Alpha Psychiatry

ORIGINAL ARTICLE
Alpha Psychiatry 2023;24(1):22-31
DOI: 10.5152/alphapsychiatry.2023.22975

Torasemide Improves the Propionic Acid-Induced Autism in Rats: A Histopathological and Imaging Study

ABSTRACT

Objective: Autism spectrum disorder is a neurodevelopmental disease in which impaired social behaviors, impaired sociality, and restricted and repetitive behaviors are seen. Bumetanide is a loop diuretic that inhibits Na^+–K^+–$2Cl^-$ cotransporter 1 and it is currently used in clinical phase studies in patients with autism spectrum disorder. In present research, it is purposed to demonstrate the beneficial effects of torasemide which is another Na^+–K^+–$2Cl^-$ cotransporter 1 inhibitor on an experimental autism model induced with propionic acid by providing imaging and brain tissue investigations.

Methods: Male Wistar rats were used in the present study (n = 30). Propionic acid of 250 mg/kg/day was administrated intraperitoneally in rats to induce autism for 5 days. Three groups were created for present study as follows: group 1, normal control (n = 10); group 2, propionic acid and saline given group (n = 10); group 3, propionic acid + torasemide-administrated group (n = 10).

Results: Torasemide group scored higher on behavioral tests compared to saline group. The brain levels of malondialdehyde, tumor necrosis factor-alpha, interleukin-2, interleukin-17, and Nuclear Factor kappa B (NF-κB), Glial fibrillary acidic protein (GFAP) were remarkably higher in propionic acid + saline group. In histopathology assessments, torasemide group had higher neuronal count of Cornu Ammonis 1, neuronal count of Cornu Ammonis 2 in hippocampus, and Purkinje cells in cerebellum. GFAP immunostaining index (Cornu Ammonis 1) and cerebellum were lower in torasemide group. Magnetic resonance spectroscopy revealed that mean lactate value was higher in propionic acid + saline group compared to torasemide group.

Conclusion: Our experimental results showed that torasemide might enhance gamma-aminobutyric acid activity. Torasemide can be considered another promising Na^+–K^+–$2Cl^-$ cotransporter 1 inhibitor in the treatment of autism with a longer half-life and less side effects after further studies.

Keywords: Autism, diuretic, torasemide, treatment

Introduction

Autism spectrum disorder (ASD) is a common neurodevelopmental disorder that is characterized by abnormal social behaviors, impaired interaction, difficulties in interests and activities, and repetitive behaviors.[1] Language development and motor abnormalities are commonly disturbed in ASD.[2] The ASD is seen as common as 1 in 150 children globally.[3] The gender difference is important in ASD and men are affected 3 times more than women.[4]

Although ADS is considered a common disorder, the exact etiology cannot be defined yet.[4] Moreover, the current therapeutic approaches are so restricted based on insufficient knowledge of etiology as well as associations between the environment and behaviors. Because obtaining brain tissue from living humans is nearly impossible in daily practice and researches, animal models are important for identifying the etiology of ASD and for creating treatment strategies. There have been several environmental agents which were used for creating animal model of ASD.[5] Propionic acid (PPA) is a potential candidate which causes

Murat Doğan[1]
Yakup Albayrak[2]
Oytun Erbaş[3]

[1]*Hekim Medical Company, İstanbul, Turkey*
[2]*Department of Psychiatry, Tekirdağ Namık Kemal University Faculty of Medicine, Tekirdağ, Turkey*
[3]*Department of Physiology, Demiroğlu Bilim University Faculty of Medicine, İstanbul, Turkey*

Corresponding author:
Yakup Albayrak
dr.fuge@hotmail.com

Received: June 1, 2022
Accepted: October 11, 2022
Publication Date: January 6, 2023

Cite this article as: Doğan M, Albayrak Y, Erbaş O. Torasemide improves the propionic acid-induced autism in rats: A histopathological and imaging study. *Alpha Psychiatry.* 2023;24(1):22-31.

several behavioral and neuro-inflammatory changes in the rat model of ASD. Actually, PPA is a metabolic outcome of enteric bacteria and it has ability to cross the gut–blood and gut–brain barriers. After transmission, PPA accumulates in the cells and leads intracellular acidification[6,7]; thus, it can affect the normal functions of numbers of neurotransmitters.[7] Currently, PPA is considered as a well-established agent for the development of experimental model of ASD.[8-14]

The imbalance between neurotransmitters which have excitatory and inhibitor properties is known to be an etiological factor for ASD.[15] Gamma-aminobutyric acid (GABA) is a pivotal inhibitory neurotransmitter in the central nervous system (CNS). The inhibitor effect of GABAergic neurotransmission is highly important for brain development as well as neurotransmission regulation. Thus, GABA has been taken great interest in the etiologies of numerous neurological and mental diseases including schizophrenia, depression, and anxiety.[16,17] The experimental studies have also demonstrated that dysfunction in GABAergic signaling pathway was significantly associated with core symptoms of ASD.[18] The high percentage of comorbidity of epilepsy with ASD also confirms this suggestion.[19]

The accumulating evidence demonstrated that the Na^+–K^+–$2Cl^-$ cotransporter 1 (NKCC1) and K^+–Cl^- cotransporter 2 (KCC2) were significantly associated with GABAergic signaling.[20] The chloride importer NKCC1 and the chloride exporter KCC2 play important roles in GABA receptors.[21] Bumetanide is an NKCC1 inhibitor, and clinical phase studies showed that use of oral solution form of bumetanide had improved the symptoms including social reciprocity and behavioral symptoms in children and adolescents with ASD.[22-25] Torasemide is a non-acid loop diuretic which also inhibits NKCC1, similar to bumetanide.[26] However, there is neither any clinical nor an experimental study that investigated the effects of torasemide on ASD symptoms. There have been 2 studies which investigated the effects of torasemide on experimental-induced seizures. Hampel et al[21], in his first study, investigated the pharmacokinetic-pharmacodynamic properties of torasemide, azosemide, and bumetanide in mouse brain. They demonstrated that all 3 agents hardly pass the blood–brain barrier (BBB); however, free brain concentrations of bumetanide and torasemide were found to be within the range of NKCC1 inhibitory activity. The later study, which was also reported by Hampel et al[27], again demonstrated that azosemide, torasemide, and butenamide had similar effects in experimental-induced epilepsy model.

This research aimed to demonstrate the beneficial effects of torasemide on the PPA-induced autism model by providing imaging and brain tissue investigations.

MAIN POINTS

- *Torasemide was found to have beneficial effects on behavioral tests.*
- *Torasemide group had favorable results compared to saline group in terms of brain neurochemical findings and histopathological comparisons.*
- *Magnetic resonance spectroscopy assessment supported the superiority of torasemide to saline group.*
- *Torasemide can be considered a promising agent in the treatment of autism.*

Methods

Animals

This study used 30 male albino Wistar rats weighing 150-200g and 10-12 weeks of age. This study was certified by Local Animal Ethics Committee (Demiroğlu Bilim University, number: 19210702). The rats which were used in present study were from Experimental Animal laboratory of Demiroğlu Bilim University.

