdU of the DG of WT and *tph2*-BDNF mice. White box in overview picture marks magnified area. Arrowheads depict co-localizing cells. Scale bar, 100 μm. (E) Percentage of DCX+ /BrdU+ cells of all BrdU+ cells in the DG is significantly increased in *tph2*-BDNF mice as compared to WT mice 28 days after the BrdU pulse. n = 6 for WT and n = 4 for *tph2*-BDNF. Unpaired student's t-test.

a significantly higher social interaction index compared to stressed WT animals and were not significantly different to the WT-no stress group ($p = 0.6587$). Animals of the *tph2*-BDNF-no stress group displayed the highest social interaction index, which was significantly higher ($p = 0.039$) than that of stressed *tph2*-BDNF animals. This demonstrates that, irrespective of the complete protective effect of BDNF overexpression in serotonergic neurons seen in the FST (Fig. 5C), *tph2*-BDNF mice do still react to stress in terms of reduced sociability. These results rather hint towards a stress-buffering than a complete stress-preventive effect of BDNF in the social interaction paradigm. Relating the behavioral phenotype of *tph2*-BDNF mice to the observed increase in 5-HT axonal outgrowth in the DG (Fig. 3), we asked whether *tph2*-BDNF animals display enhanced extracellular levels of 5-HT in the DG by performing microdialysis experiments (Fig. S5). Under basal conditions, 5-HT concentrations in the DG between the WT and *tph2*-BDNF group were not significantly different (1.44 ± 0.12 fmol/5 μl in WT and 1.67 ± 0.26 fmol/5 μl in *tph2*-BDNF mice). Moreover, social defeat stress caused an immediate increase in 5-HT release in both WT and BDNF-overexpressing mice. However, significant differences of 5-HT levels between WT and *tph2*-BDNF animals were found at the end of the social defeat and immediately after stress exposure by statistical analysis with 2-way ANOVA with repeated measures and appropriate post-hoc analysis. As shown in Fig. S5A, compared to WT mice in which 5-HT levels declined to basal levels immediately after stress exposure, *tph2*-BDNF mice showed a sustained and prolonged 5-HT release upon social stress.

To get further insights into the molecular mechanism of the stress-protecting function of serotonergic neuron produced BDNF, we performed RT/qPCR of stress-related genes in the hippocampus in no stress-control and stress conditions (Fig. 5E). Our data revealed no significant differences in glucocorticoid receptor (GR) and in mineralocorticoid receptor (MR) mRNA expression in the absence or following CSDS. Furthermore, the FK506-binding protein 51 (FKBP51), known as a selective modulator of glucocorticoid sensitivity, was unchanged in WT versus *tph2*-BDNF mice, both in stressed or non-stressed conditions. Remarkably, we found that hippocampal 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), catalyzing the generation of active steroids from its inert ketoforms, was significantly reduced ($p = 0.044$) in stressed *tph2*-BDNF animals. In addition, the neuroprotective (Koutmani et al., 2013) hippocampal corticotropin-releasing hormone (CRH) was significantly upregulated ($p = 0.043$).

