

Protective Effect of Nimodipine Against Valproic-acid Induced Biochemical and Behavioral Phenotypes of Autism

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Objective: Present study was designed to investigate behavioral and biochemical role of nimodipine in prenatal valproic acid (Pre-VPA) induced autism in rats.

Methods: Valproic acid was utilized to induce autistic phenotypes in Wistar rats. The rats were assessed for social behavior. Hippocampus and prefrontal cortex (PFC) were utilized for various biochemical assessments, whereas cerebellum was used to assess blood brain barrier (BBB) permeability.

Results: Pre-VPA rats showed reduction social interaction. Pre-VPA administration were decreased PFC levels of interleukin-10 (IL-10), and glutathione along with hippocampus cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF). Also, the animals have shown increase in PFC levels of IL-6, tumor necrosis factor- α , thiobarbituric acid reactive substance, Evans blue leakage and water content. Nimodipine countered Pre-VPA administered reduction in social interaction, CREB, BDNF, inflammation, oxidative stress, BBB permeability.

Conclusion: Pre-VPA has induced autistic phenotype, which were attenuated by nimodipine in rats. Nimodipine and other calcium channel blockers should further investigate to check the management of autism.

KEY WORDS: Cyclic AMP response element-binding protein; Nimodipine; IL-6; BDNF; Blood brain barrier; Calcium channel.

INTRODUCTION

A neurodevelopmental disorder, autism spectrum disorder (ASD) is currently being diagnosed with communication problems, repetitive behavior along with social behavior dysfunction. Shockingly, there is no affirmed treatment accessible till presently. This is following an upward example and overall budgetary weight. Males are increasingly inclined to ASD when contrasted with females. More than one percent of the populace overall is experiencing ASD [1]. Several environmental and genetic factors seem to be responsible but the exact cause still unknown. Autism has diverse genetic relations along with different environmental causes including infection, pesti-

cides and drugs [2,3]. It has suggested that maternal glyphosate exposure can mimic the autistic phenotype in animals [4]. Valproic acid is a typically used antiepileptic drug and it is used to treat depressive disorders, migraine, and neuropathic pain. Prenatal valproic acid (Pre-VPA) treatment is one of the best characterized models of autism rats with solid choice, face and predictive validity [5]. The autistic individuals have some genetic defects in calcium channel genes, which regulates the neurodevelopment [6].

It has already documented, multiple comorbid conditions including neuroinflammation and oxidative stress in children with autism [7,8]. In autistic patients, glutathione levels were higher, reduced glutathione levels were lower, and glutathione redox ratios were diminished. Additionally, animal models show low levels of glutathione (GSH) and interleukin-10 (IL-10) and high levels of thiobarbituric acid reactive (TBAR), IL-6 and tumor necrosis factor (TNF)- α [9,10]. Valproic acid is used to induce autism in rats by exposure during prenatal life 12.5th

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day. Valproic acid is widely used in the induction of autism phenotypes because it produces autistic phenotypes like the clinical features of autism. Valproic can produce core symptoms as social behavior impairment along with some other comorbid features in form of oxidative stress and neuro-inflammation [7,11-13].

L-type of calcium channels are commonly spread throughout the brain. L-type of calcium channels control neuronal excitability and combine neuronal activation with gene transcription L-type of calcium channels triggered by De Novo CACNA1D mutations in ASD have been identified [14]. Calcium-channel subunit mutations in CaV β 2 is associated with autism [15]. Nimodipine is an antagonist of calcium dihydropyridine L-type channels with a long record and a high safety score. Nimodipine binds to the pore and transmembrane of voltage receptor alpha-1 subunits and serves as a modulator for the negative allosteric channel role. Nimodipine play significant roles in dendritic and somatic calcium accumulation, gene expression and excitability [16]. Nimodipine is well described for the treatment of subarachnoid hemorrhage documented to encourage improved outcomes and less severe ischemic neurological symptoms. Nimodipine has been found to be neuroprotective in many other disorders in the brain, such as ischemic stroke, traumatic brain injury, migraine [17]. Being an antagonist of calcium channel, Nimodipine is also a strong cerebral vasodilator which act on cell membranes. It is also a molecule that is more lipophilic than nifedipine, which is also a calcium channel antagonist [17]. The neuroprotective effect of nimodipine in inflammation-mediated neurodegenerative disease was attributed to the inhibition of microglial activation, since nimodipine significantly inhibited the production of nitric oxide and further cytokines from lipopolysaccharide-stimulated cells.

There are only two study which stating that nimodipine blocked seizure-induced MeCP2 phosphorylation in developing brain [18,19]. Unfortunately, we don't have any US Food and Drug Administration (FDA) approved therapeutic to treat core symptoms of autism such as social dysfunction. Our study finds out the novel role of nimodipine in valproic acid induced autism aspect of result such as social behavior, oxidative stress, IL-6, IL-10 and TNF- α , brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB) along with blood brain barrier (BBB) permeability. It could enable the sci-

entific community to move forward in autism research.

This research examined the novel role of nimodipine; L-type of calcium channel antagonist in Pre-VPA mediated autism associated phenotypes in rats.

