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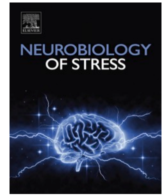

Intra-BLA alteration of interneurons' modulation of activity in rats, reveals a dissociation between effects on anxiety symptoms and extinction learning

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ABSTRACT

The basolateral amygdala (BLA) is a dynamic brain region involved in emotional experiences and subject to long-term plasticity. The BLA also modulates activity, plasticity, and related behaviors associated with other brain regions, including the mPFC and hippocampus. Accordingly, intra-BLA plasticity can be expected to alter both BLA-dependent behaviors and behaviors mediated by other brain regions. Lasting intra-BLA plasticity may be considered a form of metaplasticity, since it will affect subsequent plasticity and response to challenges later on. Activity within the BLA is tightly modulated by GABAergic interneurons, and thus inducing lasting alteration of GABAergic modulation of principal neurons may have an impactful metaplastic effect on BLA functioning. Previously, we demonstrated that intra-BLA knockdown (KD) of neurofascin (NF) reduced GABAergic synapses exclusively at the axon initial segment (AIS). Here, by reducing the expression of the tyrosine kinase receptor ephrin A7 (EphA7), we selectively impaired the modulatory function of a different subpopulation of interneurons, specifically targeting the soma and proximal dendrites of principal neurons. This perturbation induced an expected reduction in the spontaneous inhibitory synaptic input and an increase in the excitatory spontaneous synaptic activity, most probably due to the reduction of inhibitory tone. Moreover, this increased synaptic activity was followed by a reduction in intrinsic excitability. While intra-BLA NF-KD resulted in impaired extinction learning, without increased symptoms of anxiety, intra-BLA reduction of EphA7 expression resulted in increased symptoms of anxiety, as measured in the elevated plus maze, but without affecting fear conditioning or extinction learning. These results confirm the role of the BLA and intra-BLA metaplasticity in stress-induced increased anxiety symptoms and in impaired fear extinction learning but reveals a difference in intra-BLA mechanisms involved. The results also confirm the contribution of GABAergic interneurons to these effects but indicate selective roles for different subpopulations of intra-BLA interneurons.

1. Introduction

The amygdala is a key brain region that, in conjunction with the hippocampus and the prefrontal cortex, supports fear memory formation and extinction (McGaugh, 2004; Milad and Quirk, 2002; Pape and Pare, 2010). Human and animal studies have consistently demonstrated the pivotal role of the amygdala, and in particular the basolateral amygdala (BLA), in fear and anxiety-related disorders (Phelps and LeDoux, 2005; Yehuda and LeDoux, 2007; Shalev et al., 2017). The amygdala modulates neural processes in other brain areas (Abe 2001; Bergado et al., 2011), and influences the formation of emotional memory (McGaugh, 2004). Accordingly, we have reported in the past that priming the amygdala ((applying a tetanic burst stimulation to the BLA just prior to aiming to induce long-term potentiation (LTP)) affects the extent of LTP in the hippocampus and medial prefrontal cortex (mPFC) (Akirav and Richter-Levin, 1999; 2002; Richter-Levin and Maroun, 2010). Under

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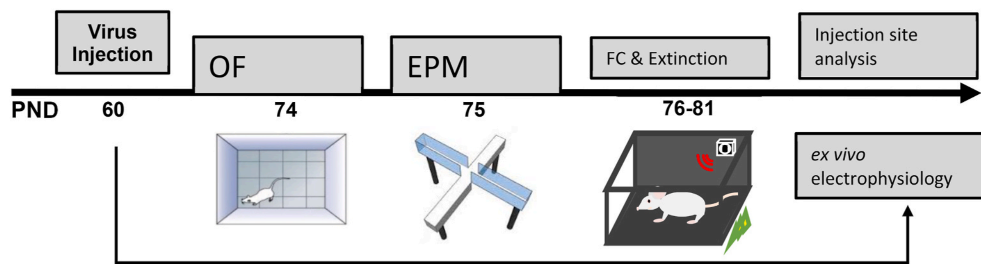

Fig. 1. Schematic layout of the experimental timeline.

extreme conditions the amygdala may lead to abnormal plasticity in other regions, effects which contribute to the pathology of trauma-related psychopathologies (Shalev et al., 2017).

There is cumulative evidence indicating a critical role of specific interneuron subpopulations in the BLA in different stages of fear formation and extinction (Bienvenu et al., 2012; Wolff et al., 2014). For instance, axo-axonic cells are recruited in response to noxious stimuli, contributing to memory consolidation and extinction learning (Seidenbecher et al., 2003; Lesting et al., 2011), and the acquisition of fear conditioning is regulated in part by bilateral disinhibitory effects of parvalbumin (PV) and somatostatin (SOM) (Wolff et al., 2014). BLA principal neurons are targeted by different classes of interneurons. The different classes of these interneurons can be distinguished by several criteria, including the target compartment on principal neurons, *i.e.* axon, soma, proximal and distal dendritic segments (Markram et al., 2004; Klausberger and Somogyi, 2008; Hensch, 2005).

Examining the impact of a specific subpopulation of interneurons requires the ability to selectively affect the functioning of that specific subpopulation, without affecting the functioning of other types of interneurons. We have developed an approach to selectively affect the functioning of a subpopulation of interneurons by compartment-specific reduction of GABAergic synapses on principal neurons (Zitman et al., 2016; Beuter et al.; Saha et al., 2017). GABAergic synapses stabilization requires the involvement of gephyrin, a postsynaptic scaffolding protein that needs to be clustered for proper assembling of GABAA receptors at the postsynaptic membrane (Kneussel et al., 1999; Yu et al., 2007; Wuchter et al., 2012; Tyagarajan and Fritschy, 2014). A previous study has identified separate transmembrane proteins and receptors involved in the stabilization of gephyrin clusters (Wuchter et al., 2012). Accordingly, we have shown that compartment-specific down--regulation of neurofascin (NF), or tyrosine kinase receptor ephrin A7 (EphA7) destabilized the preformed gephyrin clusters and the clustering of GABAA receptors in selective postsynaptic compartments *in vitro* and in adult animals *in vivo* (Wuchter et al., 2012; Kriebel et al., 2011; Beuter et al., 2016; Saha et al., 2017). Distinctively, NF knockdown in principal neurons removed GABAergic synapses specifically on axon initial segments (AIS), while EphA7 knockdown removed GABAergic synapses on somata and proximal dendritic segments (Saha et al., 2017; Beuter et al., 2016). These manipulations resulted in distinct electrophysiological and behavioral manifestations. For example, interference with GABAergic chandelier cell input at the AIS did not impact LTP and memory formation (Saha et al., 2017), while impairing the function of GABAergic basket cells on proximal dendritic and somatic compartments negatively affected dentate gyrus-LTP and hippocampal-dependent learning (Beuter et al., 2016). Thus, two classes of GABAergic interneurons innervating distinct compartments of the same principal neurons, play specific roles in controlling neuron activity and behavioral phenotype expression.

The research is a continuation of the above studies focusing on manipulating activity and plasticity within the BLA by selectively reducing the expression of EphA7 as a way to specifically affect GABAergic basket cells modulation of proximal dendritic and somatic compartments. Ephrin type-A receptor 7 is a receptor tyrosine kinase that regulates gephyrin clustering for the stabilization of basket cell innervation on proximal dendrites and somata compartments, dependent on mammalian Target of Rapamycin (mTOR) (Beuter et al., 2016). Hence, we posited that Down-regulating EphA7 expression within the BLA induces a lasting alteration, which will affect the ability of the BLA to execute its role, and to manipulate activity in its target regions, such as the prefrontal cortex. These lasting alterations are a form of within-amygdala metaplasticity, as they modify subsequent responses of the BLA (Abraham and Richter-Levin, 2018). Here, we examined how such induced intra-BLA metaplasticity, affects BLA electrophysiological responses and related behaviors.

