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RESEARCH ARTICLE

Prenatal exposure to valproic acid alters Reelin, NGF expressing neuron architecture and impairs social interaction in their autistic-like phenotype male offspring

Karunanithi Sivasangari[1] · **Koilmani Emmanuvel Rajan**[1]

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Abstract
Maternal exposure to anti-epileptic drug Valproic acid (VPA) during pregnancy increases the risk for the development of autism spectrum disorders (ASD). In this study, we have examined whether prenatal exposure to VPA will alter expression of key genes, synaptic morphology of nerve growth factor (NGF) and Reelin expressing neurons in the cortex of male offspring. To characterize in animal models, rat fetuses were exposed to VPA on 12.5 gestational day. The offspring of the VPA-exposed individuals (42%) resembles ASD-related phenotype (facial malformation, crooked-like tail, flattened paw, toenails and in-turning-ankles). Furthermore, we have observed deficit in social interaction accompanied by deregulation in expression of genes such as Caspase-3, focal adhesion kinase (FAK), Reelin, glial fibrillary acidic protein (GFAP), proliferating cell nuclear antigen (PCNA) and NGF. Subsequently, immunohistochemistry analysis revealed that exposure to VPA alters the cytoarchitecture (area, diameter) and reduced the dendritic arborization of Reelin, NGF expressing neurons in cortex. The compromised neurodevelopment by altered expression of Caspase-3, FAK, Reelin, GFAP, PCNA and NGF may cause defects in neuronal architecture, synaptic formation, synaptic plasticity and neuronal communication which could be linked with observed ASD-like phenotype and deficit social interaction.

Keywords Autism spectrum disorder · Valproic acid · Congenital malformation · Social behavior · Reelin · Neurotrophic factor

Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopment disorder associated with repetitive pattern of behaviour, deficits in reciprocal social interaction and neurocognitive dysfunction (Lord et al. 2018; Manoli and State 2021). Genetic components and environmental factors have been linked with development of ASD (Saxena et al. 2020). Valproic acid (VPA) is commonly prescribed for the treatment of migraine headaches, partial and generalized seizure (Vajda and Eadie 2014). Clinical studies have reported that VPA treatment during pregnancy was linked to increased risk of ASD in the offspring besides marked increase in cognitive deficits and congenital malformations (Roullet et al. 2013). Earlier studies have reported that prenatal exposure to VPA induced autistic-like behaviour (Nicolini and Fahnestock 2018; Miyazaki et al. 2005; Chaliha et al. 2020). In addition, animal model studies have provided insight into the effect of VPA exposure on molecules such as alterations in epidermal growth factor receptor (Jung et al. 2008), serotonin and serotonin receptor (Dufour-Rainfray et al. 2010), dopamine receptors (Schiavi et al. 2019), expression of brain-derived neurotropic factor (BDNF), N-methyl-D-aspartate (NMDA) receptor (Roullet et al. 2010), neuronal migration (Kotagiri et al. 2014), pro- and anti-apoptosis gene (Zhang et al. 2016), activation of inflammatory cytokines (Young et al. 2016), histone acetylation/chromatin remodeling (Ibi et al. 2019) and DNA damage (Aboul-Fotouh 2013). These effects are linked with induction of neurodegeneration/neuronal cell death and activation of apoptosis in different brain region including in prefrontal cortex, hippocampus and cerebellum (Young et al. 2016; Aboul-Fotouh 2013; Kataoka

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✉ Koilmani Emmanuvel Rajan
emmanuvel1972@yahoo.com

[1] Behavioural Neuroscience Laboratory, Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli 620024, Tamil Nadu, India

