Perturbation of GABAergic Synapses at the Axon Initial Segment of Basolateral Amygdala Induces Trans-regional Metaplasticity at the Medial Prefrontal Cortex

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Abstract

GABAergic synapses in the basolateral amygdala (BLA) play an important role in fear memory generation. We have previously reported that reduction in GABAergic synapses innervating specifically at the axon initial segment (AIS) of principal neurons of BLA, by neurofascin (NF) knockdown, impairs fear extinction. BLA is bidirectionally connected with the medial prefrontal cortex (mPFC), which is a key region involved in extinction of acquired fear memory. Here, we showed that reducing AIS GABAergic synapses within the BLA leads to impairment of synaptic plasticity in the BLA-mPFC pathway, as well as in the ventral subiculum (vSub)-mPFC pathway, which is independent of BLA involvement. The results suggest that the alteration within the BLA subsequently resulted in a form of trans-regional metaplasticity in the mPFC. In support of that notion, we observed that NF knockdown induced a severe deficit in behavioral flexibility as measured by reversal learning. Interestingly, reversal learning similar to extinction learning is an mPFC-dependent behavior. In agreement with that, measurement of the immediate-early gene, c-Fos immunoreactivity after reversal learning was reduced in the mPFC and BLA, supporting further the notion that the BLA GABAergic manipulation resulted in trans-regional metaplastic alterations within the mPFC.

Key words: amygdala, medial prefrontal cortex, metaplasticity, neurofascin, reversal learning

Introduction

Emerging evidence from behavioral and electrophysiological studies on rodents indicate that amygdala plays critical role in orchestrating fear response (Phelps and LeDoux 2005; Pape and Pare 2010). However, amygdala circuits act via dynamic interactions between several other wired brain structures (Pitkänen et al. 2000; Johansen et al. 2012). Consequently, fear memories rely on the interplay between the basolateral amygdala (BLA),
the hippocampus and the medial prefrontal cortex (mPFC). In this fear memory network, hippocampus modulates contextual aspects of fear learning (Maren 2001) while reciprocal connections between the amygdala and medial prefrontal cortex (mPFC) are extensively involved in fear extinction (Sotres-Bayon and Quirk 2010). BLA projection neurons are both directly and indirectly connected to the hippocampus (Pikkarainen et al. 1999). Pharmacological and electrophysiological manipulations of the amygdala alter hippocampus-dependent memory (Roosendaal et al. 1996; McGaugh 2000) and hippocampal LTP (Akirav and Richter-Levin 1999; Abe 2001). Similarly, BLA and mPFC projection neurons are also bidirectionally connected and BLA projection neurons can alter the neuronal firing of mPFC neurons (Perez-Jaranay and Vives 1991). BLA-mPFC pathway concurrently undergoes dynamic and plastic changes following fear acquisition and fear extinction. Most importantly, normal fear extinction training induces increased synaptic plasticity in BLA-mPFC pathway (Vouimba and Maroun 2011).

Previously we could demonstrate that knockdown of the cell adhesion molecule NF led to a selective reduction of GABAergic synapses restricted to the axon initial segment (AIS) of BLA principal neurons. Electrophysologically, synaptic plasticity in a form of LTP within the BLA was impaired. Behaviorally, such specific modulation of GABAergic local circuit activity within BLA resulted in an impairment of fear extinction (Saha et al. 2017). Based on these observations and on previous findings indicating that altered BLA may modify the way the BLA modulates plasticity in other brain areas (Akirav and Richter-Levin 1999; Vouimba and Richter-Levin 2005; Richter-Levin and Maroun 2010; Bergado et al. 2011), we hypothesized that local modification of activity within the BLA by NF knockdown could alter the way the BLA modulates fear memory formation in other brain regions. In the current study, we thus investigated the impact of such local BLA manipulation on LTP in the BLA-mPFC pathway. LTP in the BLA-mPFC pathway was found to be impaired. While this impairment could result from the alteration within the BLA, it could potentially also reflect alterations within the mPFC, which is known to be pivotal for extinction learning (Milad and Quirk 2002). In order to verify whether this BLA-mPFC LTP impairment was due to local manipulation in BLA or a result of changes induced by BLA within the mPFC, we have further investigated the impact of such local BLA manipulation on LTP in the ventral Subiculum-mPFC pathway, a pathway excluding the amygdala. Ventral subiculum-mPFC LTP was also found to be impaired, even while the BLA was inactivated, indicating metaplastic alterations of mPFC synapses, independent of the activity within the BLA itself. These metaplastic alterations within the mPFC as a result of local manipulations within the BLA suggest a new form of metaplasticity, that is, trans-regional metaplasticity.

Traditionally, metaplasticity refers to modulation of the probability to induce synaptic plasticity in a neural circuit by prior activation of the system (Abraham and Bear 1996), and is typically measured by changes in the threshold for the induction of LTP or LTD (Abraham 2008). As previously shown by Richter-Levin and Maroun (Richter-Levin and Maroun 2010) metaplasticity can be demonstrated also in the mPFC. Elevated platform stressor or electrical activation of the BLA prior to high-frequency stimulation to the vSub resulted in a form of metaplasticity, expressed as subsequent impaired LTP induction in the mPFC. Apart from this metaplasticity at the synaptic level, there is also behavioral metaplasticity (reviewed in Schmidt et al. (2013)), where different factors such as developmental programing and epigenetic changes may give rise to subsequent alterations in behavioral responses. Here in the present study, we could demonstrate how selective manipulation of GABAergic synapses in the BLA generates trans-regional metaplasticity in mPFC.

We have further examined reversal learning in the water maze (WM), which is considered to be another mPFC-dependent behavior (de Bruin et al. 1994; Dalley et al. 2004). The result indicated that trans-regional metaplasticity leads to impaired cognitive flexibility in neurofascin knockdown rats. Additionally, we have checked c-Fos immunoreactivity as a proxy for neuronal activation in mPFC, right after the reversal learning. In agreement with the above results, reduced activation of c-Fos in mPFC was found, supporting the notion of trans-regional metaplastic changes induced by BLA within the mPFC. To examine whether this form of trans-regional plasticity was selective to the BLA-mPFC pathway or more general, we examined the impact of a similar manipulation within the BLA on dentate gyrus (DG) plasticity and related behavior. Synaptic plasticity in the DG, spatial object recognition (SOR) and contextual fear conditioning were not affected by NF knockdown in the BLA.

We thus demonstrate for the first time how a moderate change of a specific GABAergic synapse in one region results in trans-regional alteration in the subsequent responsiveness of other region at both the behavioral and electrophysiological levels.

