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Alleviation of *N*-Methyl-D-Aspartate Receptor-Dependent Long-Term Depression via Regulation of the Glycogen Synthase Kinase-3β Pathway in the Amygdala of a Valproic Acid-Induced Animal Model of Autism

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Abstract The amygdala plays crucial roles in socio-emotional behavior and cognition, both of which are abnormal in autism spectrum disorder (ASD). Valproic acid (VPA)-exposed rat offspring have demonstrated ASD phenotypes and amygdala excitatory/inhibitory imbalance. However, the role of glutamatergic synapses in this imbalance remains unclear. In this study, we used a VPA-induced ASD-like model to assess glutamatergic synapse-dependent long-term depression (LTD) and depotentiation (DPT) in the amygdala. We first confirmed that the VPA-exposed offspring exhibited sociability deficits, anxiety, depression-like behavior, and abnormal nociception thresholds. Then, electrophysiological examination showed a significantly decreased paired-pulse ratio in the amygdala. In addition, both NMDA-dependent LTD and DPT were absent from the amygdala. Furthermore, we found that the levels of glycogen synthase kinase3β (GSK-3β) phosphorylation and β-catenin were significantly higher in the amygdala of the experimental animals than in the controls. Local infusion of phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin into the amygdala reversed the increased phosphorylation level and impaired social behavior. Taken together, the results suggested that NMDA receptor-related synaptic plasticity is dysfunctional in VPA-exposed offspring. In addition, GSK-3β in the amygdala is critical for synaptic plasticity at the glutamatergic synapses and is related to social behavior. Its role in the underlying mechanism of ASD merits further investigation.

Keywords Autism spectrum disorder · *N*-Methyl-D-aspartate receptor · Valproic acid · Long-term depression · Amygdala · GSK-3β

Introduction

Autism spectrum disorder (ASD) is a neuropsychiatric disorder characterized by impaired social interactions, communication deficits, and repetitive behaviors [1–3]. At present, it is widely accepted that environmental factors play critical roles in the incidence of ASD. Exposure to valproic acid (VPA) during gestation leads to a significantly higher incidence of ASD-associated developmental deficits [4, 5]. Prenatal exposure to another environmental teratogen, namely bis(2-ethylhexyl) phthalate (DEHP), which is widely used to make food wrap and toys, also increases the risk of ASD [6, 7]. Interestingly, phthalate can be hydrolyzed to 2-ethylhexanol and then metabolized to 2-ethylhexanoic acid, which is an isomer of VPA [8]. Therefore, a VPA-induced ASD-like rat

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model can be used as a tool to investigate the underlying mechanism of ASD [9].

Altered synaptic plasticity is considered a major criterion when characterizing VPA-induced ASD-like rat models as well as other genetic ASD models [10–12]. An increased synaptic excitatory/inhibitory (E/I) ratio is present in these ASD models [13–15]. Some genetic ASD animal models have demonstrated *N*-methyl-D-aspartate receptor (NMDAR)-dependent long-term depression (LTD) impairment in the hippocampus, suggesting that NMDARs are involved in the underlying mechanism of ASD [11, 16, 17]. Interestingly, enhanced plasticity has also been noted in other brain areas in VPA-exposed offspring [14, 18, 19]. In the cortex, the excitatory connections have been noted to be more plastic, displaying enhanced long-term potentiation (LTP) of the strength of the synapses [14]. Our previous study showed that the LTP in the lateral amygdala (LA) is significantly increased in VPA-exposed offspring at the thalamic-amygdala synapses [19]. In vitro, inhibition of amygdala pyramidal neurons was found to be deficient, and disruption of inhibitory circuits is considered to be one cause of some autistic phenotypes in the VPA-exposed model [18]. Clinical studies have revealed that patients with ASD have comparatively higher amygdala volumes and a lower amygdala activation during mental tasks than controls [20, 21]. Previous studies have reported that bilateral amygdala lesions decrease social interaction in rhesus monkeys [22, 23]. Therefore, amygdala dysfunction could be related to the core social and cognitive symptoms present in patients with ASD at an early developmental age [24–27].

Glycogen synthase kinase 3 (GSK3) is a multifunctional serine/threonine (ser/thr) kinase that has two isoforms, GSK-3α and GSK-3β. It regulates neurodevelopmental processes such as cell structure, microtubule dynamics, gene expression, and survival via substrate phosphorylation [28, 29]. GSK3 activity is inhibited through the phosphorylation of serine 21 in GSK-3α and serine 9 in GSK-3β [30]. Previous studies have reported that alteration of GSK3 activity is associated with psychiatric and neurodegenerative diseases, including Alzheimer's disease, schizophrenia, and ASD [29, 31–33]. Another study revealed a strong association between GSK-3β and NMDA-dependent LTD in the hippocampus [34]. The inhibition of GSK-3β is mediated by the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway, which has also been found to regulate NMDA-dependent LTD. During LTD, GSK-3β is activated by dephosphorylation of Ser-9 and inhibition of PI3K, suggesting that induction of NMDAR-dependent LTD decreases the phosphorylation of GSK-3β [34–36]. In this study, we examined the amygdala NMDA receptor-dependent LTD and DPT in VPA-induced ASD rat offspring to determine the role of the amygdala in ASD. We also investigated the possible role of GSK-3β in the regulation of amygdala E/I imbalance and autistic phenotypes.

Materials and Methods

Animals

All procedures were approved by the Experimental Animal Review Committee at National Yang-Ming University and were found to be in accord with both the Guide to the Care and Use of Laboratory Animals and the National Institute of Health guidelines (USA) with respect to the care and use of animals during experimental procedures. Sprague-Dawley (SD) rats were housed four to five to a cage in a temperature-controlled (24 °C) animal colony under a 12:12-h light/dark cycle with food and water available ad libitum. All behavioral procedures took place during the light cycle. Sodium salt of valproic acid (NaVPA; Sigma-Aldrich) was dissolved in 0.9 % saline to obtain a concentration of 150 mg/ml, pH 7.3. Pregnant female SD rats received a single intraperitoneal injection of valproic acid (500 mg/kg) on E12.5, while the control group received 0.9 % saline (500 mg/kg) [37]. Dams were housed individually and allowed to raise their own litters until weaning. Both behavioral and electrophysiological experiments were conducted on P28–35.