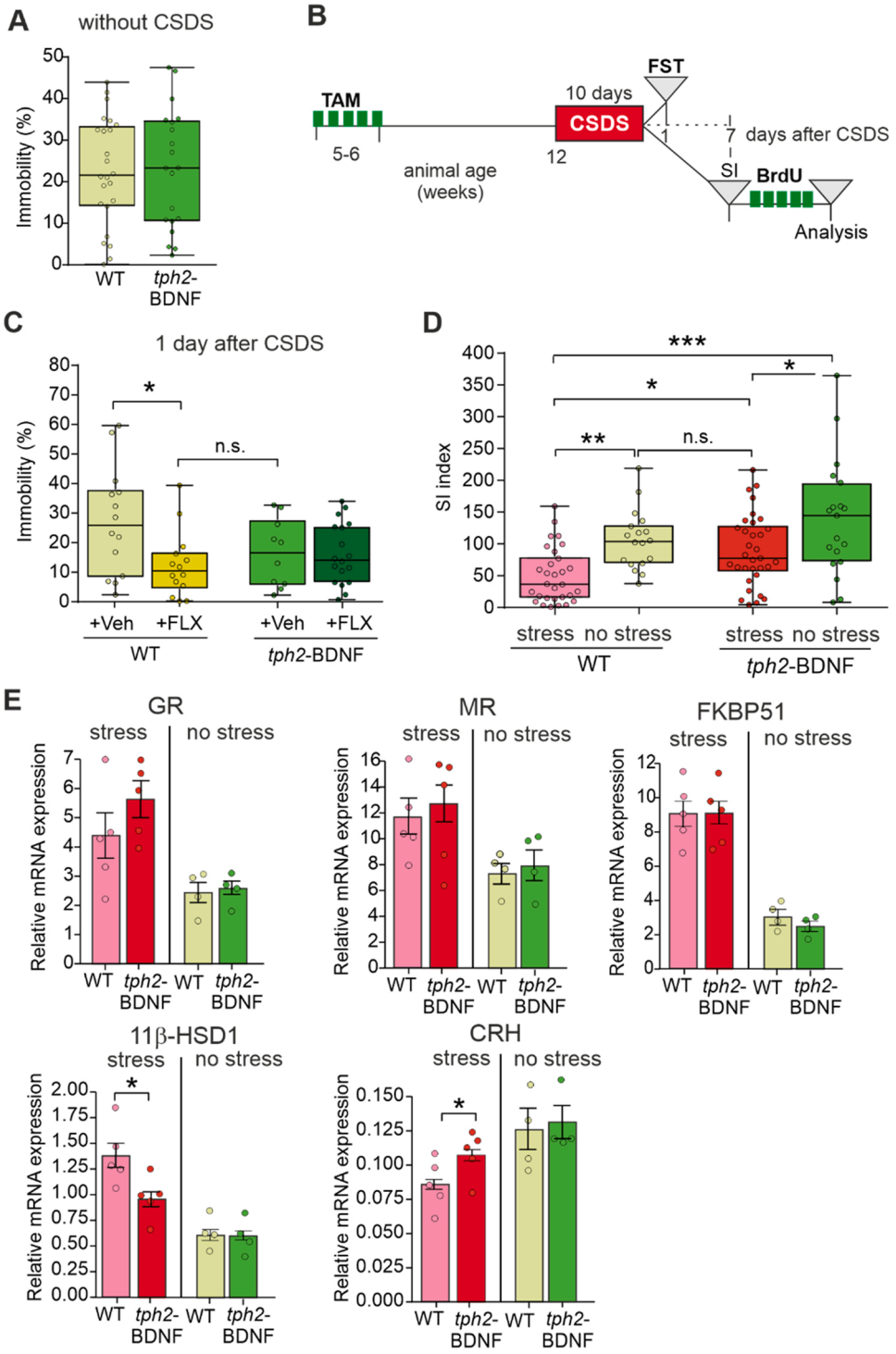

Fig. 5. Protection against CSDS in *tph2*-BDNF mice. (A) Without being exposed to chronic social defeat stress (CSDS), animals do not show a differential response of depressive-like behavior in the FST (n = 24 WT, n = 20 *tph2*-BDNF mice). (B) Scheme of animal experiment depicting 10 days of CSDS procedure and subsequent animal testing in FST (1 day after CSDS) or in social interaction (SI; 7 days after CSDS). After SI, BrdU was injected once per day over 5 days. Analysis of cell proliferation took place one day after the last BrdU pulse. (C) Chronically stressed animals overexpressing BDNF are similarly immobile as stressed WT animals 30 min after injection of the antidepressant fluoxetine (FLX) ; vehicle (Veh). n = 14 WT+Veh, n = 14 WT+FLX, n = 10 *tph2*-BDNF+Veh, n = 18 *tph2*-BDNF+FLX mice. Significant for treatment in 2-way ANOVA ($p = 0.025$) with interaction of genotype*-treatment $p = 0.041$; consecutive 1-way ANOVA $p = 0.0248$ with Tukey's post hoc tests * $p = 0.023$; n.s., not significant. (D) Social interaction test after CSDS paradigm. Social interaction index (SI index) of stressed *tph2*-BDNF mice was not significantly different from the WT-no stress group but to the WT-stress group ($p = 0.0449$). n = 30 WT-stress, n = 18 WT-no stress, n = 33 *tph2*-BDNF-stress, n = 19 *tph2*-BDNF-no stress mice. 2-way ANOVA (stress $p = 0.0027$ and genotype $p = 0.0172$) and Tukey's post hoc tests. * $p < 0.05$, ** $p < 0.01$. *** $p < 0.001$. (E) RT/qPCR of hippocampal tissue of stressed and non-stressed WT and *tph2*-BDNF animals. mRNA expression was quantified by the $2^{-\Delta Ct}$ method relative to the housekeeping gene GusB. Stressed (n = 5) and non-stressed animals (n = 4) were from different experiments, therefore unpaired student's t-test with * $p < 0.05$ was applied.

