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Correlation studies on pathological changes in brain with neurotransmitters and behavioural changes in Balb/c mice

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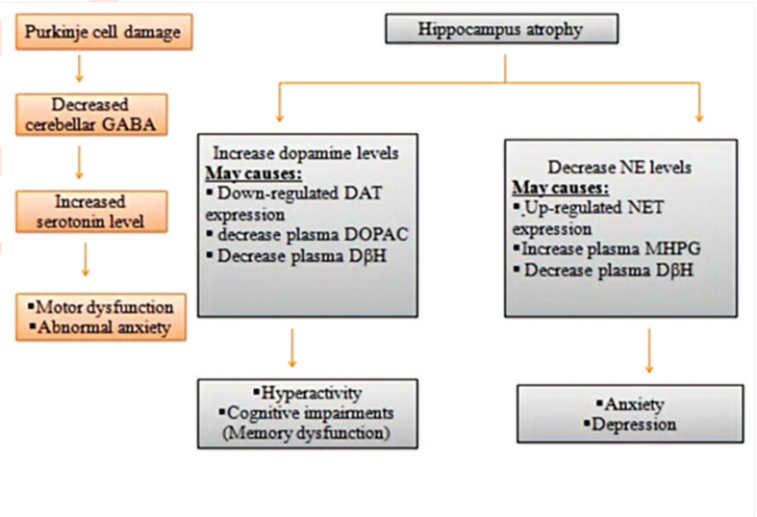
Abstract

Aim: To determine the plasma neurotransmitters simultaneously and to find any correlation with pathological changes in the hippocampus and Purkinje cells and their relation with behavioral changes in Balb/c mice.

Methodology: In the present study, both sexes of Balb /C mice were divided into two groups (4 males and 4 females; n = 8): Both the groups were given a single dose of either saline or sodium valproate (400mg kg^{-1}) respectively through subcutaneous injection on PND 14. Behavioural tests were conducted on mice pups on various postnatal days till 40th day. On PND 41, blood samples were collected from all the animals for quantification of the neurotransmitters (serotonin, dopamine, and noradrenalin) in plasma, animals were sacrificed by cervical dislocation and whole brain was isolated for histological examination of the Purkinje cells and hippocampus.



Results: Sodium valproate exposed animals showed loss of motor skill development (delayed negative geotaxic response), increased locomotor activity, increased anxiety, and retardation in water maze performance, and lower social interaction. Histopathological evolutions of cerebellum purkinje cells and hippocampus showed 40-50% atrophic cells in sodium valproate animals compared to control animals.

Interpretation: The results of the present study indicate that Sodium valproate changes specific brain cell population in Balb/C mice, which might be the reason for the altered neurotransmitter levels, leading to behavioural changes in these animals.

Key words: Altered behaviour, ASD animal model, Plasma neurotransmitters, PND 14 BALB/c, Sodium valproate

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Introduction

Autism spectrum disorders (ASD) are heritable neurodevelopmental disorders that occur in 1 to 2% children, generally below the age of three, with differing symptoms and severity (Balaji and Sinha, 2018). ASD is characterized by impaired social interactions, impaired verbal and non-verbal communication, and the appearance of unusual stereotypic, and sometimes self-injurious, behaviours (George *et al.,* 2006).The causes of autism are unclear, but various factors, such as genetic aberrations, environmental insults, and social factors, contribute to the development of autism (Pragnya *et al.,* 2014). The etiology of autism increases oxidative stress, hyperserotonaemia, and loss of Purkinje cell integrity in the cerebellum (Balaji and Sinha, 2018). Neurotransmitters are endogenous chemicals, also called as chemical transmitters or chemical messengers binding neurons that play a key role in normal brain development, memory, motor activity, and behaviour regulation development (Cetin *et al.,* 2015). Several studies have been conducted in humans on this subject reporting neurotransmitters, corresponding mostly to the pathogenesis of ASD and are serotonergic, GABAergic and glutamatergic systems.