Surgery

Two 22-gauge stainless steel tubes were implanted in the LAs of the male VPA-exposed offspring under ketamine (100 mg/kg, i.p.) and anesthesia. Two cannulas were implanted bilaterally into the LA (anteroposterior, −2.8 mm; mediolateral, ±4.5 mm; dorsoventral, −7.0 mm) [38]. The cannulas were fixed to the skull with dental cement. A 28-gauge dummy cannula was inserted into each cannula to prevent clogging. The VPA-exposed offspring were allowed to recover from the surgery for 1 week. Wortmannin (2.5 μg/side, dissolved in 20 % DMSO) (Tocris) was infused bilaterally into the LA at a rate of 0.1 μl/min, with a volume of 1 μl per side. The VPA-exposed offspring were then returned to their home cages until assessment of social behavior was performed.

Behavioral Testing

A behavioral trace of rat movement during the experiments was recorded using Smart software (version 3.0; Panlab, S.L.U., Spain).

Three-Chamber Social Interaction Test

The social interaction test involving an unfamiliar and a familiar rat was adapted from the study by Crawley [39] and performed using 4–6-week-old saline- and VPA-exposed offspring. The test took place in an environment unknown to the rat being tested, in the form of a cage with three communicating compartments. At the beginning of the sociability test, the test rat was placed in the central compartment. In the right compartment (designated "chamber S1"), a stranger rat was placed under a small plastic box (27 × 13 × 20 cm). Stranger rats were randomly selected from saline-exposed rats of the same gender as the test rat. The left compartment, i.e., the empty compartment (designated "chamber E"), contained a plastic box with no contents. After 5 min, designated the habituation period, the sociability test was performed over a 5-min duration.

Social Interaction

The social interaction test was conducted as previously described using a plastic box (45 × 45 × 45 cm) [40]. After a habituation period, one VPA-exposed offspring and one stranger rat were placed into the box for a period of 20 min. The frequency percentage of social interaction in the saline- and VPA-exposed offspring was measured in terms of sniffing, mounting, following, and crawling under any body part, all of which were taken as indicators of social engagement.

Elevated Plus Maze (EPM)

The elevated plus maze test was used to assess anxiety [41]. The elevated plus maze apparatus consisted of four arms (112 × 112 cm), two open arms and two closed arms. All arm platforms were elevated 31 cm from the floor. At the start of a trial, the rat was placed in the center of the elevated plus maze and allowed to explore the maze freely. Behavior was observed for 10 min, and the percentage of time spent in the open arms was recorded.

Open-Field Test

The open-field test was used to assess anxiety-like behavior [42]. The apparatus consisted of a novel square box (45 × 45 × 45 cm). Each rat was placed in the corner of the open field, and the time spent in each square and in the central zone over a period of 5 min was determined.

Marble-Burying Test

The marble-burying test was used to assess obsessive-compulsivity (an anxiety-related behavior) [43]. A clean cage (19 × 10.5 × 8 cm) was prepared with 4-cm corncob bedding material containing 20 embedded marbles. After 20 min, the number of marbles that remained buried in the corncob bedding was recorded.

Novelty-Suppressed Feeding (NSF)

Novelty-suppressed feeding (NSF) is used to assess depression-like behavior [44]. The rats were food-deprived for 24 h prior to the test, with free access to water. On the day of testing, the rats were placed in a novel square box (45 × 45 × 45 cm) with a pre-weighed food pellet placed at its center. In the test, the latency time prior to the rats beginning to chew the food was recorded. The maximum time allowed for the test was 15 min.

Tail-Flick Test (TFT)

A tail-flick analgesia meter (Columbus Instruments) with an 8-V high-intensity lamp (6 A) was used to measure pain sensitivity [45]. The rats were placed in plastic restrainers in order to observe the movement of the tail away from the lamp. The baseline latency was approximately 2 ± 0.25 s, and the cut-off time was 10 s in order to prevent tail skin damage [46]. Tail-flick measurements were taken three times at 30-s intervals.

Brain Slice Preparation and Electrophysiology Recording

The rats were killed by rapid decapitation. Their brains were removed and placed in a beaker containing cold (4 °C) oxygenated (saturated with 95 % O_2 and 5 % CO_2) artificial cerebrospinal fluid solution (ACSF). The ACSF solution had the following composition (mM): 87 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 4 $MgCl_2$, 23 $NaHCO_3$, 1.25 NaH_2PO_4, and 25 glucose. Extracellular recordings from LA projection neurons were performed at ~32 °C in a superfusing chamber. To record the field excitatory postsynaptic potential (fEPSP) in the LA, a concentric bipolar stimulating electrode (FHC; Bowdoinham, ME, USA) was placed in the external capsule and a capillary glass recording electrode filled with 3 M NaCl solution was placed in the LA region. The paired-pulse facilitation (PPF) ratio (second/first fEPSP slope) was measured using two paired stimuli with interpulse intervals (IPI) of 20, 40, 60, 80, and 100 ms.

