Article

Discovery Medicine 2025; 37(199): 1465–1478
https://doi.org/10.24976/Discov.Med.202537199.128

The Possible Therapeutic Effect of Huperzine A on the Cerebellar Cortex of a Rat Model of Autism: Behavioural and Histopathological Study

Saad Misfer Alqahtani[1], Khlood Mohammed Mehdar[2,*]

[1]Department of Pathology, College of Medicine, The University Hospital, Najran University, 61441 Najran, Kingdom of Saudi Arabia
[2]Department of Anatomy, Faculty of Medicine, Najran University, 61441 Najran, Kingdom of Saudi Arabia
*Correspondence: Khlood.Mehdar02@gmail.com (Khlood Mohammed Mehdar)
Published: 20 August 2025

Background: This study aimed to investigate the possible therapeutic benefits of Huperzine A on autistic behaviours and histological alterations in the cerebellum of a rat model of autism.

Methods: This study utilized 30 male neonatal rat pups (10 per group) to investigate the effects of valproic acid on the rat cerebellum. Pregnant Wistar female rats received valproic acid (VPA) on the 12.5th gestation day, and their pups were administered VPA on postnatal day 14, while the control group received saline on the corresponding days. It was determined that the experimental group received Huperzine A (0.80 µmol/kg) administered via intraperitoneal injection from postnatal day 14 to postnatal day 40. The elevated plus-maze, rotarod, and Morris water maze assessments were employed to investigate autism-like behaviours. Furthermore, on postnatal day 41, the brains of all puppies were excised and prepared for histological examination, and hippocampus homogenates were obtained for the quantification of acetylcholinesterase (AChE) in cerebellar tissue homogenates.

Results: Huperzine A (HupA) treatment reduced anxiety in VPA-induced autism spectrum disorder (ASD) rats. The elevated plus-maze revealed that, at postnatal day (PND) 40, significant decreases in open-arm entries and time were observed ($p < 0.0001$). Non-significant effects at PND 21 ($p = 0.99$, $p = 0.98$). Moreover, HupA improved memory in ASD rats. Morris water maze test revealed that escape latency significantly decreased ($p < 0.0001$) at PND 39. Memory retrieval improved with HupA ($p < 0.0001$). Non-significant escape delay increased at PND 38 ($p = 0.24$). HupA also improved motor coordination in ASD rats. Rotarod test showed a significant endurance time decrease in ASD rats ($p < 0.0001$). HupA mitigated effects at PND 25 and PND 26 ($p < 0.0001$) but not at PND 24 ($p = 0.10$). HupA increased AChE levels in ASD rats. Group II had significantly lower AChE ($p < 0.0001$) than controls. Group III showed a non-significant increase versus controls ($p = 0.0698$). Huperzine A treatment considerably improved the cerebellar histological and immunohistochemical structural alterations.

Conclusions: These findings stress the critical impact of Huperzine A on neurobehavioral alterations, acetylcholinesterase, and cerebellar histological alterations in ASDs.

Keywords: valproic acid; Autism; Huperzine A; acetylcholinesterase; cerebellum; rats

Introduction

Deficits in social communication and engagement are hallmarks of the neurological disease known as autism [1]. It belongs to a diverse collection of conditions known as autism spectrum disorders (ASDs), which share many characteristics but vary in severity, age of onset, and presentation [2–4]. Although difficulties in social communication and interaction, restricted interests, and repetitive behaviours are characteristic of ASD, the disorder manifests in diverse forms [5].

The abnormal physiology and histology of the cerebral cortex have been the focus of the great bulk of investigations on autism spectrum disorder [6,7]. There is strong evidence now that one of the primary afflicted organs in ASD may be the cerebellum [8,9]. Movement, coordination, and cognitive function are all correlated with Purkinje neurons in the cerebellum [10]. The consistent presence of changes in the cerebellum revealed by neuropathologic investigations in ASD [11,12]. Reduction in the Purkinje cell population and mean size, which is the cerebellum's output neuron. Numerous studies have demonstrated that individuals with autism exhibit a reduction in the number of Purkinje cells in the cerebellum [13,14]. These changes to the cerebellum pose a risk to the pathophysiology of autism.

The short-chain fatty acid 2-propylpentanoic acid, also known as valproic acid (VPA), is a common antiepileptic medication and mood stabilizer. By inhibiting histone deacetylase (HDAC) activity, VPA affects neurotransmission and gene expression via epigenetic chromatin remodeling [15]. Clinical research has connected VPA consumption during pregnancy to many problems, including a height-

ened incidence of neural tube defects such as spina bifida, developmental delays, and cognitive impairments [1,16–18]. Children exposed to antiepileptic medicines, such as VPA, during pregnancy have an 8–18 times higher chance of developing ASD than children who were not exposed to these drugs during pregnancy, according to recent studies on the role of environmental variables in the etiology of ASD [19–21].

Many investigations have found that the cholinergic system in the brains of people with ASD is dysregulated [22–24]. The post-mortem brains of people with autism spectrum disorder showed down-regulation of the M1 muscarinic receptor and many nicotinic receptor subunits (α3, α4, α2), but up-regulation of the α7 subunit [25,26]. All this research suggests that the cholinergic system in the brains of people with ASD may be dysregulated. Conversely, marked-available acetylcholinesterase (AChE) inhibitors, whether derived from botanical sources or chemical manufacture, exhibit numerous drawbacks, including limited bioavailability and other gastrointestinal adverse effects [27]. Consequently, it is imperative to investigate alternate AChE inhibitors sourced.

Huperzia serrata, a traditional Chinese medicinal plant, naturally yields Huperzine A (HupA), a Lycopodium alkaloid. Since HupA's activity as an efficient AChE inhibitor was discovered, it has garnered a lot of interest [28,29]. HupA could also successfully pass through the blood-brain barrier (BBB). A growing amount of preclinical research has shown that HupA exerts a substantial neuroprotective effect on central nervous system diseases, especially neurodegenerative disorders [30,31]. HupA additionally reduces traumatic brain injury-related brain damage and neuronal death [32]. However, HupA's potential as a pharmacological therapeutic target for ASD arises from its ability to modulate the cholinergic system.

The objective of this study was to investigate the possible therapeutic benefits of Huperzine A on autistic behaviours and histological alterations in the cerebellum of a rat model of autism.

Materials and Methods

Animals

We acquired twenty pregnant Wistar rats, aged 12 to 15 weeks and weighing between 210 and 250 grams, from the Laboratory Animal Center in Jeddah, Saudi Arabia, along with their littermates for the control group and ASD model. The animals were granted full access to standard feed and potable water. Consistent efforts were made to maintain the animals' living conditions in a clean and standard state. This research was approved by the institution's Bioethics and Research Committee (Number: 442-36-7263).

Prenatal and Postnatal VPA Administration ASD Rat Model

In this study, we generated an ASD rat model via prenatal and postnatal injection of VPA (Valproic acid (100 mg/mL), sodium salt, Abcam, cat# 120745) in 0.9% saline (pH 7.3) [33]. On the 12.5th day of embryonic development (E 12.5), pregnant rats were given an intraperitoneal (ip) injection of VPA 500 mg/kg dissolved in 0.9% saline [33]. On E 12.5, female controls were given saline. Every pregnant rat was allowed unlimited access to water and chow pellets in a delivery chamber that followed a regular 12-hour light-dark cycle, was kept at room temperature (22.2 degrees Celsius), and had a humidity level of 55.5%.

When calculating postnatal days, the day of birth was considered (postnatal day (PND)) 0. The mothers remained with their pups, both male and female. To control for potential effects of hormone shifts, subsequent research will only include males. Thirty male progenies were randomly housed in five-cage sets, with each set undergoing a 12-hour light-dark cycle. The newborn rats weighed 20–40 grams on the first day of the trial. On postnatal day 14, rats in group II (autism spectrum disorder) were injected subcutaneously with 400 mg/kg of freshly made VPA [34].

Experimental Design

Group I (Control group): 10 male offspring of control pregnant females were subdivided into:

- **Subgroup Ia (Naïve group)**: Two offspring of rats were allowed to reach PND 40 untreated.
- **Subgroup Ib (Saline treated groups)**: three offspring rats received a single subcutaneous dose of saline (NaCl 0.9%) at PND 14, then an ip injection from PND 14 to 40, once daily.
- **Subgroup Ic (HupA group)**: From PND 14 to PND 40, five offspring rats were given an ip injection of 0.5 mg/kg of body weight HupA (98% pure; Huperzine A was purchased from Sigma-Aldrich, St. Louis, MO, USA) once daily. To make each dose, the medicine was first dissolved in 10 mg/mL of normal saline to create a stock solution, and then the solution was diluted with more normal saline until it reached the correct concentration. As mentioned earlier, the dosage of HupA was determined by aiming for concentrations that would have a clinically meaningful impact [32].

Group II (ASD rat model): Ten male offspring born to pregnant females who were given a single dosage of VPA during pregnancy were randomly split into:

- **Subgroup IIa (VPA group)**: At PND 14, five offspring rats were injected subcutaneously with VPA and then left untreated until PND 40, the end of the experiment at PND 40.
- **Subgroup IIb (VPA + saline group)**: At PND 14, five offspring rats received an i.p. injection of saline (NaCl 0.9 percent) and a subcutaneous injection of VPA till the end of the experiment at PND 40.

Group III (VPA + HupA treated group): 10 male offspring rats of ASD pregnant females, which received a prenatal dose of VPA and received post-natal subcutaneous injection of VPA at PND 14 and HupA via i.p. injection from PND 14 to 40, once daily.

Behavioural Analysis

Effect on Anxiety (Elevated Plus-Maze Test)

Rats have a predisposition to eagerly explore new environments, therefore, this conflict test uses that tendency against the unpleasant features of an elevated open runway [35]. Tests for anxiety-related behaviour were conducted using the elevated plus maze, which is normally set 50 cm above the floor. There were two open arms and two closed arms of the elevated plus maze, each measuring 50 cm in length, 12 cm in width, and 30 cm in height. The rats were positioned in the maze's hub, facing the open arm. After each round of animal testing, the equipment was disinfected with 70% ethanol. The test was monitored for 5 minutes, and data were collected on how often participants used the open arm and how long they stayed there. It was recorded how many times the open arm was entered and how many times it was left open. To get the percentage, we took the total amount of time the arms were open and divided it by 100. Then, we divided that number by the total number of times the arms were closed. Using the same procedure, but this time using the measure for entries, we were able to get the percentage of open-armed entries [33,35]. This test was performed on PND 21 and PND 40.