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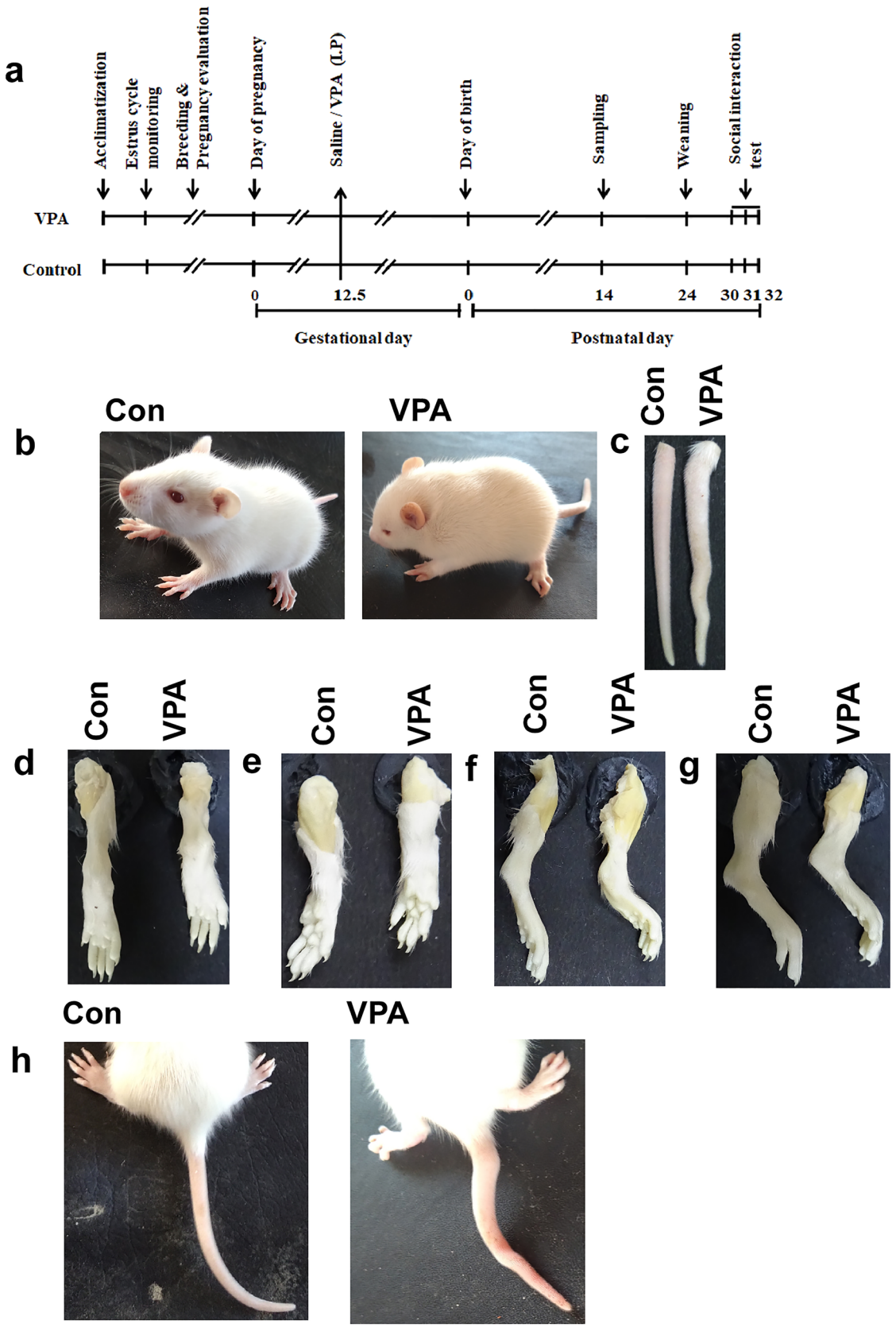
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Estrus cycle monitoring
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Day of pregnancy
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◂**Fig. 1** The experimental schedule and offspring's phenotype comparison of experimental groups. **a** Experimental timeline showing the sequence of event and behavioural testing of the experimental group animals underwent. Representative image showing facial dysmorphism (**b**) flattened forehead, crooked-like tail structure (**c**), flattened paw and toenail of malformed forelimb (left: **c**; right: **e**), alignment and orientation of hind limb are malformed (**h**), and showing in-turning (pronated) ankles (left: **f**; right: **g**) in VPA group compared to control

et al. 2013). Caspases-3 has been recognized as important protease in executioner–phase of apoptosis, balancing act of caspase fine tune the process of cellular differentiation and proliferation (Burguillos et al. 2011), cellular remodeling including dendritic pruning, neurite growth (Westphal et al. 2010), homeostatic synaptic plasticity (Li et al. 2019). Earlier studies have showed that Reelin is a large secreted glycoprotein that suppresses the apoptotic process and plays an important role in neuronal migration, cytoskeleton stability, axonal and dendritic outgrowth via multiple independent and interconnected pathways (Wasser and Herz 2017). Furthermore, the level of Reelin alters the focal adhesion kinase (FAK) and steroid receptor co-activator (Src) signaling through the activation of glial fibrillary acidic protein (GFAP) (Wei et al. 2011). Activated FAK and GFAP further influence the neuronal cell adhesion, migration, proliferation and survival (Rico et al. 2004; Watanabe et al. 2008). Additionally, FAK has several functional roles including cortical basement membrane assembly, remodeling, neuronal migration, axonal/dentritic branching and synapse formation (Wasser and Herz 2017; An et al. 2018). FAK acts as a mediator in induction of nerve growth factor (NGF), subsequently modulates structural properties of maturing neuron, and the architecture of synapses (Monje et al. 2012). In parallel cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) target genes, including proliferating cell nuclear antigen (PCNA) and NGF (Cazzalini et al. 2014) and its expression is positively correlated. VPA has been commonly used to generate ASD model (Nicolini and Fahnestock 2018; Miyazaki et al. 2005; Chaliha et al. 2020). This study was designed to test whether prenatal exposure to VPA has altered the genes involving the regulation of migration, proliferation, differentiation, maintenance, and survival of neurons and synaptic morphology of NGF and Reelin expressing neurons in ASD-like phenotype male offspring.

Materials and methods

Animals

Adult female Wistar rats (*Rattus norvegicus*, 250 ± 15 g) two or three were housed in a rectangular polypropylene cage (43 cm × 27 cm × 15 cm) with paddy husk bedding. Animals were maintained in controlled laboratory condition (24 ± 2 °C, 60% relative humidity, 12 h light/dark cycle with lights on at 07:00 h) with food and water ad libitum. They are allowed to mate during their late pro-estrus stage of the estrus cycle, and when the spermatozoa were observed in the vaginal cytology is considered as gestational day—0 and then housed in individually. All the experimental procedures were approved by the Institutional Animal Ethics Committee of Bharathidasan University, Tiruchirappalli, India (Approval No. BDU/IAEC/RE01/2019 dated 30 November 2019) following guidelines laid down by The Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India, India.

Experimental grouping

Pregnant rats were randomly assigned into two groups (i) Control dams ($n = 6$) received single dose of 0.9% saline [Intraperitoneally, (i.p)]; (ii) Valproic acid (VPA) group dams ($n = 14$) received single dose of sodium valproic acid (i.p) (NaVPA; Sigma, MKBS5723V) on gestational day 12.5. VPA was dissolved in saline for the concentration of 250 mg/ml and the dosing volume was 2.0 ml/kg (500 mg/kg) was adjusted according to the body weight of animal (File and Hyde 1978).

Experimental design

The day of birth is noted as postnatal day (PND)—0, all the pups were periodically examined for their phenotype, general health, body weight, food and water consumption throughout the experimental period. For this study, 34 phenotypically identified pups were selected from 20 litters. On PND-14, brain samples were processed for western blot analysis ($n = 6$ from each group) and immunohistochemistry ($n = 3$ from each group). A subset of individuals ($n = 8$ from each group) were tested for social interaction during their adolescent stage (PND-30-32) (Fig. 1a).