METHODS

Animals

Adult albino Wistar rats were kept in polypropylene cages in Amity University (No. 1327/PO/ReBi/S/10/CPCSEA) at a temperature of $25 \pm 2^\circ\text{C}$ with relative humidity of $50 \pm 5\%$. The animals had free access to water and standard laboratory pellet chow diet (Ashirwad Industries, Punjab, India). Animals were exposed to the natural light and dark cycle with 12 hours of light (starting at 07:00 hours and ending at 19:00 hours) followed by 12 hours of dark (starting at 19:00 hours and ending at 07:00 hours). The Institutional Animal Ethics Committee of Amity University, Uttar Pradesh, India, has approved all experiments (CPCSEA/IAEC/AIP/2019/01/19).

Drugs Chemicals and Reagents

Analytical and laboratory grade chemicals and reagents were used in the present study. Sodium salt of valproic acid was taken from Sun Pharma Pvt. Ltd. (Gurugram, India). Evans blue, ethylene glycol tetra acetic acid (EGTA) was purchased from SISCO Research Laboratory Pvt. Limited, Mumbai, India. Lowry's reagent, N-naphthylethylenediamine and 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich (Bengaluru, India). Hydrogen peroxide and pyridine was obtained from Rankem Laboratories Pvt. Ltd. (Gurugram, India).

Protocol design

In total five groups of animals, with each group containing eight ($n = 8$) animals were used for the protocol of the study. Choice of animals was based on research already published, effectively using albino Wistar rats to model experimental ASD like condition [20]. The timeline, groups, and parameters assessed for present study are seen in Figure 1.

Fertilization was ascertained using a vaginal examination for the presence of sperm cells, this day was considered as gestational day 1. Pregnant dams were housed individually till the day of parturition and weaning of the pups. The pups were weaned and then distributed ran-

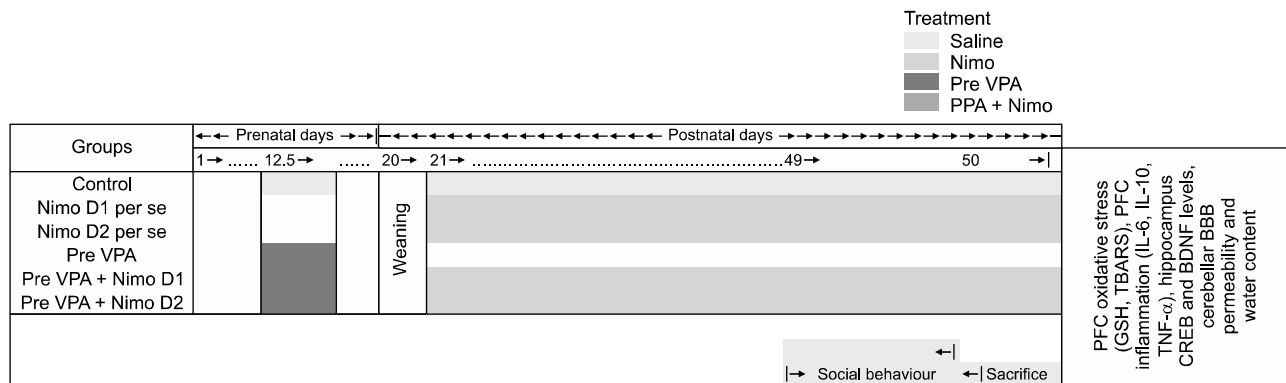


Fig. 1. Schematic representation of experimental protocol.

E, embryonic day; PFC, prefrontal cortex; P, postnatal day; Pre-VPA, prenatal valproic acid; D1, dose 1; D2, dose 2; Nimo, nimodipine; GSH, glutathione; TBARS, thiobarbituric acid reactive substance; IL, interleukin; TNF, tumor necrosis factor; CREB, cAMP response element-binding protein; BDNF, brain-derived neurotrophic factor.

domly into their respective groups on post-natal day 20 [20,21]. Nimodipine is a high lipophilic compound, it crosses the blood-brain barrier and can reach the brain and cerebrospinal fluid. Nimodipine has already tested for various central conditions including subarachnoid hemorrhage, cerebral ischemia-reperfusion injury, multiple sclerosis [22-24]. Valproic acid and nimodipine doses were selected as per our preliminary investigations and previous studies [13,22,25].

Group I-Control group: Pregnant dams were injected with single dose of 0.9% saline (3.3 ml/kg) on gestational 12.5th day. The received male pups were taken as control group.

Group II and III-Nimodipine per se: Rats received Nimodipine D1 (5 mg/kg, i.p.) and Nimodipine D2 (10 mg/kg, i.p.) from post-natal 24th day to till end of the study.

Group III-Prenatal valproic acid (Pre-VPA) group: Pregnant dams were injected with single dose of sodium valproate (500 mg/kg, i.p.) on gestational 12.5th day.

Group IV and V-Pre-VPA + Nimodipine D1 and D2 group: Males exposed to Pre-VPA were randomly selected and received Nimodipine D1 (5 mg/kg, i.p.) and D2 (10 mg/kg, i.p.) from postnatal day 21st to 50th (Fig. 1).