Effect on Memory (Morris Water Maze (MWM) Test)

The MWM test is commonly used to evaluate memory impairment in laboratory rodents. The MWM (TSE Systems, Chesterfield, MO, USA) is an open, circular pool that is half filled with water and has a smooth, flat inside surface. The MWM is divided into four sections: the "target" section, the "left" section, the "right" section, and the "opposing" section. The water in the target quadrant is 30 centimetres in depth and 26.1 degrees Celsius in temperature. The submerged hiding platform served as the objective, and the rats were free to swim around it in their search. The rats were first habituated to the training setting before the experiment began so that they could learn to navigate to the location of the platform using only distant signals. Each rat was given two minutes to swim around the pool without the aid of the platform. Each rat attempted 20 attempts over the course of the next four days (days 1–4 of the MWM testing), with four tries per day. The platform was hidden from the animal's underwater eyes by disguising it with a pool of non-toxic dye [36]. The rats in each experiment were given up to 60 seconds to swim to the underwater platform [37]. The water maze's placement in the lab was kept constant with respect to other items, and the blind observer maintained a fixed position. The escape latency was determined by timing how long it took the rat to make it to the platform. On day 5, we conducted the probe trial to evaluate the quality of our reference memory [38].

Effect on Motor Coordination (Rotarod Test)

This procedure is useful for diagnosing motor coordination impairment in mice and rats by evaluating their motor coordination and motor learning [39]. The rotarod test is commonly used to evaluate the motor function of laboratory rodents by having them walk on a spinning drum. The rotarod is used to determine how long an animal can balance on a rotating drum at a given rotational velocity. The rats participated in three separate 5-minute-long tests in which they were exposed to a rotary rod spinning at 40 revolutions per second (rpm). The rats were accustomed to spending three minutes on the fixed drum before the training sessions began. A daily one-minute habituation period was performed right before each session. Between each animal test, the instruments were cleaned. To facilitate learning in animals, the rate of rotation was slowed to 10 revolutions per minute (rpm; surface speed of 2.8 meters per minute). After each of its up to five falls, the animal was promptly returned to the drum. When the animal stayed on the drum for 180 seconds, nobody noticed that it had fallen off. In order to gauge long-term retention, the exam was administered once a day for four days in a row. Photocells automatically recorded the latency to fall, and the total latencies on the rod were examined daily. Long-term memory was assessed by administering the exam four times in one day, beginning on PND 24 and ending on PND 26 [40].

Brain Sample Preparation

Animals were sacrificed at PND 41. The animals were anesthetized using an intraperitoneal injection of urethane at a dose of 1.5 g/kg. Then, animals underwent transcardiac perfusion with 600 mL of saline and 600 mL of paraformaldehyde at a concentration of 4%. The brains were cut in half as soon as they were removed. As soon as it had been perfused, the brain was separated into two pieces, one for biochemical assessment, which was then frozen at –80 °C for later study. The other was to be examined for histological purposes.

Biochemical Measurements in Tissue Homogenates

To homogenize hippocampus tissues, lysis buffer was used, which consisted of 320 mM sucrose, 50 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.5 mM sodium orthovanadate, 5 mM -glycerophosphate, and protease inhibitors. The samples were then incubated on ice for 30 minutes and centrifuged at 13,000 g for 10 minutes. The protein concentration in the supernatant was determined by utilizing the Bradford test. Acetylcholinesterase (AChE, MBS2709297, MyBioSource, Dokki, Giza, Egypt) activity was determined using the colorimetric assay of Ellman *et al.* [41] as described in Bajgar *et al.* [42]. Commercially available kits from MyBioSource (Dokki, Giza, Egypt), were

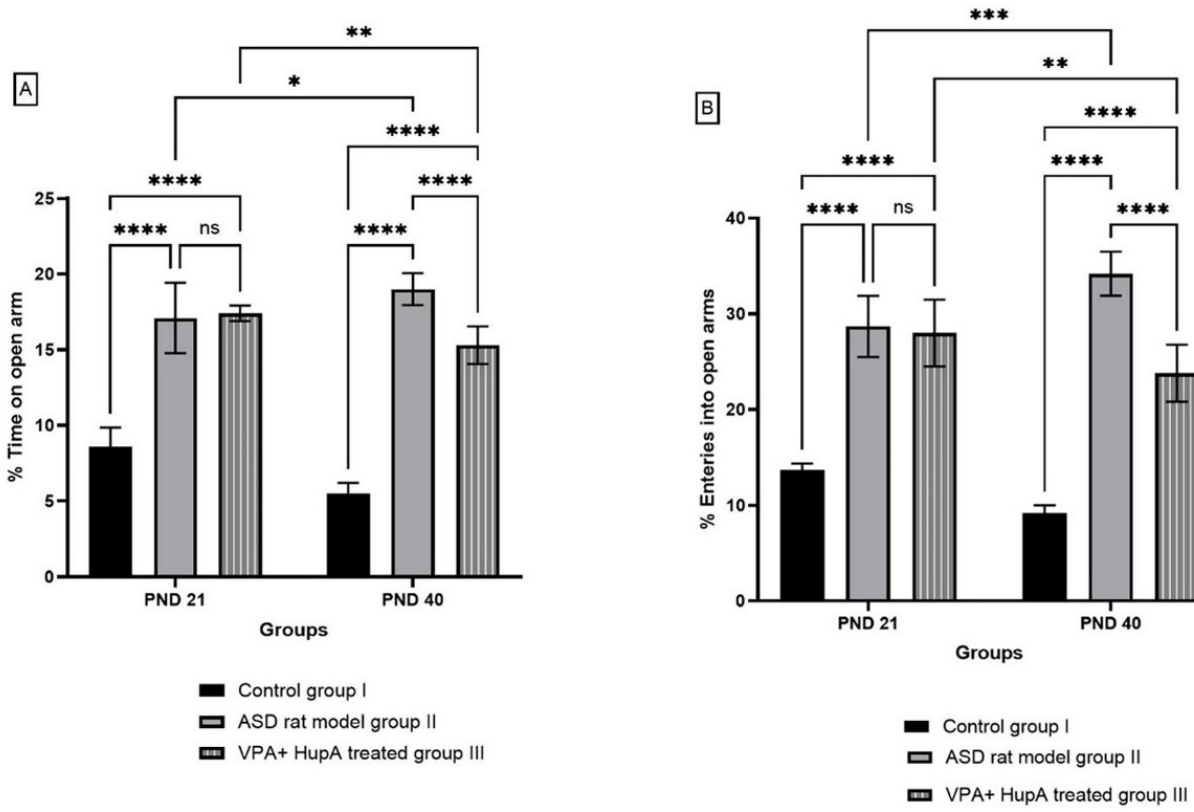

Fig. 1. Effect of Huperzine A (HupA) on (A) % time on open arm and (B) % entrance on open arm in the elevated plus maze test in valproic acid (VPA) induced rat model of autism. After running the data through one-way analysis of variance (ANOVA), the statistical analysis was completed with Tukey's post hoc test. The mean ± SD (n = 10) is used to represent the values. Not significant (ns). Noticeable variation at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

used for colorimetric testing of these markers in accordance with the manufacturer's instructions. The concentration of AChE was reported in micromoles per milligram, while the concentration of other oxidative stress markers was reported in nanomoles per milligram of protein.

Light Microscopic Examination

Additionally, the brain was sliced coronal. Specimens were dehydrated in alcohol solutions of increasing strength before being fixed for 24 hours in 10% neutral buffered formalin. Their subsequent preparation for histological and immunohistochemical examination included washing and embedding in paraffin. The last step was to deparaffinize the tissue sections, microtome them to a thickness of 5 microns, and then stain them with Haematoxylin and eosin (H&E) [43].

Immunohistochemical Study

The astrocytes were seen using immunohistochemistry for glial fibrillary acidic protein (GFAP). The antibody used was a rat monoclonal antibody at a 1/100 dilution (cat# 665759, Sigma-Aldrich, St. Louis, MO, USA), as described earlier. The Avidin-Biotin Peroxidase method was used to analyze hippocampal sections. A 30-minute pre-treatment with 0.1 percent hydrogen peroxide inhibited endogenous peroxidase. Deparaffinized and paraffin-embedded tissues were then incubated with the primary antibody. After that, tissue slices that were mounted on slides were treated for 30 minutes with a biotinylated anti-rabbit secondary antibody (1:2000) (cat# BA-9200; Vector Lab., Burlingame, CA, USA). Prior to dehydrating, cleaning, and mounting the sections, Mayer's haematoxylin was used as a counterstain. We followed the identical steps as before, but this time we used phosphate-buffered saline (PBS) instead of the primary antibody to make negative control sections [44].

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) version 26.0 (SPSS Inc. of Chicago, IL, USA) was utilized for all analyses. The graphs were generated using GraphPad Prism 10 (GraphPad Inc. in San Diego, CA, USA). Data is shown using mean ± standard deviation (mean ± SD) graphs. One- and two-way analysis of variance (ANOVA) as well as Tukey's post hoc multiple comparisons test were used to assess the results. To determine statistical significance, a *p*-value less than 0.05 was utilized.