Experimental Stages

For this study, 30 male Wistar rats were taken. Propionic acid of 250 mg/kg/day was given intraperitoneally to 20 rats to induce autism for 5 days. Ten rats were selected as control. The PPA-exposed rats were grouped as follows: group 2 (PPA + saline, n = 10): PPA via oral gavage 1 mL/kg/day 0.9% NaCl saline group; group 3 (PPA + torasemide, n = 10) PPA via oral gavage 1 mg/kg/day torasemide (Sutril tablets 5 mg, Adeka). The rats in study groups were treated for 15 days. By the fifteenth day, the behavioral tests were tested in all groups. Animals were assessed by magnetic resonance (MR) spectroscopy procedure under ketamine anesthesia (50 mg/kg).

Behavioral Tests

Three-Chamber Sociability Test: This test was performed on rats as previously described with minor modifications.[28,29]

Open-Field: The open field test was performed in present study as previously described.[28,29]

Passive Avoidance Learning: This behavioral test was performed according to previous data.[30]

Magnetic Resonance Imaging Protocol and Magnetic Resonance Spectroscopy

Conventional Magnetic Resonance Imaging: A 3.0 T MRI/magnetic resonance spectroscopy (MRS) scanner (Magnetom, Siemens Healthcare, Germany) was used for evaluation. Conventional MR sequences were performed as previously described.[31]

Magnetic Resonance Spectroscopy: Magnetic resonance spectroscopy was administrated as previously described[31] and Magnetom software was used to assess the raw data which were stored in a workstation.[31]

Hippocampus and Cerebellum Histopathology

The Cornu Ammonis (CA) 1 and CA 3 regions of hippocampus were selected for the investigations of hippocampus. The brains of rats were removed and stored for 3 days in 10% formaldehyde in 0.1 M phosphate-buffer saline. The histopathological examinations were performed according to previous descriptions.[32] GFAP immunohistochemistry was performed by primary antibodies against GFAP (Abcam, Inc., Mass, USA; 1/1000) for 24 hours. Olympus BX51 microscope was used for obtaining the histopathological images. GFAP immunostaining index was calculated as follows: GFAP-positive cells were counted at 40× magnification in randomized sections (3-4) for each rat. An imaging system (Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA) was used for the assessments.[8,33]

Brain Biochemical Analysis

The brain levels of nerve growth factor (NGF), TNF-α, interleukin (IL)-2, IL-17, and lactate levels in the brain were assessed by enzyme-linked

immunosorbent assay kits. Confirmation was made for all investigations. A microplate reader was used for the measurement of the Absorbances (MultiscanGo, Thermo Fisher Scientific Laboratory Equipment, NH, USA).[33]

Measurement of Brain Lipid Peroxidation

Lipid peroxidation was assessed by measuring the levels of malondialdehyde (MDA). Malondialdehyde levels were analyzed with a standard calibrator using tetraethoxypropane.[33]

Measurement of Brain Protein Levels

Bradford's method was used for all measurements of proteins.

Statistical Analysis

The obtained data were assessed by Statistical Package for Social Sciences version 15.0 for Windows (SPSS Inc.; Chicago, IL, USA). For defining normality and homogeneity of variance, Shapiro–Wilk's and Levene's tests were used. Analysis of variance test was used to compare the numerical data between the groups. Tukey test was used for post hoc analysis. The results are presented as mean ± standard deviation. The value of $P < .05$ was accepted as statistically significant.

Results

Behavioral Tests

Sociability test (time spent with stranger rat percent) mean point was higher in PPA + torasemide and control groups compared to PPA + saline group ($P = .0006$, $P = .008$, and $P < .007$, respectively). The control group had higher scores on sociability test compared to PPA + torasemide group ($P = .048$). The mean score of open field test (number of ambulation) was found to be higher in control and PPA + torasemide groups compared to PPA + saline group ($P = .001$, $P = .0009$, and $P = .04$, respectively) and it was similar between control and PPA + torasemide groups ($P = .051$). The mean scores of passive avoidance learning (PAL) latency were also higher in control group and PPA + torasemide group compared to PPA + saline group ($P = .001$, $P = .007$, and $P = 041$, respectively). The control group had higher scores on PAL test compared to PPA + torasemide group ($P = .044$) (Table 1).

Results of Brain Tissue Investigations

Brain MDA level was found to be higher in PPA + saline group compared to control group and PPA + torasemide group ($P = .0001$, $P = .0006$, and $P = .031$, respectively). It was also higher in PPA + torasemide group compared to control group ($P = .001$). Brain TNF-α level was higher in PPA + saline group compared to control group and PPA + torasemide group ($F = 8.99$; $P = .0001$, $P = .0007$, and $P = .043$, respectively) and it was higher in PPA + torasemide group compared to control group ($P = .045$). Brain IL-2 level was found to be higher in PPA + saline group than in control and PPA + torasemide groups ($P = .0008$, $P = .0009$, and $P = .021$, respectively). Brain IL-2 level was higher in PPA + torasemide group compared to control group ($P = .001$). Brain IL-17 level (pg/mg protein) was found to be higher in PPA + saline group compared to control group and PPA + torasemide group ($P = .0005$, $P = .0007$, and $P = .030$, respectively). Brain IL-17 level was also higher in PPA+ torasemide group than in control group ($P = .034$). Brain NF-KB level was higher in PPA + saline group than control and PPA + torasemide groups ($P = .0007$, $P = .0008$, and $P = .010$, respectively) and it was found to be higher in PPA + torasemide group compared to control group ($P = .004$). Brain lactate level was higher in both PPA + saline and PPA+ torasemide groups compared to control group ($P = .0004$; $P = .0005$, and $P = .0006$, respectively) and it was higher in PPA + saline group compared to PPA + torasemide group ($P = .001$). Brain NGF level was higher in control group and PPA + torasemide group compared to PPA + saline group ($P = .0001$, $P = .0001$, and $P = .0002$, respectively). It was also found to be higher in PPA + torasemide group compared to control group ($P = .0009$). The results are presented in Table 2.

