

## Original Article

# Cognitive dysfunction and bumetanide treatment in a valproate-induced rat model of autism

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**Abstract:** Cognitive dysfunction is an important aspect of the clinical characteristics of autism; however, research has often focused on social cognition. Learning and memory disorders often occur in autistic children, which are particularly under examined. Using cognitive and behavioral measures in our study, these disorders were endorsed in a valproate-induced rat model of autism. We identified increased levels of serum 5-hydroxytryptamine (5-HT) and gamma-aminobutyric acid (GABA), clear neuropathological changes, and enhanced protein expression levels of the N-methyl-D-aspartate (NMDA) receptor subunits NR2A and NR2B (NR2A, NR2B) and calcium/calmodulin-dependent protein kinase II (CaMK II), which suggests that these changes may have a role in the cognitive dysfunction of autism. Bumetanide has increasingly been used for the experimental treatment of autism. Thus, we treated model rats with Bumetanide and identified improvements in cognitive and behavioral measures. The current findings suggest that bumetanide may have potential long-term effects in the treatment of autism. Moreover, the effects of this drug may have a functional role through inhibition of the NKCC1 transporter.

**Keywords:** Autism, cognitive dysfunction, valproic acid, bumetanide

## Introduction

Autism is a pervasive developmental disease that draws increasing attention because of its high morbidity [1] and poor prognosis [2]. The defining features include impairments in sociability, language, communication and a range of interests and activities [3]. Cognitive dysfunction is also an important aspect of autistic symptoms. The cognitive features of autism spectrum disorder (ASD) range from differences in basic sensory perceptual processing to high-level complex social cognition [4]. Memory is an important aspect of cognitive function. Research findings regarding memory disorders in patients with autism have been inconclusive. Damage to the episodic memory of individuals with autism is selective [5] and can only be found when confronted with complex visual and aural material in a test that requires the ability to understand and recall. Semantic memory in individuals with autism also changes along with the properties of the stimuli [6]. Furthermore, working memory [7], which is relatively com-

plete when confronted with a simple task, has defects that will appear when the complexity increases. Individuals with autism have poor procedural memory [8, 9] and often cannot complete simple daily activities, such as tying their own shoes, even after repeated learning opportunities. While individuals with autism may have superb mechanical memories in certain areas related to music, characters, and numbers, these memories are isolated and unlinked to real life. Individuals with autism also have difficulties in recalling things that have previously occurred in their lives [10], and their cued recall is often invalid [11]. Social obstacles are a core symptom of autism, and social cognitive defects are often barriers in the process of socializing autism patients. Individuals with autism may have difficulty recognizing the faces [12-14] and expressions [15, 16] of other individuals; thus, they are unable to understand the information conveyed and make an appropriate response. Autistic individuals may also have difficulties under-

standing themselves, leading to the impairment of long-term unity [17] and the misattribution of their role in interpersonal relationships.

With the cognitive obstacles observed in humans that were previously discussed, the pathogenesis remains unknown. Therefore, we designed these experiments using a valproate-induced autism rat model, with the expectation that one or more core symptoms of autistic human cognitive obstacles could also be demonstrated in rats. The mechanism of VPA-induced alterations may occur through interference with the development and maturation of the nervous system [18, 19]. In our experiment, we assessed these potential alterations via biochemical and pathological examinations. Using this approach, we anticipated we would identify a preliminary mechanism that may underlie the specific cognitive obstacles associated with behaviors related to autism.

Bumetanide has increasingly been used for the experimental treatment of autism, which receives positive feedbacks. Individuals with autism who were treated with bumetanide for 3-6 months performed significantly better on tests, including the ASC scale and the CARS scale [20], and exhibited significantly improved accuracy in facial emotional labeling and increased brain activation in areas involved in social and emotional perception [21].