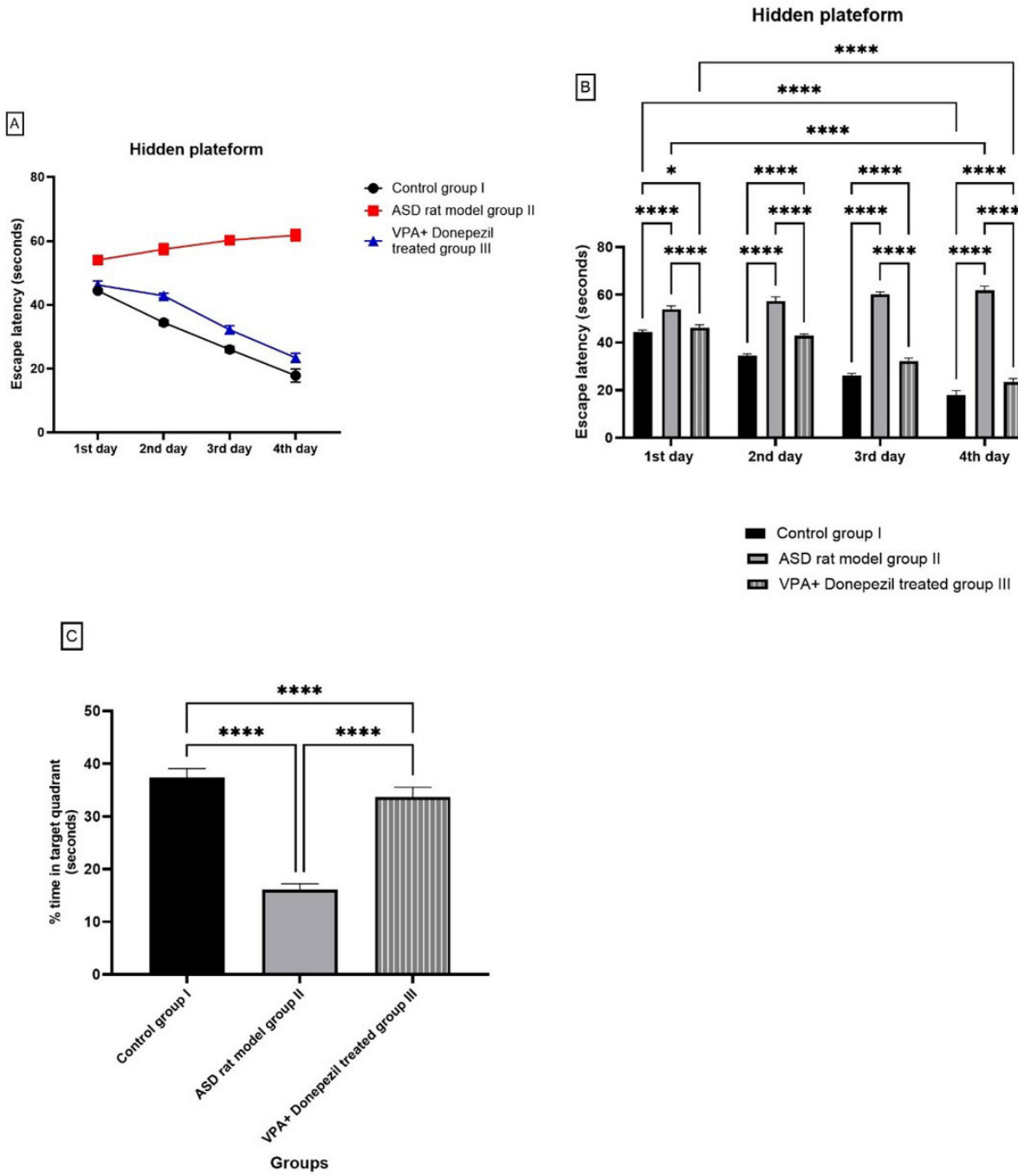

Fig. 2. (A) Line graph and (B) Bar chart showing effect of HupA on the escape latency (seconds) to reach the hidden platform in all experimental groups and (C) % time that the rats spent in target quadrant (seconds) in the Morris water maze in VPA induced rat model of autism. After running the data through one-way ANOVA, the statistical analysis was completed with Tukey's post hoc test. The mean ± SD (n = 10) is used to represent the values. There is a notable distinction between * $p < 0.05$ and **** $p < 0.0001$.

Results

Assessment of the Effects of HupA on Anxiety in a Rat Model of VPA-Induced Autism

One of the behavioural methods useful for gauging anxiety-related behaviour is the elevated plus maze test. Since rats avoid the open arms, the most important variables in this test are the frequency of entry and the amount of time spent there (Fig. 1). At PND 21 and PND 40, the rat model with ASD (group II) that was given VPA exhibited increased anxious behaviour. In comparison to the control group I, the ASD group II had a considerably higher num-

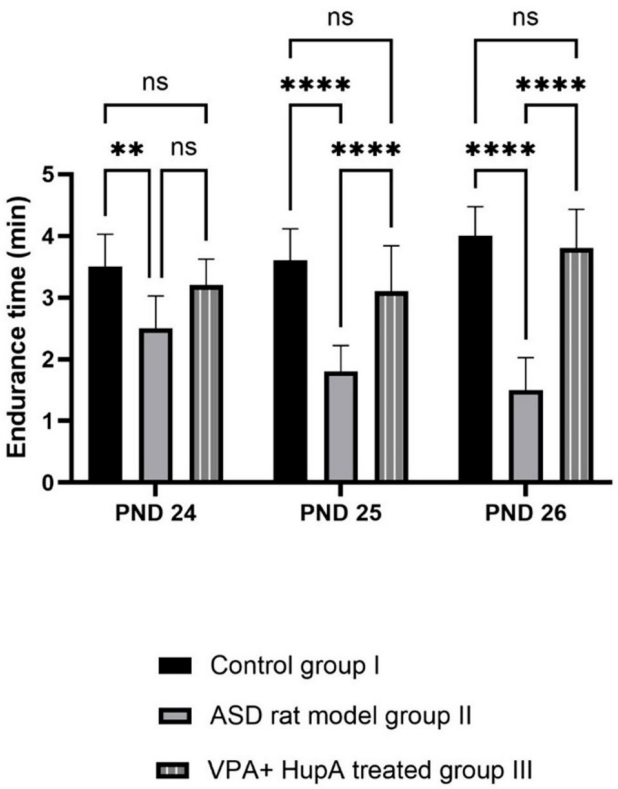

Fig. 3. Effect of HupA on Endurance time in the rotarod test in the VPA-induced rat model of autism. After running the data through one-way ANOVA, the statistical analysis was completed with Tukey's post hoc test. The mean ± SD (n = 10) is used to represent the values. Not significant (ns). There is a strong distinction at ** $p < 0.01$ and **** $p < 0.0001$.

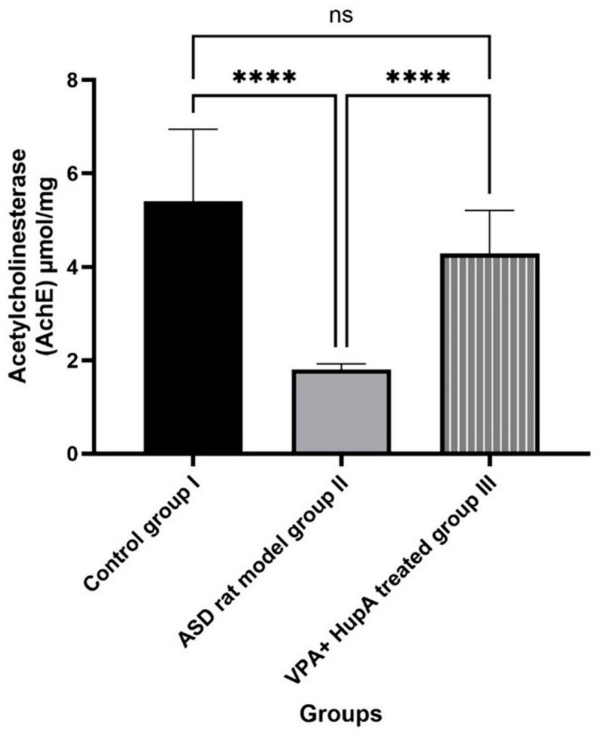

Fig. 4. Effect of HupA on the biochemical markers in hippocampus tissue homogenate of the VPA-induced rat model of autism. Acetylcholinesterase (AChE) µmol/mg. After running the data through one-way ANOVA, the statistical analysis was completed with Tukey's post hoc test. The mean ± SD (n = 10) is used to represent the values. Not significant (ns). ****$p <$ 0.0001 indicates a significant difference.

ber of entrances into the open arms at PND 21 and PND 40 ($p < 0.0001$ and $p < 0.0001$, respectively). In addition, compared to the control group I at PND 21 and PND 40, group II spent considerably more time in open arms ($p <$ 0.0001 and $p < 0.0001$, respectively).

Interestingly, compared to the ASD rat model group II, the VPA + HupA treatment group exhibited a non-significant decrease ($p = 0.99$, $p = 0.98$) for the number of admissions into the open arms and the amount of time spent in the open arms at PND 21. When comparing the VPA + HupA-treated group III to the ASD rat model group II at PND 40, there was a substantial decrease ($p < 0.0001$, $p <$ 0.0001) in both the amount of time spent in open arms and the percentage of entrances into the open arms. At PND 40, ASD group II showed a statistically significant increase (p = 0.0001 for number of entries and $p = 0.025$ for amount of time spent in open arms) compared to PND 21, when group II was first evaluated. It is worth noting that there were notable declines in HupA therapy at PND 40 compared to PND 21 ($p = 0.005$, $p = 0.009$, respectively).

Assessment of the Effects of HupA on Memory in a Rat Model of VPA-Induced Autism

We used MWM testing on rats to examine the impact of prenatal and postnatal VPA exposure on learning and memory, as well as to confirm any potential amelioration of HupA, as shown in (Table 1 and Fig. 2). The present study's findings showed that, except for the ASD rat model group II, the escape latency decreased as the days progressed in all groups (Fig. 2A).

There was a statistical difference in the mean on escape latency on the MWM test between all control rats (group I), rats given VPA (group II), and rats given VPA + HupA (group III) from PND 36 to PND 39, as shown in Fig. 2B.

In group II (ASD rat model), the latency to reach the hidden platform increased significantly ($p < 0.0001$) in the PND 39 versus PND 36 and PND 37. In contrast to PND 38, PND 39 showed a non-significant increase ($p = 0.24$) in escape delay. Fascinatingly, as compared to the ASD rat model (group II), the control rats (group I) showed a significantly shorter escape latency ($p < 0.0001$) on all days starting from the first (PDN 36, 37, and 38) to the fourth (PDN 39). Furthermore, on the 4th day (PND 39), ASD

Table 1. Assessment of the effects of huperzine A on behavioral changes in a rat model of valproic acid (VPA)-induced autism.