Behavioral analysis

The three-chambered apparatus was constructed based on the specification (Hughes et al. 2020) and social interaction test (SIT) was carried out to analyze sociability. SIT was conducted for all experimental groups during PND-30, 31, 32. SIT was conducted for two sessions: animals were transferred to the experimental room 1 h before SIT to acclimatize. On PND-30 rat pups were individually placed in the centre chamber (CC) for 5 min to habituate by closing the two doorways to Chamber 1 (C1) and Chamber 2 (C2). On PND-31, individuals were trained to interact with stranger- 1 (ST-1) by placing in C1 and empty cylindrical

chrome wire cage (EC) was placed in C2. After 5 min, the two doorways were opened and the subject was allowed to explore the apparatus and interact with ST-1 for 10 min. To avoid the olfactory cues, the apparatus was wiped clean with 75% ethanol immediately after every behavioural test. During training and testing, the subject's interaction with the ST-1 and EC time spent in each chamber was video recorded and analyzed.

Protein isolation

On PND-14, animals were sacrificed whole brain was rapidly removed and placed on an ice-cold petri dish. The cortex region was dissected (File and Hyde 1978) and homogenized in ice-cold homogenizing buffer with protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA). The homogenate was incubated in ice for 30 min, followed by centrifuged at 4 °C (10,000×g) for 30 min. The supernatants were collected in a fresh tube and again centrifuged at 4° C (12,000×g) for 15 min; samples were stored as aliquots at − 80 °C. Protein concentration was determined by Bradford method (Cat#5000006; Bio-Rad laboratories Inc., USA) using a Biophotometer Plus (Eppendorf Inc., Germany).

Western blot

Equal concentration (60 µg) of total protein was resolved on Sodium dodecyl sulphate Polyacrylamide (SDS-PAGE) gel (10%) and transferred to polyvinylidine difluoride membrane (PVDF) (Millipore, IPVH00010, Burlington, MA, USA) using Trans-Blot® Tribo™ blotting system (BioRad Laboratories Inc, USA). After the transfer, membranes were blocked with 1X TBS-T with 5% non-fat milk for 2 h at room temperature. Membrane was incubated with any one of the following primary antibody Caspases-3 (ABP50855,1:1000), NGF (ABP53296, 1:1000), Reelin (AB78540, 1:1000), GFAP (ABM0021, 1:2000), FAK (CST 3285,1:1000), PCNA (Abbkine, A01040, 1:1000), ß-actin (SC47778, 1:1000) for 12 h at 4 °C. Membrane was washed with 1XTBS-T and incubated with alkaline phosphate (ALP) conjugated goat anti-rabbit (MERK, 62110080011730, (1:2000) or goat anti-mouse (MERK, 621100480011730, 1:2000) secondary antibody for 5 h. 5 bromo-4-chloro-3-indolyl phosphate di-sodium salt (BCIP) and nitro blue tetrazolium chloride (NBT) (Merk Life science, ES006) was used to detect the ALP activity to following the manufacturer's instruction. Images were obtained by Molecular Imager and each band trace quantity was measured (Chemi Doc XRS system, Image Lab 2 software (2.0) Bio-Rad laboratory Inc, USA). The trace quantity of each target gene band was normalized with internal control β-actin bands, following that fold changes were calculated by dividing normalized values of VPA group by control group (Plaza-Briceño et al. 2020).

Immunohistochemistry

Whole brain was rapidly dissected out and processed (Mukilan et al. 2018). Embedded brain was cut into sagittal position (5 µm) using microtome (Weswox optic-MT-1090 A). Sections were deparaffinized with Xylene at 60 °C for 10 min and dehydrated with isopropanol for 15 min. Then, endogenous peroxidase activity was blocked by incubating in a solution (10% H_2O_2 and 10% methanol mixed with 1X PBS) then treated with 0.1% trypsin in 0.1% $CaCl_2$ at 37 °C for 10 min. Sections were incubated in Bovine Serum Albumin (BSA, 2.0%) for 1 h at 4 °C to block non-specific staining. Sections were incubated with Reelin or NGF antibody for overnight at 4 °C and then washed with 1XPBS and incubated with anti-rabbit Horseradish peroxidase (HRP) (SC-2030) for overnight at 4 °C. Immunodetection was done by using the chromogen, 3, 3' diaminobenzidine (DAB) peroxidase development kit (Vector Laboratories, Inc. USA). Counter stain was performed by incubating in Hematoxylin for 10 min and mounted with (DPX). Images were obtained using inverted microscope (Nikon Ts2FL). Reelin and NGF expressing neuron's area and diameter was calculated using (DS-Fi3) NIS-Element Software (from 6 neurons/sections; 4 section/sample; $n = 3$ from each group).

Statistics

Data were expressed as mean ± standard error of the mean. KyPlot (ver 5.0) was used to plot the values (SEM) as a graphical representation. The unpaired *t* test was used to compare parametric values between groups (Sigma stat version 11.0). The level of significant difference between groups $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Results

Prenatal VPA exposure induced congenital malformation in offspring

In this study, we have observed fetus desorption (35%) in dams exposed to VPA. 65% of dams gave live birth and out of which 42% offspring were malformed. Range of autistic phenotype was observed, such as a broader upper forehead (typical facial dysmorphism related to ASD) (Fig. 1b) and crooked-like tail deformation was commonly observed in all individuals (Fig. 1c). In forelimb, flattened paw, toenails are malformed relative to control (left limb: Fig. 1d; right limb: Fig. 1e) and also branchydactylyl and clinodactylyl like defect were observed. The hind limb of VPA-exposed

group shown in-turning (pronated)-ankles (left limb: Fig. 1f; right limb: Fig. 1g; Fig. 1h).