Hippocampus and Cerebellum Histopathology

Neuronal count CA1 was higher in control and PPA + torasemide groups compared to PPA + saline group ($P = .0004$, $P = .0006$, and $P = .013$, respectively). It was also higher in control group compared to PPA + torasemide group ($P = .003$). Neuronal count CA3 was higher in control and PPA + torasemide groups compared to PPA + saline group ($P = .0001$, $P = .0004$, and $P = .0003$, respectively). Neuronal count CA3 was found to be similar between control and PPA + torasemide groups (P = .621). GFAP (CA1) immunostaining index was found to be lower in control and PPA + torasemide groups compared to PPA + saline group ($P = .0003$, $P = .0004$, and $P = .0005$, respectively). It was found to be similar between control and PPA + torasemide groups ($P = .699$). GFAP immunostaining index (CA3) was also lower in control group and PPA + torasemide group than PPA + saline group ($P = .007$, $P = .008$, and $P = .031$, respectively). GFAP immunostaining index (CA3) was also higher in PPA + torasemide group compared to control group ($P = .037$). Purkinje count cerebellum was found to be higher in control group and PPA + torasemide group ($P = .006$, $P = .008$, and $P = .030$, respectively). Purkinje count cerebellum was found to be similar between PPA + torasemide group and control

Table 1. Comparison of the Results of Behavioral Test Between Groups

	Normal Control (n = 10)	PPA and Saline (n = 10)	PPA and Torasemide (n = 10)	Post Hoc Tukey
Sociability test: time spent with stranger rat percent (%)	61.8 (9.5)	30.7 (5.1)	51.9 (6.6)	**P = .008[a]** **P = .007[b]** **P = .048[c]**
Open field test: number of ambulation	13.5 (2.3)	6.9 (1.2)	10.1 (2.5)	**P = .0009[a] P = .040[b]** **P = .051[c]**
Passive avoidance learning (PAL) latency (Sec.)	285.2 (23.5)	101.5 (56.8)	211.2 (42.4)	**P = .007[a]** **P = .041[b]** **P = .044[c]**

[a]Normal control and PPA and saline; [b]PPA and torasemide and PPA and saline; [c]Normal control and PPA and torasemide.
Significant values are presented as bold characters.
PPA, propionic acid.

Table 2. Comparison of the Results of Brain Tissue Analysis Between Groups

	Normal Control (n = 10)	PPA and Saline (n = 10)	PPA and Torasemide (n = 10)	Post Hoc Tukey
Brain MDA level (nmol/g protein)	54.8 (9.2)	179.5 (11.7)	115.5 (9.5)	***P* = .0006[a] *P* = .031[b] *P* = .001[c]**
Brain TNF-alpha level (pg/mg protein)	14.1 (2.6)	113.1 (15.5)	68.7 (8.2)	***P* = .0007[a] *P* = .043[b] *P* = .045[c]**
Brain IL-2 level (pg/g protein)	2.1 (0.2)	265.3 (19.5)	166.1 (17.8)	***P* = *P* = 0.0009 [a] *P* = . 021 [b] *P* = .001[c]**
Brain IL-17 level (pg/g protein)	225.8 (12.3)	558.1 (16.6)	367.7 (21.1)	***P* = .0007 [a] *P* = .030 [b] *P* = .034 [c]**
Brain NF-KB level (pg/g protein)	17.5 (1.8)	232.4 (21.3)	104.1 (6.2)	***P* = .0008 [a] *P* = .011 [b] *P* = .004 [c]**
Brain lactate level (mmol/100 g wet weight)	1.12 (0.01)	3.36 (0.2)	2.62 (0.4)	***P* = .0006[a] *P* = .0005[b] *P* = .001[c]**
NGF level (pg/mg protein)	82.5 (5.7)	41.9 (4.5)	50.1 (7.7)	***P* = .0001[a] *P* = . 002[b] *P* = .0009[c]**

[a]Normal control and PPA and saline; [b]PPA and torasemide and PPA and saline; [c]Normal control and PPA and torasemide.
IL, interleukin; MDA, malondialdehyde; NGF, nerve growth factor; TNF, tumor necrosis factor alpha. Significant values are presented as bold characters.

group (*P* = .521). GFAP immunostaining index (cerebellum) was found to be lower in control group and PPA + torasemide group (*P* = .008, *P* = .009, and *P* = .023, respectively) compared to PPA + saline group. GFAP immunostaining index (cerebellum) was similar between the PPA + torasemide and control groups (*P* = .519). Figures 1, 2, 3, and 4 demonstrate the histopathology examinations (Figure 1-4) (Table 3).

Magnetic Resonance Spectroscopy

Mean lactate value (percentage of normal control) was higher in PPA + saline group compared to control group and PPA + torasemide group (*P* = .0003, *P* = .0007, and *P* = .0004, respectively) and it was higher in PPA + torasemide group compared to control group (*P* = .019) (Figure 5) (Table 3).

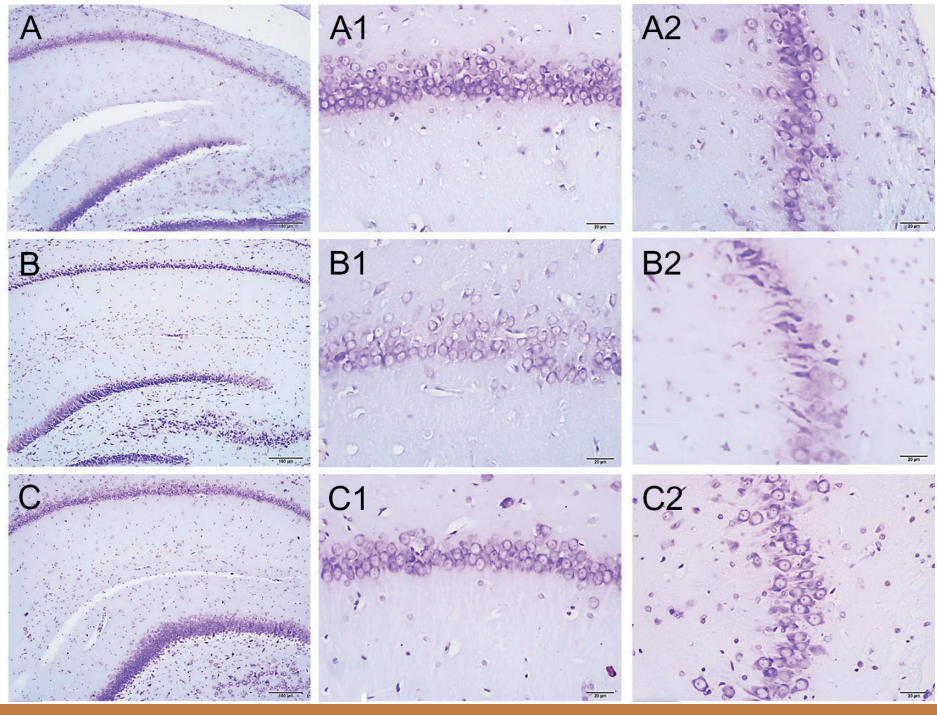

Figure 1. CA3 and CA1 regions of hippocampus in Crystal violet stain 4 at 40× magnification. A-A1-A2: normal control group male rats (CA3 and CA1). Normal pyramidal neuron. B-B1-B2: PPA and saline group male rats have neural body degeneration and decreased neural count and dysmorphological changes (CA3 and CA1). C-C1-C2: PPA and torasemide group male rats have increased neural count and improved neural morphology changes (CA3 and CA1). PPA, propionic acid.

