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CELLULAR AND MOLECULAR BIOLOGY

BHLHE40 modulates post-traumatic stress disorder behaviors with the involvement of the PI3K/AKT signaling pathway

ADILA AJI, RENA AIHEMAITI, SHAOHONG ZOU, ALIMUJIANG MAISIYITI, CHENG ZHANG, RUONAN LIU & XIAOKAITI SULIDAN

Abstract: Post-traumatic stress disorder (PTSD) is closely related to the exposure to traumatic events and results in the structural and functional changes of hippocampus. Human basic helix-loop-helix family member e40 (BHLHE40) was reported to be implicated with neuron maturity and neuronal differentiation. The present study aimed to reveal the role of BHLHE40 on single-prolonged stress (SPS) model of PTSD in mice. The morris water maze test, open field test and contextual fear test were conducted to assess memory deficits, anxiety-like behaviors, and freezing of mice. Western blot was performed to identify proteins and reveal their levels in hippocampal tissues. We found that mice receiving SPS exhibited increased anxiety-like behaviors, memory deficits, and prolonged freezing time. The protein levels of BHLHE40 were downregulated in the hippocampal tissues of SPS mice. SPS reduced the protein levels of glutamate receptors, while overexpression of BHLHE40 promoted glutamate receptor protein levels in SPS mice. Moreover, BHLHE40 overexpression activated the PI3K/AKT pathway. BHLHE40 overexpression ameliorated the SPS-induced PTSD-like behavioral deficits. Overall, BHLHE40 promotes glutamate receptor protein levels to ameliorate PTSD-like behaviors with the involvement of the PI3K/AKT pathway. This novel discovery may provide a potential target for the improvement of PTSD.

Key words: BHLHE40, Post-traumatic stress disorder, PI3K/AKT pathway, single-prolonged stress.

INTRODUCTION

Post-traumatic stress disorder (PTSD), a complicated mental disease, is closely related to the exposure to traumatic events (Bisson et al. 2015). The occurrence of PTSD is about 10-11% worldwide (Kirkpatrick & Heller 2014). In addition to war, vital diseases or accidental injury contribute to the initiation of PTSD (Turcek 2017). The typical symptoms of PTSD are intense fear, anxiety, hyperarousal or emotional numbing when re-experiencing trauma-related stimuli (Yehuda 2002). A previous study suggested that these dysregulations of emotion related to fear, anxiety or stress are based on a persistent, aberrant adaptation of neurobiological systems in response to traumatic events (Bisson 2007). Furthermore, the dysfunction of neurons in hippocampus is closely associated with PTSD (Kaplan et al. 2018, Liu et al. 2018b, Lucassen et al. 2014, Shin et al. 2006). Over the past decades, great progress has been achieved on the potential mechanisms of PTSD. However, the exact pathology of PTSD remains unclear.

Human basic helix-loop-helix family member e40 (BHLHE40), a member of basic helix-loop-helix transcription factors, is also called differentiated embryonic chondrocyte

gene 1 (DEC1), enhancer of split and hairy related protein 2 (SHARP2, rat), or stimulated with retinoic acid 13 (STRA13, mouse) (Boudjelal et al. 1997, Rossner et al. 1997, Shen et al. 1997). A previous study showed that BHLHE40 is widely expressed in various tissues and involved in the regulation of cell proliferation, differentiation, apoptosis, biological rhythm, and lipid metabolism (Kato et al. 2014, Li et al. 2016). BHLHE40 has anti-apoptotic potential by increasing survivin protein expression (Li et al. 2006). BHLHE40 overexpression selectively inactivates procaspase 3, 7, 9 to antagonize apoptosis induced by serum starvation (Li et al. 2002). In addition, knockdown of endogenous BHLHE40 reverses the promotive effect of TGF-β on cell survival in breast cancer (Ehata et al. 2007). More importantly, BHLHE40 was reported to be implicated with neuron maturity and neuronal differentiation. SHARP1 and SHARP2 (BHLHE40) are associated with synaptic plasticity in the subregions of central nervous system (Rossner et al. 1997). Additionally, STRA13 (BHLHE40) overexpression improves neuronal differentiation (Boudjelal et al. 1997). Recently, the downregulation of DEC1 (BHLHE40) was reported to promote neurotoxicity and aggravate neurological deficit induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in a mice model of Parkinson's disease (Zhu et al. 2017b).

Phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway, a highly conserved pathway, regulates multiple signaling cascades and plays an important role in neuronal survival (Kim et al. 2011, Zhu et al. 2017a). Additionally, the PI3K/AKT signaling pathway is inactivated in hippocampal tissues of PTSD rat models (Zhang et al. 2020). The Akt signaling pathway can ameliorate PTSD symptoms by promoting synaptic plasticity and glutamate transmission (Liu et al. 2018a). Glutamate receptors, the predominant excitatory neurotransmitter receptors in the mammalian brain, determine the synaptic transmission efficiency (Barnes et al. 2020, Zarebidaki et al. 2020), and play crucial roles in learning and memory behaviors (Krugers et al. 2010, Van den Oever et al. 2008). Increasing evidence relates abnormalities in the glutamatergic system to stress response and PTSD (Pitman et al. 2012, Popoli et al. 2011). When re-experiencing trauma-related stimuli, PTSD subjects have an injured glutamate system and fail to maintain adequate glutamate transmission, which causes heightened over-attention, stress reactions and fear levels (Yang et al. 2015).

In our study, we investigated the role of BHLHE40 in single-prolonged stress (SPS) mice model of PTSD. Protein levels of glutamate receptors in hippocampal tissues of mice were detected, and the effects of BHLHE40 on glutamate receptors were explored. Furthermore, the regulatory effects of BHLHE40 on the PI3K/AKT signaling pathway were identified. This novel discovery may provide a potential target for the improvement of PTSD.

MATERIALS AND METHODS

Animals and ethics statement

Adult male C57BL/6J mice (7-9 weeks) were purchased from Vital River Co. Ltd. (Beijing, China) and were housed in a temperature-controlled room (24 ± 1°C) under 12-hour light/dark cycles (6:00 AM to 6:00 PM) with *ad libitum* access to food and water for 1-week for acclimatization. All experimental procedures were approved by the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the Animal Care and Use Committee of Mental Health Center of Xinjiang in China.

The single prolonged stress (SPS) operation

SPS mice models were established as previously described (Azevedo et al. 2020, Song et al. 2018, Wang et al. 2012, Yu et al. 2013). In brief, the mice were restrained for 2 h in a BD Falcon© 50-mL conical tube with a screw-on top and air holes located 1/2 cm apart. Next, the mice were put in an 18-cm diameter, 50-cm height clear acrylic cylinder individually, and the mice were forced to swim in cylinder filled with water (22-24°C) to 2/3 of the height of the cylinder for 20 min. After a 15-min recuperation period, mice were exposed to diethyl ether till they lost consciousness. Next, the mice were immediately placed in their cages and left undisturbed for 7 days. The mice in sham group were kept in cages adjacent to the SPS mice during the treatment without food and water. The treatment schedule and behavioral test design is shown in Figure 1.

Adeno-associated virus (AAV) injection

AAV (serotype 2, characterized by poor-immunogenicity; Vigene Biosciences, shanghai, China) containing the coding sequences (provided in Supplementary Material) of BHLHE40 or empty AAV vectors (pAV-MeCP2-GFP; 4272 bp) as the negative controls were injected into mice. First, mice were intraperitoneally injected with a mixture of ketamine (7.0 mg/mL) and xylazine (0.44 mg/mL) dissolved in 0.9% saline for anesthesia. With the head of each mouse fixed in a stereotactic apparatus, 0.5 μL of AAVs (10^{12} v.g/mL) were bilaterally microinjected to the bilateral hippocampal CA1 region (2.0 mm anteroposterior from bregma; ± 1.5 mm mediolateral; 1.70 mm dorsoventral). Four weeks after AAV injection, mice were used for behavioral tests. After behavioral tests, the rats were sacrificed, and the hippocampal tissues were collected for western blot analysis.