**Materials and Methods**

**Animals**

Male Sprague–Dawley rats (Harlan Laboratories, Jerusalem, Israel) weighing 200–224 g on arrival were group-housed at room temperature (21 ± 2 °C) on a 12 h light/dark cycle (lights on at 07:00 hours), with water and food pellets ad libitum. Experimental procedures began after 5 days of acclimation to the vivarium. All experiments were conducted in accordance with the NIH guidelines for the care and use of laboratory animals and were approved by the University of Haifa ethical committee.

**Stereotaxic Lentivirus Injection**

Rats received bilateral microinjections of either lentiviral vectors (construct represented in Fig. S1A, for more details (Saha et al. 2017)) expressing miRNA directed against NF (NF1707) or the control miRNA (CTR) into the BLA (detailed in vitro and in vivo validation and functional characterization of the constructs are available in Saha et al. (2017)). Deeply anesthetized rats (10% Ketamine, 100 mg/kg, (Richter Pharma, Wells, Austria) and 2% medetomidine, 10 mg/kg (Orion Pharma, Espoo, Finland), both i.p.) were mounted on a stereotaxic frame (Stoelting instruments) under control of body temperature and antibiotic (15% Vetrinoxim, 0.2 mL/kg, s.c.; Vetmarket, Petah Tikva, Israel) and analgesic (Calmagime, 0.3 mL/kg, s.c.; Vetmarket) treatments. Body temperature was maintained throughout surgery with an adjustable warming pad. A midline incision was made down the scalp, and craniotomy was made using a dental drill. Next, a 33 G stainless steel cannula mounted on a microsyringe (NanoJil, World Precision Instruments) were lowered bilaterally into the BLA at the following coordinates: −2.8 mm AP, ± 4.7 mm ML from bregma; −7.7 mm DV from brain surface, according to Paxinos and Watson (2006). After 5 min of rest in the target area, 2 μL of viral vector suspension were injected at 0.15 μL/min (UMP3 microsyringe pump and Micro4 controller, World Precision Instruments) and the microsyringe was slowly withdrawn 10 min after the injection. After closing the scalp by suture, Antisedian...
(10 mg/kg s.c) was injected and animals were allowed to recover for at least 14 days before assessment of viral knockdown or behavioral testing commenced.

After injection of lentiviral vectors for the knockdown of NF, we have performed 4 types of experiments in this study. In Experiment 1, after recovery from viral vector injection, animals went through a battery of behavioral tests including open field test (OFT), elevated plus maze (EPM) and fear conditioning and extinction. Next, animals were anesthetized for in vivo electrophysiological recordings conducted at the medial prefrontal cortex (mPFC) while stimulating the BLA (see schematic illustration in Fig. 1A).

In Experiment 2, another set of animals were injected with NF knockdown and CTR knockdown virus. After 21 days of recovery in vivo electrophysiological recording were done within mPFC while stimulating electrode was placed in ventral subiculum (vSub) (Fig. 1B).

In Experiment 3, animals were injected and after recovery, they went through 8 days of WM task for the evaluation of reversal learning. In an extended part of this experiment, a subset of animals from both groups were perfused 90 min after the reversal learning on day 8 and later immunohistochemical analysis were done for c-Fos in mPFC and BLA (Fig. 1C).

In Experiment 4, another set of animals were injected with NF and CTR knockdown viral vector in the BLA. About 21 days after the recovery from the surgery, animals were divided randomly in 3 subsets. One set of animals were used for SOR task, while the second set was used for contextual fear conditioning and the third set of injected animals went through in vivo electrophysiological recording from the DG, stimulating in the perforant path (Fig. 1D).

Figure 1. Overview of all the experiments performed in the study. (A) Schematic representation of Experiment 1 displaying the flow of experimental procedures pursued at different postnatal day (PND) (OFT, EPM). (B) Scheme of experimental timeline for Experiment 2 for in vivo electrophysiological recording in vSub-mPFC pathway. (C) Scheme of experimental timeline for Experiment 3 for WM task. (D) Scheme of experimental timeline for Experiment 4, where after 21 days of viral injection animals went through either SOR or contextual fear conditioning or in vivo electrophysiological recording in perforant path-DG pathway.
Moreover, after completion of all the experiments, bilateral viral EGFP expression was evaluated (see Figs. S3–S5, S8, S10–S12 for representative EGFP expression within BLA).

All the detailed procedures of this study are described below.

Behavioral Analysis

Open Field Test
The open field was used to assess the locomotor activity and anxiety-like behavior as previously described in (Saha et al. 2017). Briefly, the apparatus consists of a square Plexiglas (black) box (90 × 90 × 50 cm) positioned in a dimly lit, ventilated, sound-attenuated room. At the time of testing, after 5 min habituation to the room in a standard cage rats were placed at the corner of the open field facing the wall and was allowed to explore the novel environment for 5 min while their behavior was recorded and analyzed via an EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands).

EPM Test
Anxiety-like behavior was assessed in the EPM as previously described in (Saha et al. 2017). After 5 min habituation to the room, rats were placed in the center of a cross-shaped maze (112 × 8 cm) with 2 opposing open and closed arms (walls 30 cm high) raised 50 cm above the floor in a brightly lit room. Rats were allowed to explore the maze freely for 5 min, while behavior was recorded and analyzed via an EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands).

Cued Fear Conditioning and Extinction
Cued fear conditioning and extinction took place in a sound-attenuating chamber (Panlab, Harvard Apparatus, Barcelona, Spain) containing either context A (24 × 26 × 27 cm box, grid-floor, black walls, full light, cleaned with double distilled water) or context B (transparent cylinder in white 24 × 26 × 27 cm box, white floor cover, cleaned with 70% ethanol). Procedures were adapted from (Vouimba and Maroun 2011). After 2 days of habituation in context B (10 min each day), fear conditioning commenced on day 3 in context A. On the day of conditioning, after 2 min of exploration, rats received 3 tones (conditioned stimulus, CS; 4 kHz, 30 s, 80 dB sound pressure level), each paired with a foot-shock (unconditioned stimulus, US; 0.6 mA, 1 s) at an inter-trial interval (ITI) of 2 mins. After 24 h of conditioning, rats underwent fear extinction training for 3 consecutive days. On each day of extinction, rats spend 22 min in context B during which 10 CS tones (ITI 1.5 min) were delivered in the absence of foot-shocks. The animal’s movement throughout the sessions was monitored via a high-sensitivity weight-transducer system connected to the grid-floor. Freezing, that is, the absence of all movement except for respiration, was quantified offline via the Packwin Software (Panlab, Harvard Apparatus, Barcelona, Spain) during CS presentation. For the extinction analysis, 2 CSs were later averaged to “Blocks”.