2. Materials and methods

2.1. 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 h), with water and food pellets ad libitum. Experiments were conducted between 09:00–16:00 under dim light. Experimental procedures began after 5 days of acclimatization to the vivarium. Animals were randomly allocated to experimental groups. Experimenters were unaware of the group allocation during behavioral test performance. 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.

2.2. Experimental procedure

The experimental design is depicted in Fig. 1. Rats were assigned to two experimental paradigms.

2.3. Experiment 1. animal behavior

Following arrival and acclimation, all rats were randomly assigned to two groups. The control group was injected with an ineffective control shRNA sequence (shCTR, n = 14) while the second group was transduced with one of two shRNA sequences for knockdown of EphA7 (sh1737 or sh392; n = 18 (12 and 6, respectively)) to exclude off-target effects. Both constructs have proven high effectiveness in hippocampal cells and *in vivo* (Beuter et al., 2016). The lentiviral constructs, described previously (Beuter et al., 2016), express EGFP under the control of the CamKII promoter. Following 14 days of recovery, rats were tested in the open field at post-natal day 74 (OFT; PND 74), in the elevated plus maze (EPM; PND 75) (Ardi et al., 2016; Saha et al., 2017) and then by fear conditioning (FC) to an auditory cue (PND 76–81). Extinction of fear was assessed by re-exposure to the tone on 3 consecutive post-training days (adapted from Vouimba and Maroun, 2011; see Fig. 1 for experimental timeline. After completion of the behavioral experiments, animals were perfused and viral injection sites were assessed (EGFP expression). Only animals with successful bilateral EGFP expression in the BLA were included in the study. Likewise, knockdown of EphA7 in

the BLA was verified by quantitative immunohistochemistry analysis of EphA7 protein staining *in vivo*.

2.4. Experiment 2. Ex vivo electrophysiology

For *ex vivo* electrophysiology, a separate set of rats was examined which underwent exactly the same lentiviral injection protocol used in Experiment 1, but were taken for *ex vivo* electrophysiological assessment EPH (n = 14) and CTR (n = 12)). At PND 81, following anesthesia animals were transcardially perfused and brain slices were prepared to assess cellular excitability by electrophysiology (PND 81) using standard procedures (see Supplementary Materials and Fig. 1 for experimental timeline)). Intrinsic properties were recorded in current clamp mode (modified from (Kaphzan et al., 2013).

2.5. Behavioral tests

Open Field test and *Elevated Plus Maze (EPM) test* were used to assess rat-specific anxious behavior, hereafter referred to as ‘anxiety’.

Open Field test. The open field was used to assess the locomotor activity and anxiety 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 were 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).

Elevated Plus Maze (EPM) test. Anxiety behavior was assessed in the elevated plus maze as previously described (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 two opposing open and closed arms (walls 30 cm high) raised 50 cm above the floor in a brightly lit room. Rats were allowed to freely explore the maze for 5 min, while behavior was recorded and analyzed via an EthoVision XT10 tracking system (Noldus, Wageningen, Netherlands).

Cued Fear conditioning & extinction. Cued fear conditioning (FC) 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 two 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 three 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, 1s) at an inter-trial interval (ITI) of 2 min. After 24 h, rats underwent fear extinction training for 3 consecutive days. On each day of extinction, rats spend 22 min in Context B during which 10 C S 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, i.e., 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, each two successive CSs were averaged as ‘Blocks’.

2.6. Stereotaxic virus injection

Lentiviral particles (2 × 10^7 TU/ml) were produced as previously described (Beuter et al., 2016). Rats (PND 60) received bilateral microinjections of either lentiviral vectors expressing shRNA directed against EphA7 (Eph 1737 or Eph 392) or CTR vector (see details of the construct in Supplementary Fig. 1) into the BLA. 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). Bilateral stereotaxic injections of lentiviral suspensions into the BLA, were performed using a 33G stainless steel cannula mounted on a microsyringe (Nanofil, World Precision Instruments (See Supplementary Methods).

2.7. Histology

After completion of the electrophysiological recordings and the behavioral experiments, rats were killed with an overdose of pentobarbital and transcardially perfused with 200 ml of 0.9 % sodium chloride, followed by 200 ml of 4 % paraformaldehyde (4 °C) in 0.01 M phosphate-buffered saline (PBS; all solutions from Sigma, Rehovot, Israel). Brains were removed, post-fixed overnight at 4 °C in the same fixative, and immersed in a graded series of PBS sucrose solutions (10 %, 20 %). Free-floating 40 μm coronal sections were collected with a cryostat (Leica) in PBS containing 0.02% sodium azide and stored at 4 °C until use. Sections were mounted on glass slides, air dried and coverslipped with ImmuMount embedding media (Thermoscientific, Rehovot, Israel). Enhanced green fluorescent protein expression served as marker for bilateral infection of the BLA with either control (CTR) or EphA7 knockdown (Eph) lentiviral vector. Only rats with bilateral infection restricted to the BLA were included in the electrophysiological and behavioral analysis.

For analysis of EphA7 and gephyrin expression perfusion-fixed brains of animals that had received either control (shCTR, n = 3) or EphA7 KD viral suspensions (shEphA7, n = 2) were washed in PBS and cut into free-floating sections using a vibrating microtome set at 50–70 μm section thickness (Vibratome VT1000S; Leica Biosystems). After heat mediated antigen retrieval for 30 min at 80 °C in sodium citrate buffer (pH = 8.5), the slices were blocked and permeabilized for 2 h at room temperature in 0.5% Triton X-100/1 × BMB Blocking Reagent (Roche, Mannheim, Germany)/PBS. Further processing of the brain slices and immunohistochemical staining was performed as described previously (Beuter et al., 2016). The following primary antibodies were used: EphA7 (Abcam, Cat. No. ab136095, 1:1000), gephyrin (Synaptic Systems, Cat. No. 147011, 1:500).

2.8. Image acquisition and analysis

Confocal image stacks of stained slices were recorded with a Cell Observer SD spinning disk confocal microscope equipped with a 63 × Plan-Apochromat oil immersion objective, NA 1.4 (Carl Zeiss Microscopy). Protein expression levels of EphA7 and gephyrin were quantified with ZEN 2 (Carl Zeiss Microscopy). To this end, mean gray value intensities of EphA7 or gephyrin immunoreactivity in 20 μm^2 somatic regions of interest (ROI) of EGFP positive neurons were quantified with the help of ZEN’s measure function. Regions of interest were placed at neuronal somata in confocal z-planes which showed maximum diameters of neuronal somata. Image acquisition as well as analysis were performed in a blinded fashion.

2.9. Statistical analysis

All results are presented as mean ± SEM, and statistical analyses were performed with SPSS 21 (IBM) or GraphPad Prism 10 (GraphPad Software). For histological analysis, the data was tested for Gaussian distribution using the D’Agostino and Pearson test, followed by an unpaired *t*-test for difference between the two groups. All behavioral and electrophysiological results were analyzed using independent sample *t*-test and mixed model of repeated measures of ANOVA with appropriate Greenhouse-Geisser or Huynh-Feldt corrections for sphericity issues when necessary. For comparisons of cumulative distributions, we performed a nonparametric K-S (Kolmogorov–Smirnov statistic) test.