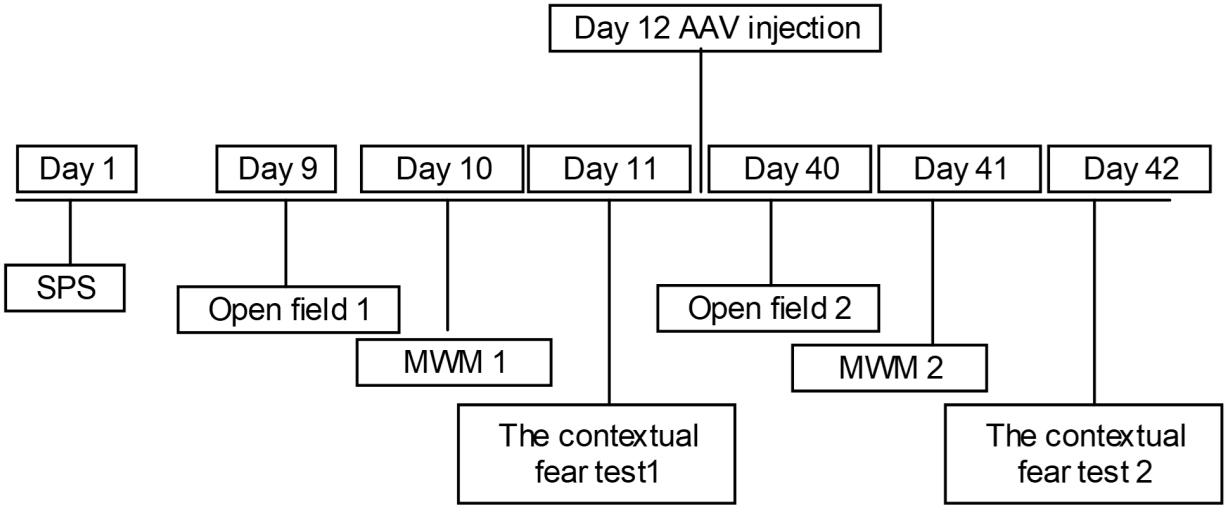

Figure 1. The design of the current study. To investigate whether SPS mice develops PTSD-like behaviors, the mice were randomly divided into sham (n = 18) and SPS (n = 36) groups. Open field test, MWM and contextual fear test were conducted on mice on day 9, 10 and 11, separately, after SPS induction. On day 12, some of the mice (n = 9 in sham group; n = 9 in SPS group) were sacrificed, and the hippocampal tissues were collected to detect the BHLHE40 protein levels. To investigate the functions of BHLHE40 on PTSD-like behaviors of mice, the remaining SPS mice were divided into 4 groups: sham (n = 9), SPS (n = 9), SPS+AAV-NC (n = 9), SPS+AAV-BHLHE40 (n = 9). Some of the mice were injected with AAV-NC and AAV-BHLHE40 on day 12. After injection of AAV for 28, 29, 30 days, open field test, MWM and contextual fear test were conducted, respectively. After injection of AAV for 30 days (42 days after SPS induction), mice were sacrificed, and the hippocampal tissues were collected.

Morris water maze (MWM) test

The MWM test was conducted to measure the memorizing and spatial learning abilities of mice according to a previous study (Broussard et al. 2018). The test includes a platform trial and a probe trial and was conducted in a standing pool (diameter: 120 cm; height: 40 cm) filled with water (depth: 30 cm; temperature: 22-24°C). Before the first trial, the visible escape platform was placed 1 cm above the water surface. For platform trial, mice were placed in a random point with their noses facing the wall, and the escape latency (the time that mice reached the platform) of mice was automatically recorded by a video-tracking system. If the mice were unable to find the hidden platform in 2 minutes, they would be helped to stand on the platform for 20 s. After the last acquisition trial, the probe test was performed. For probe trial, the platform was removed, and mice were placed at a new position with their noses facing the wall to swim at random. The total distance of mice spent in the target quadrant in 120 s was recorded and calculated.

Open field test

The open field test was used to evaluate anxiety-like behaviors of mice as previously described (Feng et al. 2020, Pi et al. 2019). In the present study, mice were put in the center of a cubic chamber (36 × 36 × 36 cm). The mice were permitted to move for 5 min under dim white light. The time and distance of mice that spent in central area were recorded and analyzed by an automatic analyzing system (Intelligent Recognition & Communications Biotech Co. Ltd, Suzhou). The inner surface was cleaned with 70% ethanol between adjacent sessions.

The contextual fear test

The contextual fear test was conducted as previously described (Feng et al. 2020). In brief, mice were placed in a contextual chamber (242 × 242 × 300 mm operant chambers) for an acclimation period (180 s). Then, mice were treated with a pure tone (28 s, 1 kHz, 90 dB) and co-terminated with a foot shock (2 s, 0.8 mA) using a stainless-steel grid floor (Med Associates Inc, USA) three times. Afterwards, mice were kept in the chamber for another 2 min. After 24 h of fear conditioning training, mice were replaced in the contextual chamber for 5 min without tone or foot shock exposure, and the freezing time of mice was automatically recorded using a FreezeScan software (CleverSys Inc). The freezing response upon re-exposure to the shock context is a measure of conditioned associative fear memory reflecting the response to trauma-related cues as a symptom of PTSD (Siegmund & Wotjak 2007). Freezing was defined as the absence of any movement except for respiration.