Social Interaction Assessment

The three-chambered task was modified from equipment in behavioral neuroscience to measure rodent social behavior in a simple way. Normal-developing children play with friends, but children with autism tend to play alone, with a toy, in a corner, rather than with other

children. On postnatal day 49th–50th day, social interaction was evaluated by Three-Chamber Sociability and Social Novelty Test apparatus (30 cm long and 70 cm wide which divided into three identical chambers) for the rat. We have used this method in our previous research adopted from Kim *et al.* [26], 2011 with slightly modification. Concisely, the test arena measured 76 × 30 × 35 cm and was divided into three equal chambers with an access point between each chamber. Animals were given free access to all the chambers and Each trial started with the animal placed in the central chamber. To encourage exploration of the side chambers, all animals were accustomed to the apparatus for 5 minutes prior to starting of the test trial. After ending of the habituation period, the rats were tested in the sociability phase lasting for 10 minutes. Animals to be placed under a wire cage were spent 30 minutes at the wire cage, prior to initiation of the sociability phase. In the sociability phase, a stranger animal was placed under the wired cage in either (left or right) side chamber, while in the other chamber an empty cage would be placed. In order to avoid side preferences, the placement of wired cages was randomized and the chamber with the stranger animal and the empty cage were called as stranger chamber and empty chamber, respectively. Upon conclusion of the sociability phase the social preference phase was initiated 2 hours after the last animal trial. In the social preference phase each animal was allotted 10 minutes to look at the complete arena. During this phase, the animal earlier considered as stranger, was now rendered familiar and introducing another novel animal into the paradigm, along with the familiar

animal. The two chambers now would be called as familiar and novel chamber. The time spent by test animals in both the side chambers was measured. Sociability index and social preference index were calculated according to the following formula [13,27,28].

$$SI = \frac{\text{Total time in stranger chamber}}{\text{Total time in empty chamber}}$$

$$SPI = \frac{\text{Total Time in novel chamber}}{\text{Total time in familiar Chamber}}$$

Preparation for Biochemical Assessments

On postnatal day 50th, a high dose of thiopental sodium (90 mg/kg, i.p.) was used to isolate the prefrontal cortex (PFC) tissue by anaesthetization. The homogenate supernatant was collected for various biochemical estimates as defined in the procedures below. Absorption was taken with a spectrophotometer (PerkinElmer, Waltham, MA, USA). Prefrontal cortex, hippocampus and cerebellum are the main brain regions implicated in ASD pathogenesis and neurochemical disorders [28,29]. So, for this study, we selected prefrontal cortex and cerebellum brain regions.

Oxidative Stress Assessments

GSH and lipid peroxidation (TBAR) are known as oxidative stress markers, so in this study, we evaluated these markers in prefrontal cortex [13].

Glutathione (GSH)

GSH is an important tripeptide thiol antioxidant and levels its intracellular concentration is an evaluation parameter of oxidative stress. The quantitative estimation of reduced form of GSH was assessed using spectrophotometer (PerkinElmer) at 412 nm [20]. In a test tube, PFC supernatant and trichloroacetic acid (10 percent w/v) were combined at 1:1 ratio and the test tubes were centrifuged (1,000 g at 4°C for 10 minutes). Supernatant (0.5 ml) and 0.3 M disodium hydrogen phosphate (2 ml) were mixed. The spectrophotometric absorbance was observed at 412 nm following the addition of .25 ml of freshly prepared DTNB (DTNB dissolved in 1 percent w/v sodium citrate). For standard curve plotting, 10–100 µM of the reduced form of glutathione was employed. The effects of reduced glutathione consistent with mg of protein were expressed as micromoles.

Lipid peroxidation

Measurement of lipid peroxidation can be accomplished using thiobarbituric acid reactive substance (TBARS) in biological fluids. Lipid peroxidation is the reaction between free radicals attacking double bonds in carbon and carbon in lipids, involving the removal of hydrogen from carbon and the placement of oxygen. Lipid peroxidation was assessed as close to our previous investigations [20]. The PFC supernatant sample (0.2 ml) was taken in test tube. In addition, 8.1 percent sodium dodecyl sulphate (0.2 ml), 30 percent acetic acid pH 3.5 (1.5 ml) and 0.8 percent thiobarbituric acid (1.5 ml) were added, followed by distilled water up to 4 ml. The test tubes were put in the incubator at 95°C for 1 hour. The incubated mixture was then cooled by adding distilled water (1 ml) followed by adding n-butanol-pyridine mixture (15:1 v/v) to 5 ml. The centrifuge tubes were handled at 4,000 g for 10 minutes. The spectrophotometric absorbance was taken on developing pink color. 1, 1, 3, 3-tetra methoxy propane (1–10 nM) was used for plotting standard calibration curve. Nano moles per mg of protein were used for expression of result.