2.7 Ventral and dorsal DG neural stem/progenitor cell proliferation buffers against CSDS and positively correlates with social interaction

To address the question whether the stress resilience-promoting effect of BDNF overexpression in serotonergic neurons might correlate with enhanced neurogenesis, we next analyzed the rate of proliferation of neural stem/progenitor cells in the ventral and dorsal hippocampus (Fig. 6A and B; experimental timeline in Fig. 5B). Irrespective of the position along the dorsoventral axis, more BrdU+ cells were observed in *tph2*-BDNF mice than in WT mice (2-way ANOVA genotype effect $p < 0.0001$, ventral DG; $p < 0.0003$, dorsal DG; stress-effect ($p = 0.0086$, ventral DG; $p = 0.0327$ dorsal DG)). Most strikingly, stressed *tph2*-BDNF animals displayed significantly more BrdU+ cells in the ventral (355 ± 37 WT, 859 ± 94 *tph2*-BDNF) and dorsal (610 ± 135, 1337 ± 146 *tph2*-BDNF) part of the DG than stressed WT mice ($p = 0.0044$, ventral DG; $p = 0.028$, dorsal DG). In conclusion, these results suggest that the BDNF-induced increased rate of adult neurogenesis in the whole DG is reflecting the stress-protective effect of BDNF in behavior. Furthermore, linear regression analysis revealed a positive correlation between social interaction index and proliferation of adult neural stem/progenitor cells, independent of genotype and stress, both in the ventral and dorsal part of the DG (Fig. 6C and D).