Western blot

The mice were killed respectively at 12 or 42 days after SPS. The brains of mice were quickly resected, and the hippocampal tissues of mice were isolated and immediately maintained at -80°C. The total proteins from mice hippocampus were extracted (Beyotime Biotechnology, China). Then, the protein samples (50 μg/lane) from each group were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Later, protein samples were transferred onto a polyvinylidene fluoride membrane, and were then blocked with 5% defatted milk for 2 h. Afterwards, the membrane was incubated with Glutamate Receptor 1 antibody (1:1000, ab31232), Glutamate Receptor 2 antibody (1:1000, ab206293), Glutamate Receptor 3 antibody (1:1000, ab40845), Glutamate Receptor 4 antibody (1:1000, ab115322), BHLHE40 antibody (1:1000, ab23797), PI3Kp110α antibody (1:1000, ab40776), phosphorylated AKT (ser473)

antibody (1:1000, ab8932), AKT antibody (1:1000, ab8805) and GAPDH antibody (1:1000, ab9485) at 4°C overnight, and then incubated with secondary antibodies at room temperature for 2 h. The antibodies were purchased from Abcam (Cambridge, UK). Then, blots were placed to the autoradiography by ECL reagents after washing in Tris buffered saline with Tween. The protein bands were visualized by the Gel Image Analysis System and quantified by ImageJ software (National Institute of Health, USA).

Statistical Analysis

The data were analyzed by SPSS 19.0 software and shown as the means ± standard deviation. The unpaired student's *t* test was employed to compare the differences between the groups. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was applied for the comparison of more than two groups. *p* value less than 0.05 had statistical significance.

RESULT

Successful establishment of SPS model mice and the downregulation of BHLHE40 protein levels in hippocampal tissues of SPS mice

As shown in Fig. 2a ($t(16)=10.804$, $p<0.001$), the escape latency of mice in visible platform trial was prolonged by treatment of SPS. The total distance that mice spent in target quadrant was shorter in SPS group compared with that in sham group in the probe trial ($t(16)=3.64$, $p=0.002$, Fig. 2b). In addition, according to the results of the open field test, compared to sham operated