Enzyme-linked Immunosorbent Assay (ELISA) Method for IL-6, IL-10 and TNF-α, BDNF and CREB

ELISA is a technique used to measure the levels of antigens in biological samples. These were assessed by enzyme-linked immune-sorbent assay- sandwich method in PFC and hippocampus area of brain. The IL-6, IL-10 and TNF-α were estimated by using RayBio® Rat ELISA kits were used for the assessments. The procedure mentioned in the product information leaflet was completely followed and samples were run in triplicate for optical density measurement, average optical density was considered for final calculation of concentration. Briefly, IL-6, IL-10, TNF-α, BDNF, and CREB quantitation was made in PFC supernatant at 450 nm on 96-well percolated to specific antibodies plates. Supernatant and standard were taken out in to well. The immobilized antibody was bound with IL-6, IL-10, TNF-α, BDNF, and CREB present in sample, respectively. Washing was given to the tubes, and the biotinylated anti-Rat IL-6, IL-10, TNF-α, BDNF, and CREB antibodies were applied to the plates. Unbound biotinylated antibody was removed by washing the well. The horseradish peroxidase (HRP) conjugated streptavidin was added to the wells. After washing a TMB substratum

solution was applied to the wells. A blue color formation showed the IL-6, IL-10, TNF, BDNF, and CREB have linked in sample. The stop solution was pipette to the wells which changed the color to yellow. Result were expressed in pg/ml and ng/ml [30].

BBB Permeability and Water Content

BBB permeability and water content performed by the concentration of Evans blue dye in the cerebellum as previously reported methods [27,31]. Concisely, 4% of dye/ Evans blue (intra-peritoneal; 4 ml/kg) was injected to subject and was permitted to circulate for 2 hours. Before collecting the samples, the subjects (anesthetized) were trans-cordially perfused with saline to flush out the remaining dye in the vessels. Weighed cerebellum for quantitative spectroscopic calculations. In short, the cerebellum precisely measured. The cerebellum processed at pH 7.4 dye extraction in 3.5 ml of 0.1 mol/L phosphate buffer saline by a homogenizer. Then, for protein precipitation, 6 ml of 60 percent trichloroacetic acid was added. This processed cerebellum was vortexed after 30 minutes of cooling for 2 minutes. The cerebellum was processed for 40 minutes through a centrifuge at 4,000 rpm to obtain pellets. The dye levels were measured at 610 nm via a spectrophotometer. The findings were expressed using a standardized curve of Evans blue as μg of Evans blue/g of cerebellum tissue. For 48 hours wet-weighted cerebellum tissue was put in the oven at 105°C. Weighted out after 48 hours of dry cerebellum. Wet-dry method was used to calculate the volume of water in the cerebellum. The percentage water content was calculated as: $(\text{wet weight} - \text{dry weight}) / \text{wet weight} \times 100\%$ and results shown in percentage of water content [31].

Statistical Analysis

Sigma Stat (v3.5) was used for statistical analysis. The findings were demonstrated in the form of mean \pm standard deviation. Statistics for all variables were analyzed using two-way ANOVA followed by a post-test by Bonferroni. This at $p < 0.05$ was considered statistically important.

RESULTS

Social Behavior

Sociability, sociability index, social preference, and social preference index

Pre-VPA has decreased time went through in stranger chamber and expanded time went through in empty chamber, with contrast to control rats, which shows lower sociability in Pre-VPA treated rats. Administration of Nimodipine has significantly mitigated Pre-VPA associated reduction of time went through in stranger chamber followed by increased time went through in the empty chamber. Pre-VPA treated rats, has shown lower sociability index when compared to control rats, which was markedly attenuated by Nimodipine (Fig. 2).

Pre-VPA administration has decrease time went through in novel chamber and expand time went through in the familiar chamber, with contrast to control rats, which shows lower social preference in Pre-VPA treated rats. Administration of Nimodipine has significantly mitigated Pre-VPA associated reduction of time went through in novel chamber and increased time went through in the familiar chamber (Fig. 2). Pre-VPA treated rats have shown lower the social preference index, contrast to control rats, which was significantly mitigated by Nimodipine.

PFC Neuro-inflammation, Oxidative Stress and Hippocampus CREB and BDNF

Pre-VPA has increased PFC Ca⁺ levels, PFC inflammation (IL-6 levels, TNF- α levels and decreased IL-10 levels), oxidative stress (decrease GSH levels and increase TBARS levels) along with decreased hippocampus CREB and BDNF. Nimodipine treatment has significantly mitigated Pre-VPA induced increased PFC inflammation (IL-6 levels, TNF- α levels and decreased IL-10 levels), oxidative stress (decrease GSH levels and increase TBARS levels) along with decreased hippocampus CREB and BDNF (Table 1, Figs. 2 and 3).

Blood Brain Barrier Permeability and Water Content in the Cerebellum

A considerably higher Evans blue concentration and water content were present in the cerebellum of Pre-VPA exposed rats in contrast to control rats. Treatment with Nimodipine expressively mitigates Pre-VPA induced in-