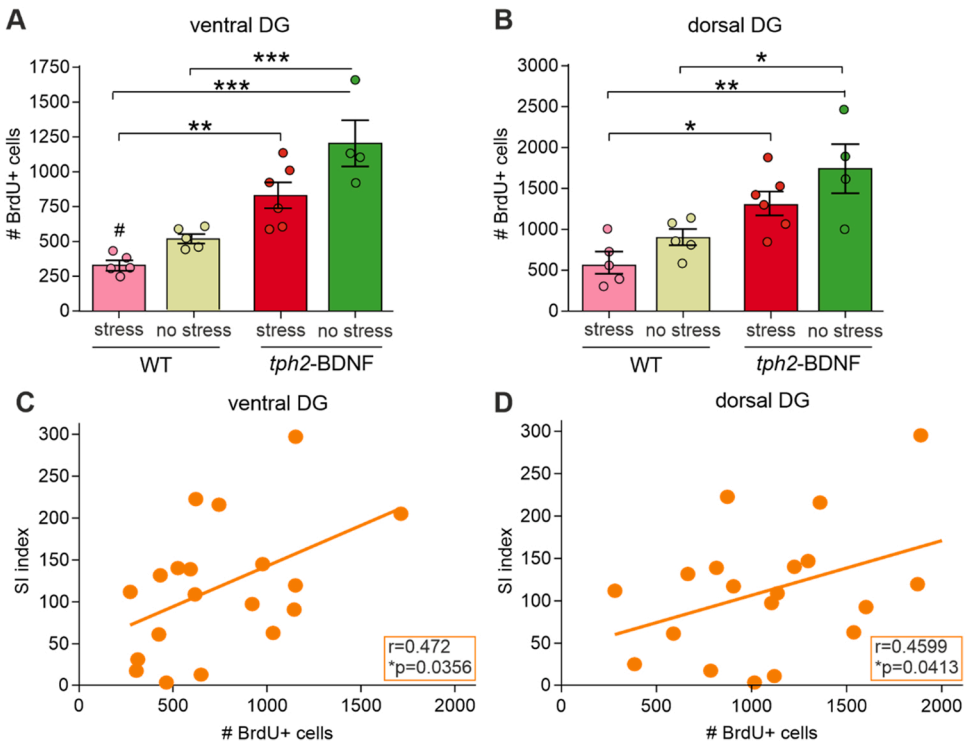

Fig. 6. Ventral and dorsal DG cell proliferation after CSDS and correlation with social interaction index. In the ventral (A) and dorsal part (B) of the DG, neural stem/progenitor proliferation is significantly increased in stressed *tph2*-BDNF compared to WT stressed animals. n = 5 WT-stress, n = 5 WT-no stress, n = 6 *tph2*-BDNF-stress, n = 4 *tph2*-BDNF-no stress mice. 2-way ANOVA and Tukey's post hoc tests. * $p < 0.05$, ** $p < 0.01$. *** $p < 0.001$; # significantly different to no stress-WT, 2-tailed unpaired student's t-test, $p = 0.0339$. (C, D) Correlation between social interaction index (SI index) and number of BrdU+ cells. Linear regression analysis demonstrates a positive correlation between SI index and proliferation of adult neural stem/progenitor cells (number of (#) BrdU+ cells) in the ventral and dorsal part of the DG. n = 20 animals of the two different genotypes (WT and BDNF), stressed and non-stressed (5 stress-WT, 5 no stress-WT, 6 stress-*tph2*-BDNF, 4 no stress-*tph2*-BDNF mice).

3. Discussion

We showed here that BDNF overexpression by adult 5-HT neurons is protective and buffers CSDS-induced behavioral responses. Concomitantly, we observed increased serotonergic axonal sprouting and upregulated adult neurogenesis in the DG, which might constitute one of the mechanisms in the observed stress protection.

Serotonergic BDNF overexpression hindered further antidepressant action of FLX, which might be explainable by the fact that not only BDNF, but also FLX binds to the tropomyosin-related kinase B (TrkB) receptor (Casarotto et al., 2021). For this reason, it might be conceivable that excess of BDNF could saturate TrkB receptors, and hence TrkB-mediated FLX-response. Decreased immobility in the FST was only detectable when animals were stressed, but not under baseline conditions. This is in accordance with other reports demonstrating that in non-stressed animals, lack of BDNF expression in the DRN does not have an impact on the response to antidepressants (Adachi et al., 2016) nor increases anhedonia and behavioral despair, but that it enhances susceptibility to subchronic unpredictable stress when specifically knocked-out in 5-HT neurons (Meng et al., 2020). The highly sensitive method of RNAscope ISH allowed us to detect BDNF mRNA expression in the majority of adult TPH2-positive neurons of the raphe nuclei. Our finding is supported by a recent single-cell transcriptomic study, which demonstrated BDNF expression in SERT-positive neurons, but suggested low abundance of BDNF-expressing serotonergic neurons in the mRN (Ren et al., 2019). Quantitative discrepancy to our results could be of technical origin, since RNAscope ISH allows single molecule detection and guarantees visualization of genes with very low expression, whereas single-cell RNA sequencing depends on the amounts of RNA starting material, sequencing depth, normalisation, and set tresholds, which can induce detection limits. Furthermore, SERT-mediated recombination during brain development as done in Ren et al. (2019), could mark neurons with a transient serotonergic phenotype that capture 5-HT but do not synthesize it later in life (Gaspar et al., 2003; Lebrand et al., 1998). Therefore, it is conceivable that for all sorted neurons a serotonergic identity in adulthood was not guaranteed, which might have led to fewer BDNF expressing cells in single-cell RNA seq as compared to the method we used.