Synaptic Plasticity

Low-frequency stimulation (LFS) producing LTD was elicited by 900 trains of stimuli (1 Hz, 1 s at 1-min intervals) for 15 min at the same stimulation intensity used for baseline measurements. The chemical LTD induction solution consisted of the above-described ACSF and 20 μM NMDA (Tocris, St. Louis, MO). After 3–5 min of chemical LTD induction, the sections were washed with ACSF for 5 min, and

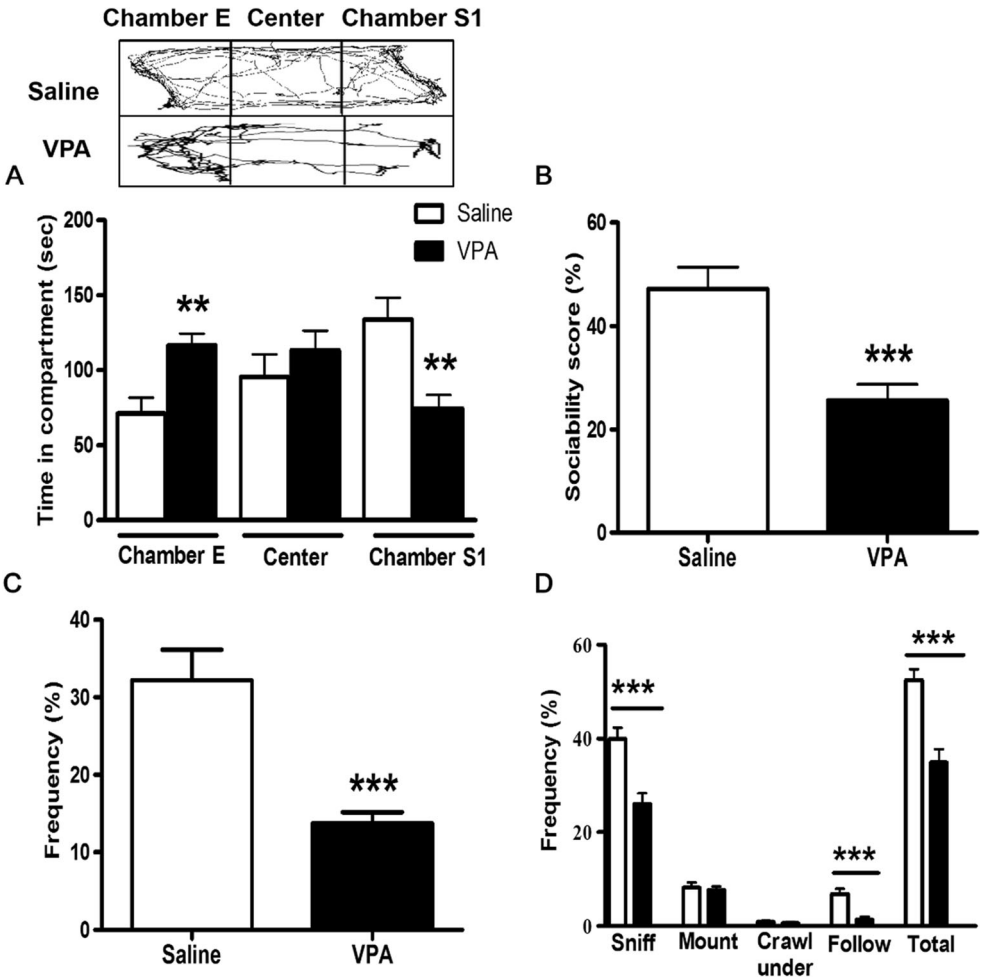

Fig. 1 VPA-exposed offspring showed reduced sociability in the social behavior test. **a** The VPA-exposed offspring spent less social time in chamber S1 and more time in chamber E than the saline-exposed offspring. **b** Sociability scores were calculated as the time spent in chamber S1/ total examination time. The results showed lower sociability in the VPA-exposed offspring than in the saline-exposed offspring. **c** The VPA-exposed offspring showed a lower frequency of social interaction with stranger rats. **d** The frequencies of sniffing, mounting, following, and crawling were measured. Data represent mean ± SEM for each experimental condition. $^{**}p < 0.01$, $^{***}p < 0.001$ (saline, $n = 12$; VPA, $n = 12$)

the fEPSPs were monitored for an hour. In the DPT experiments, the LTP was induced by high-frequency stimulation (HFS) at the test pulse intensity, which consisted of two 1-s trains of stimuli separated by an inter-train interval of 20 s at 100 Hz. After 20 min of LTP induction, LFS (1 Hz, 15 min) was applied to depotentiate the responses.

Western Blotting Assay

Brain tissues were dissected and lysed in a lysis buffer containing 1 % Triton X-100, 0.1 % SDS, 50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 mM enylmethylsulfonyl fluoride, supplemented with a complete protease inhibitor cocktail. Following sonication, lysates were centrifuged at 12,000 rpm for 30 min to obtain supernatants. The protein concentrations of the supernatants were measured using a Bradford assay, and equal amounts of protein were separated by SDS-PAGE electrophoresis, transferred to Immobilon-P membranes (Millipore), and incubated in 5 % non-fat dry milk for 60 min. Membranes were incubated with anti-GAPDH (1:10,000; Abcam, Cambridge, UK), β-catenin (1:5000; Cell Signaling Technology, Boston, MA, USA), GSK-3β (1:5000; Abcam, Cambridge, UK), and p-GSK-3β (1:2000; Cell Signaling Technology, Boston, MA, USA) antibodies overnight at 4 °C, then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactivity was detected using ECL Plus detection reagent (PerkinElmer, Boston, MA, USA). Films were exposed for different periods to ensure the optimum density was achieved without saturation, and this exposure was followed by densitometry. Protein levels were first normalized to internal control levels for each sample and then measured as fold changes with respect to the controls.