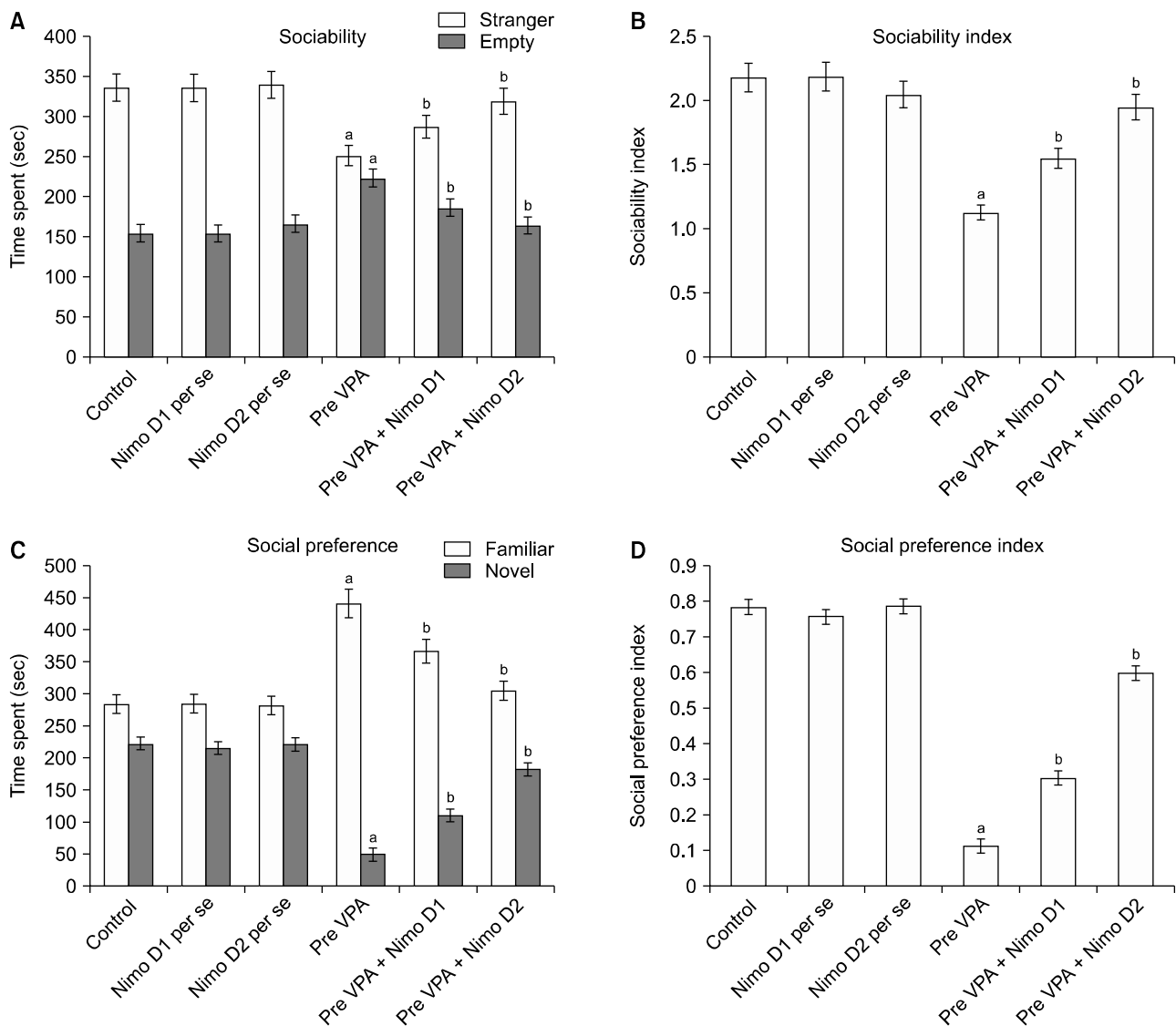


Fig. 2. (A) Sociability, (B) sociability index, (C) social preference, and (D) social preference index on Three-Chamber Sociability and Social Novelty Test apparatus. (A) Stranger: ^a $F_{1,42} = 106.277$; ^b $F_{2,42} = 31.543$; Empty: ^a $F_{1,42} = 228.979$; ^b $F_{2,42} = 68.632$. (B) Sociability index: ^a $F_{1,42} = 502.727$; ^b $F_{2,42} = 131.026$. (C) Familiar: ^a $F_{1,42} = 502.086$; ^b $F_{2,42} = 60.493$; Novel: ^a $F_{1,42} = 1,477.037$; ^b $F_{2,42} = 421.444$. (D) Social preference index: ^a $F_{1,42} = 2,585.419$; ^b $F_{2,42} = 444.524$.

Pre-VPA, prenatal valproic acid; D1, dose 1; D2; dose 2; Nimo, nimodipine.

Results are mean \pm standard deviation, two-way ANOVA followed by Bonferroni's post-test. ^a $p < 0.05$ vs. control rats, ^b $p < 0.05$ vs. Pre-VPA treated rats.

creased concentration of Evans blue and water content in the cerebellum (Fig. 4), which suggest amelioration in Pre-VPA induced BBB dysfunction.