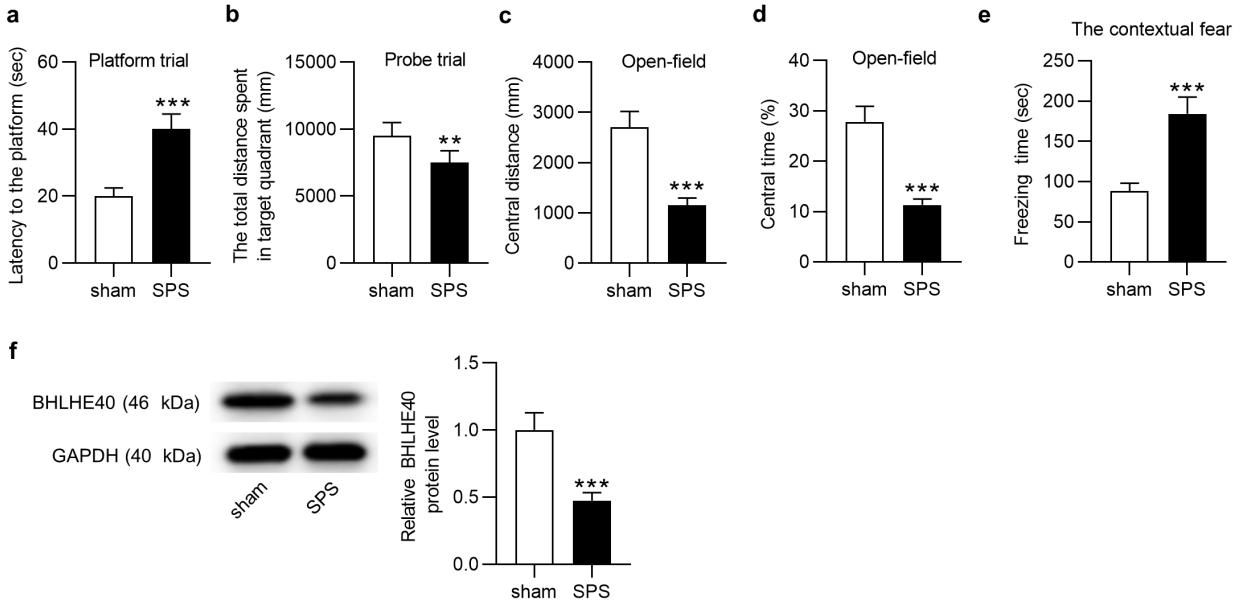

Figure 2. Successful establishment of SPS model mice and the downregulation of BHLHE40 protein levels in hippocampal tissues of SPS mice. (a) The escape latency that the mice (n=9/group) of the sham/SPS group spent in the visible platform trial on day 10. This experiment was conducted 3 times. (b) Total distance that the mice (n=9/group) of the sham/SPS group spent in target quadrant in the probe trial on day 10. This experiment was conducted once. (c-d) The distance and time that sham and SPS mice (n=9/group) spent in the central area in open field test on day 9. This experiment was conducted once. (e) Fear conditioning test of contextual fear memory assessment in sham and SPS groups (n=9/group) on day 11. This experiment was conducted once. (f) The protein levels of BHLHE40 in hippocampal tissues of SPS or sham mice (n=9/group) were determined by western blot. This experiment was conducted 3 times. Student's *t* test was applied for data analysis in Figure 2. $^{**}p< 0.01$, $^{***}p< 0.001$.

mice, SPS mice traveled shorter central distance and spent less central time (2c: t(16)=19.955, p<0.001; 2d: t(16)=12.363, p<0.001, Fig. 2c-d). Moreover, mice exhibit increased fear response to SPS in contextual fear test. As illustrated in Fig. 2e (t(16)=12.913, p<0.001), the freezing time of mice in SPS group was longer than those in sham group in the contextual fear conditioning paradigms. All these data indicated that SPS model was successfully established and can be used for following assays. At last, we measured the protein levels of BHLHE40 in hippocampal tissues of mice and found that the BHLHE40 protein levels were lower in SPS group than that in sham group (t(16)=10.927, p<0.001, Fig. 2f).

BHLHE40 overexpression promoted glutamate receptor protein levels and activated the PI3K/AKT signaling pathway

To begin with, we significantly overexpressed BHLHE40 protein levels by injection of AAV-BHLHE40 in mice (F(3,32)=832.201, p<0.001, Fig. 3a). Effects of BHLHE40 on glutamate receptors were assessed. According to the western blot, the protein levels of glutamate receptors including glutamate receptor 1, glutamate receptor 2, glutamate receptor 3 and glutamate receptor 4 were reduced by SPS, and such results were partially reversed by BHLHE40 overexpression (GluR1: F(3,32)=139.966, p<0.001; GluR2: F(3,32)=89.293, p<0.001; GluR3: F(3,32)=42.95, p<0.001; GluR4: F(3,32)=138.745,