Statistical Analysis

All values are expressed as the mean ± SEM. For the LTD experiments, statistical analysis was performed using the non-parametric Mann-Whitney *U* test. The significances of differences between groups were calculated using Student's *t* test, and Newman–Keuls post hoc comparisons were used to

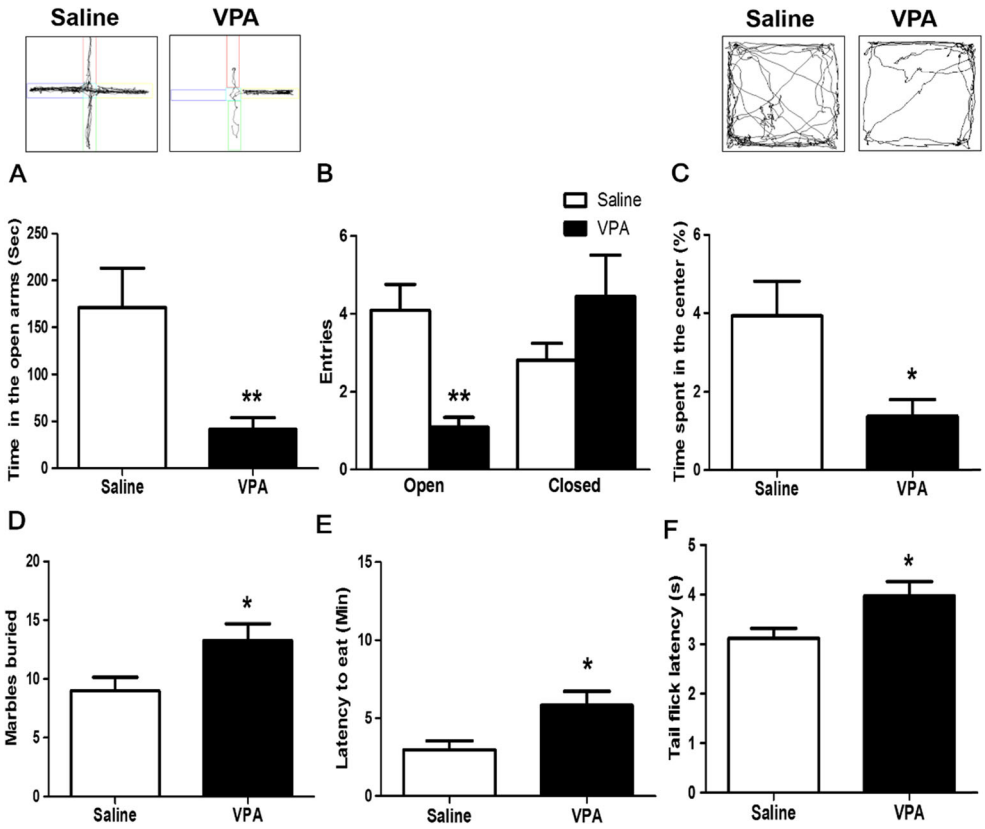

Fig. 2 VPA-exposed offspring exhibited anxiety and repetitive-like behaviors. **a** The VPA-exposed offspring tended to spend less time in the open arm than the saline-exposed offspring. **b** The number of entries into the open and closed arms in the EPM test. The VPA-exposed offspring displayed significantly fewer entries into the open arm. **c** The VPA-exposed offspring spent significantly less time in the center during the open-field test. **d** The VPA-exposed offspring buried more marbles than the saline-exposed offspring. **e** The VPA-exposed offspring showed higher latency in the time taken to approach a food pellet in the NSF test. **f** The tail-flick test showed an increased tail-flick latency in the VPA-exposed offspring. Data represent mean ± SEM for each experimental condition. $^{*}p < 0.05$, $^{**}p < 0.01$ (saline, $n = 12$; VPA, $n = 12$)

analyze the differences in the results of behavioral tests, protein levels, and electrophysiology responses. Probability values (p) of less than 0.05 were considered to represent significant differences.

Results

The VPA-Induced ASD-Like Animal Model Demonstrated Social Impairment

The VPA-exposed offspring spent slightly less time with stranger rats (chamber S1—70.44 ± 10.14, $n = 12$) and more time in chamber E (115.90 ± 10.23, $n = 12$) than the saline-exposed offspring (chamber S1—133.90 ± 14.48, $n = 12$, $p < 0.01$; chamber E—65.08 ± 13.70, $n = 12$, $p < 0.01$; Fig. 1a). Sociability scores were significantly lower in the VPA-exposed offspring (25.93 ± 3.85, $n = 12$) than the saline-exposed offspring (46.21 ± 5.70, $n = 12$, $p < 0.01$; Fig. 1b). The frequency of social interaction was 13.75 ± 1.43 ($n = 12$) and 32.17 ± 3.97 ($n = 12$) in the VPA- and saline-exposed offspring, respectively (Fig. 1c). In the social interaction test, the VPA-exposed offspring displayed less sniffing (26.33 ± 2.50, $n = 12$) and following (1.50 ± 0.60, $n = 12$) than the saline-exposed offspring (sniffing—40.92 ± 2.33, $n = 12$, $p < 0.001$; following—7.16 ± 1.11, $n = 12$, $p < 0.001$; Fig. 1d). These results demonstrated impaired social interaction in the VPA-induced ASD animal model.

VPA-Exposed Offspring Displayed Higher Anxiety, Repetitive-Like Behaviors, and Nociceptive Sensitivity

The results of the elevated plus maze (EPM) test revealed that the VPA- and saline-exposed offspring spent 41.60 ± 12.41 s ($n = 12$) and 171.20 ± 41.93 s ($n = 12$), respectively, in the open arms of the maze (Fig. 2a). In addition, the VPA-exposed offspring tended to make fewer entries (1.09 ± 0.25, $n = 12$) into the open arms of the EPM than the saline-exposed group (4.50 ± 0.73, $n = 12$, $p < 0.01$; Fig. 2b). We then used the open-field test to confirm anxiety-like behavior. As shown in Fig. 2c, the VPA-exposed offspring spent less time (2.35 ± 0.46 %, $n = 12$) in the center than the saline-exposed offspring (4.98 ± 0.75 %, $n = 12$, $p < 0.05$). In the marble-burying test, the VPA-exposed offspring buried 13.27 ± 1.434 ($n = 12$) marbles (Fig. 2d), while the saline-exposed offspring buried 9.00 ± 1.15 ($n = 12$) marbles. In the novelty-suppressed feeding test (NSF), the VPA-exposed offspring showed higher latency (5.82 ± 0.88, $n = 12$) before eating the food pellet than the saline-exposed offspring (2.96 ± 0.55, $n = 12$, $p < 0.05$; Fig. 2e). Furthermore, the tail-flick test showed higher tail-flick latencies in the VPA-exposed offspring (3.97 ± 0.29,

$n = 12$) than the saline-exposed offspring (3.12 ± 0.21, $n = 12$, $p < 0.05$), suggesting a significantly higher nociceptive threshold in the VPA-exposed offspring (Fig. 2f).