DISCUSSION

Several neural circuits and brain regions trigger social behaviour in humans and animals, and it is crucial to their survival and neurodevelopment. Social behaviour is root-

ed in understanding and interpreting social cues; individuals must recognize, process, and interpret social cues, as well as responding appropriately to them. Even though the neurophysiological substrates of abnormal social behavior are unknown, humans and animals suggest that the excitatory/inhibitory imbalance of neuronal activity might contribute to the physiological explanation of abnormal social behavior [32]. There is a strong correlation among social behavior impairment, oxidative stress,

Table 1. Effect of various agents on prefrontal cortex oxidative stress and inflammation

Groups	Oxidative stress		Inflammation		
	GSH	TBARS	IL-10	IL-6	TNF- α
Control	19.21 \pm 0.961	4.88 \pm 0.342	98.00 \pm 4.900	93.70 \pm 4.685	43.40 \pm 2.170
Nimo D1 per se	18.22 \pm 0.922	4.6 \pm 0.337	81.50 \pm 5.025	82.71 \pm 4.524	44.11 \pm 2.130
Nimo D2 per se	18.4 \pm 0.920	4.81 \pm 0.321	100.50 \pm 5.101	90.47 \pm 4.432	42.60 \pm 2.121
Pre-VPA	13.31 \pm 0.656 ^a	12.75 \pm 0.893 ^a	47.50 \pm 2.375 ^a	194.76 \pm 9.738 ^a	122.99 \pm 6.150 ^a
Pre-VPA + Nimo D1	14.85 \pm 0.743 ^b	10.23 \pm 0.716 ^b	77.00 \pm 3.850 ^b	169.07 \pm 8.454 ^b	80.30 \pm 4.015 ^b
Pre-VPA + Nimo D2	17.94 \pm 0.897 ^b	7.33 \pm 0.513 ^b	88.88 \pm 4.444 ^b	125.63 \pm 6.282 ^b	70.56 \pm 3.528 ^b

Results are mean \pm standard deviation.

GSH: ^aF_{1,18} = 163.265 ^bF_{2,18} = 685.007. TBARS: ^aF_{1,18} = 1,257.099 ^bF_{2,18} = 175.039. IL-6 levels: ^aF_{1,18} = 973.189 ^bF_{2,18} = 84.89. IL-10 levels: ^aF_{1,18} = 219.928; ^bF_{2,18} = 138.116. TNF- α levels: ^aF_{1,18} = 1,105.281 ^bF_{2,18} = 364.755.

Pre-VPA, prenatal valproic acid; Nimo, nimodipine; D1, dose 1; D2, dose 2; GSH, glutathione; TBARS, thiobarbituric acid reactive substance; IL, interleukin; TNF, tumor necrosis factor.

Two-way ANOVA followed by Bonferroni's post-test. ^ap < 0.05 vs. control rats, ^bp < 0.05 vs. Pre-VPA treated rats.

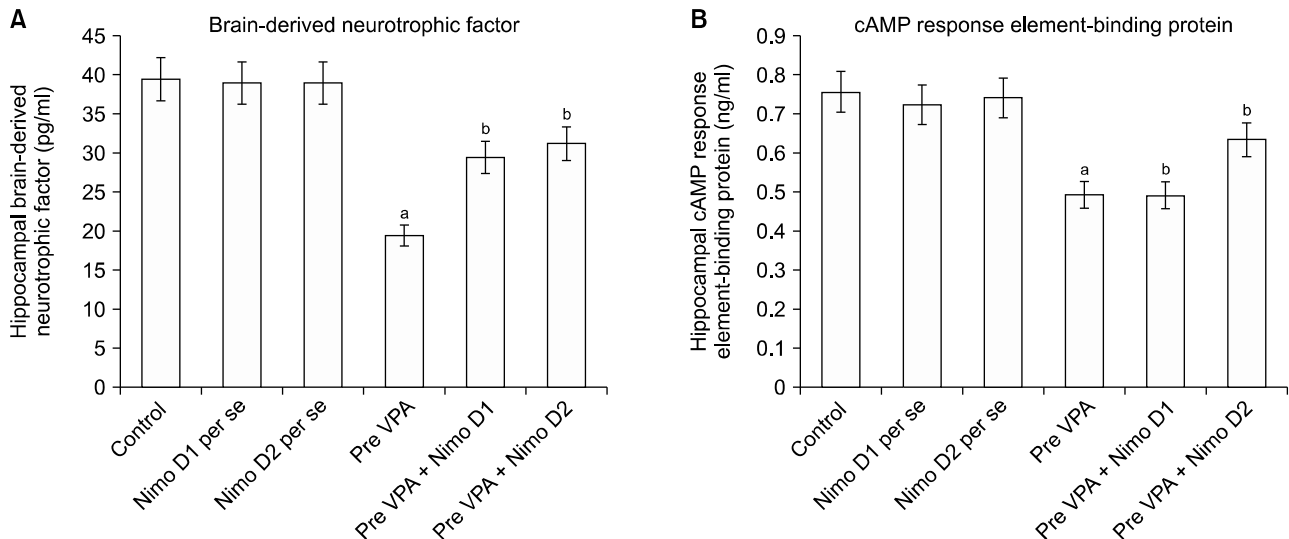


Fig. 3. Effect of various agents on hippocampal (A) brain-derived neurotrophic factor (BDNF) and (B) cAMP response element-binding protein (CREB). (A) ^aF_{1,18} = 476.678; ^bF_{2,18} = 78.896. (B) ^aF_{1,18} = 199.643; ^bF_{2,18} = 29.509.

Pre-VPA, prenatal valproic acid; D1, dose 1; D2, dose 2; Nimo, nimodipine.