Detection of low GABA levels in platelets of ASD children and post-mortem studies have demonstrated significant decrease in GABAA and GABAB receptor subunit in different brain regions (Cetin *et al.,* 2015). Decreased levels or signaling of GABA have shown hyperexcitability state leading to cognitive dysfunction. Glutamate receptors have been extremely expressed in the hippocampus and cerebellum in ASD patients (Cetin *et al.,* 2015). Yang *et al.* (2014) hypothetically demonstrated that serotonin levels increased in blood and decreased in brain of ASD patients. Decreased levels of Dopamine β Hydroxylase (DBH) activity in serum with increased levels of norepinephrine vice versa, indicates relationship of dopamine and norepinephrine with ASD (Cetin *et al.,* 2015). Other supportive studies have shown increased catecholamine levels of cerebrospinal fluid, blood and urine in ASD patients (Martineau *et al.,* 1994; Gillberg and Svennerholm, 1987). Moreover, increased levels of urinary homovalinic acid in ASD patients who had shown degradation amount of dopamine (Cetin *et al.,* 2015). Robinson *et al.* (2001) demonstrated that mothers of children suffering from ASD have low serum DBH levels and interpreted as causing potential risk factor for ASD by creating a non-ideal intrauterine environment (leading to reduced norepinephrine and increased dopamine levels).

Based on the past reports, increased levels of whole blood serotonin have often been found in autistic patients, suggesting an association of serotonin in the pathophysiology of autism (Anderson *et al.,* 1990). Sodium valproate is an anti-epileptic drug known to cause fetal-valproate syndrome (Pragnya *et al.,* 2014). Sodium valproate exposure has been reported to cause autism-like neurobehavioral defects in pre- and postnatal rodents, analogous to the motor and cognitive deficits observed in autism patients (George *et al.,* 2006; Yochum *et al.,* 2008). Postnatal day (PND) 14 is considered as a sensitive period in animal life, during which neuronal migration, differentiation, myelination, and gliogenesis occur in the cerebellum, striatum, and in the hippocampus (Rice and Barone, 2000). Treatment with sodium valproate on PND14 in rodents causes intrusions and neurodevelopment regressions leading to behavioural retardations (George *et al.,* 2006; Yochum *et al.,* 2008; David *et al.,* 2011). Besides, the administration of sodium valproate on PND 14 in rodents has shown to cause apoptosis of such cells in the cerebellum and hippocampus (Yochum *et al.,* 2008). Therefore, in this study, an attempt was made to determine the plasma neurotransmitters simultaneously and to find any correlation with pathological changes in the hippocampus and Purkinje cells and their relation with behavioural changes in the animal.

Materials and Methods

Experimental animals: Female lactating BALB/c mice with pups were obtained from the National Centre for Laboratory Animal Sciences (NCLAS) and this study was reviewed and approved by the Institutional Animal Ethics Committee (P13F/IAEC/NIN/5/2018/SNS/BALB/c-10+10 pups), National Institute of Nutrition (NIN), Tarnaka, Hyderabad, India. These mice were housed in a room with an environmentally controlled temperature and humidity and a 12hr light/dark cycle, and they had free access to food and water.

Experimental design: On 13th PND, animals were divided into two groups, *viz.,* the negative control group (4 males and 4 females; n = 8) and the positive control group (4 males and 4 females; n = 8). Sodium valproate was dissolved in saline and prepared at a concentration of 80 mg ml^{-1}. With a single dose, the negative control animals were injected with saline only and injected the positive control animals with sodium valproate (400mg kg^{-1}) through a subcutaneous route on PND 14 (Pragnya *et al.,* 2014). The volume and dose were administered depending upon the body weight of animals. The mice pups underwent behaviour testing to evaluate loss of motor skills, locomotor activity, anxiety, learning and memory tests, social behaviour by negative geotaxis, actophotometer, elevated plus maze, morris water maze tests and social interaction tests on various postnatal days up to 40th day, respectively. All the behavioural studies were conducted during daytime between 09:00 and 15:00 hrs.

Behavioural studies

Negative geotaxis: Between 14th PND and 19th PND, the negative geotropism was tested by placing the mice downwards on a wire grid at an inclination of 45^{o} in an environment having well-controlled temperature. The latency to change its direction by turning 180^{o} and the capacity to tread up along the inclination were recorded at a maximum time period of 30 sec (George *et al.,* 2006).