WM Reversal Learning Task
To assess the reversal learning, the WM task was performed in a circular black tank (150 cm diameter; depth: 60 cm) filled with water (±23 °C, around 30 cm depth) and placed in a dimly lit room with extrinsic visual clues (adapted from Cerqueira et al. (2005, 2007)). The circular tank was divided into 4 imaginary quadrants (Q1, Q2, Q3, and Q4) and a hidden platform (12 cm diameter, submerged 2 cm below the surface of the water) was placed in the center of one of them. Swimming tracks were recorded and analyzed via an EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands). Rats were trained continuously for 8 consecutive days for 4 trials of 2 min daily. Every day the trials (see Fig. S7B for schematic presentation of trial sequence) started by placing the rats in the tank, facing the wall of the maze, at a different starting point (in any of the quadrants in each session). Trials were finished once the rat found out the hidden platform; if not after 2 mins of swimming rats were gently guided to the platform. Upon finding out the platform rats were trained to stay on the platform for 30 s. After this 30 s rats were dried with a towel and then rested for 1 min until the next trial starts.

Working Memory
First 4 consecutive days (first to fourth day) spatial working memory was assessed in which the platform was hidden in randomly chosen different quadrant (Fig. S7A). The distance traveled and the time spent to reach the platform (escape latency) was evaluated.

Reference Memory
From day 4 the platform was hidden at the same quadrant until day 7 (Fig. S7A). This was performed to assess the reference memory. The relevance of this reference memory was to ensure that the rats correctly learned the position of the platform before reversal learning for the assessment of behavioral flexibility.

Reversal Learning
On eighth day the hidden platform was moved exactly opposite (new) from the seventh day quadrant (old) (Fig. S7A). Total distance traveled and duration spent on these 2 “new” and “old” quadrant indicated the reversal performance.

For the analysis of the working memory, the daily trial-to-trial progression of the distance swum to reach the platform was averaged for the different platform locations. While in case of reference memory, day-to-day progression was averaged across the 4 daily trials for the same platform location. Heat maps of reversal learning have been generated via EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands).

Spatial Object Recognition
To evaluate the object place recognition memory, we have used a modified SOR task based on the protocol of (Barbosa et al. 2012, 2013). Prior to the commencement of the behavioral task, all the animals were handled daily for 4 days. All the animals were habituated to the open field arena (90 × 90 × 50 cm) in the absence of any object for 2 × 5 min exploration a day for 2 days. On the third day, 2 identical objects (glass bottle filled with sand) were introduced in the open field arena on a specific location (adjacent quadrants) and animals were allowed to explore the objects for 5 min. After a retention interval of 1 h, the location of 1 object was shifted to an opposing quadrant (novel location) and 5 min of exploration were allowed. Exploration and contacts to the objects were recorded and analyzed via an EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands).

Contextual Fear Conditioning and Extinction
Contextual fear condition and extinction protocol were adapted from Awad et al. (2015). The apparatus was the cued fear conditioning chamber described above. On the day of conditioning
all the animals were allowed to explore the context (24×26×27 cm box, grid-floor, black walls, and full light, cleaned with 70% ethanol wipes) for 2 min. Next, they received 3 sets of foot-shocks delivered through the grids at intensity of 0.6 mA for 0.5 s. The whole conditioning session lasted ~8 min. The chamber was cleaned with 70% ethanol wipes and dried with paper towels after each rat. From 24 h after conditioning, the rats were introduced to a 10 min of non-reinforced exposure session by placing them in the chamber without receiving any electrical shock, every 24 h for 3 days (T1-T3). Animal’s movement or freezing, that is, the absence of all movement except for respiration, was quantified offline via the Packwin Software (Panlab, Harvard Apparatus, Barcelona, Spain).

### In Vivo Electrophysiology

Deeply anesthetized rats (40% urethane, 5% chloral hydrate in saline; 0.5 mL/100 g, i.p.) were placed in a stereotaxic frame (Stoelting, USA) with body temperature maintained at 37 ± 0.5 °C. Small holes were drilled into the skull to allow the insertion of electrodes into the brain. A single recording microelectrode (glass; tip diameter, 2–5 μm; filled with 2 M NaCl; resistance, 1–4 MΩ) was slowly lowered into the mediofrontal cortical structure (mPFC) (anteroposterior, 3.0–3.3 mm anterior to bregma; 0.7–1.0 mm lateral; 3.8–4.8 mm below the pial surface. A bipolar 125 μm stimulating electrode was inserted into the BLA (anteroposterior, –3 mm relative to bregma; lateral, 5 mm; ventral, –7.6 mm) (adapted from Schayek and Maroun 2015) or into the subicular region of ventral hippocampus (vSub) (anteroposterior, –6.3 mm relative to bregma; lateral, 5.0 mm; ventral, –7.5 mm) (adapted from Richter-Levin and Maroun 2010). For DG recording (similar standard recording electrode was slowly lowered into DG coordinates: 4.0–4.2 mm posterior to bregma, 2.4–2.6 mm lateral to the midline, depth 3.5–3.7 mm) while the stimulating electrode was placed in the perforant path (coordinates: 7.5–8.0 mm posterior to bregma, 4.0–4.2 mm lateral to the midline, depth 3.2–4.0 mm). Actural depth of the electrodes for each animal was adjusted to yield maximal response (excitatory post-synaptic potential) recorded in the DG. The evoked responses were digitized (10 kHz) and analyzed using a Cambridge Electronic Design (version 7) 1401-plus data acquisition system (Cambridge, UK) and its Spike2 software. Test stimuli (monopolar or biphasic pulses for DG; 100 μs duration) were delivered at 0.1 Hz. After positioning the electrodes, the rats were left for 30 min before commencing the experiment.

### LTP Induction

LTP was induced using the Theta Burst Stimulation (TBS) protocol, which was applied in all groups within 1.5 h of anesthesia. Theta-like high-frequency stimulation was delivered at 100 Hz (TS-100) to the BLA, vSub or perforant pathway in 3 sets of 10 trains, each consisting of 10 pulses (inter-train interval, 200 ms; inter-set interval, 1 min). In all experiments, baseline responses were established by delivering a stimulation of sufficient intensity (300–800 μA) to elicit a response representing 40–50% of the maximal amplitude of the evoked field potentials. TBS was delivered at the same intensity as the test stimuli during establishment of the baseline responses. Evoked field potentials at the baseline intensity were recorded from the mPFC or DG for 60 min following the application of TBS. The results are presented as the mean of each 5 min period to give 12 time points. LTP was defined as an increase in the amplitude of the field post-synaptic potentials (fPSPs). Changes in fPSP amplitude were measured for each rat as a percentage change from its baseline.

### Drugs and Infusion Procedure

In extended part of Experiment 2 we have infused lidocaine (100 μg/μl) (Boehnke and Rasmussen 2001; Kantak et al. 2002) to inactivate the BLA. BLA inactivation was verified electrophysiologically in another set of animals (Fig. S6A,B). Lidocaine was infused into the BLA. During the recording via an injection cannula which was connected through PE20 tubing to a Hamilton microsyringe driven by a microinfusion pump (Harvard Apparatus, USA). Microinjections were performed using a volume of 0.5 μL/min delivered over 2 min. Intra-BLA injection of lidocaine was performed 30 min before the application of TBS.