Results are mean \pm standard deviation; two-way ANOVA followed by Bonferroni's post-test. ^ap < 0.05 vs. control rats, ^bp < 0.05 vs. Pre-VPA treated rats.

neuroinflammation and neuroproteins changes such as BDNF and CREB [7,33]. Hence, we have assessed nimodipine effects on behavior and biochemical parameters.

Calcium stimulated second messenger pathways may activate a CREB transcription factor that could reverse this pathway downstream to BDNF but calcium excito-toxicity. CREB/BDNF signalling plays a significant role in synaptic plasticity, morphology and behaviour. Phospho-CREB regulates the transcription of some neuro-protective proteins such as BDNF. BDNF regulates memory consolidation and synaptic strengthening in brain. Reduction in BDNF level causes abnormal, learning and memory

formation, and plasticity [34]. Research has documented that diminishing the levels of p-CREB can result in the diminishing of spatial memory [35]. CREB activity plays role in double cortin, dendritic development, expression of neuronal microtubule associated protein and BDNF that could result in autistic characteristic features such as altered synaptic plasticity and neuronal survival [36]. In-utero exposure to valproic acid is produced nerve cell death, synaptic function disturbance along with neuronal tube defects in offspring. Pre-VPA has known to produce the change in the CREB and BDNF level in autism. We found significant decrease levels of CREB and BDNF pro-

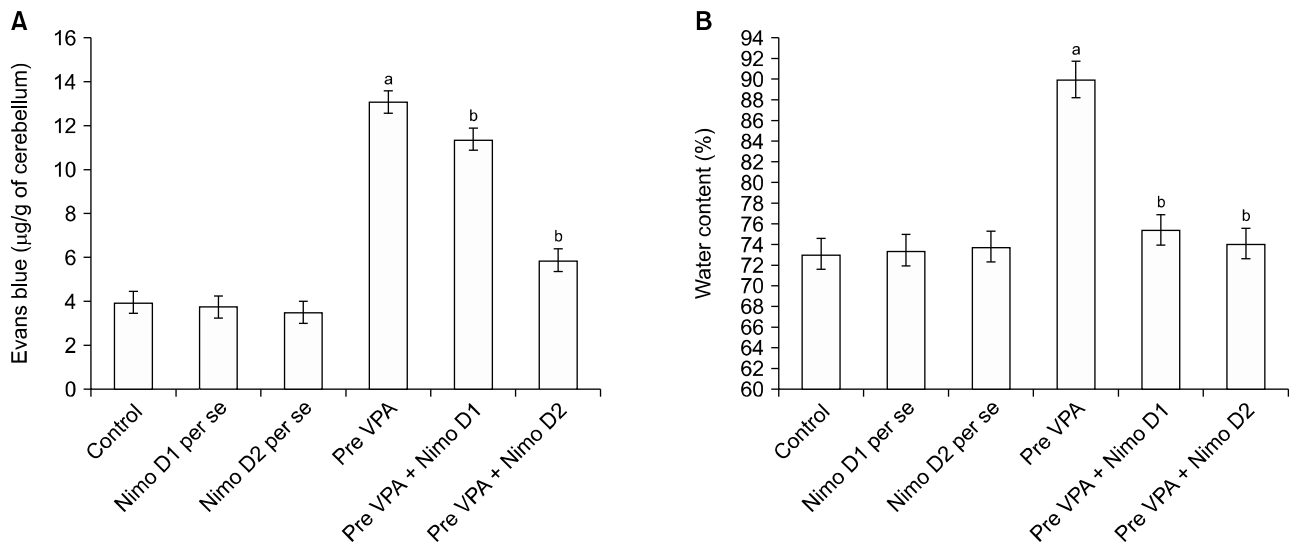


Fig. 4. Effect of various agents on blood brain barrier permeability measured by (A) Evans blue concentration and (B) water content in cerebellum. (A) $^aF_{1,18} = 971.701$; $^bF_{2,18} = 163.993$. (B) $^aF_{1,18} = 181.92$; $^bF_{2,18} = 94.856$. Cerebellum of Pre-VPA treated rat have shown higher staining compare with control rat, while Nimodipine treatment have shown lower staining compare with Pre-VPA rat. Pre-VPA, prenatal valproic acid; D1, dose 1; D2, dose 2; Nimo, nimodipine. Results are mean \pm standard deviation, two-way ANOVA followed by Bonferroni's post-test. $^a p < 0.05$ vs. control rats, $^b p < 0.05$ vs. Pre-VPA treated rats.

teins in the hippocampus of the Pre-VPA-exposed rats in parallel to previous finding [37]. Nimodipine activate TrkB phosphorylation associated increased in CREB in hippocampus and induce neuroplasticity and neuroprotective signalling events [38].