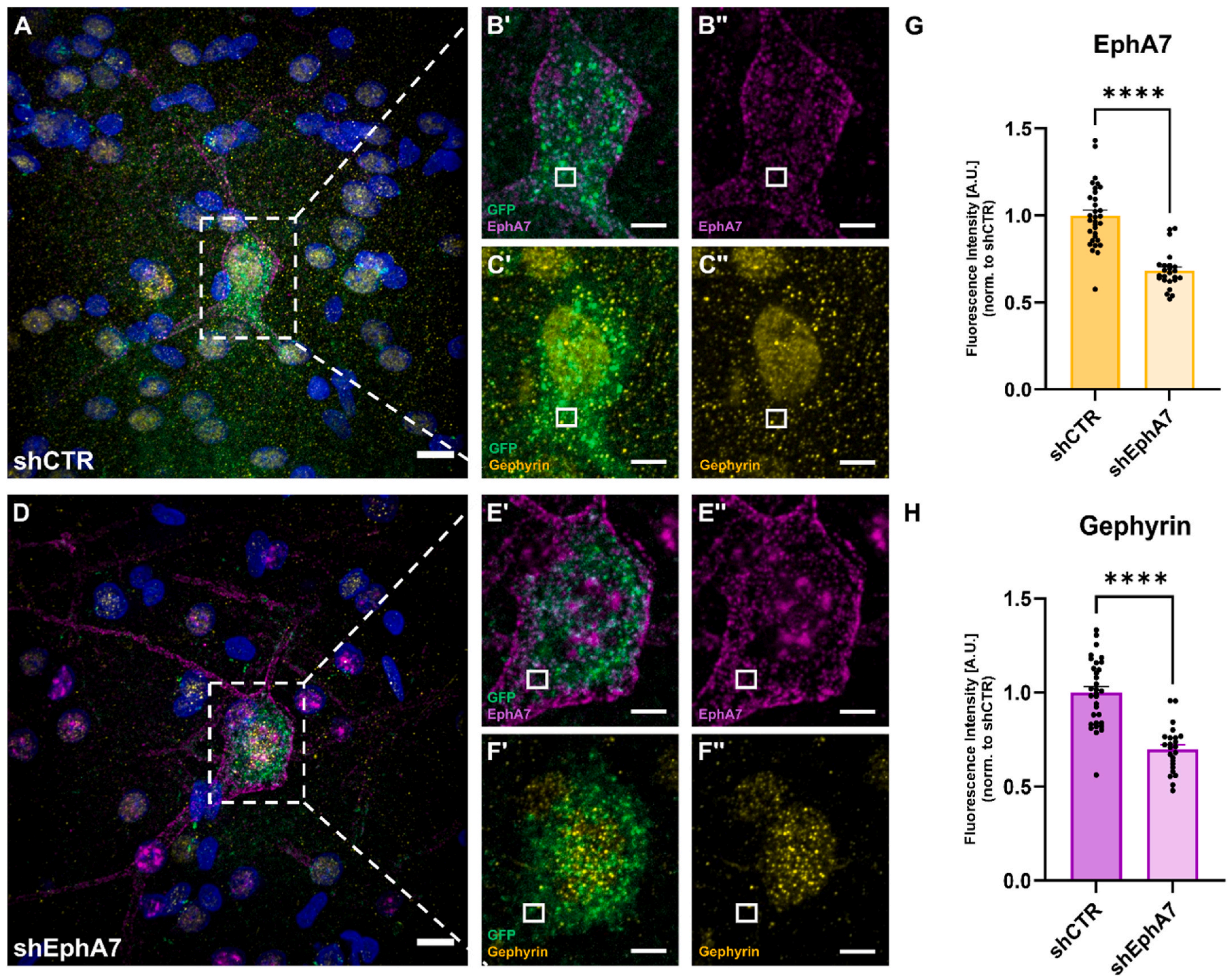

Fig. 2. Reduced EphA7 expression in the BLA accompanied by decreased gephyrin clustering.
Confocal images of rat BLA sections are shown either after injection of Lenti-shCTR-EGFP (A-C″) or Lenti-shEphA7-EGFP virus (D-F″). B' - C″ or E' - F″ depict enlarged single sections of confocal stacks shown in A, D, respectively. Red rectangles in B' - F″ depict ROIs of 20 μm^2 used for intensity measurements on somata. Overlap with nuclear locations were excluded due to cross-reactivity of the gephyrin-targeting antibody with a nuclear antigen. (G) Quantification of EphA7 fluorescence intensity on transfected, EGFP expressing neurons in BLA. shCTR $n = 32$, shEphA7 $n = 24$. (H) Quantification of gephyrin fluorescence intensity in transduced, EGFP expressing neurons in BLA. shCTR $n = 32$ collected from 3 animals, shEphA7 $n = 24$ collected from 2 animals. Data are means ± SEM. Statistical significance was assessed by one-tailed unpaired *t*-test for EphA7 intensity ($t (54) = 7.716$, $p < 0.0001$) and two-tailed unpaired *t*-test for gephyrin intensity ($t (54) = 7.146$, $p < 0.0001$). Scale bars: A & D 10 μm, B′-C'' & E′-F″ 5 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. In vivo validation of EphA7 knockdown

The effectiveness of EphA7 viral-mediated knockdown has been validated in an earlier analysis in the proximal dendritic segments and on somata of hippocampal dentate gyrus granular cells (Beuter et al., 2016). Here, we extended the research and focused on the BLA. To validate the effects of shEphA7 constructs in the BLA, we analyzed the protein expression of EphA7 and gephyrin by quantitative immunohistochemical analysis after lentiviral knockdown of EphA7 mRNA *in vivo*. Consistent with our previous study in the hippocampus, we observed a significant reduction in EphA7 expression two weeks after the injection of EphA7 shRNA compared to the control (unpaired one-tailed *t*-test, $t (54) = 7.716$, $p < 0.001$). To additionally confirm the impact of EphA7 knockdown on GABAergic synapse organization in the BLA we further assessed the expression of gephyrin protein, a post-synaptic marker of GABAergic synapses (Craig et al., 1996) in EGFP-positive infected principal cells of the BLA (Fig. 2). Loss of EphA7 protein following EphA7 shRNA expression led to a parallel reduction of gephyrin levels in BLA neurons essentially as observed in dentate gyrus granule cells, previously (Fig. 2G and H; two-tailed *t*-test $t (54) = 7.146$, $p < 0.001$; Beuter et al., 2016). Given that the shRNA-mediated knockdown of EphA7 in the BLA mirrored the observations previously made in the hippocampus, we proceeded with electrophysiological and behavioral analyses to further investigate its effects.

3.2. EphA7 knockdown reduces cellular excitability in BLA

Given the involvement of EphA7 in inhibitory GABAergic transmission, we evaluated the effects of EphA7 knockdown on inhibitory input to pyramidal cells. As expected, isolated brain slices from EphA7