Actophotometer for locomotor activity: This experiment was conducted between PNDs 34 and 37. Each mouse's locomotor activity was recorded individually using an actophotometer

(Columbus instruments, OptoVarimex). The actophotometer was equipped with infrared red emitters located on the X-and Y-axis with an equivalent number of receivers on the opposite walls. The photo beam break was regarded as a measure of locomotive activity. The locomotive activity was assessed by counting the number of beam breaks over a time interval of 10 min (Pragnya *et al.,* 2014).

Elevated plus maze: The elevated plus maze instrument composed of wood and used for evaluating anti-anxiety in mice. It consisted of two open arms (25 cm×5 cm) and two closed arms of same dimensions with opaque walls of 15cm height. The whole instrument was at an elevated position, *i.e.,* 55 cm from ground level. At first instance, each mouse was allowed a period of 10 min to adjust to the environmental conditions. On 40th postnatal day, each mouse was placed on the central square of maze, 5 cm × 5 cm, facing one of the closed arms, and each was monitored by recording its entries into the open arms and the time spent in the open arms, each of them was utilized for data analysis, as described previously (David *et al.,* 2011).

Morris water maze for learning and memory: The water maze equipment consisted of a large circular pool filled with water (69 cm in diameter and 29 cm in height). A platform was submerged 1 cm below the surface of water (7 cm in diameter). The tub was painted white on the inner side, filled with three quarters full of water maintained at 23–26°C, and finished opaque with non-toxic white latex paint. The mice were trained for four days of spatial training, each of which consisted of four tests (30sec interval). Each trial began with mice facing the wall in one of four possible positions. In each trial, we measured latency to locate the platform. If a mouse failed to find the platform within 90 sec, it was guided towards it. Each mouse stayed for 15 sec on the platform before being removed (David *et al.,* 2011).

Social behaviour: On PND 40 social behaviour was measured with a cage-mate. Subjects were placed in a new cage (42 x 42 x 21 cm). The test cage was illuminated by a red light bulb of 40W, mounted 60 cm above them. The night before the experiment, mice were separated and housed individually, to enhance later social interactions. Mice were matched for their sexual orientation and weight. Pairs of either treated or control mice were placed inside the apparatus for 15 min. Behaviours such as anogenital sniffing, social grooming, crawling under / over were observed during the testing of mouse movement and it was videotaped and recorded (Sandhya *et al.,* 2012).

Neurotransmitters estimations: Sample collection: On PND 41, blood samples were collected from the retro-orbital plexus of each mouse into a tube containing sodium EDTA solution. The plasma was separatd by centrifugation at 4000xg for 10 min at 4°C and stored at -80°C for ELISA analysis.

Estimation of neurotransmitters (serotonin, dopamine, and norepinephrine) in plasma: The plasma concentrations of neurotransmitters (serotonin, dopamine, and norepinephrine) were measured with commercially available ELISA kits. We procured mice plasma serotonin (Catalogue No: E-E- 0033 Lot No: JURBQR2YV4), dopamine (Catalogue No: E-E-0046 Lot No: 4MLCYU4JPG), and norepinephrine (Catalogue No: E-EL-0047 Lot No: HTXKX1VMCB) from Elabscience, USA. These neurotransmitters were analyzed according to instructions of the manufacturers. The assay principle is a competitive ELISA with colorimetric detection performed using an ELISA multimode microplate reader (Synergy H1 hybrid reader, Biotek) at 450 nm.

Histopathology: After blood collection on PND 41, all animals were sacrificed by cervical dislocation and their brains were isolated immediately and were placed in 10% neutral formalin solution before processing them and embedding them in paraffin. Sagittal sections of brain tissues (5 µm thick) were stained with haematoxylin and eosin (H&E) and analyzed under a light microscope for changes in Purkinje cells and hippocampus abnormalities.

Statistical analysis: All data were analyzed using SPSS Statistics version 19, and then presented as mean ± SD.The repeated measures ANOVA test was used for all behavioural analysis. The elevated plus maze test, social behaviour and neurotransmitter concentrations in the plasma were analyzed by ANOVA at a significance level of $p<0.05$. Spearman's test was used to determine correlation analysis between Purkinje cell and hippocampus atrophy, neurotransmitter levels and behavioural analysis.