Groups (n = 10)	Control rats group I	ASD rat model group II	VPA + HupA group III
Elevated plus-maze test			
% Entries into open arms (PND 21)	13.70 ± 0.67	28.7 ± 3.19	28.00 ± 3.49
% Entries into open arms (PND 40)	9.20 ± 0.78	34.20 ± 2.30	23.80 ± 2.97
% Time on open arm (PND 21)	8.60 ± 1.26	17.10 ± 2.33	17.40 ± 0.51
% Time on open arm (PND 40)	5.50 ± 0.70	19.00 ± 1.05	15.30 ± 1.25
Morris water maze test			
Escape latency (seconds) at PND 36	44.40 ± 0.84	54.00 ± 1.33	46.20 ± 1.22
Escape latency (seconds) at PND 37	34.40 ± 0.84	57.40 ± 1.71	42.80 ± 0.78
Escape latency (seconds) at PND 38	26.00 ± 0.94	60.20 ± 1.03	32.20 ± 1.22
Escape latency (seconds) at PND 39	17.80 ± 2.04	61.80 ± 1.81	23.40 ± 1.42
% time in target quadrant (seconds)	37.40 ± 1.64	16.10 ± 1.101	33.70 ± 1.82
Rotarod test (endurance time (min))			
PND 24	3.50 ± 0.52	2.50 ± 0.52	3.20 ± 0.42
PND 25	3.60 ± 0.51	1.80 ± 0.42	3.10 ± 0.73
PND 26	4.00 ± 0.47	1.50 ± 0.52	3.80 ± 0.63

With n = number of rats/groups, the data are shown as Mean ± standard deviation (SD). PND, postnatal day; ASD, autism spectrum disorder.

treated rats with HupA showed significant ($p < 0.0001$) attenuations in the escape latency versus the ASD rat model among all days from 1st (PND 36, 37, and 38). Interestingly, there was still a non-significantly ($p = 0.11$, $p = 0.24$) difference at the 1st day (PND 36) between control rats as compared to group III at PND 37 and 38. At the 4th day (PND 39), the ASD rat model treated with HupA (group III) showed a significant decrease ($p < 0.0001$) in the escape latency to locate a hidden platform compared to the control rats at PND 36, 37, and 38.

Memory retrieval was monitored by counting how long rats spent in the probe trial's quadrant containing the fixed platform (Fig. 2C). Less time was spent in this quadrant by the ASD rat model compared to control rats ($p < 0.0001$). In contrast, the percentage of time given in the target quadrant was significantly increased by the HupA treatment ($p < 0.0001$) when contrasted with the ASD rat model.

Evaluation of HupA's Impact on Motor Coordination in a Rat Model of Autism Induced by VPA

Fig. 3 displays the results of a rotarod test conducted between PNDs 24 and 26 to evaluate the impact of HupA on motor coordination. In comparison to the control rats in group I, the ASD-rat model (group II) exhibited a significant ($p < 0.01$) decrease in endurance time at PNDs 24 and 26. However, HupA treatment in group III didn't mitigate significant ($p = 0.10$) changes induced by VPA at PND 24, in contrast with the ASD-rat model. Interestingly, both at the PND 25 and PND 26, HupA treatment in group III did mitigate significant ($p < 0.0001$) changes induced by VPA in contrast with the ASD-rat model. There was no statistically significant difference between the control group and the HupA-treated ASD animals at PND 24 ($p = 0.94$), PND 25 ($p = 0.49$), or PND 26 ($p = 0.99$), despite the fact that the ASD animals spent less time on the rotarod test overall.

Assessment of the Effects of HupA on Acetylcholine Esterase Enzymes in a Rat Model of VPA-Induced Autism

The AChE levels in the ASD rat model group II were significantly lower (1.79 ± 0.12 nmol/mg) compared to the control group (5.40 ± 1.54 nmol/mg) and the VPA + HupA treated group (4.28 ± 0.92 nmol/mg), as shown in Fig. 4 ($p < 0.0001$). There was a small but statistically insignificant difference in AChE levels between the control group and group III (which received VPA and HupA), with a p-value of only 0.0698.

Assessment of the Effects of HupA on Histological and Immunohistochemical Changes of Cerebellar Tissue in a Rat Model of VPA-Induced Autism

Haematoxylin and Eosin-Stained Sections

Fig. 5A,B shows the three layers of the cerebellum of a control rat, which consists of molecular, Purkinje cell, and granular cells. You can see the Purkinje cells lined up in a single row between the two other layers. Large, pale-stained nuclei with prominent nucleoli were visible in the Purkinje cells. Cerebellar islands formed the granular layer in-between numerous clusters of granular cells. The molecular layer was stuffed with glial cells and lightly acidophilic neuropil.

In the ASD rat model, there was a disruption in the linear structure, as seen in Fig. 5C,D, with pronounced spongiosis and several vacuolated patches in the Purkinje cell layer. Additionally, there was interposition of numerous glial cells between and surrounding the Purkinje cells. The nuclei and cytoplasm of several Purkinje cells seemed shrunken and heavily stained. Nuclei were difficult to make out in some of the Purkinje cells, and their cytoplasm seemed to be rather acidophilic. The Purkinje

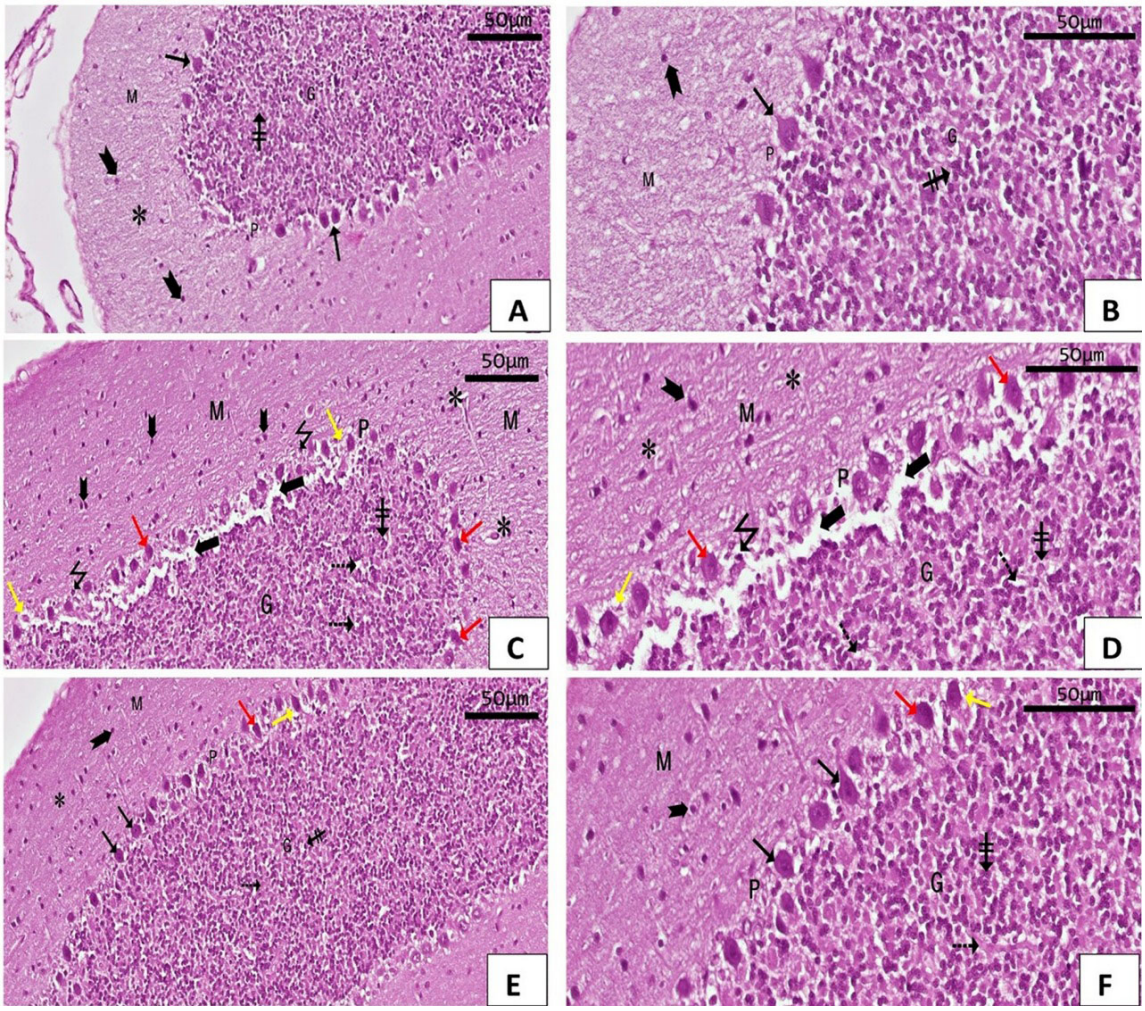

Fig. 5. Haematoxylin and eosin (H&E) staining of a parasagittal section of rat cerebellar cortex. The molecular (M), Purkinje (P), and granular (G) layers make it up. Control rat (GI; A,B): The nuclei of the Purkinje cells are big and pale-stained, and their nucleoli are conspicuous and deeply stained. Take note of how the Purkinje cells (black arrows) are arranged in a single row across the three layers. Granule cells (double stroke arrows) are arranged in distinct clusters of round cells. These cells are divided by cerebellar islands (dot arrow). Glial cells (bifid arrow) and somewhat acidophilic neuropil (*) are seen. ASD rat model (G II; C,D) disrupts the regular arrangement with noticeable spongiosis as several regions with vacuoles (yellow arrow) and glial cells interposed (zigzag arrow) between and surrounding the Purkinje cells are observed. Hardly identified nuclei and deeply acidophilic cytoplasm of many Purkinje cells (red arrows) are noticed. Take note of the fact that Purkinje cells disappeared from focal areas (thick arrow). The molecular layer (M) shows some cells with pericellular halos (bifid arrow) and wide neuropil (*). The granular cells (double stroke arrows) have a tiny size, an unclear shape, and unidentified nuclei with large gaps (dot arrow) between them. VPA + HupA-treated rat (G III; E,F) nearly appears as a control. The Purkinje cell (black arrows) retains normal linear organization. It appears that certain Purkinje cells have extremely acidophilic cytoplasm and barely discernible nuclei (red arrows). The Purkinje cell (P) layer shows very few vacuolated regions (yellow arrows). Distinct cerebellar islands (dot arrow) divide the granule cells (double stroke arrows), which look very much like the control. When compared to the control group, the molecular (M) layers show no discernible difference. Neuropil, which is slightly acidophilic, and glial cells (bifid arrow) fill it up. (H&E. A,C,E ×20 and B,D,F ×40).

cells in focal areas have noticeably disappeared. Cells with vacuolated neuropil and pericellular halos were observed in the molecular layer. There were large gaps in the granule layer. Granular cells were often tiny, had an unclear shape, and had nuclei that were difficult to make out.