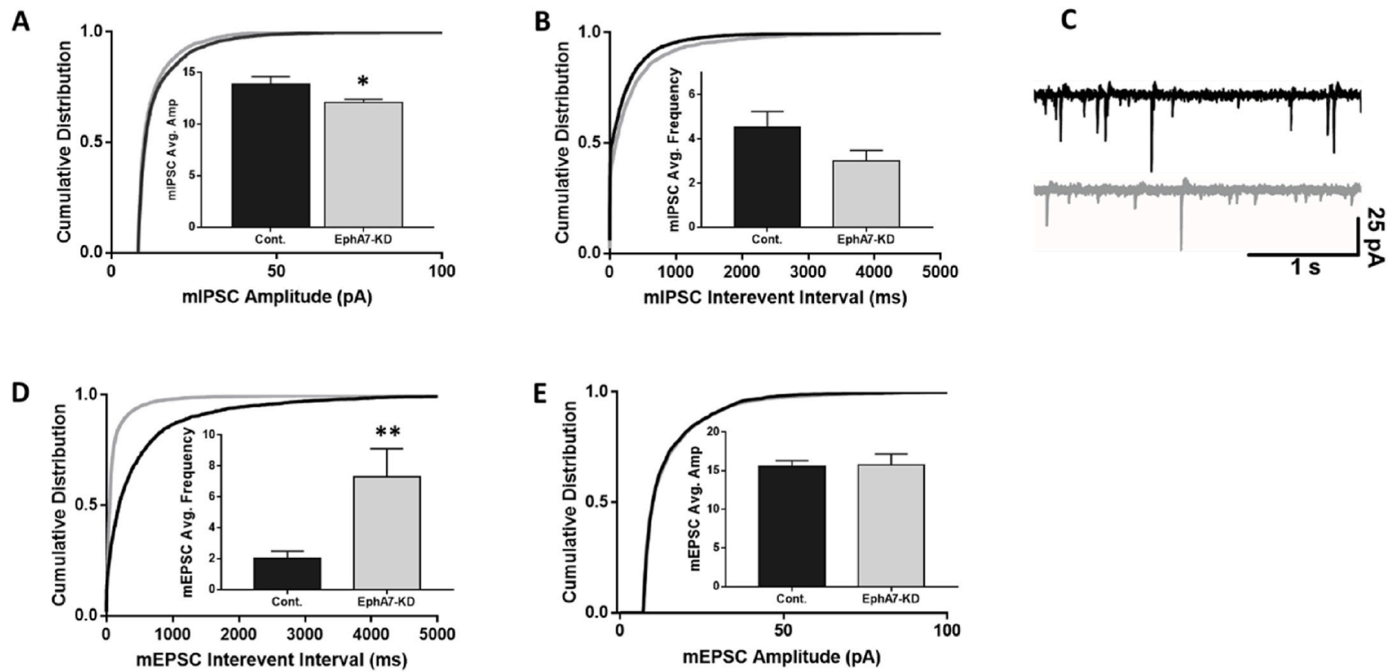

Fig. 3. Effect of EphA7 knockdown on synaptic inputs to pyramidal cells of the BLA
EphA7 knockdown elicited a reduction in the inhibitory input as manifested by the decline of both amplitude ($n = 17$) and frequency of miniature inhibitory postsynaptic currents (mIPSCs; (A,B)), compared to those in the CTR, scrambled sequence vector-infected rat derived neurons ($n = 15$). Sample traces for mIPSCs recordings under CTR and EphA7 KD vector-infected conditions are displayed in black and gray, respectively (C). EphA7 knockdown increased the glutamatergic excitatory tone as manifested by the increased miniature excitatory postsynaptic currents (mEPSCs; (D)), while not affecting mEPSCs amplitude (E). Data are shown as means ± SEM. *$p = 0.05$; **$p < 0.01$.

knockdown rats ($n = 17$) exhibited an overall reduction in the inhibitory input as manifested by the decline of amplitude of miniature inhibitory postsynaptic currents (mIPSCs) (K-S $D = 0.06336$, $p < 0.0001$; $t_{(30)} = 2.406$, $p = 0.0225$; Fig. 3A), and a suggested reduction in the frequency of mIPSCs ($t_{(30)} = 1.873$, $p = 0.0708$ Fig. 3B), with a significant rightward shift of the cumulative distribution of interevent intervals (K-S $D = 0.1062$, $p < 0.0001$) compared to that in the CTR ($n = 15$). Interestingly, EphA7 knockdown increased the glutamatergic excitatory tone as manifested by the increased miniature excitatory postsynaptic currents (mEPSCs) (K-S $D = 0.3865$, $p < 0.0001$; $t_{(28)} = 2.818$, $p = 0.0088$; Fig. 3C), while not affecting mEPSCs amplitude (K-S $D = 0.00267$, $p = 0.0689$; $t_{(28)} = 0.1362$, $p = 0.8927$; Fig. 3D–E). This effect is not unexpected, as a reduced tonic inhibition may increase the likelihood of spontaneous vesicle release (Wierda and Sørensen, 2014).

Considering the existence of a homeostatic interaction between extrinsic and intrinsic excitability, we hypothesized that EphA7 knockdown would also result in intrinsic excitability alterations. For that purpose, we have injected a series of depolarizing current steps to assess the f-I curve. This analysis revealed that projection neurons derived from EphA7 knockdown rats fired significantly less after EphA7 knockdown (Fig. 4A–B; group × current steps intensity interaction: $F_{(6,144)} = 28.12$, $P < 0.001$, and general group effect; $F_{(1,24)} = 57.60$, $P < 0.001$ in 2-way repeated measure of ANOVA).

To elucidate the cause for the reduction in excitability, we measured the various intrinsic properties of EphA7 knockdown cells in comparison to control transfected neurons. EphA7 knockdown ($n = 14$) did not affect either the resting potential or the action potential (AP) morphology as observed by similar AP amplitude and half-width (Fig. 5A-C; $t_{(24)} = 0.4024$, $p = 0.6910$; $t_{(24)} = 0.7559$, $p = 0.4571$; $t_{(24)} = 0.8677$, $p = 0.3941$, respectively). However, EphA7 knockdown decreased the input resistance ($t_{(24)} = 4.947$, $p < 0.0001$ Fig. 6C), and increased the threshold potential ($t_{(24)} = 2.39$, $p = 0.0250$; Fig. 6A), threshold current ($t_{(24)} = 6.91$, $p < 0.0001$; Fig. 6B), and the medium after-hyperpolarization (AHP; $t_{(24)} = 2.486$, $p = 0.0203$; Fig. 6D–E), all of which coincide with the observed reduction in excitability. In summary, we observed a strong reduction in intrinsic excitability.

3.3. EphA7 knockdown increases anxiety-like behavior, as measured in the EPM

Considering the role of the amygdala in both memory formation and stress response regulation, we sought out to assess EphA7 involvement in these processes. We therefore examined whether EphA7 knockdown within the BLA is sufficient to alter anxiety-related behaviors in the OF and EPM. Ephrin type-A receptor 7 knockdown ($n = 18$) and CTR ($n = 14$) did not affect locomotor activity as assessed by total distance moved (Fig. 7A–$t(30) = 0.748$, $P > 0.05$) or by percentage of distance moved in the center of the OF arena (Fig. 7B–$t(30) = 0.299$, $P > 0.05$). Likewise, the total distance moved in the EPM arena (Fig. 7C–$t(30) = 1.86$, $P > 0.05$) was comparable between EphA7 and CTR group. However, the percentage of entries to the open arms of the EPM was significantly reduced in the EphA7 knocked down cohort compared to that of the CTR (Fig. 7D–$t(30) = 2.634$, $P < 0.05$). Moreover, the extent of distance traveled in the open arms (distance index: distance in open arms/total distance) and of time spent in the open arms (duration index: time in open arms/total duration) were significantly reduced by EphA7 knockdown (Fig. 7E, $t(30) = 3.608$, $P < 0.01$ and Fig. 7F–$t(30) = 3.459$, $P < 0.01$, respectively). Taken together, these data suggest that knockdown of EphA7 increases anxiety-like response.