It is commonly known that the high affinity BDNF receptor TrkB is expressed in adult serotonergic neurons and localized to the raphe somatodendritic and the axonal compartment of ascending projections to the hippocampus (Madhav et al., 2001). Interestingly, besides providing neurotrophic support and exerting regenerative effects on lesioned serotonergic axons, BDNF was shown to promote also sprouting of mature, uninjured serotonergic axons in the adult brain (Mamounas et al., 1995). Indeed, when overexpressing BDNF in 5-HT neurons, we observed enhanced axonal fiber density of serotonergic axons in the dorsal and ventral DG. This suggests an autocrine effect of released BDNF at the DG serotonergic axon terminal leading to axonal sprouting, which however does not exclude a somatodendritic autocrine action of BDNF in the mRN. Accumulating data suggests that morphological changes of serotonergic axons in response to stress are implicated in the pathophysiology of depression (Liu and Nakamura, 2006). For instance, Austin et al. have reported reduced density of 5-HT axons in the prefrontal cortex of depressed suicidal victims (Austin et al., 2002). Vice versa, antidepressant electroconvulsive shock therapy, known to induce neurotrophic signaling through enhanced BDNF and TrkB expression, resulted in increased serotonergic fiber outgrowth in the hippocampus (Madhav et al., 2000). Therefore, it is conceivable that the stress-protective effect of overexpressed BDNF released from serotonergic neurons in our study is caused by increased 5-HT axonal sprouting, consequently leading to the observed sustained and prolonged 5-HT release upon social stress, which could buffer stress-induced loss of axonal density and reduction of neurogenesis.

The behavioral data of our naïve *tph2*-BDNF mouse line (without CSDS) revealed a connection to adult neurogenesis as a mechanism of serotonergic BDNF-induced stress-buffering action. Whereas hippocampus-dependent contextual fear learning of mice was enhanced, cued fear learning was unchanged. In other studies, spatial memory of contextual fear learning was shown to be increased by enhanced neurogenesis (Akers et al., 2014) and reduced by blocking neurogenesis. In contrast, cued fear memory was neurogenesis-independent (Saxe et al., 2006). *Tph2*-BDNF animals displayed a higher neurogenesis rate than WT littermates under non-stressed conditions and after CSDS, as

evaluated by increased neural/stem progenitor cell proliferation and enhanced neuronal differentiation into DCX+ cells. We suggest that these increases reflect at least one of the mechanism to protect from CSDS (Leschik et al., 2021; Levone et al., 2015). We suggest that enhanced serotonin release due to enhanced serotonergic DG innervation leads to the observed augmentation of neurogenesis, which could be concomitant to enhanced BDNF release by 5-HT neurons, which however needs to be proven in future studies. Furthermore, we cannot exclude direct neurogenesis-independent effects of augmented BDNF on contextual fear memory (Notaras and van den Buuse, 2020).

Increased populations of proliferating BrdU-positive cells were uniformly distributed along the dorsoventral axis of the DG, which accounts for a complementary interplay of ventral and dorsal hippocampal function (Diniz et al., 2022; Huckleberry et al., 2018; Komorowski et al., 2013). In this respect, an involvement of dorsal neurogenesis in the protection from stress-related dysfunction is plausible, facilitating the discrimination between threatening and safe contexts, thereby preventing unnecessary resource consumption in safe situations. Various publications addressed regional changes in hippocampal cell proliferation, survival and differentiation in animal models of depression (Tanti and Belzung, 2013). In fact, most studies report a dorsoventral homogenous stress effect specifically on neural stem/progenitor cell proliferation (Hawley and Leasure, 2012; Nollet et al., 2012; Oomen et al., 2010; Païzanis et al., 2010; Rainer et al., 2012), and SSRIs seem to act uniformly on dorsal and ventral adult neurogenesis (Tanti and Belzung, 2013). Accordingly, we found a positive correlation between an individual's stress response and the number of proliferating cells, which was independent of the hippocampal subregion studied. This finding is particularly important for resilience research, supporting findings which demonstrate elevated levels of adult neurogenesis as an individual resilience-conducive property to cope with stressful events (Kheirbek et al., 2012).

So far, our data to elucidate the molecular mechanism involve a downregulation of hippocampal 11β-HSD1, which is a neuronal amplifier of glucocorticoid action (Sarabdjitsingh et al., 2014; Wheelan et al., 2018), known to negatively regulate adult neurogenesis (Cameron and Gould, 1994; Chapman et al., 2013). In fact, 11β-HSD1 deficiency has been shown to augment hippocampal neurogenesis in a knock-out mouse model (Yau et al., 2007). Additionally, we observed increased hippocampal CRH in stressed *tph2*-BDNF mice. CRH is known as major mediator of adaptive response to stressors and could antagonize negative effects of glucocorticoid as it has been shown to exert direct and beneficial effects on neuronal progenitors (Koutmani et al., 2013). Further experiments should address how the observed changes in stress-related genes mechanistically impact adult neurogenesis, and which signaling pathways are involved in which particular cell types.