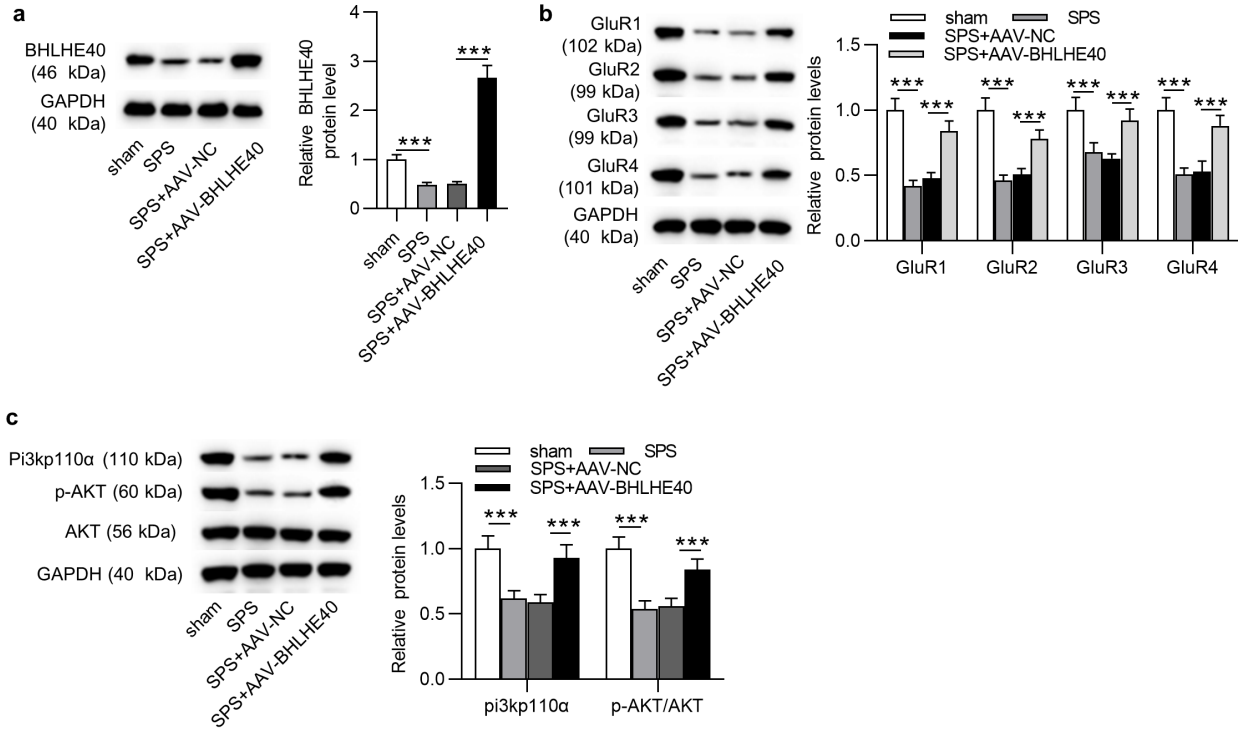

Figure 3. BHLHE40 overexpression promoted protein levels of glutamate receptors and activated the PI3K/AKT signaling pathway. (a) The overexpression efficacy of BHLHE40 in mice of sham, SPS, SPS+AAV-NC, SPS+AAV-BHLHE40 groups (n=9/group) was evaluated by western blot analysis. This experiment was conducted 3 times. (b-c) The protein levels of glutamate receptors, PI3Kp110α, phosphorylated AKT (ser473) and total AKT in mice of sham, SPS, SPS+AAV-NC, SPS+AAV-BHLHE40 groups (n=9/group) were examined by western blot analysis. This experiment was conducted 3 times. One-way ANOVA was applied for data analysis in Figure 3. ***p< 0.001.

p<0.001, Fig. 3b). Moreover, we evaluated the protein levels of PI3Kp110α, phosphorylated AKT (ser473) and total AKT. The results demonstrated that the decreased protein levels of PI3Kp110α and phosphorylated AKT (ser473) induced by SPS were rescued by overexpression of BHLHE40 (Pi3kp110α: $F(3,32)=65.276$, p<0.001; p-AKT/AKT: $F(3,32)=194.665$, p<0.001, Fig. 3c).

BHLHE40 overexpression mitigated PTSD-like behaviors of mice

Next, we explored the effects of BHLHE40 on animal PTSD-like behaviors. First, the prolonged escape latency ($F(3,32)=87.766$, p<0.001) and the shortened total distance ($F(3,32)=14.553$, p<0.001) of mice spent in target quadrant induced by SPS were rescued by BHLHE40 overexpression (Fig. 4a-b), In addition, the open field test revealed that the decreased central distance ($F(3,32)=163.421$, p<0.001) and time ($F(3,32)=172.643$, p<0.001) spent by SPS mice were neutralized by the injection of AAV-BHLHE40 (Fig. 4c-d). Finally, the contextual fear test showed that the increase of freezing time resulting from SPS was partially rescued by BHLHE40 overexpression ($F(3,32)=122.241$, p<0.001, Fig. 4e).

DISCUSSION

As a complex and debilitating neuropathology, PTSD has brought considerable troubles to life quality of the patients (Series 2019). PTSD has an overlap of comorbidity and symptoms with other disorders such as fear, anxiety, and suicidal ideation. To date, acupuncture, psychoanalytic therapy, psychotherapy, and medical treatment have been widely used to improve PTSD (Buhmann & Andersen 2017, Oh et al. 2018). Additionally, selective serotonin reuptake inhibitors (SSRIs) including sertraline, paroxetine and fluoxetine were reported to be effective for PTSD treatment (Akiki & Abdallah 2018, Bushnell et al. 2018). However, the increased prescription of SSRIs may induce suicide, especially for adolescents (Locher et al. 2017). Thus, to identify novel biomarkers for PTSD is necessary.