3.4. EphA7 knockdown in the BLA does not alter cued fear conditioning (FC) or fear extinction

Finally, we investigated EphA7 involvement in learning under stressful conditions in the FC task. Twenty-four hours after EPM test, both EphA7 knockdown ($n = 18$) and CTR ($n = 14$) animals were subjected to cued FC. During the fear acquisition phase, both the EphA7 knockdown and the CTR cohorts showed similar freezing behavior over

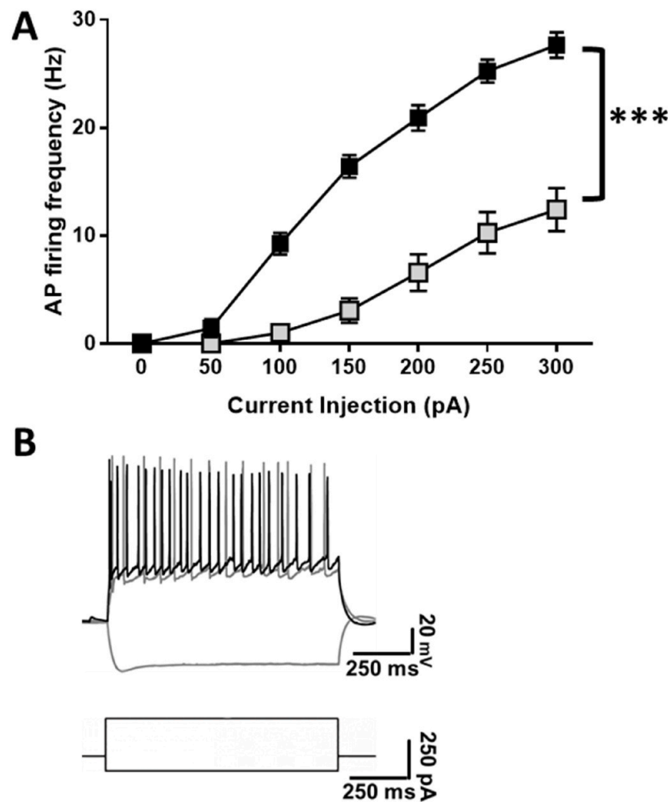

Fig. 4. EphA7 knockdown reduces overall intrinsic excitability of pyramidal cells at the BLA
Significant reduction in the frequency of AP firing in EphA7 KD vector-infected rat derived cells (n = 17) compared to that of the CTR vector derived neurons (n = 15, (A)). Sample traces for AP firing due to 250 pA depolarizing current injection under control and no EphA7-KD conditions are displayed in black and gray respectively (B). Data are shown as means ± SEM. ***p < 0.001.

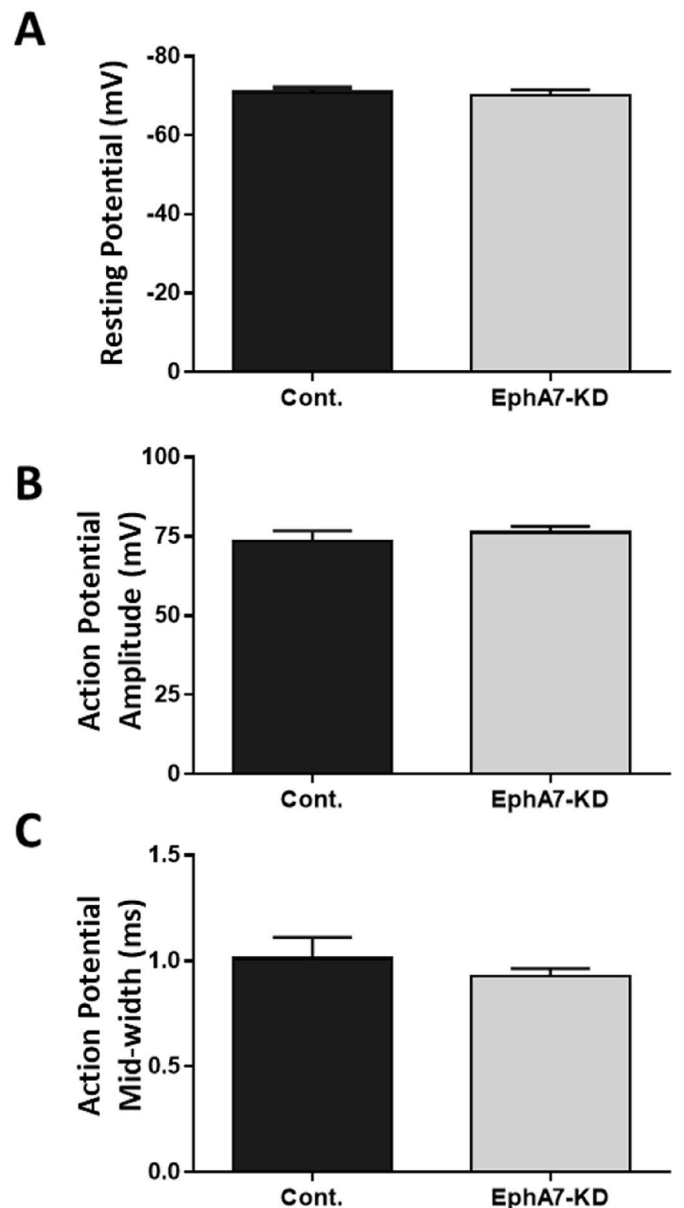

Fig. 5. EphA7 knockdown does not affect resting potential and AP morphology. EphA7 knockdown did not affect the resting potential (A) or the action potential (AP) morphology as observed by similar AP amplitude (B) and half width (C).

the three conditioned stimulus (CS)–US pairings, as assessed by repeated-measure ANOVA (Fig. 8A; $F(1,30) = 0.338$, $P > 0.05$), which remained at comparable levels in 2 min post training (Fig. 8B; $t(30) = 0.838$, $P > 0.05$). Twenty-four hours after cued fear conditioning all the animals went through 3 days of extinction sessions where all the animals received only the CS. The freezing response to the CS was comparable between EphA7 and CTR group. Mixed model repeated measure ANOVA reveal that there is no significant difference between groups (Fig. 8C; $F(1,30) = 0.003$, $P > 0.05$). Together, these results indicate that EphA7 knockdown in the BLA does not alter fear memory acquisition and extinction.

4. Discussion

In this study, we extend previous research aimed at untangling the differential mechanisms by which distinct subpopulations of GABAergic inhibitory interneurons influence behavioral outcomes. Specifically, we investigated the impact of reducing GABAergic synapses locally, at the proximal dendrites and somata compartments of principal projecting cells on fear and anxiety behaviors. The results demonstrate that targeting GABAergic synapses at this compartment, by shRNA knockdown of receptor tyrosine kinase EphA7, prompted anxiety behavior.

The amygdala is a dynamic brain region, which is responsive to emotional experiences and subject to long-term plasticity. Activity and plasticity of the BLA is considered critical for acute stress reaction and expression of fear, as well as the initial acquisition of extinction memory (Herry et al., 2008; Quirk and Mueller 2008). The BLA also has a role in modulating activity, plasticity and related behaviors associated with other brain regions, including the mPFC and the hippocampus (McGaugh, 2004; Richter-Levin, 2004; Bergado et al., 2011). Accordingly, plasticity within the BLA can be expected to alter both BLA-dependent behaviors and behaviors mediated by other brain regions which are modulated by the BLA (Bergado et al., 2011). Because of that, intra-BLA lasting plasticity may be considered as a form of metaplasticity, since it will affect subsequent plasticity within the BLA and also the ability of other brain regions, such as the hippocampus and mPFC to respond to challenges later on (Abraham and Richter-Levin, 2018). In recent years, we have begun investigating the possible implications of within-amygdala lasting plasticity for emotional behaviors and stress management. Activity within the BLA is tightly modulated by GABAergic interneurons (Martijena and Molina, 2012; Prager et al., 2016), thus inducing lasting alteration of GABAergic modulation of principal neurons within the BLA can be expected to have an impactful effect on BLA functioning. One possibility to manipulate GABAergic local circuitry activity within the BLA is by compartment-specific reduction of GABAergic input onto principal neurons. Knockdown of