To all appearances, the rat treated with VPA and HupA (G III; Fig. 5E,F) was used as a control. The granule cells were situated between cerebellar islands and seemed very much like the control group. In the molecular layers, there was no discernible difference compared to the control group. Glial cells and acidophilic neuropil were packed inside.

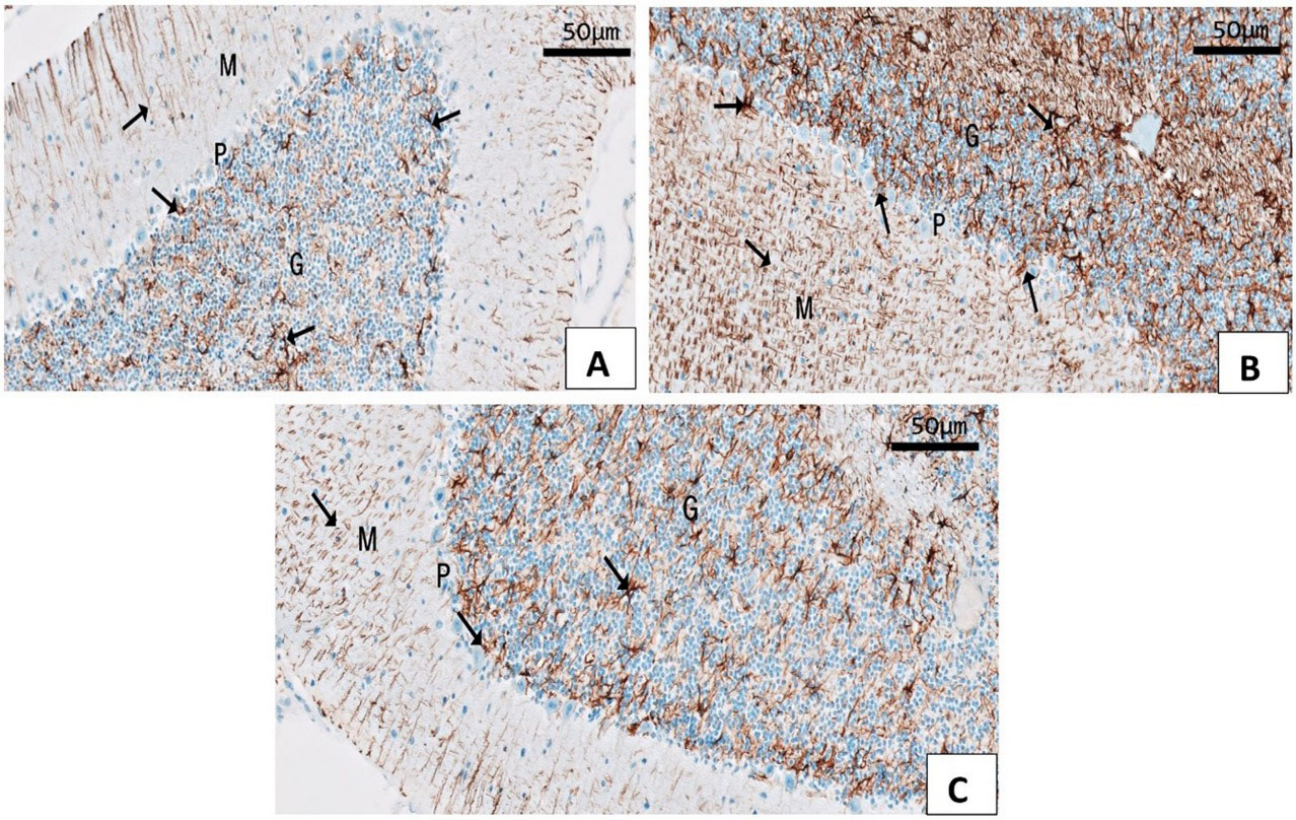

Fig. 6. The photomicrograph clearly shows the granular (G), Purkinje (P), and molecular (M) layers of the rat cerebellar cortex. (A) (control group I): normal distribution as weak positive reaction of glial fibrillary acidic protein (GFAP) positive brownish immunoreactive astrocytes (arrows). (B) (ASD rat model group II): strong positive reaction of GFAP-positive brownish immunoreactive astrocytes (arrows). Notice the long interdigitating processes of astrocytes. (C) (VPA + HupA treated group III): moderate positive reaction of GFAP-positive brownish immunoreactive astrocytes (arrows). (GFAP IHC stain, A–C ×20).

Immunohistochemically Stained Sections for Glial Fibrillary Acidic Protein (GFAP)

Immunohistochemical staining for GFAP revealed a mild positive reaction in the processes and cytoplasm of astrocytes in control rats, whereas this positive reaction was intense in ASD rat model animals in the three cerebellar layers. Rats concomitantly treated with HupA displayed a moderate reaction compared to VPA-treated animals (Fig. 6A–C).

Discussion

The cerebellum is the brain structure most consistently found abnormal in ASD. The potential role of the cerebellum in the pathophysiology of ASD has been demonstrated by a number of animal and clinical investigations [8,9,45–47]. Significant cerebellar pathology, including a decrease in Purkinje cells, was observed in individuals with ASD [47–49]. Our findings add to the growing body of evidence suggesting that prenatal VPA exposure has a neurotoxic effect on the cerebellar cortex, particularly on Purkinje cells. This agrees with previous research by Cho *et al.* [49], Shona *et al.* [50], and Wang *et al.* [8]. Furthermore, we sought to determine if the effects of prenatal and postnatal VPA on neural behavior, AChE activity, and histological cerebellar alterations might be mitigated by administering HupA (AChE inhibitors) to an animal model of autism.

The current study's findings supported the hypothesis that valproic acid exposure at any time during or after pregnancy could contribute to the onset of ASD. The behavioral changes in VPA-exposed rats and the disturbed behavior in autistic patients, including a marked decrease in social interaction and repetitive stereotyped behavior, were shown to be similar, according to several experimental studies, biochemical studies, and histopathological analyses, confirming the negative effects of the VPA animal model of autism [51–54]. Research has shown that prenatal and postnatal VPA exposures in mice might cause impairments in neurobehavioral development that are similar to those seen in people with autism [55–57]. An AChEI called HupA has a long history of use in the treatment of a wide range of symptoms, including edema, strain, muscle cramps, and bruising. So far, the effects of HupA on cognition and memory have been the focus of a number of clinical trials [58–60]. Furthermore, valuable studies on the pharmacological characteristics and structure of HupA revealed that HupA

may have a role in neuroprotection and anti-inflammation [61]. Following this, we decided to use HupA as the study's key medication.

In the current study, the ASD rat model was induced by a combined prenatal and postnatal exposure to VPA. The prenatal VPA exposure (500 mg/kg) was on gestational day 12.5. After the neural tube has closed, this time frame corresponds to the first trimester in humans [56]. Adverse behavioral effects, such as reduced social behavior, repetitive actions, hyperactivity, and impaired recognition, were found in a significant number of mouse pups who were exposed to VPA during the neural tube closure period [62,63]. Regarding VPA exposure after birth, it is administered to mice at PND 14, which is approximately the same as the third trimester of a human being's gestation, when the brain starts to grow, in order to induce developmental delays and behaviors similar to autism [55,64,65]. In addition, we only employed male rats for this analysis. In humans, the incidence of ASD is almost four times higher in males than in females [66]. This sex difference highlights the importance of taking into account sex variations in rodent models of ASD, as sex-specific ASD symptoms may emerge [67].

Increased repetitive stereotyped behavior and anxiety (as measured by the elevated plus-maze test), cognitive impairment (as measured by the Morris water maze), and motor coordination delay were all symptoms of autism that were induced by VPA exposure during pregnancy or shortly after birth (in the rotarod test). Previous experimental research corroborates VPA's ability to cause behavioural changes [55,64,65]. Furthermore, our findings provide further evidence that the patterns of behavioural alterations seen in rats are comparable to those seen in autistic humans [68]. Alternatively, research has demonstrated that VPA alters the neurobiology of the amygdala, cerebellum, and hippocampus, which in turn impacts social behaviour, locomotor activity, and cognitive ability [69].

Rats with ASD whose hippocampus were exposed to VPA both during pregnancy and after birth have elevated levels of AChE. It appears that VPA accomplishes its role by inhibiting histone deacetylase (HDAC), a negative regulator of gene expression [70]. Recent research has demonstrated that VPA can momentarily increase acetylated histone levels in developing mouse brains. This transient hyperacetylation has two effects: an increase in neocortex apoptotic cell death and a decrease in ganglionic eminence cell proliferation. Mice in this study also showed changes in behavior that are similar to those seen in people with autism [71]. One of the most prevalent comorbidities in those with ASD, especially children and adolescents, is anxiety [72]. Due to the high prevalence of anxiety in ASD, rat models of the disorder have also been subjected to rigorous evaluation for the presence of anxiety. In order to identify any anxiety-related behaviors in our results, the elevated plus maze test was utilized. Compared to control rats, VPA rats exhibited less anxiety by spending a longer time in the open arm. Nevertheless, after taking HupA, the VPA group's higher anxiety levels returned to the control group's levels. The VPA rodents, on the other hand, have been demonstrated in many experiments to exhibit anxiety as seen by their preference for the confinement of their open arms [63,71,73]. The VPA rodents were observed to spend equal amounts of time in open and closed arms in other investigations [74].

In order to study cognitive processes related to spatial learning and memory, the MWM test was employed. Impairment in spatial learning (as reflected by poor performance on the MWM spatial navigation task) is a common symptom of ASD [75]. A number of hypotheses have been advanced to explain the autism-like effects of VPA, including its ability to block sodium and calcium channels, increase levels of the inhibitory neurotransmitter GABA in the brain, and alter gene expression in developing embryonic brains through epigenetic chromatin remodelling. Synaptic plasticity, learning, and memory are all regulated by chromatin remodelling, which, when disrupted, can lead to apoptotic cell death, decreased cell proliferation in various brain locations, and, ultimately, impaired behavior [76]. Curiously, the current study found that the HupA group needed less time to locate the platform than the vehicle group, indicating that HupA reduced cognitive impairment in the ASD rat model. In a similar vein, research has demonstrated that impairments in spatial awareness and recognition memory are associated with hippocampal injuries [77]. Further evidence suggests that HupA therapy may mitigate brain ischemia-reperfusion injury-induced cognitive deficits in rats [78,79]. It was not surprising that the spatial memory of ASD rats was improved by HupA therapy in this study.