### Immunohistochemistry

About 90 min after the onset of reversal learning, rats were anesthetized with sodium pentobarbital (CTS, Kirty Malachi, Israel) and perfused intracardially with ice-cold saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS, 0.01 M, pH 7.4).

The brains were removed and stored in the same fixative for 24 h at 4 °C and subsequently immersed in 30% sucrose at 4 °C. Brains were then frozen in powdered dry ice and stored at –80 °C until sectioning. Coronal sections (30 μm) containing relevant structures such as mPFC (3.20 mm posterior to bregma), BLA (~2.80 mm posterior to bregma), and DG (~3.14 mm posterior to bregma) (Faxinos and Watson 2006) were cut using a cryostat (Leica Microsystems, Wetzlar, Germany) at –20 °C and collected in PBS for immunohistochemical processing (adapted from Ritov et al. (2016)).

Free-floating sections were washed (3 times for 10 min each) in PBS and incubated for 15 min in Background Sniper (Biocare Medical, Concord, CA, USA). Sections were then incubated with the primary antibodies for c-Fos (rabbit anti c-Fos: 1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA, catalog no. 2250 S) in 3% bovine serum albumin in PBS with 0.3% Triton X-100 (PBST) for an overnight incubation at 4 °C on a shaker. On the next day, sections were washed (3 times for 10 min each) in PBS and incubated on a shaker for 1 h with the secondary antibody Alexa Fluor 555 (1:1000 dilution, Invitrogen, Waltham, MA, USA) in PBST at room temperature. Finally, sections were washed (3 × 10 min with 0.01 M PBS), mounted on super frost glass slides, embedded with Immunomount (Sigma-Aldrich, Switzerland), cover slipped and stored at 4 °C protected from light.

Fluorescent images were taken with a Zeiss AxioScope.A1 (Carl Zeiss, Jena, Germany) epifluorescence microscope equipped with a digital camera AxioCam MRc (Carl Zeiss) using a 5× objective. In order to characterize activated cells the number of labeled c-Fos immunoactive (IR) nuclei were quantified bilaterally and averaged from 4 different sections (90 μm apart) containing mPFC, BLA and DG for each rat. Sampled areas were about 1 mm², and the number of labeled cells was manually counted in a blind manner using the ZEN lite 2012 software (Carl Zeiss).

### Statistical Analysis

Data were analyzed using the IBM SPSS (21) Statistics software (IBM, Armonk, NY, USA). All behavioral, electrophysiological and immunohistochemical results were analyzed using independent sample t-test and mixed model of repeated measures ANOVA with appropriate Greenhouse-Geisser or Huynh-Feldt corrections for sphericity issues when necessary. All results are presented as mean ± SEM.
Results

Neurofascin Knockdown Within BLA Impairs Fear Extinction Without Affecting Anxiety Measures

In Experiment 1 (see Fig. 1A), after recovery from viral vector injection rats went through the behavioral battery of OFT, EPM and cued fear conditioning and 3 days of extinction. NF knockdown did not affect locomotor activity as assessed by the total distance moved (Fig. S2A; t(20) = 0.130, P > 0.05). Moreover, the percentage of distance moved in the center of the open field arena was comparable in both groups, which confirms that anxiety-like phenotype was unaffected after the viral manipulation (Fig. S2B; t(20) = 0.461, P > 0.05). Similarly, the total distance moved in the EPM arena (Fig. S2C; t(20) = 0.795, P > 0.05) and the percentage of distance moved (Fig. S2D; t(20) = 1.891, P > 0.05) or the duration spent (Fig. S2E; t(20) = 1.609, P > 0.05) in the open arms did not show any significant differences between groups.

Next in the fear conditioning training day the baseline freezing of both CTR (n = 8) and NF (n = 13) showed similar level of low pre-conditioning freezing level in context A (Fig. S2F; t(20) = 0.372, P > 0.05). During the training phase both groups displayed increasing levels of freezing percentage during the 3 conditioned stimulus (CS)-US pairings (Fig. S2G) as assessed by repeated-measure ANOVA (effect of tones: F(1,40, 27.84) = 670.90, P = 0.001; no group effect: F(1,20) = 0.073, P = 0.790; no group × tone interaction: F(1,40, 27.84) = 0.881, P = 0.390) in context B. About 24 h after the fear acquisition, 3 days of fear extinction training started in context A. In the session 1 both CTR and NF showed high level of freezing, while in second and third session freezing percentage in CTR group reduced significantly, while NF group continued to show higher level of freezing percentage as measure by repeated-measure ANOVA (F(7,11,142.24) = 26.08, P < 0.001) with a significant block × group interaction (F(7,11,142.24) = 3.42, P < 0.01), but no general group effect (F(1,20) = 3.76, P > 0.05). (Fig. S2H).

Neurofascin Knockdown Occludes Induction of LTP in the BLA-mPFC Pathway

Next in Experiment 1, we have employed in vivo recording to assess the level of synaptic plasticity within the mPFC as it is considered to be a key region involved in fear extinction. To that end we stimulated in BLA with NF knockdown (see Fig. S3A,B for verification of virus expression) and recorded the neuronal responses (Fig. 2A) from the mPFC (see Fig. 2B for schematic representation). Upon TBS application, BLA-evoked field potentials in the mPFC were effectively potentiated in the control group, but not in the NF knockdown group. Mixed model repeated-measure ANOVA of the last 5 time points of potentiation level revealed strong difference between groups (group effect: F(1,19) = 21.72, P < 0.001; and group × time point interaction: F(1,68,32) = 0.652, P > 0.05; Fig. 2C).

Neurofascin Knockdown Within BLA Impairs Synaptic Plasticity in the vSub-mPFC Pathway

The blockade of LTP in the BLA-mPFC pathway could result from the induced altered activity in the BLA but could also result from subsequent modifications within the mPFC. To dissociate between these 2 possibilities, we examined the impact of NF knockdown within the BLA also on LTP in the mPFC induced by activating a BLA independent pathway—the vSub-mPFC pathway. If the impaired LTP in the BLA-mPFC pathway was a result of the intra-BLA alterations than vSub-mPFC pathway should not be affected. Animals were injected with viral vectors for the knockdown of NF (n = 6) or an ineffective control construct CTR (n = 6) (see Fig. S4A,B for verification of virus expression). After recovery from the injection we have employed in vivo electrophysiological recording from the mPFC while stimulating in the vSub (see Fig. 3B for schematic representation) and the neuronal responses were measured (Fig. 3A). Upon TBS application vSub-evoked field potentials in the mPFC were effectively potentiated in the control group while NF knockdown group showed impaired LTP (Fig. 3C,D). Mixed model repeated measures of ANOVA of the last 5 time points of potentiation level showed significant difference between groups (group effect: F(1,10) = 5.479, P < 0.05; and no group × time point interaction: F(1,93,19.27) = 0.545, P > 0.05). This impairment of LTP in vSub-mPFC pathway implicates that BLA-NF knockdown-induced trans-regional alterations within the mPFC which affected the ability to induce synaptic plasticity in a pathway independent of the BLA, that is, the vSub-mPFC pathway.