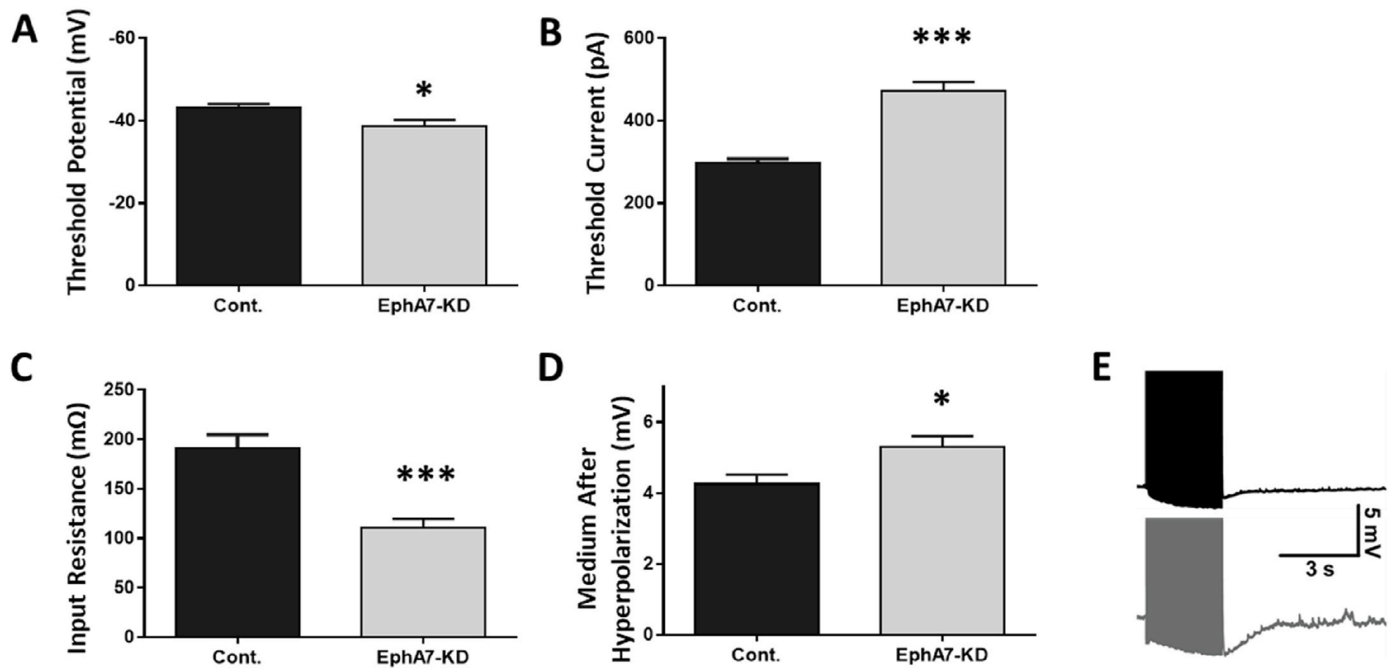

Fig. 6. EphA7 knockdown alters intrinsic excitability of pyramidal cells at the BLA
Threshold potential (A), threshold current (B), and the medium after-hyperpolarization (D) were increased in EphA7 knockdown derived cells ($n = 14$) together with a decrease in input resistance (C). Data are shown as means ± SEM. *$p = 0.05$; ***$p < 0.001$.

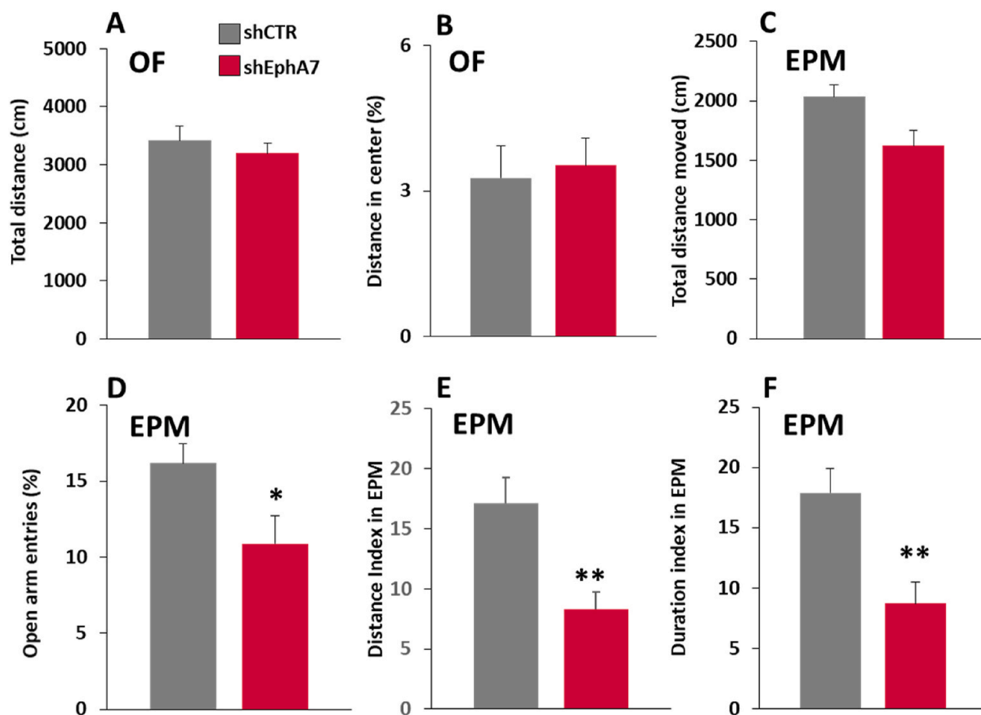

Fig. 7. Effect of EphA7 knockdown in the BLA on locomotor activity and anxiety.
Locomotor activity measured by the total distance moved in the open field arena did not differ between the shCTR ($n = 14$) and shEphA7 ($n = 18$) groups (A). The % of distance that the animals spent in the center of the open field arena was also not altered (B). The total distance traveled in the elevated plus maze (EPM) was similar in shCTR and shEphA7 groups (C). The % of entries to the open arms in the EPM arena was significantly reduced in shEphA7 knockdown group (D). Distance and duration indices in the EPM (% open arm distance or duration/total distance or duration) are significantly different between groups (E,F). Data are shown as means ± SEM. Stars indicate statistical significance between groups (*$P < 0.05$, **$P < 0.01$).

neurofascin (NF), a transmembrane receptor involved in the stabilization of gephyrin clustering (Kriebel et al., 2011; Wuchter et al., 2012), reduced GABAergic synapses exclusively at axon initial segments (AIS) within the BLA (Saha et al., 2017) and dentate gyrus (DG) (Zitman et al., 2016) which is accompanied by electrophysiological alterations in both brain regions. Here, by reducing the expression of EphA7, we selectively