We postulated that the utilization of the AChE inhibitor HupA might alleviate autistic symptoms in the VPA-induced autistic animal model, since elevated AChE levels could have a negative impact on brain ACh levels. In the current study, the ASD rat model was treated daily with HupA from PND 14 to PND 40. The results of this study were in line with those of Ohba *et al*. [60], who studied how Japanese H. serrata extract affected memory and AChE activity inhibition in a mouse model of scopolamine-induced cognitive impairment. By inhibiting the enzyme that hydrolyses acetylcholine, HupA reverses cognitive deterioration. Consequently, it increases the concentration of acetylcholine in the presynaptic area of the cholinergic neuron [80].

The aberrant alterations that occur during embryological development, such as maternal exposure to certain chemicals, can cause abnormal changes in the cerebellum [81]. As such, the cerebellum serves as an ideal model for researching numerous facets of neurological development. Our histological and immunohistochemical findings showed that exposure to VPA during pregnancy caused Purkinje cells in the cerebellar cortex to undergo apoptosis and degenerative alterations. These findings were con-

sistent with earlier research showing that exposure to VPA during pregnancy caused a significant deterioration of the cerebellar Purkinje and granular cells [50,82,83]. In the current research, the granular layer displayed pericellular unstained haloes and hemorrhage, whereas the molecular layer of the offspring of women treated with VPA showed numerous conspicuous vacuolated regions and pyknotic nuclei of Purkinje cells. Other research made a similar finding, stating that VPA increased granular cell destruction by causing aggregation and the loss of the majority of the cortical architecture [82,83]. Some authors referred to the vacuolation that developed inside the Purkinje cell and molecular layers as spongiform alterations [84]. Possible causes of neuronal degeneration include intercellular biochemical problems, such as altered morphological cell integrity, abnormal protein synthesis, impaired secretory and detoxification processes, or inhibition of oxidative phosphorylation in Purkinje cell mitochondria [85]. As demonstrated by the improvement in the histopathological examination, the current study's administration of HupA reduced the harmful effects of VPA on the affected group's cerebellar cortex. These results were consistent with research by Zhang *et al.* [61], in which the authors assessed the possible protective benefits of HupA on the cuprizone-induced demyelination model of multiple sclerosis.

One distinguishing feature of adult astrocytes is the presence of GFAP, the most abundant intermediate filament protein in these cells. Activated microglia have been found to be crucial in the development of autism spectrum disorder [86]. Activated microglia are the primary source of reactive oxygen species in brain disease, and their neurotoxic effects and the subsequent release of inflammatory cytokines contribute to neurodegeneration across a variety of brain regions [83]. The current study's findings validated neurodegenerative and apoptotic changes in the cerebellum, as shown by an increase in GFAP positivity, using histological and immunohistochemical evaluations in a rat model of autism spectrum disorder. We found that GFAP was overexpressed in the cytoplasm of astrocytes in the Autistic rat model as compared to the control group. This expression was observed in both the cell body and processes. These results were previously seen by histology and immunohistochemistry studies in the cerebellum of autistic animal models [50,82,83]. A compensatory mechanism following VPA neurotoxicity could include astrocyte activation, as shown by a statistically strong positive GFAP immune reactivity. The production of hazardous reactive oxygen and nitrogen species in response to neurodegeneration turned these neuroglial cells into cells with the ability to cause cell death [50]. The current study demonstrated that HupA has a protective effect by showing that the GFAP immunohistochemistry reaction improved significantly after HupA treatment with VPA.

Conclusions

These findings highlight the significant effect of Huperzine A on neurobehavioral changes, acetylcholinesterase, and cerebellar histology changes in the rat model of autism caused by VPA. This suggests that Huperzine A could be a valuable therapeutic drug for the treatment of autism spectrum disorders.

Availability of Data and Materials

Data are available on request from both authors.

Author Contributions

The authors confirm contribution to the paper as follows: study conception and design: KMM, SMA; analysis and interpretation of results: KMM, SMA; draft manuscript preparation and critical revision: KMM, SMA. Both authors reviewed the results and approved the final version of the manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Approval of this research was secured from the institutions' bioethics and research committee with the reference number no. 442-36-7263.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] American Psychiatric Association (ed.) Diagnostic and statistical manual of mental disorders: DSM-5. American Psychiatric Association: Washington, DC. 2013.

[2] Young H, Oreve MJ, Speranza M. Clinical characteristics and problems diagnosing autism spectrum disorder in girls. Archives De Pediatrie: Organe Officiel De La Societe Francaise De Pediatrie. 2018; 25: 399–403. https://doi.org/10.1016/j.arcped.2018.06.008.

[3] Mohammadi S, Asadi-Shekaari M, Basiri M, Parvan M, Shabani M, Nozari M. Improvement of autistic-like behaviors in adult rats prenatally exposed to valproic acid through early suppression of NMDA receptor function. Psychopharmacology. 2020; 237: 199–208. https://doi.org/10.1007/s00213-019-05357-2.

[4] Hodges H, Fealko C, Soares N. Autism spectrum disorder: definition, epidemiology, causes, and clinical evaluation. Translational Pediatrics. 2020; 9: S55–S65. https://doi.org/10.21037/tp.2019.09.09.

[5] Scheggi S, Guzzi F, Braccagni G, De Montis MG, Parenti M, Gambarana C. Targeting PPARα in the rat valproic acid model of

autism: focus on social motivational impairment and sex-related differences. Molecular Autism. 2020; 11: 62. https://doi.org/10.1186/s13229-020-00358-x.

[6] Hashemi E, Ariza J, Rogers H, Noctor SC, Martínez-Cerdeño V. The Number of Parvalbumin-Expressing Interneurons Is Decreased in the Prefrontal Cortex in Autism. Cerebral Cortex (New York, N.Y.: 1991). 2017; 27: 1931–1943. https://doi.org/10.1093/cercor/bhw021.

[7] Oblak AL, Gibbs TT, Blatt GJ. Reduced GABAA receptors and benzodiazepine binding sites in the posterior cingulate cortex and fusiform gyrus in autism. Brain Research. 2011; 1380: 218–228. https://doi.org/10.1016/j.brainres.2010.09.021.

[8] Wang R, Tan J, Guo J, Zheng Y, Han Q, So KF, *et al*. Aberrant Development and Synaptic Transmission of Cerebellar Cortex in a VPA Induced Mouse Autism Model. Frontiers in Cellular Neuroscience. 2018; 12: 500. https://doi.org/10.3389/fncel.2018.00500.

[9] Kelly E, Meng F, Fujita H, Morgado F, Kazemi Y, Rice LC, *et al*. Regulation of autism-relevant behaviors by cerebellar-prefrontal cortical circuits. Nature Neuroscience. 2020; 23: 1102–1110. https://doi.org/10.1038/s41593-020-0665-z.

[10] Schmahmann JD. The cerebellum and cognition. Neuroscience Letters. 2019; 688: 62–75. https://doi.org/10.1016/j.neulet.2018.07.005.

[11] Varghese M, Keshav N, Jacot-Descombes S, Warda T, Wicinski B, Dickstein DL, *et al*. Autism spectrum disorder: neuropathology and animal models. Acta Neuropathologica. 2017; 134: 537–566. https://doi.org/10.1007/s00401-017-1736-4.

[12] Sherkatghanad Z, Akhondzadeh M, Salari S, Zomorodi-Moghadam M, Abdar M, Acharya UR, *et al*. Automated Detection of Autism Spectrum Disorder Using a Convolutional Neural Network. Frontiers in Neuroscience. 2020; 13: 1325. https://doi.org/10.3389/fnins.2019.01325.

[13] Adorjan I, Ahmed B, Feher V, Torso M, Krug K, Esiri M, *et al*. Calretinin interneuron density in the caudate nucleus is lower in autism spectrum disorder. Brain: a Journal of Neurology. 2017; 140: 2028–2040. https://doi.org/10.1093/brain/awx131.

[14] Whitney ER, Kemper TL, Bauman ML, Rosene DL, Blatt GJ. Cerebellar Purkinje cells are reduced in a subpopulation of autistic brains: a stereological experiment using calbindin-D28k. Cerebellum (London, England). 2008; 7: 406–416. https://doi.org/10.1007/s12311-008-0043-y.

[15] Nicolini C, Fahnestock M. The valproic acid-induced rodent model of autism. Experimental Neurology. 2018; 299: 217–227. https://doi.org/10.1016/j.expneurol.2017.04.017.

[16] Vajda FJ, O'brien TJ, Hitchcock A, Graham J, Cook M, Lander C, *et al*. Critical relationship between sodium valproate dose and human teratogenicity: results of the Australian register of antiepileptic drugs in pregnancy. Journal of Clinical Neuroscience: Official Journal of the Neurosurgical Society of Australasia. 2004; 11: 854–858. https://doi.org/10.1016/j.jocn.2004.05.003.

[17] Wyszynski DF, Nambisan M, Surve T, Alsdorf RM, Smith CR, Holmes LB, *et al*. Increased rate of major malformations in offspring exposed to valproate during pregnancy. Neurology. 2005; 64: 961–965. https://doi.org/10.1212/01.WNL.0000154516.43630.C5.

[18] Clayton-Smith J, Bromley R, Dean J, Journel H, Odent S, Wood A, *et al*. Diagnosis and management of individuals with Fetal Valproate Spectrum Disorder: a consensus statement from the European Reference Network for Congenital Malformations and Intellectual Disability. Orphanet Journal of Rare Diseases. 2019; 14: 180. https://doi.org/10.1186/s13023-019-1064-y.

[19] Taleb A, Lin W, Xu X, Zhang G, Zhou QG, Naveed M, *et al*. Emerging mechanisms of valproic acid-induced neurotoxic events in autism and its implications for pharmacological treatment. Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie. 2021; 137: 111322. https://doi.org/10.1016/j.biopha.2021.111322.

[20] Bristot Silvestrin R, Bambini-Junior V, Galland F, Daniele Bobermim L, Quincozes-Santos A, Torres Abib R, *et al*. Animal model of autism induced by prenatal exposure to valproate: altered glutamate metabolism in the hippocampus. Brain Research. 2013; 1495: 52–60. https://doi.org/10.1016/j.brainres.2012.11.048.