In order to further ensure independency from the impact of the BLA we examined the ability to induce LTP in the vSub-mPFC pathway while transiently inactivating the BLA. First, we verified the effectiveness of lidocaine-induced BLA inactivation by measuring the blockade on BLA-mPFC LTP in naive animals. Lidocaine (100 μg/μl) was infused into the BLA and results showed that after TBS application in BLA naive (Naive-Lido (n = 5)) animals with intra-BLA lidocaine failed to generate potentiation in the mPFC compared to the Naive-control (n = 5) animals (Fig. S6B). Mixed model repeated-measure ANOVA of the last 5 time points of potentiation level showed significant difference between groups (group effect: F(1,8) = 53.10, P < 0.001; and no group × time point interaction: F(1,30, 10.41) = 0.274, P > 0.05).

We then recorded from the mPFC while stimulating the vSub from control and BLA-inactivated groups (CTR-Lido (n = 6) and NF-Lido (n = 6)) (see Fig. S5A,B for verification of virus expression). CTR-Lido group developed effective mPFC potentiation while in the NF-Lido group mPFC LTP was impaired, despite BLA inactivation (Fig. 3D). Mixed model repeated measures of ANOVA of the last 5 time points of potentiation level showed significant difference between groups (group effect: F(1,10) = 12.156, P < 0.01; and no group × time point interaction: F(1,40) = 0.116, P > 0.05) (Fig. 3E). Moreover, we have also performed mixed model repeated-measure ANOVA of the last 5 time points of potentiation level between all 4 groups (CTR, NF, CTR-Lido, NF-Lido) of Experiment 2. The result shows a strong group effect (group effect: F(3,20) = 6.124, P < 0.01; and no group × time point interaction: F(12,80) = 0.781, P > 0.05). Post-hoc comparisons between the 4 groups revealed that NF knockdown group was significantly different from CTR and CTR-Lido groups (< 0.05), while not significantly different from the NF-Lido group (P > 0.05).

This result confirms that NF knockdown within the BLA leads to lasting trans-regional alterations within the mPFC which affected the ability to induce LTP within the mPFC independent of the alterations within the BLA. This trans-regional alteration in the ability to induce plasticity may be termed trans-regional metaplasticity.

Neurofascin Knockdown Impaired Cognitive Flexibility in Reversal Learning While Working and Reference Memory was Unaffected

To evaluate the impact of NF knockdown on mPFC related behavior we have utilized in Experiment 3 a modified version of the WM paradigm, which is considered to be dependent on mPFC functioning (Cerqueira et al. 2005, 2007) after NF

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knockdown in BLA (see Fig. S8 A for verification of virus expression) (see Fig. 1C for experimental timeline). There was no difference between the groups (NF (n = 14); CTR (n = 15)) in the working memory evaluation task (Fig. 4A), assessed during the first 4 days of training, (F(2.18, 248.33) = 55.11, P < 0.001). Repeated measure of tested by ANOVA indicated no significant difference group × trial interaction (F(2.18, 248.33) = 2.23), P = 0.105) and no general group effect (F(1, 114) = 1.06, P = 0.305).

Both groups did not differ also in the reference memory task (Fig. 4B) measure during days 4–7. ANOVA for repeated measures revealed significant days effect F(1.60, 182.60) = 23.11, P < 0.001 but no group × days interaction (F(1.60, 182.60) = 0.17, P = 0.80) and no general group effect (F(1, 114) = 0.38, P = 0.54). In contrast, in the behavioral flexibility task of reversal learning measured on eighth day CTR (n = 15) effectively learned the new location of the platform while the NF (n = 14) knockdown group exhibited impaired preference, with a tendency to persevere and search the old location quadrant. The CTR group crossed significantly longer distance in the new quadrant (Q1) (Fig. 4D t(98.28) = 3.02, P < 0.01) compared to NF knockdown group. In contrast, the NF knockdown group exhibited significantly longer distance in the old quadrant Q3 from day 7 (t(114) = 3.18, P < 0.01). Similarly, the NF knockdown group spent less time in the new quadrant (Q1) compared to CTR (Fig. 4E, t(98.98) = 3.03, P < 0.01), and spent significantly more time in old quadrant Q3 (t(114) = 3.28, P = 0.001). “Heat maps” (average of all the animals from both CTR (n = 15) and NF (n = 14) knockdown group) presented in Figure 4C clearly demonstrate the deleterious effect of NF knockdown on reversal learning. The CTR group started looking for the hidden platform in the old quadrant (Q3) on trial 1, but in trial 3 and 4 they clearly preferred the new location of the platform (Q1). In contrast, the NF knockdown group kept showing their preference to the old quadrant Q3 from trial 1 to trial 4.

Neurofascin Knockdown Reduces c-Fos Activation in mPFC and BLA After Reversal Learning