4. Materials and Methods

4.1. Generation of R26-CAG-flox-stop-BDNF knock-in mouse line

R26-CAG-flox-stop-BDNF knock-in recombinant allele was generated in V6.5 mouse embryonic stem cells (Eggan et al., 2001; Rideout et al., 2000) as previously described (Leschik et al., 2013). Briefly, the coding sequence of mouse pre-pro-BDNF–GFP was fused to the long 3′UTR of mouse BDNF (NCBI RefSeq ID: NW_001030694.1, corresponding region: 70780131–70783754) comprising the two BDNF-3′UTR polyadenylation (pA) sites (corresponding regions: proximal pA site 70780414–70781175; distal pA site 70783428–70783668) and cloned into a Rosa26-targeting vector (Remedi et al., 2009; Soriano, 1999). Positive ESC clones were injected in C57BL/6 host blastocysts and implanted into pseudopregnant female mice to obtain first chimeric and then founder animals (produced by Genoway, Lyon, France).

4.2. Experimental Animals

Homozygous R26-CAG-flox-stop-BDNF knock-in mice were bred with the TPH2-CreER^{T2} mouse line bearing a tamoxifen-inducible CreER^{T2} recombinase expressed under the regulatory elements of the mouse brain-specific TPH2 (tryptophan hydroxylase 2) gene (Weber, 2009). Experimental animals (C57BL/6 N background) were either heterozygous mutant for the CreER^{T2} allele (named *tph2*-BDNF) or littermate controls wild-type for the CreER^{T2} allele (named WT) with both being homozygous for the R26-CAG-flox-stop-BDNF allele. Only male mice (animal age: 5 weeks at start of experiments) were used in the study. Animals were housed under standard conditions in a temperature (23–24 °C) and humidity-controlled room on a 12 h/12 h light/dark cycle with water and food ad libitum. All experiments were carried out in accordance with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes and approved by the Ethical Committee on animal care and use of Rhineland-Palatinate, Germany (Landesuntersuchungsamt Koblenz, permit number G13–1–095 and G 18–1–013).

4.3. Tamoxifen and BrdU administration

Five week-old experimental animals were administered tamoxifen (TAM) (Sigma-Aldrich, St. Louis, MO, USA) at 60 mg/kg/d for 5 days intraperitoneally (i.p.). Daily, first a 100 mg/ml stock solution was prepared by dissolving TAM in 33 % DMSO/33 % EtOH/33 % Tween-80, which was then 1:10 diluted with 0.9 % NaCl to obtain the working solution of 10 mg/ml. CreER^{T2}-transgene and wild-type animals were treated with TAM to exclude any treatment induced effect. To examine the proliferation, survival, and differentiation of targeted cells, mice were i.p. injected for 5 days with bromodeoxyuridine (BrdU, 50 mg/kg/d) (Sigma-Aldrich, St. Louis, MO, USA) 6 weeks after TAM treatment. Mice were killed either 1 day (proliferation, survival analysis) or 28 days (differentiation/doublecortin analysis) after BrdU injections.