To explore the development of PTSD, previous studies have constructed a variety of models including the restraint stress model, the inescapable shock model, the predator-stress model, the SPS model and the social defeat stress model to mimic PTSD (Aspesi & Pinna 2019). In our study, we established SPS mice models to explore the role of BHLHE40 in PTSD. According to previous studies (Aspesi & Pinna 2019, Flandreau & Toth 2018), SPS can imitate the physiological challenges of PTSD. The enhancement of hypothalamic-pituitary-adrenal (HPA) negative feedback may contribute to a rise of glucocorticoid receptor (GR) expression in hippocampus 7 days after the exposure to stress. Furthermore, other research revealed that SPS operation contributed to behavioral abnormalities to imitate PTSD symptoms like hyperarousal or contextual freezing. In our study, the SPS caused increased anxiety levels, acoustic startle responses and contextual freezing time. Hippocampus, a region involved in memory, learning and contextual fear extinction, has been confirmed to be correlated with the progression of PTSD (Coburn 2018). Additionally, the structural or functional alterations of hippocampus have been observed in the pathophysiology of PTSD (Wingenfeld & Wolf 2014). We detected BHLHE40 protein levels in hippocampal tissues and found that BHLHE40 protein levels were lower in SPS group than sham group.

The glutamate receptor system consists of three subfamilies, two ligand-gated ion channels (ionotropic receptors), the N-methyl-d-aspartate receptor (NMDAR) and the