Prenatal VPA exposure induced social interaction deficit in adolescent rat

We have examined whether or not prenatal VPA exposure induces effects of ASD-like social behaviour. The sociability of individuals was determined during their adolescent stage. VPA-exposed individuals spent significantly less time in the stranger chamber than the control group ($t = 33.46$; $p < 0.001$), but there was no significant difference in time spent at the empty cage between control and VPA group ($t = 2.48$; $p > 0.05$). Whereas the control group spent significantly more time at the stranger-1 chamber than empty chamber [$t = 16.23$; $p < 0.001$], but there was no difference in VPA group ($t = 3.11$; $p > 0.05$) (Fig. 2a). The sociability index was significantly lower in the VPA group than the control [$t = 19.46$; $p < 0.001$] (Fig. 2b). Observed behavioural data suggest that prenatal exposure to VPA induces impairment in social interactions.

Exposure to VPA altered the expression of genes involving in neuronal development

We have examined the expression status of different molecules involving neuronal maturation, differentiation and survival during development. Caspases-3 is one among the molecules; its expression was induced by VPA exposure (Fig. 3a). Estimated level of Caspase-3 was significantly higher than the control group ($t = 28.36$; $p < 0.001$) (Fig. 3b). Subsequently, Caspase-3 mediated expression of FAK and Reelin was examined. The level of FAK expression was significantly reduced in the VPA group than the control group ($t = 34.92$; $p < 0.001$) (Fig. 3c). Similarly, the level of Reelin was significantly lower in VPA group compared to control ($t = 9.04$; $p < 0.01$) (Fig. 3d). FAK has been known to alter the expression of GFAP, as expected, the level of GFAP was significantly lower in VPA group compared to control ($t = 11.24$ $p < 0.001$) (Fig. 3e). Level of PCNA was significantly higher in VPA-exposed group than the control group ($t = 24.17$; $p < 0.001$) (Fig. 3f). Subsequently, increased level of PCNA can further elevate the level of NGF expression.

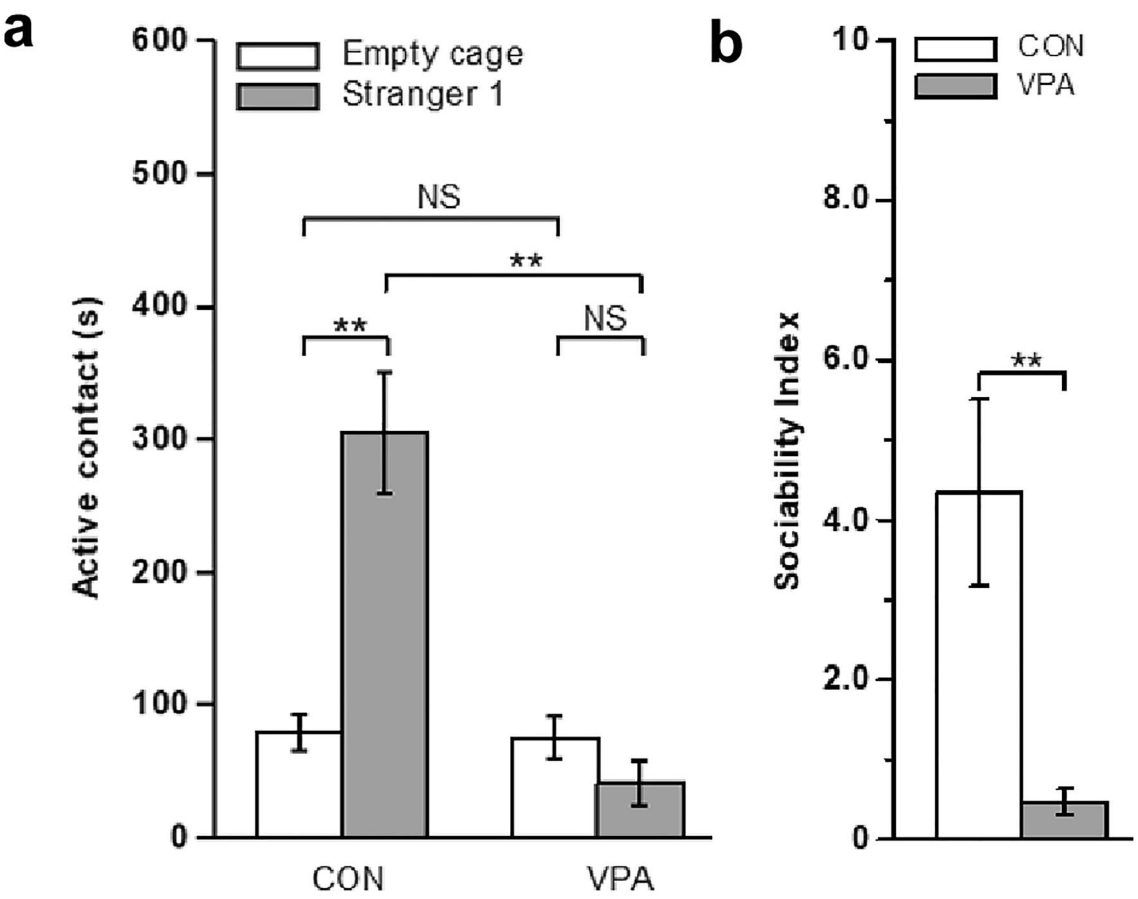

Fig. 2 Prenatal exposure to valproic acid (VPA) impaired social interaction in their offspring during adolescent age. VPA-exposed rat offspring active contact with the Stranger-1 (**a**) and the sociability index (**b**) was significantly low compared to control. Data are expressed in mean ± S.E.M, ($n = 8$). **Indicate statistically significance $P < 0.01$ and NS (not significant) between the experimental groups