[21] Bromley RL, Mawer GE, Briggs M, Cheyne C, Clayton-Smith J, García-Fiñana M, *et al*. The prevalence of neurodevelopmental disorders in children prenatally exposed to antiepileptic drugs. Journal of Neurology, Neurosurgery, and Psychiatry. 2013; 84: 637–643. https://doi.org/10.1136/jnnp-2012-304270.

[22] Petersen AK, Ahmad A, Shafiq M, Brown-Kipphut B, Fong CT, Anwar Iqbal M. Deletion 1q43 encompassing only CHRM3 in a patient with autistic disorder. European Journal of Medical Genetics. 2013; 56: 118–122. https://doi.org/10.1016/j.ejmg.2012.11.003.

[23] Chilian B, Abdollahpour H, Bierhals T, Haltrich I, Fekete G, Nagel I, *et al*. Dysfunction of SHANK2 and CHRNA7 in a patient with intellectual disability and language impairment supports genetic epistasis of the two loci. Clinical Genetics. 2013; 84: 560–565. https://doi.org/10.1111/cge.12105.

[24] Mikhail FM, Lose EJ, Robin NH, Descartes MD, Rutledge KD, Rutledge SL, *et al*. Clinically relevant single gene or intragenic deletions encompassing critical neurodevelopmental genes in patients with developmental delay, mental retardation, and/or autism spectrum disorders. American Journal of Medical Genetics. Part a. 2011; 155A: 2386–2396. https://doi.org/10.1002/ajmg.a.34177.

[25] Kim JW, Seung H, Kwon KJ, Ko MJ, Lee EJ, Oh HA, *et al*. Subchronic treatment of donepezil rescues impaired social, hyperactive, and stereotypic behavior in valproic acid-induced animal model of autism. PloS One. 2014; 9: e104927. https://doi.org/10.1371/journal.pone.0104927.

[26] Anand R, Amici SA, Ponath G, Robson JI, Nasir M, McKay SB. Nicotinic acetylcholine receptor alterations in autism spectrum disorders–biomarkers and therapeutic targets. IntechOpen: London, UK. 2011.

[27] Zaki AG, El-Sayed ESR, Abd Elkodous M, El-Sayyad GS. Microbial acetylcholinesterase inhibitors for Alzheimer's therapy: recent trends on extraction, detection, irradiation-assisted production improvement and nano-structured drug delivery. Applied Microbiology and Biotechnology. 2020; 104: 4717–4735. https://doi.org/10.1007/s00253-020-10560-9.

[28] Hu Q, Zhang R, Dong X, Yang D, Yu W, Du Q. Huperzine A ameliorates neurological deficits after spontaneous subarachnoid hemorrhage through endothelial cell pyroptosis inhibition. Acta Biochimica et Biophysica Sinica. 2024; 56: 645–656. https://doi.org/10.3724/abbs.2024037.

[29] Guo X, Wu Y, Wang Q, Zhang J, Sheng X, Zheng L, *et al*. Huperzine A injection ameliorates motor and cognitive abnormalities via regulating multiple pathways in a murine model of Parkinson's disease. European Journal of Pharmacology. 2023; 956: 175970. https://doi.org/10.1016/j.ejphar.2023.175970.

[30] Mei Z, Hong Y, Yang H, Sheng Q, Situ B. Huperzine A protects against traumatic brain injury through anti-oxidative effects via the Nrf2-ARE pathway. Iranian Journal of Basic Medical Sciences. 2021; 24: 1455–1461. https://doi.org/10.22038/IJBMS.2021.58169.12932.

[31] Friedli MJ, Inestrosa NC. Huperzine A and Its Neuroprotective Molecular Signaling in Alzheimer's Disease. Molecules (Basel, Switzerland). 2021; 26: 6531. https://doi.org/10.3390/molecules26216531.

[32] Mei Z, Zheng P, Tan X, Wang Y, Situ B. Huperzine A alleviates neuroinflammation, oxidative stress and improves cognitive function after repetitive traumatic brain injury. Metabolic Brain Disease. 2017; 32: 1861–1869. https://doi.org/10.1007/s11011-017-0075-4.

[33] Fereshetyan K, Chavushyan V, Danielyan M, Yenkoyan K. Assessment of behavioral, morphological and electrophysiological changes in prenatal and postnatal valproate induced rat models of autism spectrum disorder. Scientific Reports. 2021; 11: 23471. https://doi.org/10.1038/s41598-021-02994-6.

[34] Wagner GC, Reuhl KR, Cheh M, McRae P, Halladay AK. A new neurobehavioral model of autism in mice: pre- and postnatal exposure to sodium valproate. Journal of Autism and Developmental Disorders. 2006; 36: 779–793. https://doi.org/10.1007/s10803-006-0117-y.

[35] Moy SS, Nadler JJ, Young NB, Perez A, Holloway LP, Barbaro RP, *et al*. Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. Behavioural Brain Research. 2007; 176: 4–20. https://doi.org/10.1016/j.bbr.2006.07.030.

[36] Alghamdi BS. The Effect of Melatonin and Exercise on Social Isolation-Related Behavioral Changes in Aged Rats. Frontiers in Aging Neuroscience. 2022; 14: 828965. https://doi.org/10.3389/fnagi.2022.828965.

[37] Jiang H, Ashraf GM, Liu M, Zhao K, Wang Y, Wang L, *et al*. Tilianin Ameliorates Cognitive Dysfunction and Neuronal Damage in Rats with Vascular Dementia via p-CaMKII/ERK/CREB and ox-CaMKII-Dependent MAPK/NF-*κ*B Pathways. Oxidative Medicine and Cellular Longevity. 2021; 2021: 6673967. https://doi.org/10.1155/2021/6673967.

[38] Alqurashi GK, Hindi EA, Zayed MA, Abd El-Aziz GS, Alturkistani HA, Ibrahim RF, *et al*. The Impact of Chronic Unpredictable Mild Stress-Induced Depression on Spatial, Recognition and Reference Memory Tasks in Mice: Behavioral and Histological Study. Behavioral Sciences (Basel, Switzerland). 2022; 12: 166. https://doi.org/10.3390/bs12060166.

[39] Ornoy A, Weinstein-Fudim L, Ergaz Z. Prevention or Amelioration of Autism-Like Symptoms in Animal Models: Will it Bring Us Closer to Treating Human ASD? International Journal of Molecular Sciences. 2019; 20: 1074. https://doi.org/10.3390/ijms20051074.

[40] Schneider T, Przewłocki R. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology. 2005; 30: 80–89. https://doi.org/10.1038/sj.npp.1300518.

[41] Ellman GL, Courtney KD, Andres V, Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology. 1961; 7: 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

[42] Bajgar J, Hajek P, Slizova D, Krs O, Fusek J, Kuca K, *et al*. Changes of acetylcholinesterase activity in different rat brain areas following intoxication with nerve agents: biochemical and histochemical study. Chemico-biological Interactions. 2007; 165: 14–21. https://doi.org/10.1016/j.cbi.2006.10.006.

[43] Suvarna KS, Layton C, Bancroft JD. Bancroft's theory and practice of histological techniques E-Book, Immunohistochemical techniques. Bancroft's Theory and Practice of Histological Techniques (Eighth Edition). Elsevier: AMSTERDAM. 2019; 337–394. https://doi.org/10.1016/B978-0-7020-6864-5.00010-4.

[44] Ramos-Vara JA, Kiupel M, Baszler T, Bliven L, Brodersen B, Chelack B, *et al*. Suggested guidelines for immunohistochemical techniques in veterinary diagnostic laboratories. Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc. 2008; 20: 393–413. https://doi.org/10.1177/104063870802000401.

[45] D'Mello AM, Stoodley CJ. Cerebro-cerebellar circuits in autism spectrum disorder. Frontiers in Neuroscience. 2015; 9: 408. https://doi.org/10.3389/fnins.2015.00408.

[46] Rogers TD, Dickson PE, McKimm E, Heck DH, Goldowitz D, Blaha CD, *et al*. Reorganization of circuits underlying cerebellar modulation of prefrontal cortical dopamine in mouse models of autism spectrum disorder. Cerebellum (London, England). 2013; 12: 547–556. https://doi.org/10.1007/s12311-013-0462-2.

[47] van der Heijden ME, Gill JS, Sillitoe RV. Abnormal Cerebellar Development in Autism Spectrum Disorders. Developmental Neuroscience. 2021; 43: 181–190. https://doi.org/10.1159/000515189.

[48] Skefos J, Cummings C, Enzer K, Holiday J, Weed K, Levy E, *et al*. Regional alterations in purkinje cell density in patients with autism. PloS One. 2014; 9: e81255. https://doi.org/10.1371/journal.pone.0081255.

[49] Cho HS, Kim TW, Ji ES, Park HS, Shin MS, Baek SS. Treadmill exercise ameliorates motor dysfunction through inhibition of Purkinje cell loss in cerebellum of valproic acid-induced autistic rats. Journal of Exercise Rehabilitation. 2016; 12: 293–298. https://doi.org/10.12965/jer.1632696.348.

[50] Shona SI, Rizk AA, El Sadik AO, Emam HY, Ali EN. Effect of valproic acid administration during pregnancy on postnatal development of cerebellar cortex and the possible protective role of folic acid. Folia Morphologica. 2018; 77: 201–209. https://doi.org/10.5603/FM.a2017.0100.

[51] Kumar H, Sharma B. Memantine ameliorates autistic behavior, biochemistry & blood brain barrier impairments in rats. Brain Research Bulletin. 2016; 124: 27–39. https://doi.org/10.1016/j.brainresbull.2016.03.013.

[52] Cezar LC, Kirsten TB, da Fonseca CCN, de Lima APN, Bernardi MM, Felicio LF. Zinc as a therapy in a rat model of autism prenatally induced by valproic acid. Progress in Neuropsychopharmacology & Biological Psychiatry. 2018; 84: 173–180. https://doi.org/10.1016/j.pnpbp.2018.02.008.

[53] Wu H, Wang X, Gao J, Liang S, Hao Y, Sun C, *et al*. Fingolimod (FTY720) attenuates social deficits, learning and memory impairments, neuronal loss and neuroinflammation in the rat model of autism. Life Sciences. 2017; 173: 43–54. https://doi.org/10.1016/j.lfs.2017.01.012.