4.4. Immunohistochemistry

Animals were transcardially perfused with 4 % paraformaldehyde and the hippocampus cryosectioned into 30 μm sections. Immunohistochemistry was performed as previously described (Zimmermann et al., 2018). For BrdU-immunohistochemistry, cell nuclei were stained with DRAQ5 (Thermo Fisher Scientific, Waltham, MA, USA). The following primary antibodies were used: rat anti-BrdU (1:100, Abcam, Cambridge, UK; RRID:AB_305426), rabbit anti-GFP (1:500, kind gift of Matthias Klugman, Sydney, Australia), mouse anti-GFP (1:100, Abcam, Cambridge, UK; RRID:AB_298911), guinea pig anti-DCX (doublecortin, 1:500, Merck Millipore, Billerica, MA, USA; RRID:AB_1586992), mouse anti-Cre recombinase (1:500, Merck Millipore, Billerica, MA, USA; RRID:AB_2085748); mouse anti-TPH2 (1:200, Thermo Fisher Scientific, Waltham, MA, USA; RRID:AB_2848913), mouse anti-SERT (1:500,; RRID:AB_2622241), rabbit anti-SERT (1:500, Sigma-Aldrich, St. Louis, MO, USA; RRID:AB_10603631). When GFP was detected without antibodies, sudan black was used to quench autofluorescent background signals after immunostaing. After dipping sections in dH_2O and subsequently in 70 % ethanol, sections were incubated with 0.05 % Sudan black for 10 min. Prior to mounting with Mowiol, sections were washed with 70 % ethanol for 5 min followed by a final 5 min washing step with dH_2O.

4.5. Microscopic analysis of histology

BrdU-positive cells were quantified in the hippocampus of each animal based on the Cavalieri principle of stereology (Cruz-Orive, 1997). Every eighth atlas-matched, coronal hippocampal section located between 1.2 and 3.2 mm posterior to bregma was used for immunostaining

to cover the whole hippocampus. To make a distinction between the dorsal and ventral part, 5 sections located in region 1.2–2.3 mm posterior to bregma, and 3 sections in region 2.3 – 3.2 mm posterior to bregma were analyzed. Slides were observed under a Leica DM5500 fluorescence microscope (Leica camera, Wetzlar, Germany). Image acquisition for cell quantification was performed by the use of a Zeiss Axiovert LSM 710 (Carl Zeiss, Oberkochen, Germany) laser scanning confocal microscope with 40x magnification and a high number line averaging to obtain a better signal to noise ratio. Tile scans comprising the whole dentate gyrus per analyzed section and z-stacks spanning the total 30 μm z-plane of hippocampal section were recorded with 1 μm optical section. Manual counting of immunopositive cells occurred by the use of maximum intensity projections.

4.6. Serotonergic fiber analysis

For each DG region four high power confocal images of anti-SERT immunostaining were taken on two adjacent coronal hippocampal sections either located in the dorsal or ventral part of the hippocampus, three animals per genotype were used. Imaging was performed in a way that at either location the suprapyramidal and the infrapyramidal blade of the subgranular zone was included in one image by 40 x magnification, comprising as few as possible mature neurons of the DG granule cell layer. Z-series of 68 stacks with a step-size of 0.15 μm were acquired at 1024 × 1024 pixel resolution, pixel size 0.21 μm. 3-D reconstruction analysis was performed by Imaris x64 9.7.0 software (Bitplane, Zürich, Switzerland) in the resulting cuboids of xyz= 213 × 213 x 10 μm with the semi-automatic filament tracing tool. Total filament length and volume were extracted from Imaris output analysis.

4.7. Stress paradigm and behavior

4.7.1. Chronic social defeat stress

Experimental mice, 12 weeks old, were submitted to chronic social defeat stress (CSDS) for 10 consecutive days. Every day, each experimental mouse was introduced into the home cage of an unfamiliar resident for 2 min and was physically defeated. Resident mice were CD1 retired breeders selected for their attack latencies reliably shorter than 30 s upon 3 consecutive screening tests. After 2 min of physical interaction, residents and intruders were maintained in sensory interaction for 24 h using a stainless steel mesh partition, dividing the resident home cage in two halves. On every day, experimental mice were exposed to a new resident home cage. Control animals were housed by in pairs, one on each side of partition, and were handled daily. Mice were single-housed in a novel cage before further experimentation.

4.7.2. Social interaction

Social interaction (SI) was tested one week after CSDS. The experimental animal was placed into an open field box (40 ×40 cm) with an empty wire mesh cage (diameter 10 cm, height 20 cm) at one wall of the box and allowed to explore the arena for 2.5 min and was then placed back to its home-cage. Afterwards, an unfamiliar CD-1 aggressor was placed into the mesh cage, the experimental animal was re-introduced to the box and allowed to explore for another 2.5 min. Time in the interaction zone, defined as a circular 8 cm wide zone around the mesh cage, was recorded automatically with the tracking software Ethovision XT (Noldus, Wageningen, Netherlands) at illumination level 30 lux using an analogue video camera. The time the experimental animal has spent in the interaction zone when the aggressor was absent (empty mesh cage) was compared to the time spent in the interaction zone when the aggressor was present in the mesh cage. Social interaction index (SI) was then calculated according to the formula: SI = (Interaction time with mouse)/(Interaction time with no-mouse) x 100.