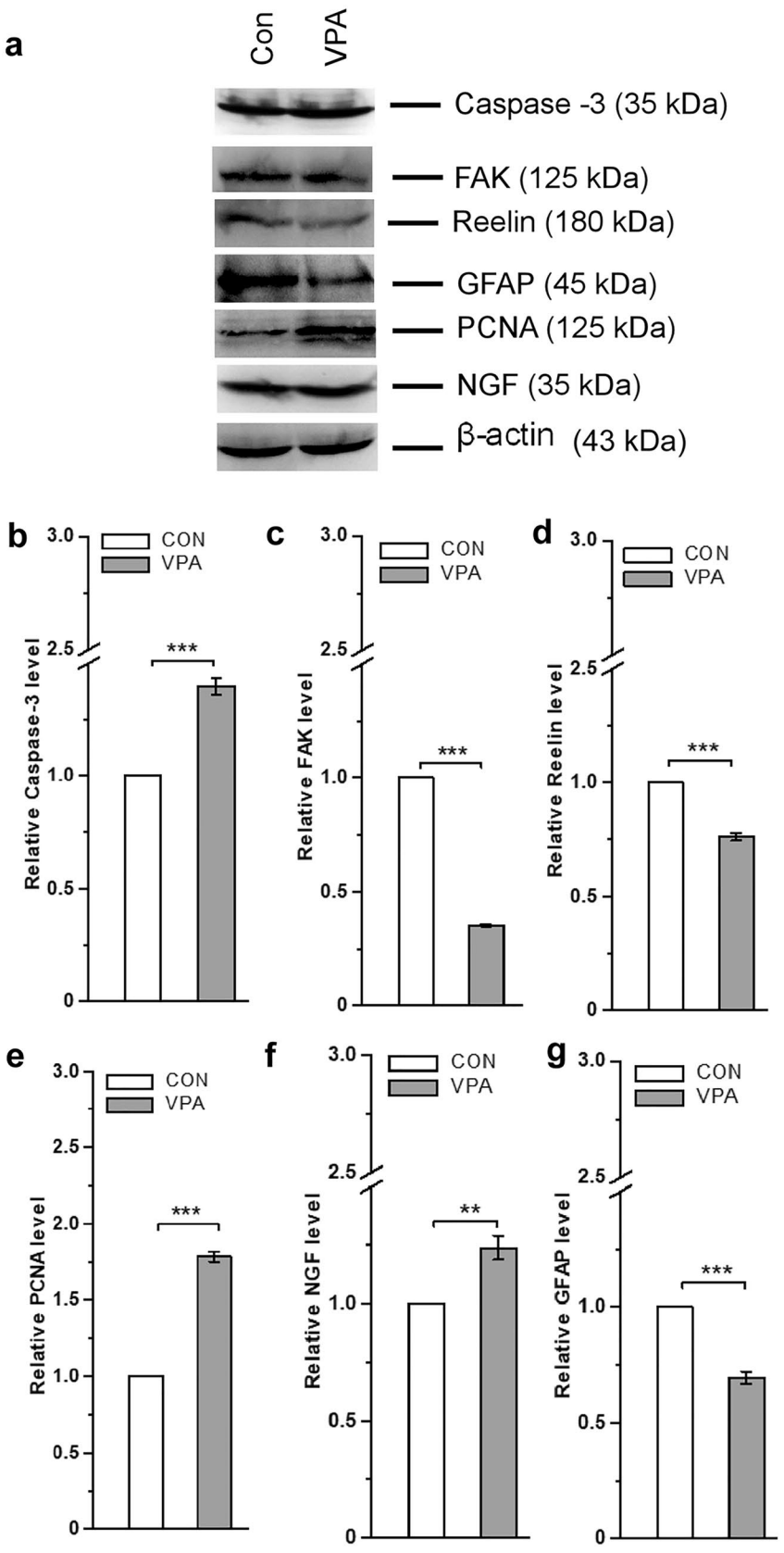

Fig. 3 Prenatal valproic acid (VPA) exposure alters the expression of genes involving in regulation of neuronal development. **a** Representative western blots showing the expression pattern of Caspase-3, Focal adhesion kinase (FAK), glial fibrillary acidic protein (GFAP), proliferating cell nuclear antigen (PCNA) and nerve growth factor (NGF). Level of Caspase-3 (**b**), PCNA (**e**) and NGF (**f**) expression was elevated, but the level of FAK (**c**), Reelin (**d**), GFAP (**g**) was decreased in VPA-exposed offspring compared to control group. Data are expressed as mean ± S.E.M, ($n = 6$). *** Indicates significant difference $P < 0.001$ between groups

The level of NGF was significantly higher in VPA-exposed individuals than the control ($t = 7.32$; $p < 0.01$) (Fig. 3g). The observed signaling molecules suggested that exposure to VPA suppress molecules involving in cellular development.

Prenatal VPA alter the Reelin, NGF expression and neuronal morphology

We have observed the reduction of Reelin expression in VPA-exposed rats' cortex region and neurons morphological structure were also altered (Fig. 4a, b). In control, Reelin expressed neurons exhibit a polarized shape, whereas in VPA-exposed group, Reelin expressing neuronal morphology, axon elongation and dendritic arborization were altered (Fig. 4c). Similarly, we have observed difference in distribution of NGF expressing neurons between control and VPA-exposed rats. Exposure to VPA alters the distribution, density (Fig. 5a, b) and cytoarchitecture/morphology of NGF expressing neurons, including induction of vesicle formation (Fig. 5c). Quantitative analysis showed that Reelin, NGF expressing neurons morphology was altered, the area of Reelin ($t = 15.52$; $p < 0.001$), NGF ($t = 15.54$; $p < 0.001$) (Fig. 6c), and diameter of Reelin ($t = 17.48$; $p < 0.001$) and NGF ($t = 16.74$; $p < 0.001$) (Fig. 6d) expressing neuron area was significantly less in VPA-exposed group than control.