Results and Discussion

In the present study, on using the sodium valproate induced mice model of Autism, it was found that Purkinje cells atrophy and other hippocampal aberrations that occured during autism were correlated with the plasma neurotransmitter levels. Further, it was also found that the plasma neurotransmitter levels were correlated with the behavioural abnormalities. In this study Balb/c mice were used because they are less reactive to social contact on sodium valproate-induced Autism than other inbred mouse strains like C57/129 mice (Pragnya *et al.,* 2014). In accordance with previous research reports in this study, sodium

Table 1: Effect of VPA on anxiety using elevated plus maze apparatus

Group	Time spent in open arms (Sec)	Number of entries into open arms
Control	102.75±11.32	10.00±1.06
Sodium Valproate	77.00±12.64*	6.87±1.64*

Data expressed as mean ± SD, n = 8 per group (4 males and 4 females). Significance at *P < 0.05 vs. control

Table 2: Effect of VPA on neurotransmitters concentrations in plasma

Group	Serotonin (ng ml^{-1})	Dopamine (pg ml^{-1})	Norepinephrine (ng ml^{-1})
Control	79.87±14.14	195.03±10.91	4.35±0.58
VPA	129.88±13.64*	264.54±17.65*	2.40±0.49*

Serotonin, dopamine, and norepinephrine levels in plasma. Values are presented as mean ± SD, n = 8 per group (4 males and 4 females). Significance at *P < 0.05 vs. control

Table 3: Spearman's Rho correlation between purkinje cells atrophy and serotonin, neuro and social behaviour activity

Parameter	Group	N	r	P	
Serotonin (ng ml^{-1})	Control	8	0.581	0.131	P^{a} n.s
	VPA	8	0.946**	0.001	P^{a} S
Negativegeotaxis	Control	8	0.425	0.294	P^{a} n.s
(PND 19)	VPA	8	0.125	0.768	P^{a} n.s
Anogenital sniffing	Control	8	-0.247	0.555	N^{b} n.s
	VPA	8	-0.834**	0.01	N^{b} S
Crawl under/over	Control	8	-0.249	0.552	N^{b} n.s
	VPA	8	-0.781*	0.022	N^{b} S
Social grooming	Control	8	-0.577	0.134	N^{b} n.s
	VPA	8	-0.977**	0.02	N^{b} S

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level; [a]Positive Correlation; [b]Negative Correlation

Table 4: Spearman's Rho correlation between hippocampus atrophy and dopamine, hyperactive and memory impairment

Parameter	Group	N	r	P	
Dopamine (pg ml^{-1})	Control	8	0.082	0.846	P^{a} n.s
	VPA	8	0.964**	0.001	P^{a} S
Hyperactivity	Control	8	-0.412	0.310	N^{b} n.s
(Actophotometer) (PND 37)	VPA	8	0.667	0.071	P^{a} n.s
Memory impairment	Control	8	0.334	0.419	P^{a} n.s
(Water maze test) (PND 40)	VPA	8	-0.151	0.721	N^{b} n.s

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level; [a]Positive Correlation; [b]Negative Correlation

Table 5: Spearman's Rho correlation between hippocampus atrophy and norepinephrine and anxiety activity

Parameter	Group	N	r	P	
Norepinephrine	Control	8	-0.082	0.846	N^{b} n.s
(ng ml^{-1})	VPA	8	0.185	0.660	P^{a} n.s
Time spent in-	Control	8	-0.082	0.846	N^{b} n.s
Open arms (sec)	VPA	8	0.581	0.131	P^{a} n.s
Number of entries-	Control	8	0.087	0.838	P^{a} n.s
In open arms	VPA	8	0.283	0497	P^{a} n.s

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level; [a]Positive Correlation; [b]Negative Correlation

valproate exposed animals showed loss of motor skill development (delayed negative geotaxic response) (Fig.1a), increased locomotor activity (Fig. 1b), increased anxiety (Table. 1), and retardation in water maze performance (Fig. 1c), and decreased social interaction (Fig.1d) (Pragnya *et al.,* 2014). In accordance with previous reports, cerebellar and hippocampus damage may cause motor dysfunction, anxiety, learning and social deficits (Mirza and Sharma, 2019; Edalatmanesh *et al.,* 2013; Elnahas *et al.,* 2021). As