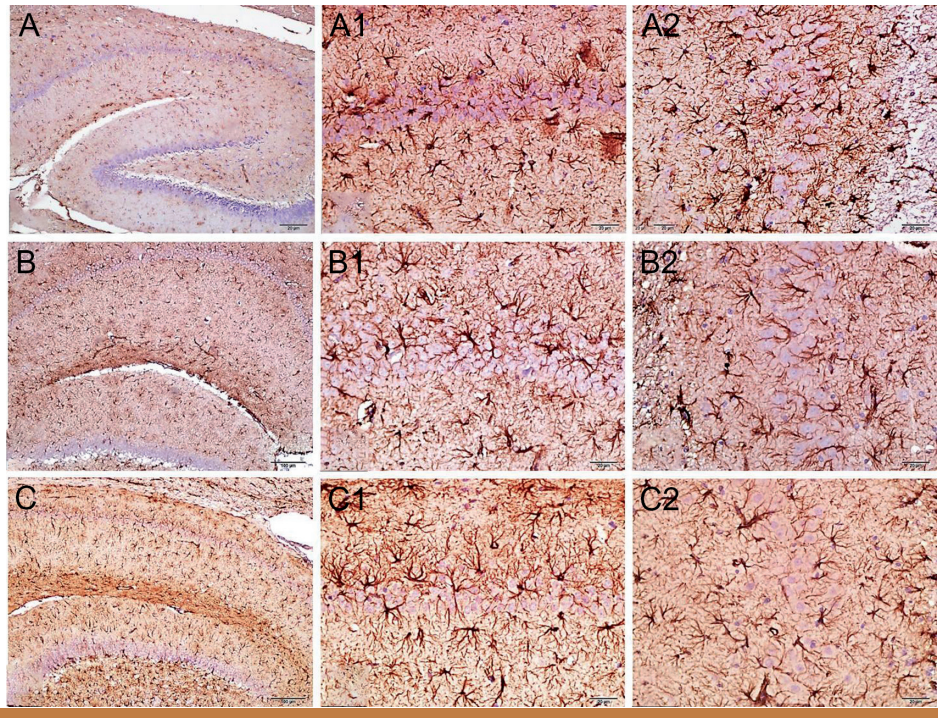

Figure 2. CA3 and CA1 of hippocampus at 40× magnification. Astrogliosis was characterized by GFAP immunostaining (Brown staining). A-A1-A2: normal control group male rats (CA3 and CA1), B-B1-B2: PPA and saline group male rats have increased glial activity (CA3 and CA1). C-C1-C2: PPA and torasemide group male rats have decreased glial activity (CA3 and CA1). PPA, propionic acid.

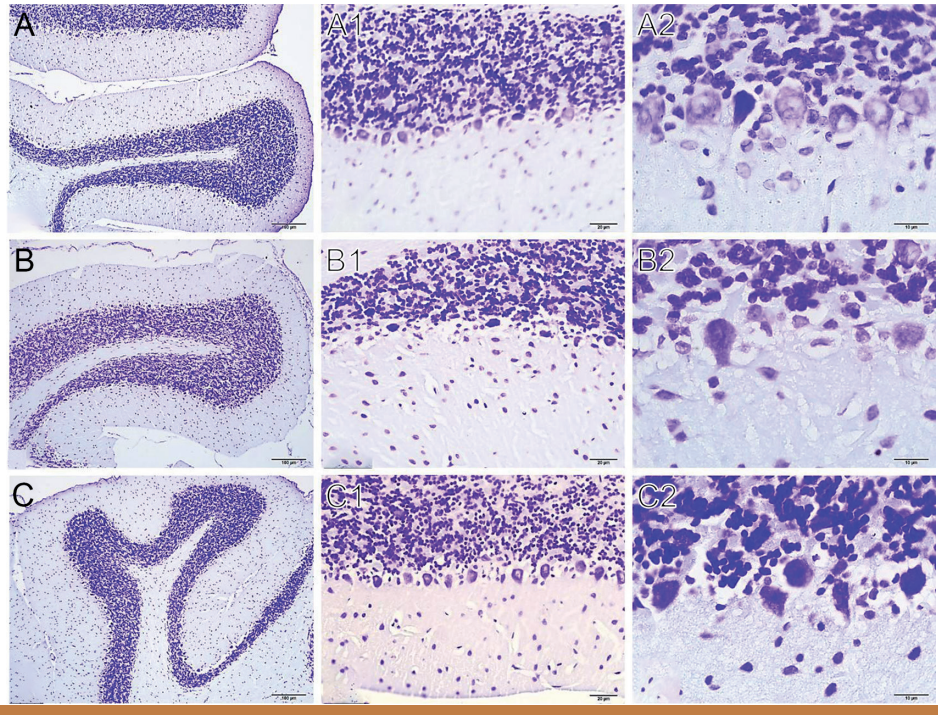

Figure 3. Cerebellum at 4×, 40×, 100× magnifications in crystal violet stain. A-A2-A3: normal control group male rats' cerebellum and normal Purkinje neuron. B-B1-B2: PPA and saline group male rats have decreased count and dysmorphological Purkinje neuron. C-C1-C2: PPA and torasemide group male rats have increased count and improved neural morphological changes Purkinje neuron. PPA, propionic acid.

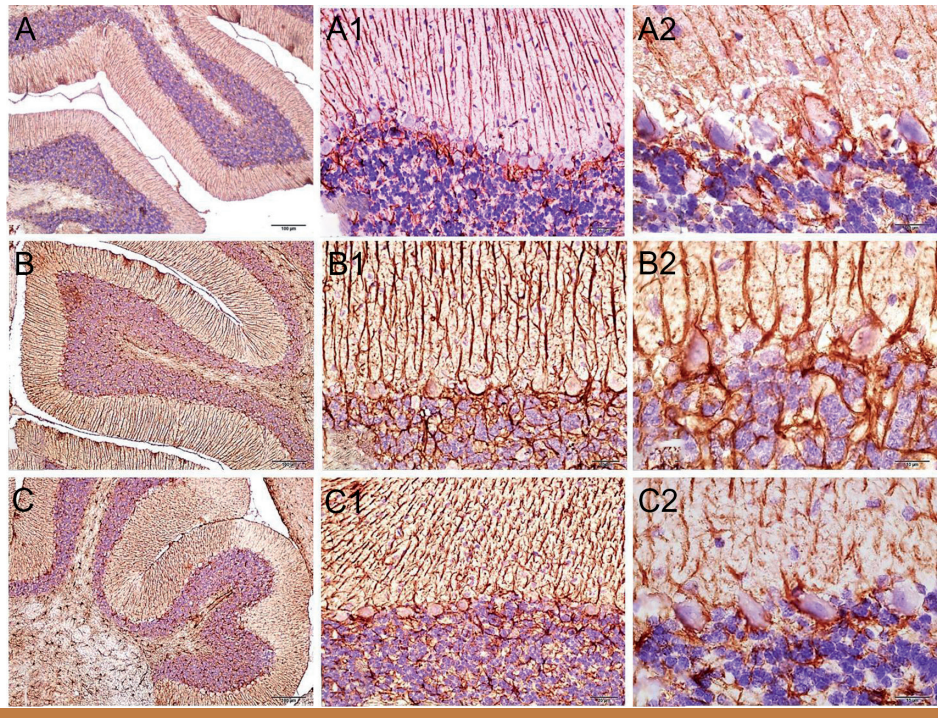

Figure 4. Cerebellum at 4×, 40×, 100× magnifications. Astrogliosis was characterized by GFAP immunostaining (Brown staining). A-A1-A2: normal control group male rats' cerebellum and normal Purkinje neuron. B-B1-B2: PPA and saline group male rats have increased glial activity cerebellum. C-C1-C2: PPA and torasemide group male rats have decreased glial activity cerebellum. PPA, propionic acid.