Discussion

Earlier clinical study data showed that pregnant women treated for epilepsy with anti-epileptic drug valporate (500–1000 mg/day) causes congenital malformation (10.0–14.5%) in children (Meador et al. 2008; Tomson et al. 2018; Vossler 2019). In animal models, exposure to different doses of VPA (350–800 mg/kg) during pregnancy leads to autistic-like features (behavioural, anatomical, physiological and molecular) in the offspring (Roullet et al. 2013; Narita et al. 2010; Anshu et al. 2017; Cezar et al. 2018; Elnahas

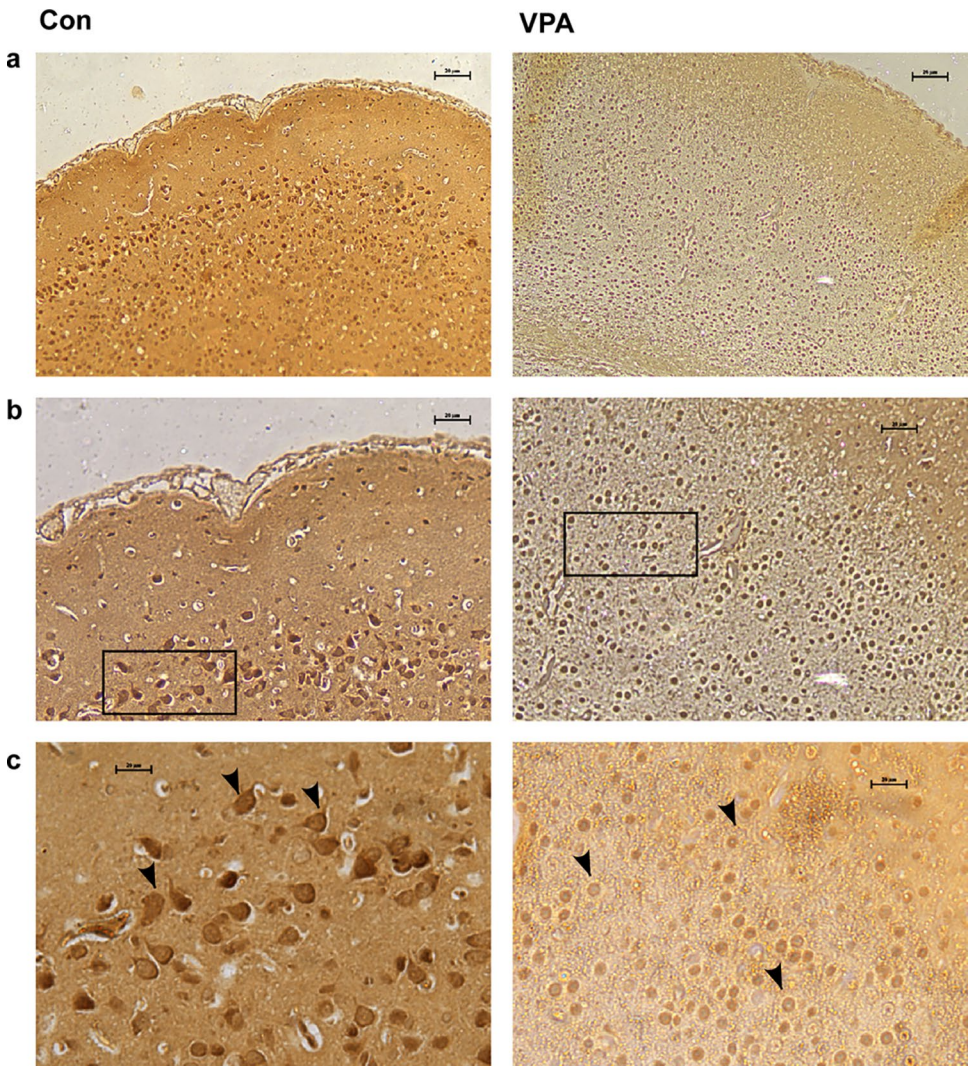

Fig. 4 Neuronal phenotype of reelin expressing neuron in cortex region of control and valproic acid (VPA) exposed rat offspring. Light micrograph image (×4) showing the reeling expressing neurons in cortex (**a**), higher magnification (×20) of selected region (**b**) and higher magnification (×40) of the box in "**b**" (**c**). Arrow indicates VPA exposure induced changes in cytoarchitecture, defects in axon and dendritic arborization of reelin expressing neurons. Scale bars: 20 µm (**a**–**c**)