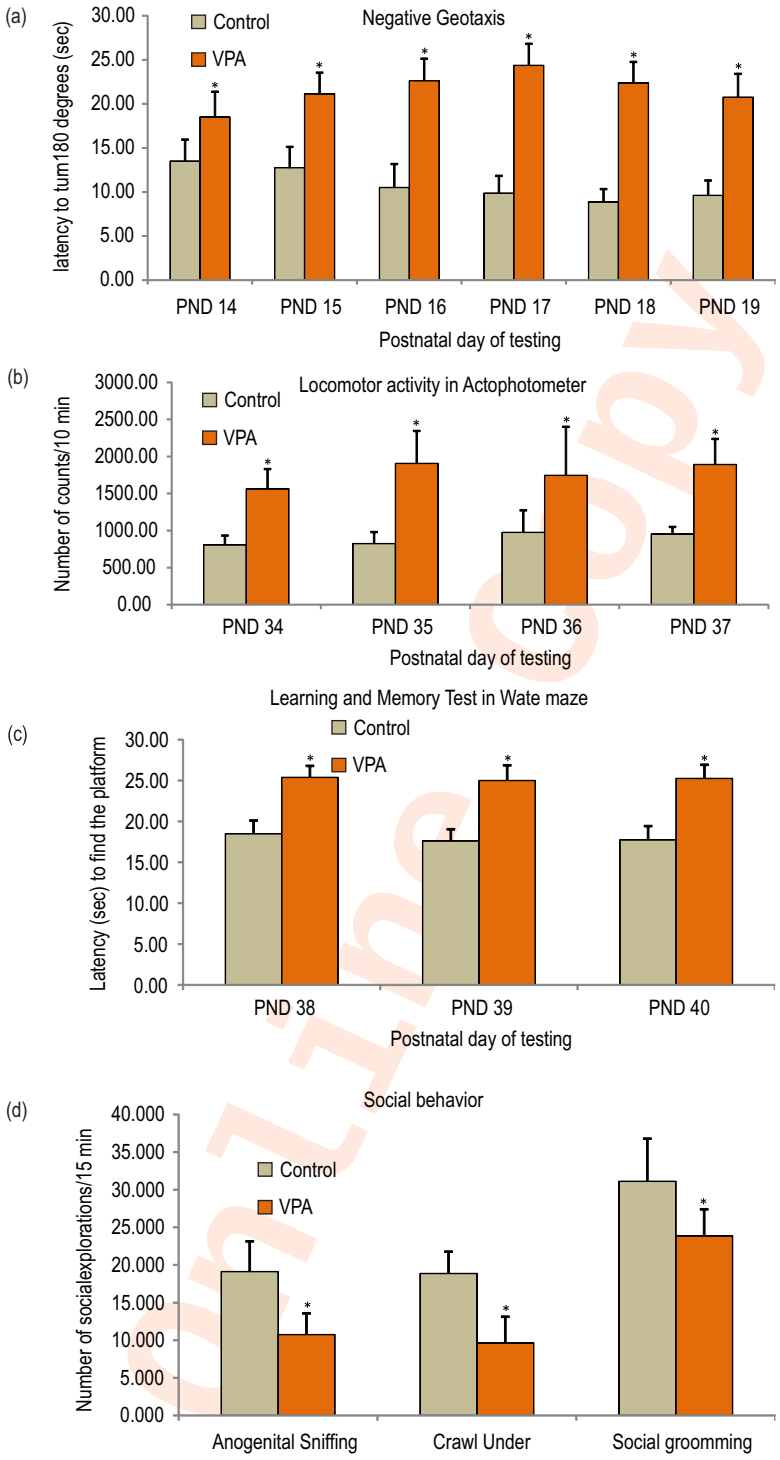

Fig. 1: (a) Capability of BALB/c mice to exhibit the negative geotaxis response with administration of sodium valproate (VPA, 400 mg kg^{-1} s.c.) on postnatal day 14. Values are presented as mean ± SD, n = 8 per group, **P* < 0.05 vs. control. (b) Locomotion due to hyperactive nature following administration of sodium valproate (VPA, 400 mg kg^{-1} s.c.) on postnatal day 14 and tested on postnatal day 34–37. Data expressed as mean ± SD, n = 8 per group, **P* < 0.05 vs. control. (c) Escape latency on a hidden platform of the water maze in animals treated with sodium valproate (VPA, 400 mg kg^{-1} s.c.) on postnatal day 14 and tested on postnatal day 38–40. Data expressed as mean ± SD, n = 8 per group, **P* < 0.05 vs. control. (d) Exposed-matched pairs were watched for social practices in an open field condition over a15 minutes following administration of sodium valproate (VPA, 400 mg kg^{-1} s.c.) on postnatal day 14 and tested on postnatal day 40. Data expressed as mean ± SD, n = 8 per group, **P* < 0.05 vs. control.