Basal Synaptic Transmission and Presynaptic Influences in the VPA-Induced ASD Animal Model

To determine the basal synaptic transmission, we examined the stimulus–response relationships of field excitatory postsynaptic potentials (fEPSPs) in the cortico-lateral amygdala of the VPA- and saline-exposed offspring. There were no significant differences in the evoked fEPSPs between the VPA-exposed offspring (4.08 ± 1.03, $n = 12$; $p = 0.6597$) and the saline-exposed offspring (3.58 ± 0.85, $n = 12$; Fig. 3a). We further investigated paired-pulse facilitation (PPF) in the VPA- and saline-exposed offspring. The synaptic transmission evoked by the PPF ratio at interpulse intervals of 20, 40, 60, 80, and 100 ms was assessed. As illustrated in Fig. 3b, the PPF ratios were significantly lower at each stimulation interval in the VPA-exposed offspring ($n = 10$) than in the saline-exposed offspring ($n = 10$; 20 ms—1.10 ± 0.03 in VPA-exposed offspring, 1.28 ± 0.05 in saline-exposed offspring, Mann-Whitney U test, $p = 0.02$; 40 ms—1.01 ± 0.04 in VPA-exposed offspring, 1.20 ± 0.04 in saline-exposed offspring, Mann-Whitney U test, $p = 0.02$; 60 ms—1.03 ± 0.02 in VPA-exposed offspring, 1.16 ± 0.03 in saline-exposed offspring, Mann-Whitney U test, $p = 0.01$; 80 ms—0.98 ± 0.03 in VPA-exposed offspring, 1.09 ± 0.02 in saline-exposed offspring, Mann-Whitney U test, $p = 0.02$; 100 ms—1.04 ± 0.03 in VPA-exposed offspring, 0.96 ± 0.02 in saline-exposed offspring, Mann-Whitney U test, $p = 0.03$).

VPA-Exposed Offspring Showed NMDA-Dependent LTD and NMDA-Dependent DPT Impairment of the Amygdala

LTD in amygdala neurons was induced by LFS (1 Hz, 900 pulses) for 15 min, a technique that has been used previously to induce NMDA-dependent LTD in the hippocampus [47]. We observed that LTD was completely prevented in the VPA-exposed offspring (93.72 ± 5.71 %, $n = 10$; saline-exposed offspring—69.35 ± 4.18 %, $n = 10$, $p < 0.01$; Fig. 4a, d). We also used NMDA to induce chemical LTD, and this was found to be dysfunctional in the amygdala of the VPA-exposed offspring (90.68 ± 5.33 %, $n = 10$) but not in the saline-exposed offspring (69.31 ± 4.94 %, $n = 10$, $p < 0.05$; Fig. 4b, d). LFS administered 15 min after the application of high-frequency stimulation (HFS) resulted in DPT of the LTP. The fEPSP response of the saline-exposed offspring returned to near baseline after LFS (84.20 ± 3.68 %, $n = 10$), while that of the VPA-exposed offspring remained above baseline (124.50 ± 2.7 %, $n = 10$, $p < 0.001$; Fig. 4c, d).

The Increased GSK-3β Phosphorylation and Decreased Sociability Were Reversed after Bilateral Infusion of Wortmannin into the Amygdala

The NMDA receptor induction of LTD occurs via increased GSK-3β activity, leading to dephosphorylation of GSK-3β at

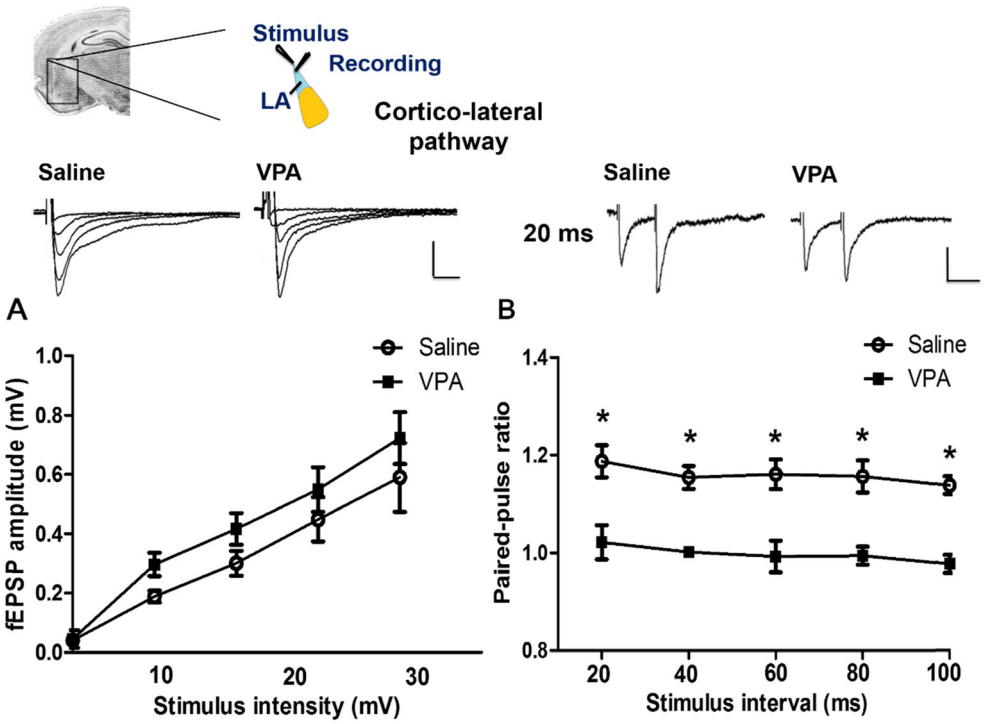

Fig. 3 Input–output curves of the fEPSP and paired-pulse facilitation of the amygdalas of saline- and VPA-exposed offspring. **a** The results revealed no significant difference in the stimulus–response curve between the saline- and VPA-exposed offspring. **c** The paired-pulse ratio was observed at stimulus intervals of 20, 40, 60, 80, and 100 ms. The paired-pulse ratio was lower in the VPA-exposed offspring than the saline-exposed offspring. Data represent means ± SEM for each experimental condition. *$p < 0.001$ (saline, $n = 10$; VPA, $n = 10$); scale 5 ms and 0.4 mV