In an extended part of Experiment 3, to test whether NF knockdown within the BLA alters the neuronal activation in BLA but also in the mPFC after reversal learning, we measured in a subset of animals (CTR (n = 6) and NF (n = 7)) the expression levels of the immediate-early gene c-Fos as a proxy for neuronal activity (Liang et al. 2015). After reversal learning c-Fos activation was lower in the BLA of the NF knockdown group compared to controls (Fig. 5D; t(11) = 3.44, P < 0.01). Importantly,
Figure 3. Neurofascin knockdown leads to reduced synaptic plasticity in the vSub-mPFC pathway. (A) Representative evoked field potential responses recorded in the mPFC of rats injected with CTR (n = 6) or NF (n = 6) knockdown group before (gray) and after TBS (black) in the vSub to induce LTP. (B) Graphical representation of the rat brain showing the coronal levels of the mPFC and the ventral subiculum (vSub) investigated in this set of electrophysiology. In vivo electrophysiological recordings were obtained from mPFC while the stimulating electrode was placed in vSub. (C) TBS to the vSub pathway induced a significant potentiation of mPFC field potentials in CTR (n = 6) rats, which was reduced in NF (n = 6) knockdown rats. (D) Representative evoked field potential responses recorded in the CTR (n = 6) or NF knockdown (n = 6) before (gray) mPFC while the BLA was inactivated and after TBS (black) in the vSub to induce LTP in the mPFC. (E) While the BLA is inactivated TBS to the vSub pathway induced a significant potentiation of mPFC field potentials in CTR-Lido (n = 6) rats, which was significantly reduced in NF-Lido (n = 6) knockdown rats. (The statistics were performed between 4 groups described in the main text. For the clarity of presentation, we have divided in 2 separate sub figures). Data are shown as means ± SEM. Asterisks indicate significant differences (**P < 0.001).
Figure 4. Neurofascin knockdown affects the spatial reversal learning without altering the working and reference memory in the WM. (See Fig. S7A for the graphical sketch of task). (A) Working memory measured on the first 4 days of WM task showed no difference between CTR (n = 15) and NF (n = 14) knockdown groups. (B) Similarly, reference memory evaluated from day 4 to day 7 also showed comparable effect in both group. (C) Heat maps showing the combined traces of all the animals from both CTR and NF knockdown group during day 8 trial 1 to trial 4. NF knockdown group spent more time in the old quadrant searching for the hidden platform while CTR group learned the new location. (D) Reversal learning task on day 8 showed clear deficit in NF knockdown group as measured via % of distance swam in 4 different quadrants. NF knockdown group showed significantly more preference to the old quadrant (Q3) while CTR group spent more time in new quadrant (Q1). (E) Measurement of % of time spent in old (Q3) and new (Q1) also confirmed NF knockdown group spent more time in the old quadrant (Q3). Data are shown as means ± SEM. Asterisks indicate significant differences (**P < 0.01).
also in the mPFC c-Fos positive cell numbers were significantly lower in the NF knockdown group compared to the CTR group (Fig. 5B; t(5.27) = 3.88, P < 0.05). We further counted c-Fos positive cells separately within infralimbic (IL) and pre-limbic (PL) parts of the mPFC (Fig. 5A,B). c-Fos positive cell numbers were reduced in both part of the mPFC in the NF knockdown group (Fig. 5A; PL: t(6.53) = 5.002, P < 0.01 and Fig. 5B; IL: t(11) = 3.02, P < 0.05). These findings indicate that the deficit in behavioral flexibility is correlated with decreased responsiveness of the mPFC after reversal learning task. Interestingly, we have also compared the level of c-Fos positive cells in another area known to be modulated by the BLA, that is, the DG and found no difference between 2 groups (Fig. 5F; t(5.27) = 0.33, P = 0.77).

Spatial Object Memory was Unaltered After Neurofascin Knockdown
The results so far suggest that NF knockdown within the BLA results in trans-regional alterations or trans-regional metaplasticity in the mPFC. We next set out to verify whether this effect was specific to the BLA-mPFC axis or would similar alterations be found in other brain areas modulated by the BLA. We have already found that hippocampus-dependent aspects of the VM task were not affected (Fig. 4A,B) and the c-Fos activation within the DG was not affected (Fig. 5F). We set out to examine the impact of BLA-NF knockdown on other hippocampus-dependent behaviors. We thus examined the impact of the BLA manipulation on the animals’ performance in a SOR task (part of Experiment 4) in which hippocampus is more extensively involved. Animals were injected with NF (n = 11) or CTR (n = 9) virus (see Fig. S10A,B for viral expression) before the commencement of this task. During the testing phase both CTR and NF group showed similar level of preference, spending more time around the object placed in a new, shifted location (2 way repeated-measure ANOVA: duration percentage: F(1,18) = 26.25, P < 0.001, no duration×group interaction: F(1,18) = 0.754, P > 0.05), post-hoc pairwise comparison of CTR (familiar vs. novel: P > 0.01 and NF [familiar vs. novel: P > 0.01]) (Fig. 6A). No significant differences were found between NF and CTR group in the time spent in the old location or in the novel location (t(18) = 0.87, P > 0.05). Similarly, comparable number of contacts towards the object placed in a new location were found for both groups (t(18) = 0.39, P > 0.05) (Fig. 6B). Together these results suggest that BLA-NF knockdown does not alter spatial object memory.

Neurofascin Knockdown Within the BLA Does Not Affect Contextual Fear Memory and Extinction
To further explore if the manipulation of GABAergic synapse within BLA alters other hippocampal dependent behaviors, we examined the impact of this modification on contextual fear conditioning and extinction which are considered to depend much on the hippocampus (Phillips and LeDoux 1992). NF (n = 7) and CTR (n = 5) virus injected animals (see Fig. S11A,B for viral expression) were subjected to contextual fear conditioning. About 24 h after the application of foot-shock fear memory was assessed in the training context. Both NF (n = 7) and CTR (n = 5) groups showed similar levels of freezing (t(10) = 0.472, P = 0.65), suggesting that BLA-NF knockdown does not alter contextual fear memory (Fig. 6C). In contrast, during the 3 days of extinction learning the NF knockdown group showed higher levels of freezing while the CTR group exhibited normal extinction (Fig. 6D). Repeated measure of ANOVA revealed that significant effects of days, F(2,20) = 16.43, P < 0.001 and significant interaction days × group, F(2,20) = 6.48, P < 0.01. Moreover, post-hoc pairwise comparison of CTR versus NF in T1 showed no significant difference (P > 0.05). While in T2 and T3 there was significant difference between CTR and NF (P < 0.05).

LTP Induction in the DG was not Unaltered After Neurofascin Knockdown in the BLA
Finally, we wanted to examine whether BLA-NF knockdown could affect synaptic plasticity in the DG in a similar way to the effect found in the mPFC. About 21 days after the recovery from the viral injection (see Fig. S12A,B for viral expression), in vivo electrophysiological recording was performed in both groups NF (n = 6) and CTR (n = 6), measuring field potentials in the DG in response to stimulating the perforant path (part of Experiment 4). After induction of TBS both groups showed a similar potentiation of the EPSP slope. Repeated-measure ANOVA of the last 5 time points of potentiation revealed no significant difference between NF and CTR groups (group effect: F(1,10) = 1.756, P = 0.215, and group × time point interaction: F(1,14,12.43) = 0.74, P = 0.436; Fig. 6F). Moreover, The comparison of average potentiation across time points after induction also showed no significant difference between the 2 groups (Fig. 6G, t(10) = −1.35, P = 0.221).

Discussion
In our previous study (Saha et al. 2017), we manipulated GABAergic synapses restricted to the AIS of BLA principal neurons by means of local NF knockdown. This manipulation reduced vGAT punctae and gephyrin clustering specifically at the AIS as perisomatic inhibition was unaltered. Moreover, cellular excitability in NF knockdown animals was reduced. Together, this finding suggested the functional physiological consequence of the local GABAergic manipulation. At the behavioral level, this minor BLA manipulation led to a selective impairment of fear extinction. Indeed, in the current study, we first successfully replicated previous behavioral findings, demonstrating again BLA specific NF knockdown-induced impairment of fear extinction without any alteration in anxiety responses (Saha et al. 2017). However, the fear response is mediated by a network which includes additional brain structures, such as the mPFC and the hippocampus (Pitkänen et al. 2000). In the present study, we examined whether this local BLA modification alters synaptic activity and plasticity in other related regions, focusing here on the mPFC.