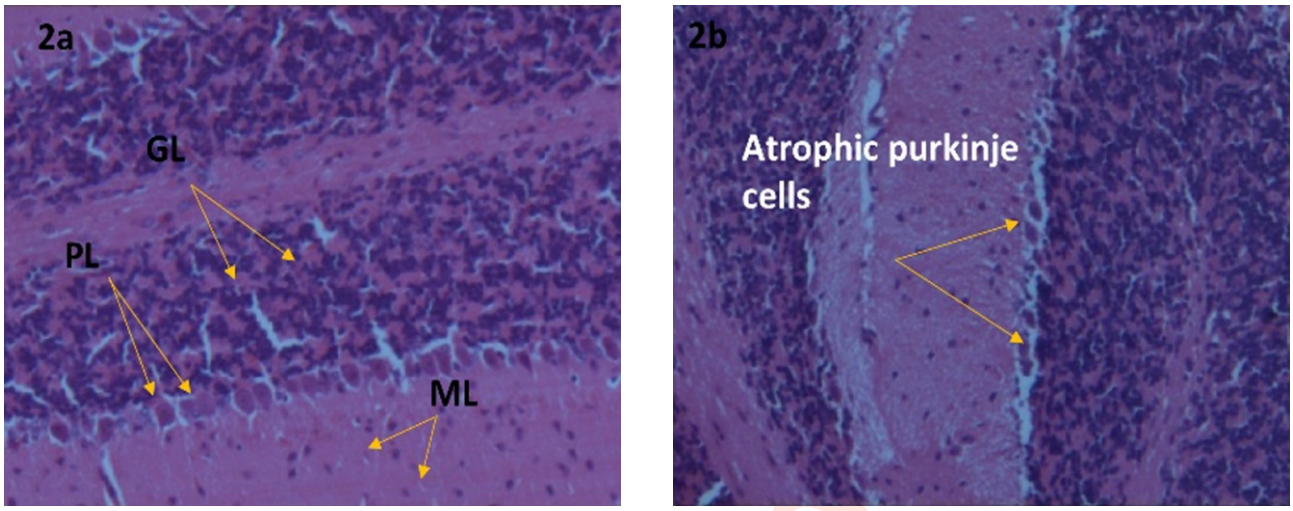

Fig. 2: A photomicrographs of 5 µm thick sagittal sections of cerebellum taken PND41 following either saline administered (H&E10× magnification) (2a) and sodium valproate injection (VPA, 400 mg kg^{-1}, s.c.H&E10× magnification) (2b) showing distribution of Purkinje cells. PL indicates the Purkinje cell layer of the cerebellum, ML depicts the molecular layer of cerebellum and P refers to Purkinje cells.