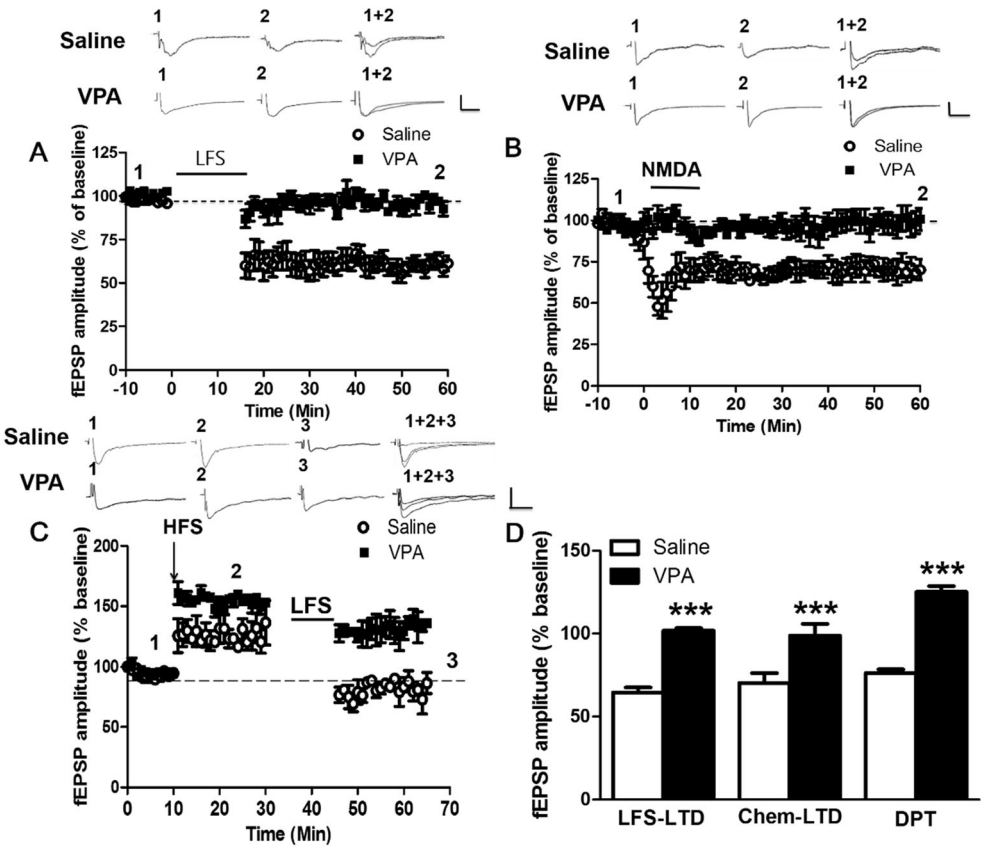

Fig. 4 NMDA-dependent LTD and DPT impairment in the amygdalas of VPA-exposed offspring. **a** The VPA-exposed offspring exhibited higher NMDA-dependent LTD induced by LFS. **b** The NMDA-induced chemical LTD was also increased in the VPA-exposed offspring. **c** LTP induction by HFS (2 × 100 Hz) and depotentiation by LFS (1 Hz, 15 min) were impaired in the VPA-exposed offspring. **d** Bar charts comparing the effects of VPA- and saline-exposed offspring on LFS, chemical LTD, and DPT induction in the amygdala. The magnitude of LTD was measured 30–40 min after the application of LFS or NMDA washout. Data represent mean ± SEM for each experimental condition. ***$p < 0.001$ (saline, $n = 10$; VPA, $n = 10$); scale 5 ms and 0.4 mV

Ser-9, which enables LTD to occur [34]. We investigated whether the phosphorylation of GSK-3β was altered in the VPA-exposed offspring and found that the phosphorylation level of GSK-3β was significantly higher in the VPA-exposed offspring than in the saline-exposed offspring (116.4 ± 7.407 % of saline, $n = 6$, $p < 0.05$; Fig. 5a). As PI3K is an upstream regulator of GSK-3β, we administered the PI3K inhibitor wortmannin (2.5 μg/side) bilaterally into the amygdala and found that this reversed the higher level of phosphorylation of GSK-3β in the VPA-exposed offspring (91.92 ± 5.832 % of saline, $n = 7$, $p < 0.01$; Fig. 5a). Furthermore, the VPA-exposed offspring had higher levels of the active form of β-catenin (113.4 ± 2.586 % of saline, $n = 7, p < 0.01$; Fig. 5b) than the saline-exposed offspring, and this was further blocked by wortmannin application (92.31 ± 4.299 % of saline, $n = 7$, $p < 0.001$; Fig. 5b). We then examined whether wortmannin rescued the impaired social behavior evident in VPA-exposed offspring. The VPA-exposed offspring were infused with wortmannin (2.5 μg/side) bilaterally into the amygdala, and the three-chamber social interaction test was performed 30 min later. Treatment with wortmannin rescued the lower sociability level of the VPA-exposed offspring (VPA + Wort—67.76 ± 3.691 %, $n = 7$, $p < 0.01$; Fig. 5c, d), suggesting that GSK-3β is involved in the abnormal cognitive regulation observed in VPA-induced ASD models.