A large body of research emphasizes the involvement of the mPFC in fear extinction (Milad and Quirk 2002; Quirk and Mueller 2008). Increased neuronal activity is found in the pre-limbic part of the mPFC (PL) in response to CS presentation during fear extinction (Burgos-Robles et al. 2009), while the infralimbic subdivision of mPFC (IL) is more responsive to recall of extinction (Milad and Quirk 2002). BLA and mPFC are reciprocally connected via a direct bidirectional pathway (McDonald 1987; Cassell et al. 1989; St Onge et al. 2012) and there is also another PL to IL indirect reciprocal projection to the BLA (Mcdonald et al. 1996). Strong crosstalk between these reciprocal synaptic connections of BLA and mPFC are believed to play a crucial role in encoding fear memory and extinction (Herry et al. 2008; Vouimba and Maroun 2011). During fear memory acquisition and extinction of that conditioned fear BLA-mPFC circuitry experiences dynamic synaptic changes. Moreover, plasticity within this circuitry allows learning about the averse events and their predictors. Synaptic potentiation within
BLA-mPFC pathway is essential for normal extinction of fear (Vouimba and Maroun 2011). LTP in the mPFC was also found to be associated with the maintenance of extinction while LTD was associated with the return of fear (Herry and Garcia 2002). Interestingly, stress modulates plasticity within the BLA-mPFC circuits, which presumably provide saliency (i.e., presence or absence of threat) to the event (Maroun and Richter-Levin 2003). Thus, the lack of LTP in BLA-mPFC pathway in the present study suggests that NF knockdown rats may have difficulty in changing the established prediction, and therefore keep predicting about the aversive event even when it stopped occurring.

Extinction learning represents behavioral flexibility as it is one form of inhibitory learning. Likewise, reversal learning is a reliable behavioral paradigm to predict cognitive flexibility in rodents (Bussey et al. 1997; Floresco and Jentsch 2011). Reversal learning represents behavioral flexibility as it is one form of inhibitory learning. Likewise, reversal learning is a reliable behavioral paradigm to predict cognitive flexibility in rodents (Bussey et al. 1997; Floresco and Jentsch 2011). Reversal

Figure 5. Neurofascin knockdown causes reduction in c-Fos activation 90 min after the reversal learning. (A) Representative image of medial prefrontal cortex (mPFC) from the coronal section of CTR and NF knockdown group showing c-Fos activation (red) in the upper panel and Dapi (blue) in lower panel (scale bars: 100 μm). (B) In mPFC number of c-Fos positive cells was significantly reduced in NF (n = 7) knockdown group compared to CTR (n = 6) group. (C) Representative image of BLA from the coronal section of CTR and NF knockdown group showing c-Fos activation (red) in the upper panel and Dapi (blue) in lower panel (scale bars: 100 μm). (D) Similar to mPFC, in BLA number of c-Fos positive cells was significantly reduced in NF (n = 7) knockdown group compared to CTR (n = 6) group. (E) Representative image of dorsal DG from the coronal section of CTR and NF knockdown group showing c-Fos activation (red) in the upper panel and Dapi (blue) in lower panel (scale bars: 100 μm). (F) In contrast to mPFC and BLA, in DG c-Fos activation was comparable in CTR (n = 6) and NF (n = 7) group. Data are shown as means ± SEM. Asterisks indicate significant differences (**P < 0.01, *P < 0.05).
Figure 6. Neurofascin knockdown in the BLA does not alter SOR memory, contextual fear memory and DG plasticity. (A) Both CTR (n = 9) and NF (n = 11) knockdown group spend significantly more percentage of time exploring the object placed in the novel location compared to the object present in the familiar location. But there is no significant difference between groups for the spatial object memory. (B) CTR and NF both group showed comparable percentage of contact number towards the object located in the novel location. (C) Both CTR (n = 5) and NF (n = 7) knockdown rats showed similar level of contextual fear memory 24 h after training. (D) In the extinction session NF knockdown group showed higher level of freezing in T1, T2, and T3 compared to CTR knockdown group. The contextual extinction of NF knockdown group is severely affected. (E) Representative evoked field potential responses recorded in the DG of rats injected CTR or NF knockdown before (gray) and after TBS (black) in the perforant path to induce LTP in the DG. (F) After induction of TBS in perforant path both NF (n = 6) and CTR (n = 6) knockdown displayed similar level of potentiation in DG field potential. (G) The average % of LTP was also unaltered in NF group compared to CTR group. Data are shown as means ± SEM. Asterisks indicate significant differences (**P < 0.01, *P < 0.05).
learning has been widely examined in various cognitive tasks such as the Morris WM (D’hooge and De Deyn 2001), and T-maze (Abada et al. 2013). Shortly, in a reversal learning task, the animal has to adapt to a new set of “rules” (de Bruin et al. 1994) and inhibit the previously learned response (Gemmell and O’Mara 1999). BLA-NF knockdown rats failed to shift to the new location during the reversal learning in the WM task. Mounting research implicated the mPFC as a key regulator for different forms of behavioral flexibility tasks (Ragozzino et al. 2003; Boulougouris et al. 2007). Animals with lesion or damage in the prefrontal cortex perseverate on the initial goal or previously learned response and exhibit difficulty to switch to the new response (de Bruin et al. 1994; Delatour and Gisquet-Verrier 2000). This alteration in spatial switching is suggested to be mediated by modification in the medial prefrontal cortex, as perseveration is one of the most functional outcomes of prefrontal deficits. It has been widely demonstrated that the medial prefrontal cortex, including PL and IL regions, are involved in spatial reversal (Lacroix et al. 2002; McDonald et al. 2008). The significant deficit in reversal learning in the present study reveals that NF knockdown within BLA not only impairs BLA-mPFC synaptic plasticity but also cognitive flexibility, which is assumed to depend on mPFC functioning. This result suggests an interesting possibility that continuous alteration of BLA functioning, as induced by the intra-BLA-NF viral vector, may result in secondary alteration in related brain areas, such as the mPFC, or a form of trans-regional metaplasticity, which affects the functioning of those regions. This trans-regional metaplasticity could possibly resulted in an impaired extinction and reversal learning both of which are indicative of cognitive inflexibility.