An inflammation of the brain, even at low levels, can adversely affect synaptic function and result in behavioral abnormalities. Pre-VPA causes neuro-inflammation via accelerate inflammatory cytokines like IL-6, TNF- α along with decreased IL-10 [39]. Pre-VPA causes neuro-inflammation followed by neuronal disorganization and reactive gliosis in frontal cortex that may be capable for social behavior impairment and cognitive impairment [40]. Studies are evident for existence of the relation between increased inflammations linked social behavior impairment, repetitive behavior in autism. Increased neuro-inflammatory markers like TNF- α and IL-6 may modulate social and repetitive behavior via deviations in the brain microvascular system [41], and equilibrium of excitatory and inhibitory state responsible for glutamate signaling [40,42,43]. The exposure to valproic acid during pregnancy resulted in the development of long-term microglial changes in brain structures. Neuroinflammation makes for pro-inflammatory cytokines that can change the neuronal homeostasis of Ca^{2+} by L-type calcium channel. Previous research has shown that nimodipine

counter the effect of IL-6, IL-10, TNF- α [44]. Nimodipine may be involved through inhibition of proinflammatory cytokines [45] and reduced calcium influx [46]. A reduction in inflammation is known to help to keep the BBB more robust. By preserving calcium homeostasis, nimodipine reduces the permeability of the blood brain barrier in rats [47,48]. This regulation of nimodipine in the neuro-inflammatory signals would be responsible for correcting behaviors for propionic acid induced ASD in rats [49]. It could be proposed that the correction of proinflammatory and anti-inflammatory cytokines in the current research may be one of the pathways for corrective behavior.

Oxidative stress is one of the sponsors for autistic characteristic in clinical as well pre-clinical findings [50-52]. Previous findings also indicating, Pre-VPA leads to increase in oxidative markers in rats [20]. Impaired glutathione associated antioxidant pathways such as methionine cycle, redox system, and translocation reported in autistic candidates [53]. Increased level of oxidative stress can leads to activation of the Wnt Signaling Pathway, which has important role in autism pathogenesis [54-56]. Valproic acid, as well as other induction models, have been used to describe oxidative stress and inflammation [7,30,57,58]. The cerebellar damage along with irregular glutathione redox grade is associated with communication problems along with social deficits in rodents and au-

tistic subjects [59,60]. Oxidative homeostasis dysregulation via catenin path activation was found responsible for the impairment of social, repetitive behavior and cognition in rats [61]. There are multiple antioxidant has shown neuroprotective effects in Pre-VPA exposed animals In present study, Nimodipine was found to reduce the TBAR levels followed by increase in GSH in Pre-VPA exposed rats. Several previous finding also suggest for antioxidant activity of nimodipine by working on antioxidant and related pathways such as activation of CREB and AKT signaling pathways [62,63]. The above line of discussion that may conclude, nimodipine possibly modulates the endogenous antioxidant pathways and results in decrease oxidative stress in Pre-VPA exposed rats.

BBB dysfunction has been found to be involved in autism which supports the gut barrier hypothesis of autism. Postmortem of the cerebellum of ASD was found to altered gene expression responsible for BBB integrity [64]. Interestingly, in our previous research, Pre-VPA exposed rats was found with increased BBB permeability measured by Evans blue dye staining [20]. Altered cytokines levels, endogenous redox mechanism, mitochondrial function, elevated neuro dermatitis levels may contributes to BBB dysfunction [65,66]. Increased in oxidative stress may be responsible for increase BBB permeability [67]. BBB permeability is mainly ruled by the tight junctions between adjacent cells and together low and high levels of Ca⁺ have been shown to have an adverse effect on these endothelial junctions. Collective intracellular Ca⁺ levels has been shown to affect in tight junction structure through apoptosis and protein kinase C-alpha [68]. Nimodipine modulates the water homeostasis and blood-brain barrier permeability possibly via maintain Ca⁺ homeostasis within the brain [69,70].

These experiments were limited by the fact that they were conducted only on male young-adult offspring. Males were believed to suffer from ASD more often than females for a long period of time. Recent works have raised more doubts about sex differences in autism, though, and strengthened discussions in this area. In spite of this, studies on the VPA model have shown both neurochemical and behavioral abnormalities in male offspring, while only marginal differences were seen in female offspring [71]. Females are presumably underreported as having autism-related disorders due to different modes of masking social deficiency. As a result, we only used male

rat in this study, which focused mainly on the behavioral and biochemical changes within the brain. Though autism's complex pathophysiology remains a mystery, significant research has been made in deciphering it and developing effective therapies. Genetic modification and environmental exposure have made it easier to develop these models like Pre-VPA but have not masked the complexity of their mechanisms. Animal models cannot fully approximate the symptoms of autism, which is a particular disorder that affects humans [72]. Nimodipine can be tested in some other induction model of autism for supporting and pushing this research towards translation study. Still, there is a huge amount of molecular level research required to elucidate protective effect of nimodipine in autism.

On the basic of the above line of discussion, L-type calcium channel blocker; Nimodipine could ameliorate Pre-VPA induced remarkable decrease social behavior followed by NADH dehydrogenase activity, SDH activity, cytochrome oxidase activity, GSH, CREB and BDNF. Furthermore, nimodipine was found to counter Pre-VPA associated increase in anxiety, PFC IL-6, IL-10, and TNF- α and TBARS. Pre-VPA has been increase in BBB permeability and water content, which was ameliorated by nimodipine treatment in rats. Further, future study is required to explore the role of nimodipine in autism.

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■ Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

■ Author Contributions

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