Table 3. Comparison of the Results of Hippocampus and Cerebellum Histopathology and MR Spectroscopy Between Groups

	Normal Control (n = 10)	PPA and Saline (n=10)	PPA and Torasemide (n=10)	Post Hoc Tukey
Neuronal count (CA1)	81.5 (3.3)	35.2 (4.1)	56.5 (4.4)	***P*=.0006**[a] ***P*=.013**[b] ***P*=.003**[c]
Neuronal count (CA3)	46.3 (1.8)	30.9 (2.2)	45.8 (3.2)	***P*=.0004**[a], ***P*=.0003**[b] ***P*=.621**[c]
GFAP immunostaining index (CA1)	34.5 (2.8)	48.5 (5.3)	35.1 (4.5)	***P*=.0004**[a] ***P*=.0005**[b] ***P*=.699**[c]
GFAP immunostaining index (CA3)	33.9 (3.9)	53.2 (4.8)	43.3 (2.9)	***P*=.008**[a] ***P*=.031**[b] ***P*=.037**[c]
Purkinje count cerebellum	17.5 (2.3)	11.1 (1.5)	15.2 (0.9)	***P*=.008**[a] ***P*=.030**[b] ***P*=.521**[c]
GFAP immunostaining index (cerebellum)	16.1 (2.8)	27.7 (1.8)	19.8 (1.7)	***P*=.009**[a] ***P*=.023**[b] ***P*=.519**[c]
MR spectroscopy lactate value (% of normal control)	100	265.1 (54.8)	149.8 (33.5)	***P*=.0007**[a] ***P*=.0004**[b] ***P*=.019**[c]

[a]Normal control and PPA and saline;[b]PPA and torasemide and PPA and saline;[c]Normal control and PPA and torasemide.
MR Lac, magnetic resonance spectroscopy lactate value (% of normal Control); NC, neuronal count; Purkinje, Purkinje count cerebellum.
Significant values are presented as bold characters.

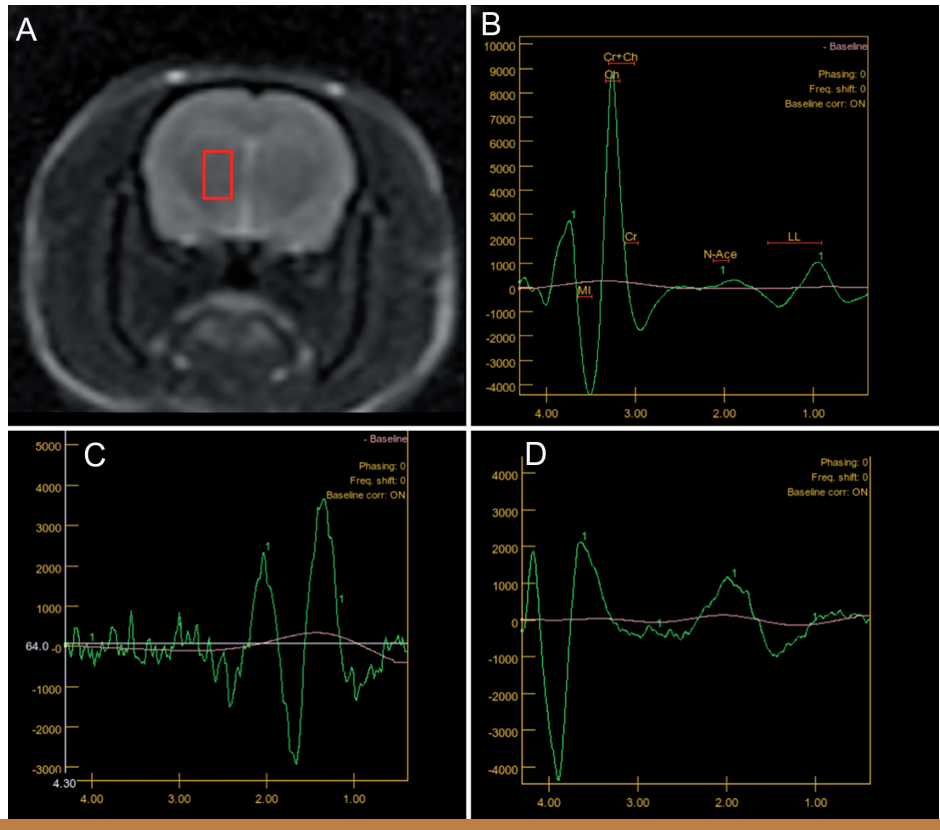

Figure 5. A-D. Magnetic resonance spectroscopy. (A): Magnetic resonance spectroscopy chosen area (Red box), (B): normal control group male rats, (C): PPA and saline group male rats, (D): PPA and torasemide group male rats. PPA, propionic acid.

Discussion

The pharmacological management of ASD does not exist yet and the current medical approaches in the management of ASD are focused on the improvement of behavioral symptoms and co-existing conditions.[34] The patients with ASD are currently attempted to be treated with educational and behavioral interventions.[35] The available pharmacological treatments are only approved for the improvement of behavioral symptoms.[36] Thus, it can be considered that any proof for the treatment of ASD will be of great interest for both individuals who are suffering from ASD and the researchers.

Bumetanide which is initially used as loop diuretic is currently being considered for the treatment of main symptoms of ASD.[22-25] In present study, we demonstrated that scores on open field test, sociability test, and passive avoidance learning were significantly higher in torasemide group compared to saline group. Our results indicate that torasemide improved ASD symptoms in experimental conditions. Gamma-aminobutyric acid signaling is the major inhibitor system in CNS and plays significant roles in cognitive functions and in the development of CNS.[18] The GABA neurons are regulated by the levels of intracellular chloride and they are able to be controlled by NKCC1 and KCC2.[37] Thus, an imbalance between excitatory and inhibitory neuronal activity is known to be related to impairments in communication skills, language, and sensory perception.[38] Increasing the activity of GABA signaling effects was reported to be associated to enhance the activity of NKCC1.[39] Hampel et al[21] compared the pharm acokinetic-pharmacodynamics properties of torasemide, azosemide, and bumetanide in mouse brain. All 3 drugs were shown to have free plasma levels which were sufficient to block NKCC1 in CNS. They concluded that azosemide and torasemide would be promising alternatives for CNS disorders with their longer half-life as well as their lower diuretic potency. There is no study that investigated the effects of torasemide on ASD. From this point, torasemide is a promising agent for improving the main symptoms of ASD with a higher half-life and without lower side effects.