[54] Wang CY, Cheng CW, Wang WH, Chen PS, Tzeng SF. Postnatal Stress Induced by Injection with Valproate Leads to Developing Emotional Disorders Along with Molecular and Cellular Changes in the Hippocampus and Amygdala. Molecular Neurobiology. 2016; 53: 6774–6785. https://doi.org/10.1007/s12035-015-9600-9.

[55] Mony TJ, Lee JW, Dreyfus C, DiCicco-Bloom E, Lee HJ. Valproic Acid Exposure during Early Postnatal Gliogenesis Leads to Autistic-like Behaviors in Rats. Clinical Psychopharmacology and Neuroscience: the Official Scientific Journal of the Korean College of Neuropsychopharmacology. 2016; 14: 338–344. https://doi.org/10.9758/cpn.2016.14.4.338.

[56] Elnahas EM, Abuelezz SA, Mohamad MI, Nabil MM, Abdelraouf SM, Bahaa N, *et al*. Validation of prenatal versus postnatal valproic acid rat models of autism: A behavioral and neurobiological study. Progress in Neuro-psychopharmacology & Biological Psychiatry. 2021; 108: 110185. https://doi.org/10.1016/j.pnpbp.2020.110185.

[57] Gedzun V, Dubynin V, Malyshev A, Sarycheva N, Svinov M. Comparing models of autism spectrum disorder based on pre- and postnatal valproate exposure: behavioral and histological assessment. European Neuropsychopharmacology. 2017; 27: S679–S680.

[58] Yin JJ, He Y, An J, Miao Q, Sui RX, Wang Q, *et al*. Dynamic Balance of Microglia and Astrocytes Involved in the Remyelinating Effect of Ginkgolide B. Frontiers in Cellular Neuroscience. 2020; 13: 572. https://doi.org/10.3389/fncel.2019.00572.

[59] Ghassab-Abdollahi N, Mobasseri K, Dehghani Ahmadabad A, Nadrian H, Mirghafourvand M. The effects of Huperzine A on dementia and mild cognitive impairment: An overview of systematic reviews. Phytotherapy Research: PTR. 2021; 35: 4971–4987. https://doi.org/10.1002/ptr.7126.

[60] Ohba T, Yoshino Y, Ishisaka M, Abe N, Tsuruma K, Shimazawa

M, *et al*. Japanese Huperzia serrata extract and the constituent, huperzine A, ameliorate the scopolamine-induced cognitive impairment in mice. Bioscience, Biotechnology, and Biochemistry. 2015; 79: 1838–1844. https://doi.org/10.1080/09168451.2015.1052773.

[61] Zhang H, Wang D, Sun J, Wang Y, Wu S, Wang J. Huperzine-A Improved Animal Behavior in Cuprizone-Induced Mouse Model by Alleviating Demyelination and Neuroinflammation. International Journal of Molecular Sciences. 2022; 23: 16182. https://doi.org/10.3390/ijms232416182.

[62] Kim KC, Kim P, Go HS, Choi CS, Yang SI, Cheong JH, *et al*. The critical period of valproate exposure to induce autistic symptoms in Sprague-Dawley rats. Toxicology Letters. 2011; 201: 137–142. https://doi.org/10.1016/j.toxlet.2010.12.018.

[63] Markram K, Rinaldi T, La Mendola D, Sandi C, Markram H. Abnormal fear conditioning and amygdala processing in an animal model of autism. Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology. 2008; 33: 901–912. https://doi.org/10.1038/sj.npp.1301453.

[64] Reynolds S, Millette A, Devine DP. Sensory and motor characterization in the postnatal valproate rat model of autism. Developmental Neuroscience. 2012; 34: 258–267. https://doi.org/10.1159/000336646.

[65] Chaliha D, Albrecht M, Vaccarezza M, Takechi R, Lam V, Al-Salami H, *et al*. A Systematic Review of the Valproic-Acid-Induced Rodent Model of Autism. Developmental Neuroscience. 2020; 42: 12–48. https://doi.org/10.1159/000509109.

[66] Maenner MJ, Shaw KA, Baio J, EdS1, Washington A, Patrick M, *et al*. Prevalence of Autism Spectrum Disorder Among Children Aged 8 Years - Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2016. Morbidity and Mortality Weekly Report. Surveillance Summaries (Washington, D.C.: 2002). 2020; 69: 1–12. https://doi.org/10.15585/mmwr.ss6904a1.

[67] Mottron L, Duret P, Mueller S, Moore RD, Forgeot d'Arc B, Jacquemont S, *et al*. Sex differences in brain plasticity: a new hypothesis for sex ratio bias in autism. Molecular Autism. 2015; 6: 33. https://doi.org/10.1186/s13229-015-0024-1.

[68] Tian J, Gao X, Yang L. Repetitive Restricted Behaviors in Autism Spectrum Disorder: From Mechanism to Development of Therapeutics. Frontiers in Neuroscience. 2022; 16: 780407. https://doi.org/10.3389/fnins.2022.780407.

[69] Vakili Shahrbabaki SS, Jonaidi H, Sheibani V, Bashiri H. Early postnatal handling alters social behavior, learning, and memory of pre- and postnatal VPA-induced rat models of autism in a context-based manner. Physiology & Behavior. 2022; 249: 113739. https://doi.org/10.1016/j.physbeh.2022.113739.

[70] Mabunga DFN, Gonzales ELT, Kim JW, Kim KC, Shin CY. Exploring the Validity of Valproic Acid Animal Model of Autism. Experimental Neurobiology. 2015; 24: 285–300. https://doi.org/10.5607/en.2015.24.4.285.

[71] Kataoka S, Takuma K, Hara Y, Maeda Y, Ago Y, Matsuda T. Autism-like behaviours with transient histone hyperacetylation in mice treated prenatally with valproic acid. The International Journal of Neuropsychopharmacology. 2013; 16: 91–103. https://doi.org/10.1017/S1461145711001714.

[72] Rosen TE, Mazefsky CA, Vasa RA, Lerner MD. Co-occurring psychiatric conditions in autism spectrum disorder. International Review of Psychiatry (Abingdon, England). 2018; 30: 40–61. https://doi.org/10.1080/09540261.2018.1450229.

[73] Chen YW, Lin HC, Ng MC, Hsiao YH, Wang CC, Gean PW, *et al*. Activation of mGluR2/3 underlies the effects of N-acetylcystein on amygdala-associated autism-like phenotypes in a valproate-induced rat model of autism. Frontiers in Behavioral Neuroscience. 2014; 8: 219. https://doi.org/10.3389/fnbeh.2014.00219.

[74] Campolongo M, Kazlauskas N, Falasco G, Urrutia L, Salgueiro N, Höcht C, *et al*. Sociability deficits after prenatal exposure to valproic acid are rescued by early social enrichment. Molecular Autism. 2018; 9: 36. https://doi.org/10.1186/s13229-018-0221-9.

[75] Zohny SM, Habib MZ, Mohamad MI, Elayat WM, Elhossiny RM, El-Salam MFA, *et al*. Memantine/Aripiprazole Combination Alleviates Cognitive Dysfunction in Valproic Acid Rat Model of Autism: Hippocampal CREB/BDNF Signaling and Glutamate Homeostasis. Neurotherapeutics: the Journal of the American Society for Experimental NeuroTherapeutics. 2023; 20: 464–483. https://doi.org/10.1007/s13311-023-01360-w.

[76] Roullet FI, Lai JKY, Foster JA. In utero exposure to valproic acid and autism–a current review of clinical and animal studies. Neurotoxicology and Teratology. 2013; 36: 47–56. https://doi.org/10.1016/j.ntt.2013.01.004.

[77] Du Y, Liang H, Zhang L, Fu F. Administration of Huperzine A exerts antidepressant-like activity in a rat model of post-stroke depression. Pharmacology, Biochemistry, and Behavior. 2017; 158: 32–38. https://doi.org/10.1016/j.pbb.2017.06.002.

[78] Yang Y, Yang J, Jiang Q. The protective effect of huperzine A against hepatic ischemia reperfusion injury in mice. Transplantation Proceedings. 2014; 46: 1573–1577. https://doi.org/10.1016/j.transproceed.2014.01.018.

[79] Mao XY, Cao DF, Li X, Yin JY, Wang ZB, Zhang Y, *et al*. Huperzine A ameliorates cognitive deficits in streptozotocin-induced diabetic rats. International Journal of Molecular Sciences. 2014; 15: 7667–7683. https://doi.org/10.3390/ijms15057667.

[80] Mezeiova E, Soukup O, Korabecny J. Huprines—an insight into the synthesis and biological properties. Russian Chemical Reviews. 2020; 89: 999. https://doi.org/10.1070/RCR4938.

[81] Wang SSH, Kloth AD, Badura A. The cerebellum, sensitive periods, and autism. Neuron. 2014; 83: 518–532. https://doi.org/10.1016/j.neuron.2014.07.016.

[82] Yadav P, Shrestha S, Kushwaha R. Effect of folic acid in prenatal Pregabalin-induced cerebellar changes in the Swiss albino mice. International Journal of Therapeutic Applications. 2018; 35: 19–27.

[83] Galal AT, Sayed SA, Mubarak WA, Farag WG. Effect of Valproic Acid on Pre and Postnatal Development of the Cerebellar Cortex of the Albino Rat and the Possible Protective Role of the Folic Acid. The Egyptian Journal of Hospital Medicine. 2022; 89: 4215–4225.

[84] López-Cervantes M, Quintanar-Stephano A, Alcauter-Solórzano S, Hernández-Pando R, Aguilar-Roblero R, Gasca-Martínez D, *et al*. Cerebellar spongiform degeneration is accompanied by metabolic, cellular, and motor disruption in male rats with portacaval anastomosis. Journal of Neuroscience Research. 2021; 99: 2287–2304. https://doi.org/10.1002/jnr.24853.

[85] Sobaniec-Lotowska ME. Ultrastructure of Purkinje cell perikarya and their dendritic processes in the rat cerebellar cortex in experimental encephalopathy induced by chronic application of valproate. International Journal of Experimental Pathology. 2001; 82: 337–348. https://doi.org/10.1046/j.1365-2613.2001.00206.x.

[86] Young AMH, Chakrabarti B, Roberts D, Lai MC, Suckling J, Baron-Cohen S. From molecules to neural morphology: understanding neuroinflammation in autism spectrum condition. Molecular Autism. 2016; 7: 9. https://doi.org/10.1186/s13229-016-0068-x.