Discussion

In this study, it was confirmed that VPA-exposed offspring exhibited a lower level of social interaction, more anxiety/repetitive-like behaviors, and higher nociceptive thresholds than the control offspring [9, 19, 48, 49]. These results support previous evidence showing that amygdala E/I imbalance plays a role in the development of ASD. The amygdala complex is the pilot site that connects the cerebral cortex, hippocampus, and striatum, and participates in socio-emotional behavior [21, 50, 51]. In our study, amygdala E/I imbalance was linked to the abnormal NMDA-dependent receptor LTD and DPT, which were associated with higher GSK-3β phosphorylation levels. We also demonstrated that local infusion of the PI3K inhibitor wortmannin into the amygdala reversed the abnormal GSK-3β phosphorylation levels and also the impairment of social behavior. Our results suggested that GSK-3β in the amygdala is critical to NMDA receptor dysfunction and related behavior in VPA-exposed offspring.

Synaptic dysfunction has been identified as a common hallmark of ASD in both human and animal models [52–54]. Mutations in the synaptic scaffolding proteins and adhesion molecules that control excitatory and inhibitory neurotransmission have been demonstrated to be linked to ASD [55, 56]. Multiple levels of evidence have highlighted that both genetic and non-genetic factors affect the significance of the

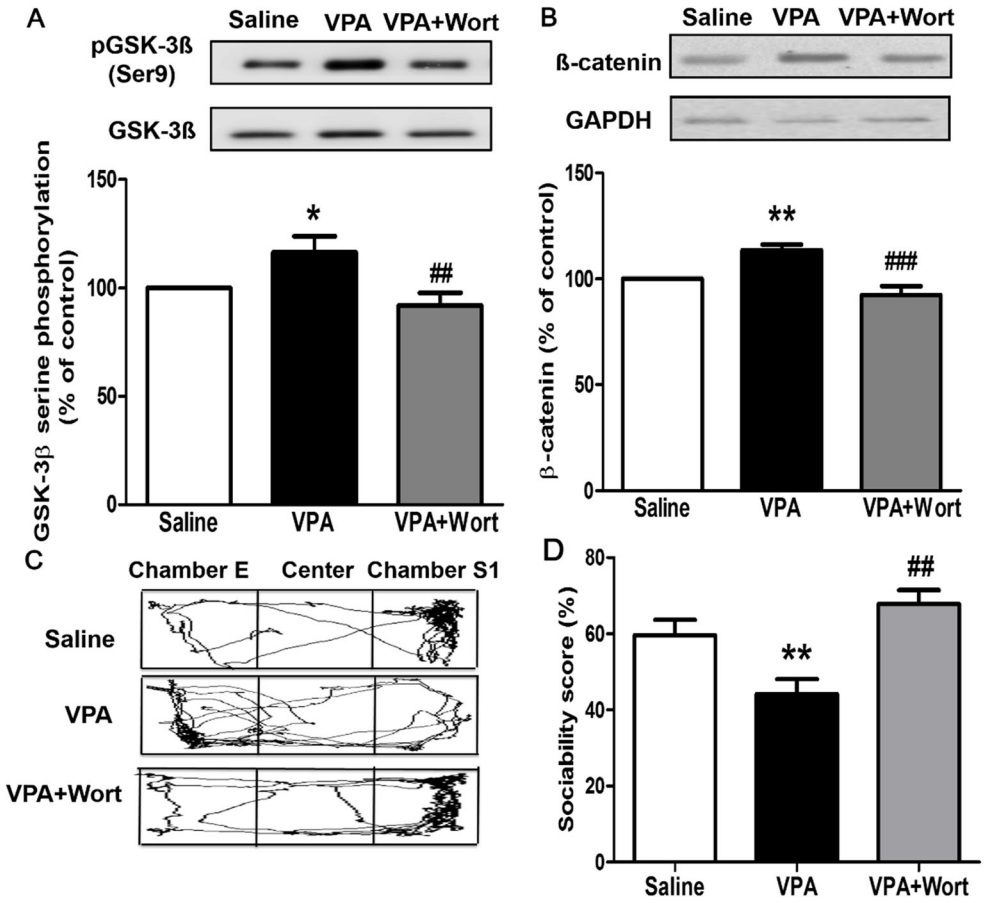

Fig. 5 The impairment of social behavior was rescued by inhibition of PI3K activity in the amygdalas of VPA-exposed offspring. Rats were divided into groups of saline-exposed offspring and VPA-exposed offspring, and the VPA-exposed offspring received bilateral amygdala infusions of wortmannin (2.5 μg/side, dissolved in 20 % DMSO) 30 min before the social behavior test. **a** The VPA-exposed offspring had higher levels of GSK-3β phosphorylation than the saline-exposed offspring, and this was reversed by administration of wortmannin bilaterally into the amygdala. **b** Bilateral infusion of wortmannin into the amygdala significantly decreased the level of β-catenin in the VPA-exposed offspring. **c** Trace of movement during the three-chamber social test recorded using Smart software. **d** The lower level of sociability of the VPA-exposed offspring, as demonstrated by the score on the social behavior test, was rescued by administration of wortmannin bilaterally into the amygdala. Data represent mean ± SEM for ea 4ch experimental condition. $*p < 0.05$, $**p < 0.01$ vs. saline; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. VPA (saline, $n = 8$; VPA, $n = 8$)

E/I imbalance in ASD [57–61]. As a histone deacetylase (HDAC) inhibitor, VPA suppresses the formation of inhibitory synapses, which further disturbs the E/I balance [62–64]. Enhancement of postsynaptic plasticity mediated by NMDARs has been noted in the neocortex of VPA-exposed offspring [14, 65]. We previously demonstrated a higher synaptic E/I ratio in the amygdala of VPA-induced ASD rat offspring as compared with controls, suggesting that hyperglutamatergic transmission in the amygdala may cause the autistic phenotypes observed in VPA-exposed offspring [19].