4.7.3. Context and cued fear conditioning

Fear conditioning was performed in a transparent plexiglas box (Med Associates, Warner Robins, GA, USA; 15 cm × 20 cm x 20 cm, cleaned with water) with grid floor. For conditioning, the mouse was placed into the conditioning chamber with house light (25 lux) turned on. After 2 min, a 28 s tone (85 dB, 10 kHz pulsing every 200 ms) was presented and directly afterwards an electric foot shock of 0.4 mA was delivered to the animals through the foot grid. After a 20 s inter-stimulus interval, a second tone and shock pairing was done. Then, mice returned to their home cage. 24 h after conditioning, fear learning was examined. The context-dependent memory test consisted of re-exposure of the animal to the conditioning context for 3 min. Cued fear learning was analyzed by placing mice into a neutral, new environment (custom-made plexiglas cylinders, 15 cm diameter, with bedding, cleaned with 70 % ethanol, 5 lux house light). After 3 min, a tone (same settings as in conditioning) was presented for 180 s. Freezing in both conditions (context and cued) was scored with the Ethovision immobility filter set at 0.5 % change of the pixels representing the mouse, with averaging over two consecutive frames (25 frames/s).

4.7.4. Forced swim test

Mice were placed in a water-filled beaker (water temperature 21 °C) and were video recorded for 6 min. In case of fluoxetine treatment, a single dose of 20 mg/kg fluoxetine hydrochloride (Biotrend Chemikalien, Köln, Germany) was i.p. injected 30 min before the forced swim test. Behavior was analyzed manually, the immobility time was defined as the duration a mouse floating in the water without struggling and making only small movements with one paw.

4.8. Immunoprecipitation and Western Blot

Immunoprecipitation (IP) was carried out with Pierce™ NHS-activated magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA) coupled to polyclonal goat anti-GFP antibody (Genscript, Piscataway, NJ, USA; RRID:AB_2622198) according to manufacturer's protocol. Tissue lysates of dissected midbrain raphe regions (pooled from 8 animals) and hippocampus were prepared as previously reported (Leschik et al., 2013) and immunoprecipitation was carried out with 2 mg protein and 50 μl antibody-coupled beads in 1 ml according to manufacturer's guidelines. After elution with 45 μl 0.1 M glycine, the whole IP-fraction was loaded onto a 7.5 % acrylamide gel. 75 μg kiBE heterozygous hippocampal lysate (Leschik et al., 2019) was used as positive control. Western Blot was performed as previously described (Leschik et al., 2013) with monoclonal mouse antibodies against human BDNF (1:200, Icosagen, Tartu, Estonia; RRID:AB_2631315), and, after stripping membranes, with monoclonal mouse anti-GFP antibodies (1:500, Roche Diagnostics, Basel, Schweiz; RRID:AB_390913). Detection was performed with supersensitive Weststar Supernova chemiluminescent substrate (Cyanagen, Bologna, Italy).

5. Statistical analysis

Data are either presented as mean ± standard error of the mean (SEM) in bar graphs or as box plots depicting the median, the 25th and 75th percentiles and max-min single data points. Statistical analysis was done using GraphPad Prism 4 and 7 (GraphPad Software Inc, La Jolla, CA, USA) and IBM SPSS Statistics 22 v software (IBM Corporation, Armonk, NY, USA). When comparing only two groups, 2-tailed unpaired student's t-test was used. Differences in experiments with four groups were tested with 2-way analysis of variance (ANOVA) for factors genotype and stress and post hoc Tukey test. In case of significant interaction (genotype * stress) consecutive 1-way ANOVA with Tukey's post hoc test were applied. Differences were assumed to be significant if $p < 0.05$, (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Unless otherwise stated, non-significant differences are not indicated. Correlations were assessed using Pearson's rank correlation test.

Further methods are described in Supplementary Information.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pneurobio.2022.102333.

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