The experimental model of ASD which was performed by intracereb roventricular delivery of PPA was shown to increase TNF-α, IL-6, and interferon-γ cytokine levels. [40,41] In the present study, it was also shown that brain levels of MDA, IL 2, IL 17, TNF-α, and NF-KB which are well-known inflammatory factors were remarkably higher in PPA + saline group compared to other 2 groups. Actually, there is not any evidence that indicates the effects of torasemide on neuroinflammation. However, several studies reported that torasemide had anti- inflammatory and anti-fibrotic effects on heart tissue.[42,43] Regarding the decreased levels of MDA, IL 2, IL 17, TNF-α, and NF-KB in torasemide group compared to saline group in the present study, it can be suggested that torasemide can have beneficial effects on the neurobiological errors in the brain tissue of ASD via the prevention of neuro-inflammation. Nerve growth factor is one of the well-known neurotrophic factor which plays a particular role in cell death and/or neurodegeneration.[44-46] The data which identified the associations between brain NGF level and ASD are restricted. Lu et al[47] reported that several NGF single-nucleotide polymorphism could be related to deficits in nonverbal communication. We also demonstrated that

torasemide group had significantly higher NGF level than saline group. These results can also be associated with anti-inflammatory action of torasemide.

Numerous evidences indicated that hippocampus is an important part of the brain in terms of etiology of ASD.[48-50] Moreover, cognitive dysfunctions were reported to be significantly associated with hippocampal neurotransmission.[51] The networks in amygdala and hippocampus are considered to be the main region for the "social brain."[52] Thus, the abnormalities in hippocampus were reported to be significantly related to core symptoms of ASD.[53] Evidences demonstrated that abnormality in glial cells could be significantly related to ASD.[54,55] Particularly, GFAP was reported to be expressed more in the ASD brain compared to healthy controls.[56] Furthermore, reduced microbiota complexity was shown to be related to impaired microglial proliferation in an experimental model.[57] Considering our results, torasemide seems to have beneficial effects on cell count as well as microglial formation in PPA-induced autism model.

The cerebellar dysfunctions were reported to be associated with ASD.[58] However, the exact etiological model cannot be explained yet. The cerebellar abnormalities in patient with ASD were considered to be related to main symptoms of ASD including social, cognitive, language, and movement symptoms.[59,60] The clinical studies also confirmed the existence of decreased cerebellar volume in patients with ASD.[61-63] Purkinje cells also take interest in terms of etiology of ASD.[64] The reduce in the amount of Purkinje cells in patients with ASD is reported to be related to alterations in cerebellar proteins which are associated with apoptosis (Bcl2 and p53),[65] astroglial activation (GFAP),[66] GABAergic signaling pathway (GAD65/67), and glutamatergic synaptic functions.[67] In the present study, the cerebellar findings in PPA + saline group support the results of previous studies. Furthermore, Purkinje cell lost and GFAP levels were found to be less in PPA + torasemide group. This novel finding provides the enhancing effects of torasemide on GABAergic neurons.

There have been several in vivo studies that investigated the brain lactate levels which is a biomarker of mitochondrial disease in ASD with magnetic resonance spectroscopy (1H).[68-70] The most current one showed increased lactate levels in patients with ASD compared to healthy subjects. In this study, it was concluded that mitochondrial dysfunction would be a neurobiological factor in ASD.[71] In the present study, we succeeded to demonstrate increased lactate levels in PPA + saline group in both tissue investigation and MRS imaging.

The comparison of bumetanide and torasemide would give stronger neurobiological, histopathological, and imaging proofs for torasemide. However, bumetanide is not available in our country; thus, we could not compare the effects of bumetanide and torasemide. This issue is a limitation of this study. However, this limitation will be an interest to further clinical and experimental studies.

Conclusion

The present study is the first to show the benefits of torasemide on the main symptoms of ASD experimentally. Our experimental results showed that torasemide might enhance GABA activity. Torasemide can be considered another promising NKCC1 inhibitor in the treatment of autism with a longer half-life and less side effects after further studies.

Ethics Committee Approval: *The experimental procedures employed in the present study were certified by the Animal Ethics Committee (Demiroğlu Bilim University, no: 19210702).*

Informed Consent: *N/A*

Peer-review: *Externally peer-reviewed.*

Author Contributions: *Concept – M.D.; Design – O.E.; Supervision – M.D.; Materials – M.D.; Data Collection and/or Processing – O.E.; Analysis and/or Interpretation – O.E.; Literature Review – M.D.; Writing Manuscript – Y.A.; Critical Review – Y.A.*

Declaration of Interests: *The authors have no conflicts of interest to declare.*

Funding: *The authors declared that this study has received no financial support.*

References

1. American Psychological Association, *American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders*. 5th ed; 2013. [CrossRef]
2. Bhat S, Acharya UR, Adeli H, Bairy GM, Adeli A. Autism: cause factors, early diagnosis and therapies. *Rev Neurosci* 2014;25(6):841-850. [CrossRef]
3. Marotta R, Risoleo MC, Messina G, et al. The neurochemistry of autism. *Brain Sci*. 2020;10(3):163. [CrossRef]
4. Lotufo Denucci B, Silva de Lima L, Ferreira Lima Mota I, et al. Current knowledge, challenges, new perspectives of the study, and treatments of autism Spectrum Disorder. *Reprod Toxicol*. 2021;106:82-93. [CrossRef]
5. Lawler CP, Croen LA, Grether JK, Van de Water J. Identifying environmental contributions to autism: provocative clues and false leads. *Ment Retard Dev Disabil Res Rev*. 2004;10(4):292-302. [CrossRef]
6. Bonnet U, Bingmann D, Wiemann M. Intracellular pH modulates spontaneous and epileptiform bioelectric activity of hippocampal CA3-neurones. *Eur Neuropsychopharmacol*. 2000;10(2):97-103. [CrossRef]
7. El-Ansary AK, Bacha AB, Kotb M. Etiology of autistic features: the persisting neurotoxic effects of propionic acid. *J Neuroinflammation*. 2012;9:74. [CrossRef]
8. Sever IH, Ozkul B, Bozkurt MF, Erbas O. Therapeutic effect of finasteride through its antiandrogenic and antioxidant role in a propionic acid-induced autism model: demonstrated by behavioral tests, histological findings and MR spectroscopy. *Neurosci Lett*. 2022;779:136622. [CrossRef]
9. Sahin K, Orhan C, Karatoprak S, et al. Therapeutic effects of a novel form of biotin on propionic acid-induced autistic features in rats. *Nutrients*. 2022;14(6):1280. [CrossRef]
10. Abujamel TS, Al-Otaibi NM, Abuaish S, et al. Different alterations in gut microbiota between Bifidobacterium longum and fecal microbiota transplantation treatments in propionic acid rat model of autism. *Nutrients*. 2022;14(3):608. [CrossRef]
11. Khera R, Mehan S, Bhalla S, et al. Guggulsterone mediated JAK/STAT and PPAR-gamma modulation prevents neurobehavioral and neurochemical abnormalities in propionic acid-induced experimental model of autism. *Molecules*. 2022;27(3):889. [CrossRef]
12. Abuaish S, Al-Otaibi NM, Aabed K, et al. The efficacy of fecal transplantation and Bifidobacterium supplementation in ameliorating propionic acid-induced behavioral and biochemical autistic features in juvenile male rats. *J Mol Neurosci*. 2022;72(2):372-381. [CrossRef]
13. Xie MJ, Iwata K, Ishikawa Y, et al. Autistic-like behavior and impairment of serotonin transporter and AMPA receptor trafficking in N-ethylmaleimide sensitive factor gene-deficient mice. *Front Genet*. 2021;12:748627. [CrossRef]
14. Sharma AR, Batra G, Saini L, et al. Valproic acid and propionic acid modulated mechanical pathways associated with autism spectrum disorder at