NMDA glutamate receptors are important in the synaptic functions underlying learning and memory, and in cognitive-related behaviors [66–68]. The mechanism of NMDAR dysfunction has been investigated in ASD models [69]. Social play and social investigation behaviors were found to be increased following systemic injection with a non-competitive NMDAR NR2A subunit antagonist and a NMDAR NR2B subunit antagonist [70]. Lower NR1 levels lead to loss of affinity behavior [71, 72]. Pharmacological modulation of NMDAR function reverses dysfunctional NMDARs and ameliorates the ASD-like behaviors observed in animal models [69, 73, 74]. The data obtained in this study demonstrated impaired social behavior and higher levels of anxious behavior in the VPA-exposed offspring, suggesting that NMDARs play vital roles in core ASD-like behaviors [75, 76]. Mutation in synaptic proteins, in particular in *SHANK*, leads to ASD-related phenotypes and NMDAR dysfunction [77]. However, whether or not synaptic scaffolding proteins and adhesion molecules affect NMDAR function in VPA-exposed offspring requires further investigation. Accumulating evidence indicates that NMDARs regulate long-term plasticity in the CNS, including LTP, LTD, and DPT [78]. In autism animal models such as

Shank2$^{-/-}$ mice, *Mecp2*$^{308/Y}$ mice, and *IRSp53*$^{-/-}$ mice, NMDAR-dependent LTD impairment has been reported [11, 17, 79]. Previous studies have shown that D-cycloserine and zinc normalize NMDA-dependent synaptic plasticity through NMDAR activation [17, 80]. In both our previous and current studies, we found that the NMDAR-dependent LTP and LTD differed in the amygdala of the VPA-exposed offspring as compared with the controls. Induction of LTD is mediated by NMDAR activation, which triggers α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) endocytosis while simultaneously increasing the expression of gamma-aminobutyric acid receptors (GABARs) [81–83]. However, the role of GABA receptors in LTD expression in VPA-exposed offspring requires further study. Our study findings were the first to demonstrate NMDAR-dependent LTD impairment in the amygdala of VPA-exposed offspring at an early developmental age.

Neuronal signaling dysregulation has been implicated in ASD [84]. GSK3 plays a pivotal role in development and neuroplasticity, and has been implicated in neuropsychiatric and neurodegenerative diseases [28, 85, 86]. GSK-3β is highly enriched within dendrites and dendrite spines in the brain, suggesting that it may play a role in synaptic function and determine neural plasticity during development [34]. GSK-3β has been found to be strongly associated with regulation of NMDA-LTD [87, 88]. GSK-3β phosphorylation at Ser-9 results in the inactivation of GSK-3β and the upregulation of β-catenin, which further prevents LTD induction [89, 90]. We demonstrated that GSK-3β is a key component of the low LTD and impaired social ability observed in the VPA-exposed offspring. We also found that the activation of NMDA receptors inhibits LTD and DPT due to an increase in the phosphorylation of GSK-3β and an increase in the expression of β-catenin in the amygdala of VPA-exposed offspring. VPA is known to inhibit the activity of GSK-3β, which in turn activates Wnt/β-catenin signaling and further impairs synaptic plasticity [31, 91]. Studies have reported that GSK-3β inhibition facilitates the induction of LTP [86, 92]. In the present study, we sought to confirm and extend the findings of our previous work showing enhanced LTP during the DPT-initiating phase in the amygdala of VPA-exposed offspring. The inhibition of GSK-3 blocks LTD through NMDA receptor-induced GluR1/2 internalization in the rat hippocampus [93]. In addition, GSK-3β is inhibited by Ser-9 phosphorylation, resulting in decreased protein phosphatase-1 (PP1) activity, which contributes to the generation of LTD [94, 95]. We identified an increase in the phosphorylation of GSK-3β at Ser-9, suggesting that the decreased activity of GSK-3β inhibits the induction of LTD, possibly via increased PP1 activity, in VPA-exposed offspring. Therefore, a GSK-3β-dependent mechanism may be essential for regulating NMDAR-dependent synaptic plasticity in VPA-exposed offspring.

Additionally, PI3K signaling is an upstream regulator of GSK-3β and is involved in mental disorders including ASD and schizophrenia [54, 96]. In *Pten* mutant mice, an animal model of ASD, increased activation of the PI3K/Akt/mTOR/S6K pathway and inactivation of GSK-3β caused impaired social behavior, anxiety-like behavior, abnormal axonal growth, and abnormal synapse numbers in the brain [97]. Whether or not GSK-3β is involved in abnormal growth of axons in VPA-exposed offspring requires further study. Previous studies using PI3K inhibitor have shown that activation of GSK-3β is a key component of neurodegenerative diseases [98]. In addition, modulation of PI3K signaling by a PI3K antagonist could rescue the phenotypes of Fragile X syndrome (FXS) [99, 100]. Our data demonstrated that wortmannin inhibits PI3K signaling, dysregulates the phosphorylation of GSK-3β, and improves sociability in VPA-exposed offspring. The dysregulation of GSK-3 activity plays an important role in sociability deficits and the aberrant synaptic plasticity of the amygdala in VPA-exposed offspring.

In this study, we uncovered the first evidence of correction of ASD-like behavior in VPA-exposed offspring by modulation of GSK-3β, which further regulates NMDARs. We identified an increase in GSK-3β phosphorylation in our model, which downregulates NMDARs in the amygdala and contributes to ASD-like behavior. Thus, modulation of NMDARs by GSK-3β may be a potential strategy for improving cognitive function in the VPA-induced autism-like model. This study was the first to describe the impaired NMDA receptor-related synaptic plasticity of LTD, which differs from that observed in other ASD-related animal models. The study findings suggested the importance of, and key role played by, NMDARs in ASD. Thus, modulation of NMDA receptors by GSK-3β may offer a potential strategy for the treatment of ASD.

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Authors' Contributions HC Lin, HF Wu and PS Chen conceived and designed the experiments. HF Wu, YJ Chen, CW Lee, and IT Chen

performed the experiments. HF Wu analyzed the data. PS Chen contributed reagents/materials/analysis tools. HF Wu and HC Lin wrote the paper.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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