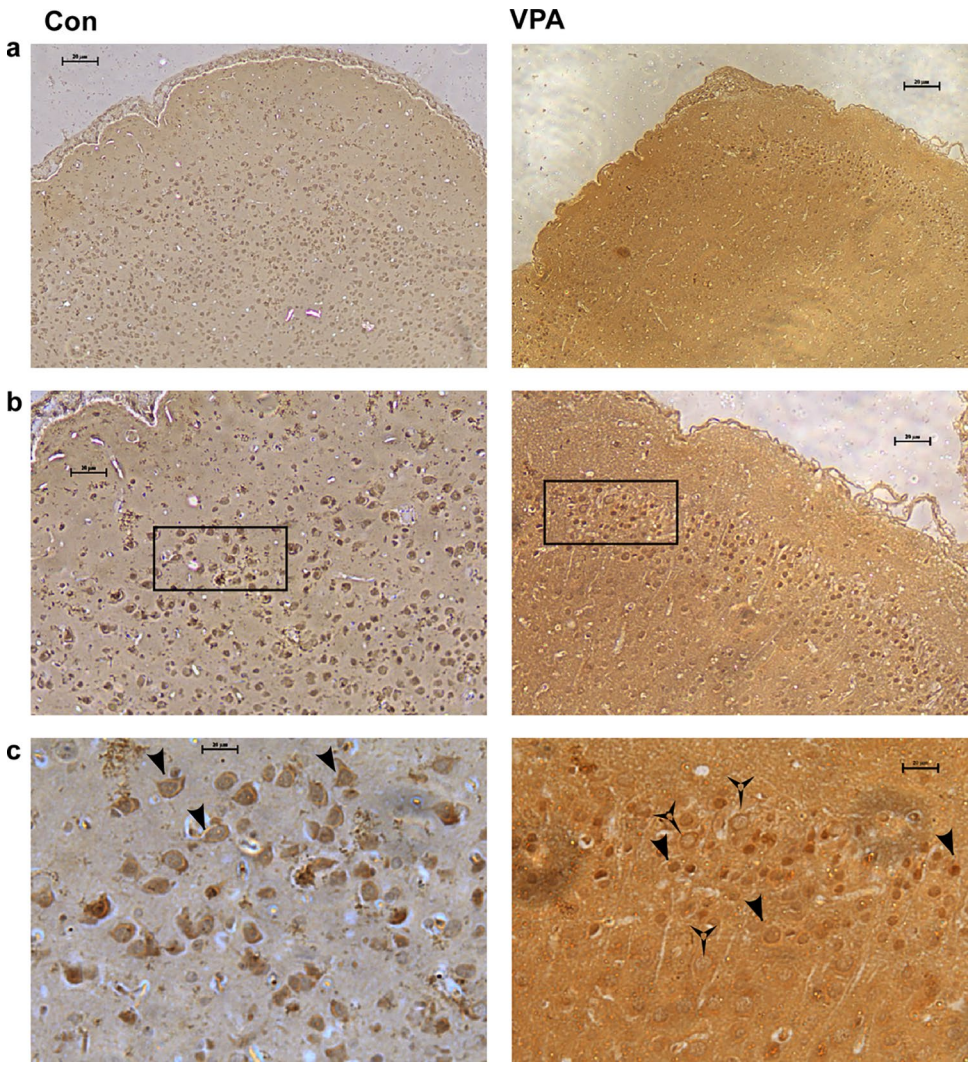

Fig. 5 Neuronal phenotype of nerve growth factor (NGF) expressing neuron in cortex region of control and valproic acid (VPA) exposed rat offspring. Light micrograph image (×4) showing the distribution pattern of NGF expressing neurons in cortex (**a**), higher magnification (×20) of selected region (**b**) and higher magnification (×40) of the box in "**b**" (**c**). Arrow indicates VPA exposure induced changes in cytoarchitecture (long arrow shows presence of large vesicle in neuron) defects in axon and dendritic arborization of NGF expressing neurons. Scale bars: 20 µm (**a**–**c**)

et al. 2022). In this study, the single dose of 500 mg/kg on gestational day 12.5 induces congenital malformation, this time window is comparable to human embryonic day 20–24 and critical phase for neural tube closer (Dufour-Rainfray et al. 2010). In line with earlier reports (Rodriguez-Pinilla et al. 2000), we have observed several malformation including facial dysmorphism, crooked-like tail, flattened paw, clindoactylyl, arachnodactyly and in-turning (pronated) ankles. As described earlier (Aldridge et al. 2011), observed facial dysmorphism in this study possibly by the imbalance in coordination between face and brain development. Exposure to the VPA during gestation has been known to induce the defects in neural tube (Kaufman 2004) and leads to development of crooked-like tail (Binkerd et al. 1998), which has been known to alter the brain development and adhesion of the neural tube. Interestingly, the observed defects such as flattened paw, orientation and extension are possibly associated with the VPA-induced changes in brain development and volume (Zigler et al. 2016). The observed phenotype in this study validates our experimental procedure and indicate that further molecular and behaviour analysis can be performed in our model. Therefore, we have conducted social interaction test VPA-exposed rat exhibits less social interaction with unfamiliar to avoid the unfamiliar stranger. This analysis showed that, restricted exploration to the unfamiliar rat is similar to the earlier clinical report (Oberman et al. 2008) and ASD models (File and Hyde 1978; Narita et al. 2010). Observed behavioural impairment and facial dysmorphism may be correlated with minor physical anomalies (MAPs) reported in clinical study (Tripi et al. 2019).

Furthermore, we have correlated the phenotype and behavioral phenotype with genes involving neural migration, maturation, dendritic arborization and synaptic formation (Gilbert and Man 2017). Caspase has been known to involve in the regulation of developmental process (Burguillos et al. 2011; Westphal et al. 2010; Li et al. 2019). Similar to our observation, prenatal expose to VPA induces the apoptosis