prenatal and neonatal exposure. *CNS Neurol Disord Drug Targets*. 2022;21(5):399-408. [CrossRef]
15. Kumar RA, Christian SL. Genetics of autism spectrum disorders. *Curr Neurol Neurosci Rep*. 2009;9(3):188-197. [CrossRef]
16. Amin Z, Mason GF, Cavus I, Krystal JH, Rothman DL, Epperson CN. The interaction of neuroactive steroids and GABA in the development of neuropsychiatric disorders in women. *Pharmacol Biochem Behav*. 2006;84(4):635-643. [CrossRef]
17. Möhler H. The GABA system in anxiety and depression and its therapeutic potential. *Neuropharmacology*. 2012;62(1):42-53. [CrossRef]
18. Cellot G, Cherubini E. GABAergic signaling as therapeutic target for autism spectrum disorders. *Front Pediatr*. 2014;2:70. [CrossRef]
19. Frye RE, Rossignol D, Casanova MF, et al. A review of traditional and novel treatments for seizures in autism spectrum disorder: findings from a systematic review and expert panel. *Front Public Health*. 2013;1:31. [CrossRef]
20. Kaila K, Price TJ, Payne JA, Puskarjov M, Voipio J. Cation-chloride cotransporters in neuronal development, plasticity and disease. *Nat Rev Neurosci*. 2014;15(10):637-654. [CrossRef]
21. Hampel P, Römermann K, Gramer M, Löscher W. The search for brain-permeant NKCC1 inhibitors for the treatment of seizures: pharmacoki netic-pharmacodynamic modelling of NKCC1 inhibition by azosemide, torasemide, and bumetanide in mouse brain. *Epilepsy Behav*. 2021;114(A):107616. [CrossRef]
22. Hadjikhani N, Zürcher NR, Rogier O, et al. Improving emotional face perception in autism with diuretic bumetanide: a proof-of-concept behavioral and functional brain imaging pilot study. *Autism*. 2015;19(2):149-157. [CrossRef]
23. Lemonnier E, Degrez C, Phelep M, et al. A randomised controlled trial of bumetanide in the treatment of autism in children. *Transl Psychiatry*. 2012;2(12):e202-e202. [CrossRef]
24. Lemonnier E, Villeneuve N, Sonie S, et al. Effects of bumetanide on neurobehavioral function in children and adolescents with autism spectrum disorders. *Transl Psychiatry*. 2017;7(3):e1056. [CrossRef]
25. Crutel V, Lambert E, Penelaud PF, et al. Bumetanide oral liquid formulation for the treatment of children and adolescents with autism spectrum disorder: design of two Phase III studies (SIGN trials). *J Autism Dev Disord*. 2021;51(8):2959-2972. [CrossRef]
26. Hampel P, Römermann K, MacAulay N, Löscher W. Azosemide is more potent than bumetanide and various other loop diuretics to inhibit the sodium-potassium-chloride-cotransporter human variants hNKCC1A and hNKCC1B. *Sci Rep*. 2018;8(1):9877. [CrossRef]
27. Hampel P, Römermann K, Gailus B, et al. Effects of the NKCC1 inhibitors bumetanide, azosemide, and torasemide alone or in combination with phenobarbital on seizure threshold in epileptic and nonepileptic mice. *Neuropharmacology*. 2021;185:108449. [CrossRef]
28. Erbas O, Erdogan MA, Khalilnezhad A, et al. Neurobehavioral effects of long-term maternal fructose intake in rat offspring. *Int J Dev Neurosci*. 2018;69:68-79. [CrossRef]
29. Sünnetçi E, Durankuş F, Albayrak Y, Erdoğan MA, Atasoy Ö, Erbaş O. Effects of the prenatal administration of tetanus toxoid on the sociability and explorative behaviors of rat offspring: A preliminary study. *Clin Psychopharmacol Neurosci*. 2021;19(1):84-92. [CrossRef]
30. Erbaş O, Akseki HS, Aktuğ H, Taşkıran D. Low-grade chronic inflammation induces behavioral stereotypy in rats. *Metab Brain Dis*. 2015;30(3):739-746. [CrossRef]
31. Yu TG, Feng Y, Feng XY, Dai JZ, Qian HJ, Huang Z. Prognostic factor from MR spectroscopy in rat with astrocytic tumour during radiation therapy. *Br J Radiol*. 2015;88(1045):20140418. [CrossRef]
32. Durankuş F, Albayrak Y, Erdoğan F, Albayrak N, Erdoğan MA, Erbaş O. Granulocyte colony-stimulating factor has a sex-dependent positive effect in the maternal immune activation-induced autism model. *Int J Dev Neurosci*. 2022. [CrossRef]
33. Solmaz V, Erdoğan MA, Alnak A, Meral A, Erbaş O. Erythropoietin shows gender dependent positive effects on social deficits, learning/memory impairments, neuronal loss and neuroinflammation in the lipopolysaccharide induced rat model of autism. *Neuropeptides*. 2020;83:102073. [CrossRef]
34. Medavarapu S, Marella LL, Sangem A, Kairam R. Where is the evidence? A narrative literature review of the treatment modalities for autism spectrum disorders. *Cureus*. 2019;11(1):e3901. [CrossRef]
35. Howlin P, Moss P. Adults with autism spectrum disorders. *Can J Psychiatry*. 2012;57(5):275-283. [CrossRef]
36. Hong MP, Erickson CA. Investigational drugs in early-stage clinical trials for autism spectrum disorder. *Expert Opin Investig Drugs*. 2019;28(8):709-718. [CrossRef]
37. Deidda G, Bozarth IF, Cancedda L. Modulation of GABAergic transmission in development and neurodevelopmental disorders: investigating physiology and pathology to gain therapeutic perspectives. *Front Cell Neurosci*. 2014;8:119. [CrossRef]
38. Lunden JW, Durens M, Phillips AW, Nestor MW. Cortical interneuron function in autism spectrum condition. *Pediatr Res*. 2019;85(2):146-154. [CrossRef]
39. Ben-Ari Y, Cherubini E. The GABA polarity shift and bumetanide treatment: making sense requires unbiased and undogmatic analysis. *Cells*. 2022;11(3):396. [CrossRef]
40. Shultz SR, MacFabe DF, Ossenkopp KP, et al. Intracerebroventricular injection of propionic acid, an enteric bacterial metabolic end-product, impairs social behavior in the rat: implications for an animal model of autism. *Neuropharmacology*. 2008;54(6):901-911. [CrossRef]
41. Foley KA, Ossenkopp KP, Kavaliers M, MacFabe DF. Pre- and neonatal exposure to lipopolysaccharide or the enteric metabolite, propionic acid, alters development and behavior in adolescent rats in a sexually dimorphic manner. *PLoS One*. 2014;9(1):e87072. [CrossRef]
42. Arumugam S, Sreedhar R, Karuppagounder V, et al. Comparative evaluation of torasemide and spironolactone on adverse cardiac remodeling in a rat model of dilated cardiomyopathy. *Cardiovasc Ther*. 2017;35(5):e12283. [CrossRef]
43. Cooper LB, Bruce S, Psotka M, et al. Proteomic differences among patients with heart failure taking furosemide or torsemide. *Clin Cardiol*. 2022;45(3):265-272. [CrossRef]
44. Skaper SD. Nerve growth factor: a neuroimmune crosstalk mediator for all seasons. *Immunology*. 2017;151(1):1-15. [CrossRef]
45. Allen SJ, Watson JJ, Shoemark DK, Barua NU, Patel NK. GDNF, NGF and BDNF as therapeutic options for neurodegeneration. *Pharmacol Ther*. 2013;138(2):155-175. [CrossRef]
46. Ciafrè S, Ferraguti G, Tirassa P, et al. Nerve growth factor in the psychiatric brain. *Riv Psichiatr* 2020;55(1):4-15. [CrossRef]
47. Lu ATH, Yoon J, Geschwind DH, Cantor RM. QTL replication and targeted association highlight the nerve growth factor gene for nonverbal communication deficits in autism spectrum disorders. *Mol Psychiatry*. 2013;18(2):226-235. [CrossRef]
48. Bristot Silvestrin R, Bambini-Junior V, Galland F, et al. Animal model of autism induced by prenatal exposure to valproate: altered glutamate metabolism in the hippocampus. *Brain Res*. 2013;1495:52-60. [CrossRef]
49. Chaddad A, Desrosiers C, Hassan L, Tanougast C. Hippocampus and amygdala radiomic biomarkers for the study of autism spectrum disorder. *BMC Neurosci*. 2017;18(1):52. [CrossRef]
50. Chaddad A, Desrosiers C, Toews M. Multi-scale radiomic analysis of subcortical regions in MRI related to autism, gender and age. *Sci Rep*. 2017;7(1):45639. [CrossRef]
51. Guo D, Peng Y, Wang L, et al. Autism-like social deficit generated by Dock4 deficiency is rescued by restoration of Rac1 activity and NMDA receptor function. *Mol Psychiatry*. 2021;26(5):1505-1519. [CrossRef]
52. Baron-Cohen S, Ring HA, Bullmore ET, Wheelwright S, Ashwin C, Williams SCR. The amygdala theory of autism. *Neurosci Biobehav Rev*. 2000;24(3):355-364. [CrossRef]
53. Xu Q, Zuo C, Liao S, Long Y, Wang Y. Abnormal development pattern of the amygdala and hippocampus from childhood to adulthood with autism. *J Clin Neurosci*. 2020;78:327-332. [CrossRef]