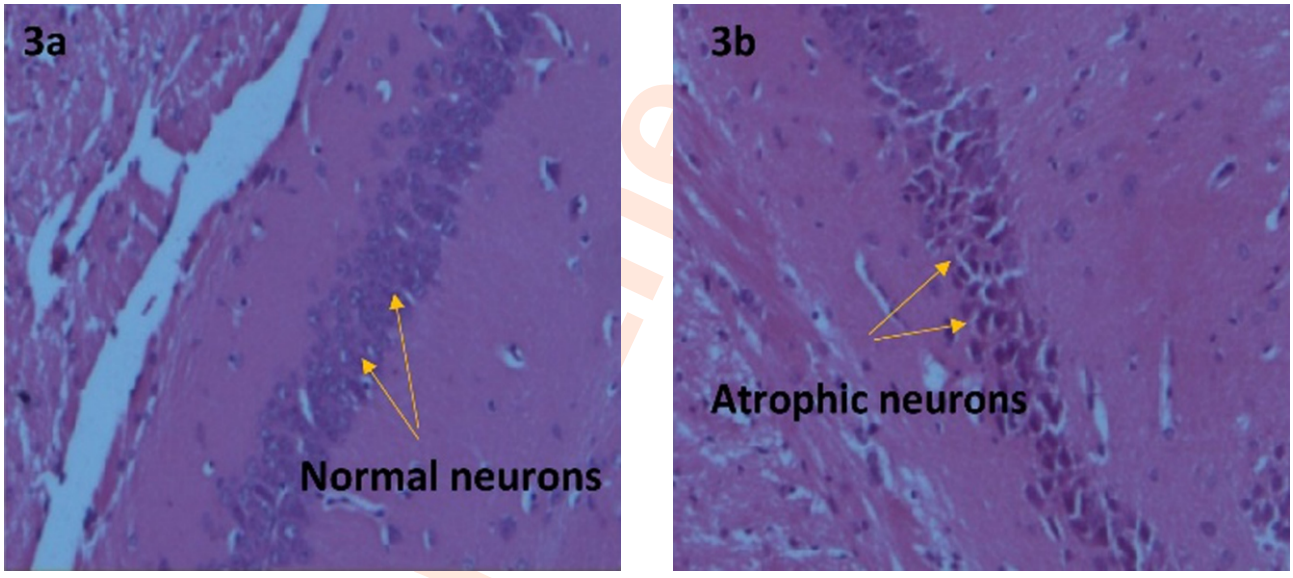

Fig. 3: A photomicrographs of 5 µm thick sagittal sections of hippocampus taken PND41 following either saline administered (H&E10× magnification) (3a) and sodium valproate injection (VPA, 400 mg kg^{-1}, s.c.H&E20× magnification) (3b).

reported earlier by Rice and Barone (2000), Pragnya *et al.* (2014) and Elnahas *et al.* (2021), the first 3 weeks of postnatal period are essential for the development of motor organization.

Humans as well as animals are found to be affected by X-ray irradiation, methylazoxy methanol, ethanol, lead, methyl mercury, or chlorpyrifos, resulting in neurotoxicity (Rice and Barone, 2000). In the present study, our primary goal was to establish a correlation between various behavioural tasks involving mainly the cerebellar and hippocampal regions of the brain in the Balb/C mice challenged with sodium valproate. Autism Spectrum Disorder (ASD) is attributable to dysfunction of neurotransmitter system, which alters neuronal cell migration, differentiation, and synaptogenesis and the overall developmental processes of the brain (Cetin *et al.,* 2015). Alterations in GAB Anergic, glutamatergic, and serotonergic

neurotransmitter systems are most commonly associated with ASD pathogenesis (Trottier *et al.,* 1999). Interestingly, abnormal neurotransmitter or it's metabolite levels within the cerebrospinal fluid (CSF) and/ or plasma of ASD patients are correlated with abnormal brain functions (Sarter *et al.,* 2006). In accordance, hyperserotonaemia is considered as the most reliable biomarker associated with ASD (Pragnya *et al.,* 2014). In this study on inducing Autism in postnatal mice by sodium valproate, an enhancement in the plasma serotonin and dopamine concentrations was observed, associated with a decrease in plasma norepinephrine levels (Table. 2). Both clinical as well as animal studies concluded that during ASD, cerebellum, frontal cortex, amygdala, cingulate cortex, and hippocampus undergo several structural abnormalities (Bristot *et al.,* 2013; Kataoka *et al.,* 2013; Courchesne, 1997). Purkinje cells represent important cerebellar connections with the limbic system and cerebral cortex, (Allen, 2006; Pragnya *et al.,* 2014). Several neurological disorders show either a decreased count or damage to the Purkinje cells, which is responsible for the abnormal behaviour symptoms seen in the autistic spectrum disorders (Allen, 2006;Pragnya *et al.,* 2014, Samimi and Edalatmanesh., 2016; Thomas *et al.,* 1998). Purkinje cells release Gama- aminobutyric acid (GABA) that exerts repressive actions on bound neurons, and thereby reducing the transmission of nerve impulses. Loss of Purkinje cells decreases the release of GABA (Gironell, 2014), which in turn increasesthe extracellular serotonin levels (Tao and Auerbach, 2000; Summavielle *et al.,* 2004). In this study, too, histopathological findings showed a substantial decrease in the Purkinje cell count (Fig. 2) and increase in plasma serotonin in sodium valproate treated mice. Here, incremental serotonin levels in the plasma may be attributed to down-regulated serotonin transporter (SERT) expression resulting in diminished reuptake of released serotonin (Sandhya *et al.,* 2012), this may contribute to motor dysfunction (loss of motor skill development) and social behavior deficits (Summavielle *et al.,* 2004; Yochum *et al.,* 2010). Moreover, the genetic strain of BTBR mice showed autism like behaviour characterized by social behavioural impairments (Ornoy *et al.,* 2019; Pangrazzi *et al.,* 2020) which mainly causes increase of serotonin neurons (Guo and Commons, 2017). This effect has been seen in sodium valproate induced autism animal model (Narita *et al.,* 2002).