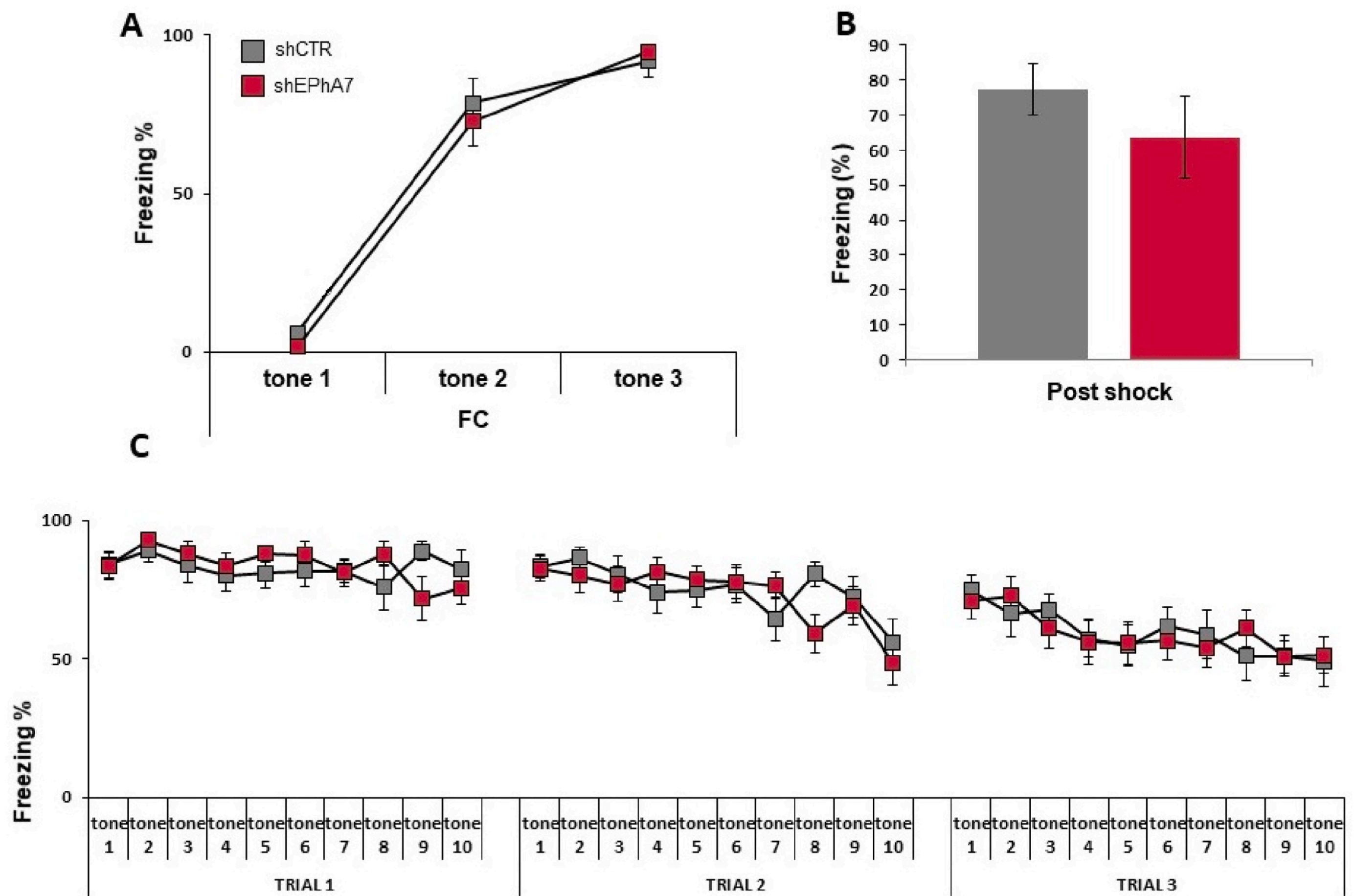

Fig. 8. Effect of EphA7 knockdown on fear memory acquisition and extinction in the BLA.
Cued fear conditioning and extinction took place in a sound-attenuating chamber in animals with bilateral expression of shCTR (n = 14) or EphA7 infection vectors (n = 18). Acquisition of fear to the auditory conditioned stimulus (CS) paired with shock, was measured as % freezing to the CS (A). The post-shock freezing levels were comparable between groups (B). Twenty-four hours after conditioning, both groups underwent 3 days of extinction (C). Data are shown as means ± SEM.

impaired the modulatory function of a different subpopulation of interneurons, specifically targeting GABAergic synapses at the soma and proximal dendrites (Wuchter et al., 2012; Beuter et al., 2016), thus leading to lasting plasticity of a different form than that with the NF KD (Saha et al., 2017). Indeed, while intra-BLA NF-KD resulted in impaired extinction learning, albeit without increased symptoms of anxiety (Saha et al., 2017), intra-BLA reduction of EphA7 expression resulted in increased symptoms of anxiety, but without affecting fear conditioning or extinction learning.

As expected, the reduction of EphA7 at the BLA reduced the inhibitory tone on the BLA's pyramidal neurons (Fig. 3A and B), subsequently modifying the entire cellular functioning of the BLA pyramidal cells. This reduction of inhibition yielded an increase in the constant excitatory synaptic activity (Fig. 3C and D). Such a lasting enhanced extrinsic excitability is probably dependent on the internal circuitry of the BLA, where the inhibitory neurons that consist ~20% of the BLA neurons, modulate the ~80% interconnected excitatory principal neurons. Since neurons entail an innate tendency to preserve an activity balance over long periods of time, it is not surprising that reduction of inhibition induced a reduction in intrinsic excitability, which could be interpreted as a homeostatic response to the overall increased extrinsic excitability. Such long-term changes are in accordance with the homeostatic response model (Turrigiano, 2012). Interestingly, most of the enhanced intrinsic excitability derives from the increased input resistance alongside an increase in the mAHP (Fig. 6). It is probable that the increase in excitatory tone enhances the calcium levels, thus modulating the activity and expression of SK channels that are the main contributors of mAHP (Sun et al., 2020). Interestingly, increased SK2 expression and reduced mAHP in the basolateral amygdala were correlated with reduced anxiety, but in our study the mAHP increase is presumed to be a homeostatic response to the enhanced synaptic excitatory tone (Mitra et al., 2009; Qin et al., 2021).

Fear memory extinction deficits induced by exposure to stress and trauma are assumed to result from increased levels of anxiety, which result from hyperactivation of the amygdala (Maren and Holmes, 2016; Singewald and Holmes, 2019). However, the dissociation found between the effects of NF-KD (impairing extinction learning but not affecting anxiety symptoms [Saha et al., 2017]) and the effects EphA7-KD (increasing anxiety symptoms, but not impairing extinction learning), suggest that increased anxiety and impaired extinction are promoted by two different intra-BLA stress-related mechanisms involving separate GABAergic inhibitory interneuron subpopulations. An anxiety behavior is modulated in part by GABAergic neurons innervating the somata and proximal dendritic segments of BLA principal cells, whereas learning of fear extinction requires GABAergic activity at the axonal initial segment. The granule cell compartment different interneurons target differentiates between chandelier cells, which specifically target the axon initial segment (AIS) [Inan and Anderson, 2014; Wang et al., 2016], and basket cells, which target the soma and proximal dendrites, but not the AIS, or distal dendrites [Ribak, 1992]. Thus, two classes of GABAergic interneurons innervating distinct compartments of the same principal neurons, play specific roles in controlling neuron activity and behavioral phenotype expression. Both Chandelier and Basket cells are Parvalbumin (PV)-positive interneurons (Bartholome et al., 2020). BLA