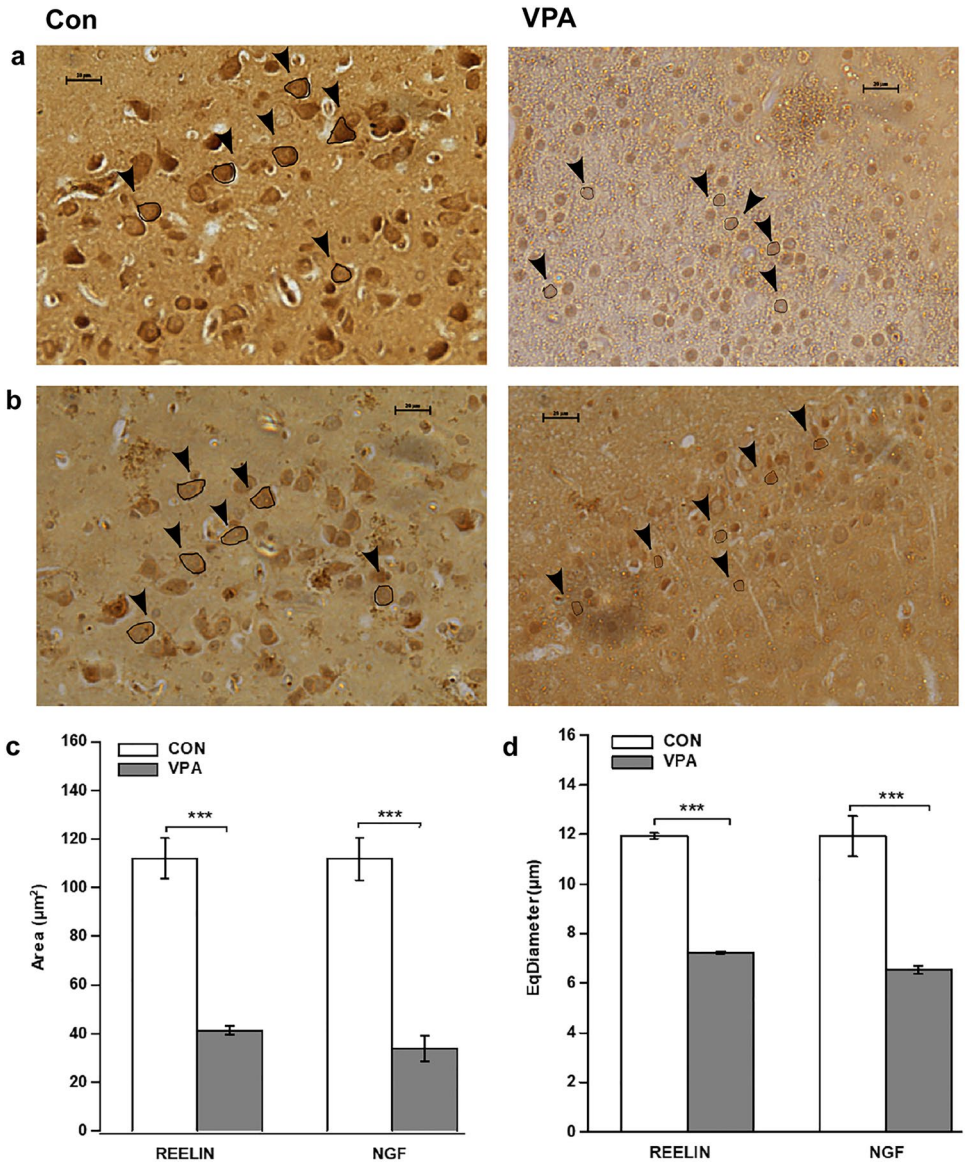

Fig. 6 Representative micrographs of paraffin-embedded coronal sections from control (left) and VPA (right) exposed group's cortex sub-region. Immunolabeled sections showing **a** reelin and **b** NGF expressing neurons. The area (**c**), diameter (**d**) of reelin/NGF expressing neurons in the cortex was significantly less in VPA group compared to control group. Data are expressed as mean ± S.E.M, ($n = 6$). ***Indicates significant difference $P < 0.001$ between groups. Scale bar: 20 µm

by induction and activation of Caspase-3, which mediate defects in neural tube fusion and adhesion (Tung and Winn 2011). Furthermore, the integrated mechanism of Caspase-3 and BCL-2 receptor has been linked to the activation of Reelin. In the signaling pathway, Reelin inhibits the apoptosis process through interacting with downstream molecules (Ohkubo et al. 2007), and play a critical role in neuronal development, migration and lamination (Fatemi et al. 2005; Folsom and Fatemi 2013), and also in social behaviour (Ohkubo et al. 2007). The expression level of Reelin activates/inactivates FAK, FAK is one of the components of cell adhesion and growth factor receptors, which critically regulates neural migration, morphology, synaptic formation and neutrite growth (Folsom and Fatemi 2013). The observed reduction of GFAP has been known to cause imbalance in excitatory-inhibitory circuits during early brain development and neuronal communication (Wei et al. 2011). Exposure to VPA elevated level of the PCNA (Watanabe et al. 2017), which is regulated by cytoskeletal protein and recognized as a marker for cell proliferation. Elevated level of PCNA further influences the expression of NGF. Our western blot analysis showed the level of NGF expression that has increased in VPA group compared to control. Elevated level of NGF has been known to cause of developmental delay in pre and postnatal period of life and considered as a potential marker for autism (Kaufman 2004).

Furthermore, VPA exposure during development has been known to alter programmed neurogenesis/cell death, neuronal proliferation, migration and development (Kataoka et al. 2013). In this study, we have observed that Reelin, NGF expressing neurons area, diameter and dendritic arborization was reduced. Observed changes possibly either direct

effect of VPA on histone acetylation/chromatin remodeling-mediated expression or through the complex network/interaction of genes during development (Roullet et al. 2013; Kotagiri et al. 2014; Ibi et al. 2019; Kataoka et al. 2013; Burguillos et al. 2011; Leemhuis and Bock 2011), in which the molecules tested in this study and their interaction may be a part.

Conclusion

In conclusion, VPA-exposed offspring shows deficit in social interaction and alter the expression of Caspase-3, PCNA, NGF, Reelin, FAK and GFAP genes, and cytoarchitecture of Reelin and NGF expressing neurons in cortex. The genes have analyzed in this study that had known to regulate programmed neurogenesis/cell death, neuronal proliferation, migration, neuronal development, dendritic arborization and synaptic plasticity, however, further studies required to clearly understand the gene targets of VPA in utero and how these changes determine the neurodevelopment.

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Author contributions KS: performed the experiment and has analyzed the data, KER: the idea, designed the experiment and drafted the manuscript. All authors agreed to the final version of the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and mentioned in the manuscript.

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