Histopathological studies showed that sodium valproate administration had atrophied 40-50% of neurons in hippocampus (CA1 and CA2) and cerebellum (Fig. 3 and 2). Furthermore, previous reports have demonstrated hippocampus disruption (Lodge and Grace, 2007; Al-Amin *et al.,* 2018) resulting in dysregulation of dopamine (Lodge and Grace, 2007) and norepinephrine (Borodovitsyna *et al.,* 2017), leading to increased amount of dopamine (Lodge DJ and Grace, 2007) and decreased amount of norepinephrine (Genestine *et al.,* 2015). Dysfunction in dopaminergic and noradrenergic signalling may be a primary cause of numerous neuropsychiatric disorders, such as schizophrenia, bipolar disorder, attention-deficit hyperactivity disorder (ADHD), anxiety, depression, schizophrenia, Parkinson's disease, Alzheimer's disease and autism (Enzo Emanuele., 2015; Genestine *et al.,* 2015). Correlation analysis revealed a positive correlation between Purkinje cells atrophy and serotonin levels; and negative correlation with social behaviour activity of mice in sodium valproate group, when compared with the control group. No significant differences were observed for the negative geotaxis (motor activity) between the sodium valproate treated and the control groups (Table 3). Moreover, a positive correlation between hippocampus atrophy and dopamine levels was also observed; and no significant difference appeared in hyperactivity and memory impairment between the sodium valproate and control groups (Table 4).

There was no significant difference between hippocampus atrophy with norepinephrine levels, time spent in open arm and number of open arm entries in sodium valporate group when compared with control group (Table 5). Although several studies have been conducted in this field of autism, and the GAB Aergic, glutamatergic, and serotonergic systems have mostly been attributed to ASD pathogenesis (Trottier *et al.,* 1999), no research to date has reported all three neurotransmitters in plasma. The strength and uniqueness of the present study is that all the neurotransmitters in the plasma were monitored, and a correlation was found between the neurotransmitter levels, hippocampus abnormalities, Purkinje cell damage and behavioural impairment of sodium valproate treated Balb/c mice.

In this study, we have shown that sodium valproate injection altered the brain cells, which perhaps lead to altered neurotransmitter levels, leading to behavioural alterations in mice.Based on our observations, we conclude that sodium valproate treated mice exhibited positive correlation with the atrophy of purkinje cells and serotonin levels and negative correlation in social behaviour.

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Add-on Information

Authors' contribution: G. Balaji: Carried out the experiments; **S.N. Sinha:** Conceived and planned the experiments; **M.V. Surekha:** Carried out the necropsy and histopathological analysis; **V. Kasturi:** Carried out the necropsy; **S.K. Mungamuri:** Carried out the experiments; **P. Shashikala:** Conceived and planned the experiments.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Female lactating BALB/c mice with pups were obtained from the National Centre for Laboratory Animal Sciences (NCLAS) and this study was reviewed and approved

by the Institutional Animal Ethics Committee (P13F/IAEC/NIN/5/2018/SNS/BALB/c-10+10 pups), National Institute of Nutrition (NIN), Tarnaka, Hyderabad, India.

Conflict of interest: The authors declare that they don't have any conflict of interest.

Data from other sources: Not applicable

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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