Bumetanide, a loop diuretic that acts by inhibiting NKCC transporters, induces a significant shift in the intracellular chloride (Cl<sup>-</sup>) concentration, which facilitates the transition of GABA from an excitatory to inhibitory neurotransmitter [22, 23]. When the GABAA receptor is activated, the Cl<sup>-</sup> channel is opened, and the flow direction of Cl<sup>-</sup> depends on the concentration of Cl<sup>-</sup> inside and outside the cell. In immature neurons, the membranes primarily express the main chloride importer (Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporter, NKCC1), which increases the intracellular Cl<sup>-</sup> levels. When GABAA receptors are activated, Cl<sup>-</sup> flows out of the cell, which causes postsynaptic membrane depolarization and excitatory effects [24]. These excitatory effects play an important role in synapse formation and nervous system plasticity [25]. However, excessive intracellular accumulation of Cl<sup>-</sup> may have negative effects that are related to various brain dysplasias. During development, the sequential expression of NKCC1 and the main

chloride exporter KCC2 cause a reduction in the intracellular Cl<sup>-</sup> concentration, and GABAergic currents shift from excitatory to inhibitory. This shifting sequence was abolished in hippocampal CA3 pyramidal neurons of VPA rats [23], with NKCC1 up-regulated, KCC2 down-regulated, and sustained excitatory GABA and hyperactive developing networks. Bumetanide significantly decreases intracellular Cl<sup>-</sup> and GABAA receptors and reinstates the GABA shifting sequence, reverses the excitatory GABA and hyperactive developing networks, and ultimately restores naïve behavior in VPA rats.

In our experiment, we assessed whether bumetanide injection treatment would improve cognitive obstacles and subsequently validated the therapeutic mechanism of bumetanide.

### Materials and methods

#### Animals

Adult Wistar rats were housed in cages in a temperature-controlled (25°C) animal colony under a 12:12-h light/dark cycle, with lights on at 7:00 AM. Female Wistar rats, with controlled fertility cycles, were mated overnight, and pregnancy was determined by the presence of spermatozoa on embryonic day 1 (E1). Sodium valproic acid (NaVPA, Sigma-Aldrich) was dissolved in 0.9% saline to obtain a concentration of 250 mg/ml. Females received a single intraperitoneal injection of 600 mg/kg sodium valproate on E12.5, and control females were injected with saline at the same time. Rats were housed individually and were allowed to raise their own offspring. The offspring were weaned on postnatal day (PND) 23; rats of each sex were housed separately. Experiments were carried out on male offspring. The offspring of females with VPA were further divided into the model and bumetanide groups. The bumetanide group received treatment with bumetanide injection (2 mg/kg, once/d) started at PND 21 for 15 days. The offspring of control females were defined as the control group. Despite the high mortality rate of the offspring of the females injected with VPA, we managed to obtain 10 male offspring for each group. Animals in the same group were maintained with five rats to a cage (475×350×200 mm<sup>3</sup>), with a controlled temperature of 21 ± 1°C and light conditions

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(lights on at 07:00, lights off at 19:00). Rats had free access to food (standard laboratory pellets) and water. All the experiments were performed in the light phase between 09:00 and 15:00. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Jilin University, Changchun, China, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

### *Behavioral tests used to calibrate the VPA model*

*Repetitive/stereotypic and exploratory activity:* Repetitive/stereotypic behaviors were assessed on PND 69. Two observers watched videos of the rats' movement for 60 min and assessed the intensity of stereotypic behavior at 5-min intervals throughout its duration according to the scoring system established by Costall and Naylor in 1973 [26].

Exploratory activity was assessed in a small open field on PND 71. The apparatus consisted of a wooden rectangular box measuring 66×57×40 cm with two holes in the shorter walls and three holes in the longer walls of the box located regularly in each wall. The number of rearings and hole-pokings (the insertion of the animal's nose into the hole) were measured during a 5-min session.

### *Social behavioral tests*

*Social behavior in prepubertal adolescence:* The test was performed under red light in a novel test cage. On PND 35, the animals were socially isolated in macrolone cages measuring 43×28×15 cm<sup>3</sup> for 3.5 h prior to the experiment. This isolation period has been shown to produce a half maximal increase in the amount of social play [27]. The test consisted of placing two rats from the same group into the test cage for 15 min. Latency to pinning (defined as one animal lying with its dorsal surface on the floor of the test cage with the other animal standing over him), total duration, and the frequency of pinning were measured.