54. Yang Y, Higashimori H, Morel L. Developmental maturation of astrocytes and pathogenesis of neurodevelopmental disorders. *J Neurodev Disord*. 2013;5(1):22. [CrossRef]
55. Kaushik G, Zarbalis KS. Prenatal neurogenesis in autism spectrum disorders. *Front Chem*. 2016;4:12. [CrossRef]
56. Edmonson C, Ziats MN, Rennert OM. Altered glial marker expression in autistic post-mortem prefrontal cortex and cerebellum. *Mol Autism*. 2014;5(1):3. [CrossRef]
57. Erny D, Hrabě de Angelis AL, Prinz M. Communicating systems in the body: how microbiota and microglia cooperate. *Immunology*. 2017;150(1):7-15. [CrossRef]
58. Sussman D, Leung RC, Vogan VM, et al. The autism puzzle: diffuse but not pervasive neuroanatomical abnormalities in children with ASD. *NeuroImage Clin*. 2015;8:170-179. [CrossRef]
59. Levisohn L, Cronin-Golomb A, Schmahmann JD. Neuropsychological consequences of cerebellar tumour resection in children. Cerebellar cognitive affective syndrome in a paediatric population. *Brain*. 2000;123(5):1041-1050. [CrossRef]
60. Gottwald B, Wilde B, Mihajlovic Z, Mehdorn HM. Evidence for distinct cognitive deficits after focal cerebellar lesions. *J Neurol Neurosurg Psychiatry*. 2004;75(11):1524-1531. [CrossRef]
61. Courchesne E, Karns C, Davis H, et al. Unusual brain growth patterns in early life in patients with autistic disorder: an MRI study. *Neurology*. 2011;76(24):2111-2111. [CrossRef]
62. Sparks BF, Friedman SD, Shaw DW, et al. Brain structural abnormalities in young children with autism spectrum disorder. *Neurology*. 2002;59(2):184-192. [CrossRef]
63. Herbert MR, Ziegler DA, Deutsch CK, et al. Dissociations of cerebral cortex, subcortical and cerebral white matter volumes in autistic boys. *Brain*. 2003;126(5):1182-1192. [CrossRef]
64. DeLong GR. The cerebellum in autism. *The Neurol Autism*. 2005. [CrossRef]
65. Araghi-Niknam M, Fatemi SH. Levels of Bcl-2 and P53 are altered in superior frontal and cerebellar cortices of autistic subjects. *Cell Mol Neurobiol*. 2003;23(6):945-952. [CrossRef]
66. Laurence JA, Fatemi SH. Glial fibrillary acidic protein is elevated in superior frontal, parietal and cerebellar cortices of autistic subjects. *Cerebellum*. 2005;4(3):206-210. [CrossRef]
67. Purcell AE, Jeon OH, Zimmerman AW, Blue ME, Pevsner J. Postmortem brain abnormalities of the glutamate neurotransmitter system in autism. *Neurology*. 2001;57(9):1618-1628. [CrossRef]
68. Chugani DC, Sundram BS, Behen M, Lee ML, Moore GJ. Evidence of altered energy metabolism in autistic children. *Prog Neuropsychopharmacol Biol Psychiatry*. 1999;23(4):635-641. [CrossRef]
69. Friedman SD, Shaw DW, Artru AA, et al. Regional brain chemical alterations in young children with autism spectrum disorder. *Neurology*. 2003;60(1):100-107. [CrossRef]
70. Corrigan NM, Shaw DWW, Richards TL, et al. Proton magnetic resonance spectroscopy and MRI reveal no evidence for brain mitochondrial dysfunction in children with autism spectrum disorder. *J Autism Dev Disord*. 2012;42(1):105-115. [CrossRef]
71. Goh S, Dong Z, Zhang Y, DiMauro S, Peterson BS. Mitochondrial dysfunction as a neurobiological subtype of autism spectrum disorder: Evidence from brain imaging. *JAMA Psychiatry*. 2014;71(6):665-671. [CrossRef]