The unaltered reference memory part of the task is in line with previous literatures indicating that lesioning the mPFC does not affect spatial reference memory while reversal learning is impaired (de Bruin et al. 1994; Lacroix et al. 2002). Although previous reports suggest an involvement of the mPFC in working memory (Granot et al. 1994; Murphy et al. 1996; Ragozzino and Kesner 1998; Taylor et al. 1999), we did not observe working memory deficits. This finding is in line with other studies using lesions in mPFC which did not observe consistent impairment of spatial working memory performance in the radial arm maze (Delatour and Gisquet-Verrier 1996; Gisquet-Verrier and Delatour 2006). Studies correlating working memory with mPFC functionality in rodents usually employ tasks with a delayed response contingency, including spatial delayed alternation (Larsen and Divac 1978; Kesner 2000) and delayed non-matching to sample (Aggleton et al. 1995; Chudasama and Muir 1997). Rats with lesions in the PL and IL are profoundly impaired in such tasks when delays are imposed (Delatour and Gisquet-Verrier 1999; Sloan et al. 2006). Thus, unimpaired working memory observed in the current study is likely to be due to the employment of a simple, without delay version of the task (Morris 1984).

Here, we have modified BLA functioning by NF knockdown, and observed impaired plasticity in the BLA-mPFC pathway as well as selectively affected behavioral flexibility. Diminished plasticity in the BLA-mPFC pathway could be easily explained by altered BLA responsiveness to the TBS, but an alternative possibility is that continuously modified activity in the BLA eventually resulted in metaplasticity within the mPFC (trans-regional metaplasticity) which affected the level of LTP within this region. This possibility, which was supported by the selective impairment in the mPFC-dependent reversal learning, could not be confirmed by examining LTP in the BLA-mPFC pathway.

To verify, whether mPFC metaplasticity may be involved, we investigated the ability to induce LTP in the mPFC by activating another pathway which does not involve the BLA—the ventral subiculum-mPFC pathway (vSub-mPFC pathway) (Richter-Levin and Maroun 2010). The vSub-mPFC pathway exhibits activity-dependent bidirectional synaptic plasticity (Jay et al. 1995; Burette et al. 1997; Parent et al. 2010). Plasticity in the vSub-mPFC pathway was found to be regulated by metaplastic effects involving other inputs to the PFC. For example, high-frequency stimulation of the BLA, which has reciprocal connections with both the ventral hippocampus and the mPFC, prevents the subsequent induction of LTP in the vSub-mPFC pathway (Richter-Levin and Maroun 2010).

Indeed, it was found that continuous alteration of BLA AIS GABAergic synapses resulted in impairment of LTP in the vSub-mPFC pathway, which does not involve the BLA. Our finding strongly supports the notion that continuous altered BLA activity may induce a form of trans-regional metaplasticity within the mPFC, which affects the responsiveness of the mPFC not only to the BLA but also to inputs from other brain regions, such as the vSub. To further support this novel notion of trans-regional metaplasticity, we repeated the LTP in the vSub-mPFC pathway experiment, but now while the BLA was inactivated. However, even under this condition NF knockdown rats exhibited reduced LTP in the vSub-mPFC pathway, indicating the contribution of within mPFC alterations induced by BLA-NF knockdown.

Several studies describe neural connection between BLA and hippocampus, including the DG (Pikkarainen et al. 1999; Bergado et al. 2007). Electrical stimulation of the BLA-evoked field potentials in the DG in anesthetized rats, verified to be mono-synaptic (Ikegaya et al. 1996). Additionally, it has also been reported that NMDA injection within BLA results in c-Fos expression within DG (reviewed in Cahill and McGaugh (1998)). The BLA modulates activity and plasticity within the DG (Akirav and Richter-Levin 1999; Abe 2001; Richter-Levin, 2004; Bergado et al., 2011). To investigate if intra-BLA GABAergic synapse modification induced trans-regional metaplasticity also in the DG we have assessed hippocampus-dependent behaviors (SOR, contextual fear conditioning and extinction) and DG LTP. Pharmacological and lesion studies have showed the significant involvement of hippocampus in the SOR task (Barbosa et al. 2012). But the NF knockdown group showed similar preference towards newly positioned object compared to control group. Similarly, contextual fear memory which is dependent on hippocampus (Maren et al. 2013) was also unaffected. Contextual fear extinction learning however was impaired, presumably reflecting again mPFC deficits (Rozesek et al. 2015). DG LTP was also not affected. These findings together with the results of working and reference memory from the WM task suggest that of the BLA-NF knockdown-induced trans-regional metaplasticity was confined to the BLA-mPFC axis.

It should be noted though that caution should be practiced when interpreting results of behavioral tests into effects within specific brain areas. Both the hippocampus and the mPFC are involved in some way in working and reference memory, in conditioning and in extinction learning, while the activity in each region contributes specific qualities to the processing of the information (Barker and Warburton 2011; Monaco et al. 2015). Furthermore, the interaction between these regions is critical for optimal performance in such tasks (Barker et al. 2017). The region specificity of the long-term effects of altering BLA functioning, and the possibility of differential specificity under different conditions should be further studied.

The results obtained from c-Fos activation further support the notion of trans-regional metaplasticity, as well as its
specificity to the BLA-mPFC axis. Expression of the immediate-early gene c-Fos is correlated with recent neuronal activation (Kaczmarek 1993; Radulovic et al. 1998; Santín et al. 2003) and is instrumental in mediating synaptic plasticity and cognition (Kaczmarek 1993, Fleischmann et al. 2003). Reversal learning usually increases the c-Fos activation in the mPFC (Sampedro-Piquero et al. 2015). Here, the observed reduction in c-Fos activation within the mPFC indicates an altered responsiveness of mPFC. Such attenuated response is likely to dampen the ability of the mPFC to mediate behavioral flexibility which is hallmark of neuropsychiatric disorders (Krueger et al. 2011). In contrast to the effect in the mPFC, the level of DG c-Fos was unchanged supporting the possibility of specificity of trans-regional plasticity to the BLA-mPFC.

Our results indicate a preference of trans-regional metaplasity in the BLA-mPFC axis, while BLA-hippocampus axis seems to exhibit no such modulation. Previous studies have reported differential activation of mPFC and hippocampus under same stress exposure (Elfving et al. 2008; Müller et al. 2011). However, it is too early to conclude whether there are indeed unique properties for the BLA-mPFC axis or whether, under different conditions trans-regional metaplasticity could be developed also in other BLA-modulated areas such as hippocampus.

Conclusion
In the current study here we have not used stress or trauma in order to manipulate BLA functioning, but rather a local and selective manipulation of GABAergic synapses at the AIS of principal neurons within the BLA. This specific manipulation is not expected to encompass all components of the stress effects. Presumably, however, it mimics a certain aspect of the long-term influence of exposure to experiences that involve significant activation of the BLA, such as stress and trauma. In that respect the current findings may contribute to our understanding of the long-term mechanisms of stress and trauma responses. We propose that trans-regional metaplasticity is important to our understanding of the dynamic nature of effects of stress and trauma. For, example, since trans-regional metaplasticity is likely to develop over time it may underlie evolving symptoms in stress-related psychopathologies, such as delayed-onset post-traumatic stress and the transformation of anxious symptoms towards post-traumatic depression (Bryant et al. 2016).

Supplementary Material
Supplementary data are available at Cerebral Cortex online.

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Notes
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