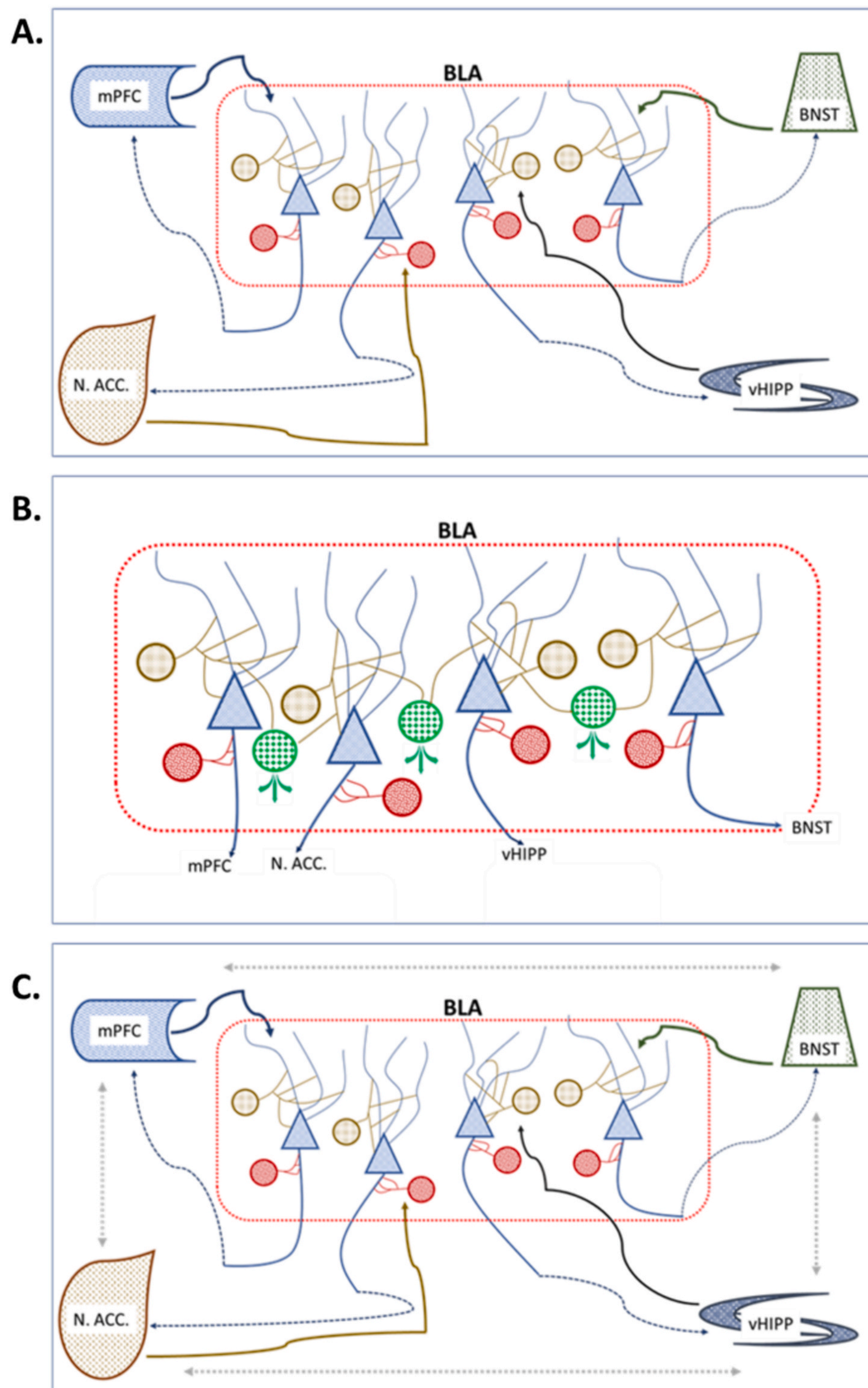

Fig. 9. The differential effect of selective intra-BLA manipulations of GABAergic modulation of principal neurons' activity on specific aspects of behaviors may be the result of altered activity in behavioral networks. A.
The main reciprocal projections to the BLA come from the medial prefrontal cortex (mPFC), the ventral hippocampus (vHIPP), the bed nucleus of the stria terminalis (BNST), and the nucleus accumbens (N. Acc.), each being part of a behavioral network associated with executive functions, contextual learning and memory, modulating fear and anxiety responses, and assessing valence, respectively (Janak and Tye, 2015; Huang et al., 2021). It is suggested that most BLA principal neurons are involved each in a specific behavioral network, thus enabling the BLA to contribute to a range of behaviors (Janak and Tye, 2015; Huang et al., 2021). B. About 5 % of the BLA principal neurons interact with more than one behavioral network. In addition, Intra-BLA interactions, involving as yet unidentified interneurons, may enable a BLA principal neuron to shift the weight of its contribution from one behavioral network to another. C. The behavioral networks involve the contribution of other brain regions, which project also to the BLA (e.g. the mPFC, vHIPP, BNST, N. Acc). Altered activity in these regions, may affect the characteristics of activity of BLA interneurons, leading to a shift the weight of these principal neurons' contribution from one behavioral network to another.

PV-positive interneurons are implicated in fear learning and extinction (Yau et al., 2021). However, whether these are the Chandelier or Basket (PV)-positive interneurons was not yet verified. Here we found that selectively impacting GABAergic synapses at the proximal dendrites/somata did not affect fear learning or extinction. We previously found that selectively impacting GABAergic synapses at the AIS did not affect fear learning but did impair fear extinction learning (Saha et al., 2017). Together, the findings suggest that the BLA Chandelier (PV)-positive interneurons play a pivotal role in fear learning and extinction, while BLA basket cells are more directly involved in aspects of anxiety responses.

Accumulating evidence suggests that within the BLA, different local networks are involved in mediating different behaviors (Janak and Tye, 2015; Hintiryan et al., 2021), but also that the same neuron may be part of several such networks (Janak and Tye, 2015). Here we propose a mechanism which may enable the same BLA neuron to take part in mediating different behaviors, in part, depending on GABAergic modulation of that neuron by different interneurons. Because it is suggested that specific BLA principal neurons receive and reciprocally project to specific target regions (Hintiryan et al., 2021), is still not clear how a change in the GABAergic modulation of BLA principal neurons may lead to differential effect on different behaviors. One possibility, described in Fig. 9, is that the BLA is a part of several behavior-specific networks, and that altering the characteristics of activity of a principal neuron, by modifying the GABAergic modulation it receives, alters the weight of its participation in that network, and by this, the pattern of activity of that network (Fig. 9B). Together with that, intra-BLA interactions, involving as yet unidentified interneurons, may enable a BLA principal neuron to shift the weight of its contribution from one behavioral network to another (Fig. 9C). Verifying these possibilities awaits further research.

To conclude, the outcome of local directed shRNA manipulation is dictated by the compromised membrane receptor, determining which neuronal compartment will be deprived of GABAergic synapses innervation. Targeting specific gephyrin-associated proteins enabled us to examine the specific contributions of intra-BLA GABAergic synapses at either the AIS (NF-KD; Saha et al., 2017) or the proximal dendrites (EphA7-KD) to anxiety behavior and to extinction learning. The results agree with the suggested role of the BLA and of metaplasticity within the BLA in enhanced stress-induced anxiety symptoms and in impaired fear extinction learning (Schmidt et al., 2013; Saha et al., 2022). The results also confirm the contribution of GABAergic interneurons to these effects (Maguire, 2014), but indicate that different subpopulations of intra-BLA GABAergic interneurons are involved in each of these effects.

CRediT authorship contribution statement

Rinki Saha: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Lisa-Sophie Wüstner:** Investigation, Formal analysis. **Darpan Chakraborty:** Writing – original draft, Validation, Investigation, Formal analysis. **Rachel Anunu:** Validation, Project administration, Investigation. **Silvia Mandel:** Writing – review & editing, Writing – original draft, Visualization. **Joyeeta Dutta Hazra:** Conceptualization. **Martin Kriebel:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation. **Hans-juergen Volkmer:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Conceptualization. **Hanoch Kaphzan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology. **Gal Richter-Levin:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

Intra-BLA alteration of interneurons' modulation of activity in rats, reveals a dissociation between effects on anxiety symptoms and extinction learning.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2024.100681.

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