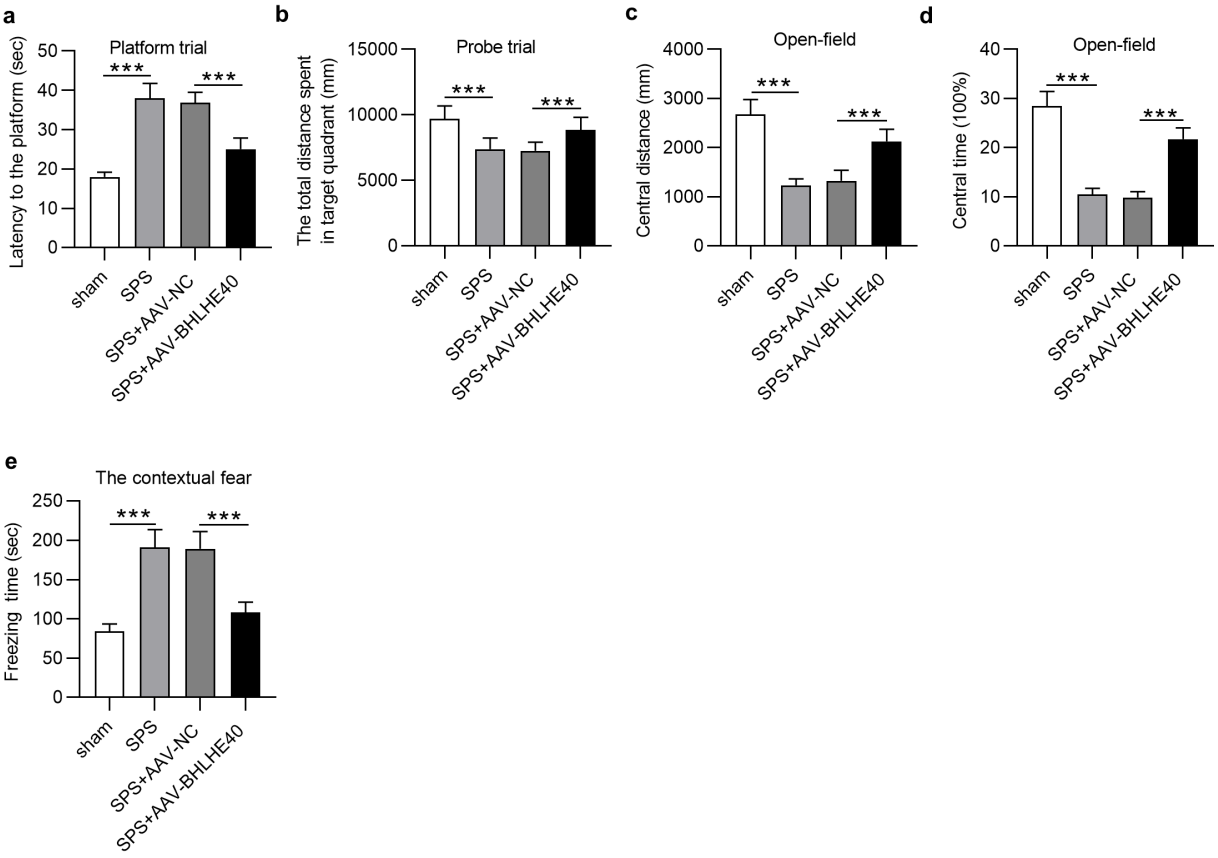

Figure 4. BHLHE40 overexpression mitigated PTSD-like behaviors of mice. (a) The escape latency of mice of sham, SPS, SPS+AAV-NC, SPS+AAV-BHLHE40 groups (n=9/group) in the visible platform trial on day 41. This experiment was conducted 3 times. (b) Total distance that mice of sham, SPS, SPS+AAV-NC, SPS+AAV-BHLHE40 groups (n=9/group) spent in target quadrant in the probe trial on day 41. This experiment was conducted once. (c-d) The distance and time that mice of sham, SPS, SPS+AAV-NC, SPS+AAV-BHLHE40 groups (n=9/group) spent in the central area in open field test on day 40. This experiment was conducted once. (e) Fear conditioning test of contextual fear memory assessment in mice of sham, SPS, SPS+AAV-NC, SPS+AAV-BHLHE40 groups (n=9/group) on day 42. This experiment was conducted once. One-way ANOVA was applied for data analysis in Figure 4. $^{***}p< 0.001$.

α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate receptors (AMPAR/KAR), and one metabotropic receptor (Hollmann & Heinemann 1994) q. Metabotropic glutamate receptors are classified into eight subtypes (mGluR1-8), which are subdivided into group I-III based on sequence homology and intracellular G-protein coupling activity (Niswender & Conn 2010). Group I is characterized by mGluR1 and mGluR5 and causes stimulation of Phospholipase C beta and activation of protein kinase C. Group II, composed of mGluR2 and mGluR3, and group III consisting of mGluR4 and mGluR6-8, are responsible for the inhibition of adenylyl cyclase, which potentiates the PI3K/Akt signaling pathway (Iacovelli et al. 2002). The metabotropic GluRs in the central nervous system mediate the postsynaptic neuronal response to glutamate, modulating both NMDAR and AMPAR activities, as well as cellular proliferation, growth, migration, survival, and calcium-mediated cellular homeostasis (Pin &

Duvoisin 1995)Montpellier, were decreased in hippocampal tissues of sham mice and were increased by BHLHE40 overexpression. BHLHE40 has been reported to be a crucial repressor of IL-10 (Huynh et al. 2018), and IL-10 restores glutamate receptor-controlled Ca 2^{+}-pathway in brain circuits (Turovskaya et al. 2020). BHLHE40 can increase the expression of CXCL12 (Teng et al. 2020), and CXCL12 facilitates glutamate synaptic activity at serotonin neurons in the rat dorsal raphe nucleus (Heinisch & Kirby 2010). Our study revealed that BHLHE40 overexpression increased expression of metabotropic glutamate receptors to reverse the increase of anxiety levels and contextual freezing time resulting from SPS in mice.

Moreover, BHLHE40 can activate the PI3K/AKT signaling pathway by promoting the phosphorylation processes of PI3K and AKT in neurons (Zhu et al. 2017b). Additionally, repetitive transcranial magnetic stimulation regulates the PTEN/Akt signaling pathway to ameliorate PTSD symptoms by promoting synaptic plasticity and glutamate transmission in the anterior cingulate cortex of rats (Liu et al. 2018a). Metabotropic glutamate receptors can activate the PI3K/Akt signaling pathway (Willard & Koochekpour 2013). Therefore, we hypothesized that BHLHE40 activates the PI3K/AKT signaling pathway in hippocampus to mitigate PTSD-like behaviors. The present study revealed that protein levels of PI3Kp110α, phosphorylated AKT were lower in hippocampal tissues of SPS mice than sham mice, while BHLHE40 overexpression increased PI3Kp110α and phosphorylated AKT protein levels.

However, our study has limitations. First, the expression status and biological roles of BHLHE40 in neurons were not explored. Second, the effects of the PI3K/AKT signaling pathway on PTSD behaviors were not evaluated. At last, the detailed molecular mechanisms of BHLHE40 downregulation in PTSD deserved further exploration.

In conclusion, BHLHE40 alleviates PTSD-like behaviors with the involvement of the PI3K/AKT signaling pathway in a glutamate receptor-dependent manner in mice. This novel discovery may provide a potential target for the improvement of PTSD.

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SUPPLEMENTARY MATERIAL

Coding sequences of BHLHE40

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