*Social behavior in adulthood:* Social behaviors of adult animals were tested on PND 75. One week prior to the experiment, rats from both

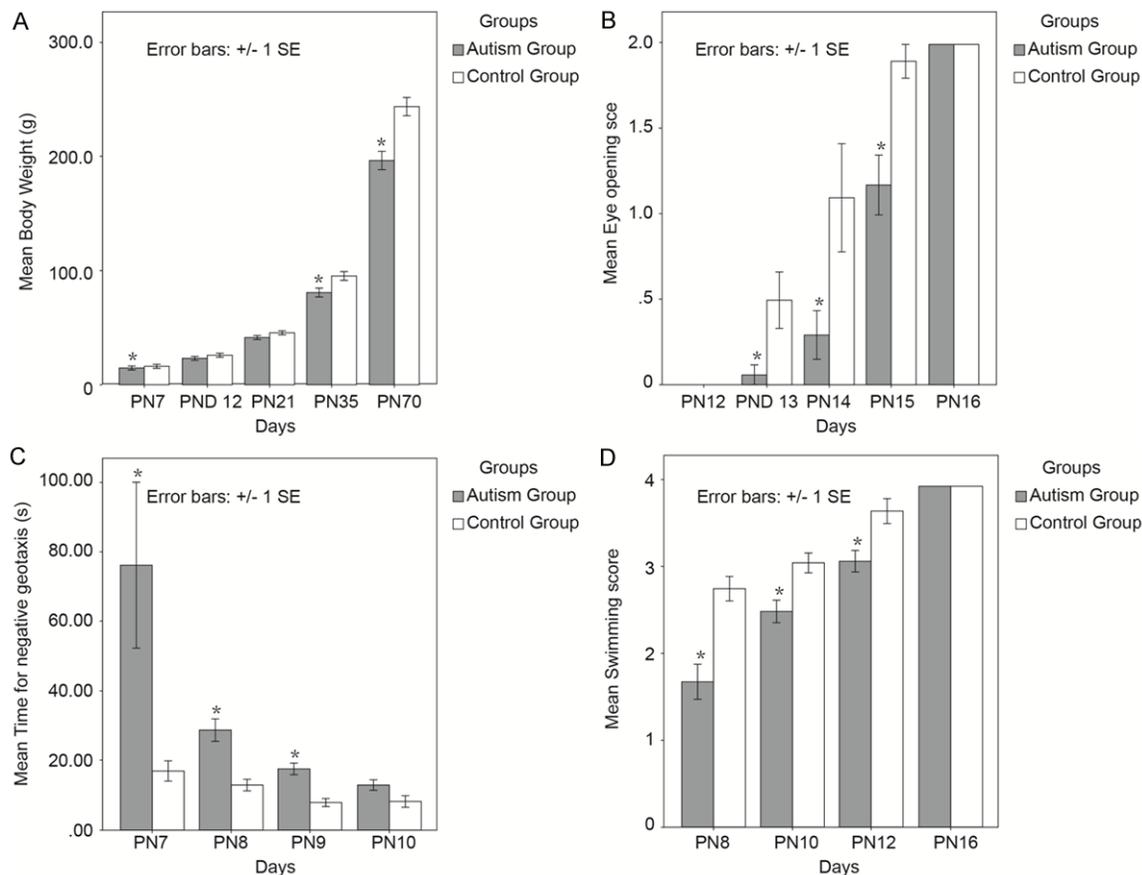
groups were socially isolated. The stimulus animals were housed in groups of five per cage. All animals were placed individually in the test cage two times per day for 5 min for 2 days prior to the experimental day, in order to reduce stress associated with the novel environment. The test consisted of placing one isolated animal and one strain, age and sex matched stimulus animal into the test cage for 10 min. Latency to social behavior, the total duration and frequency of social exploration, and contact were measured, including the following behaviors: sniffing or licking any part of the body of the conspecific, crawling or mounting (standing on hind legs and putting one or two forepaws on the back of conspecific or climbing over the conspecific), and approaching or following the conspecific.

### *Learning and memory tests*

*Passive avoidance test:* Animals were tested on PNDs 76-78. The habituation trial was performed 1 day prior to the acquisition trial. Each rat was placed in the apparatus without electric shock for 5 min, and the animal was allowed to freely explore the apparatus. The acquisition trial was performed on the first experimental day. The rats were individually placed in the light room with access to the dark room for 3 min; the dark room was designed to administer a shock (50 Hz, 0.2 mA) when the animal reached the grid floor. The initial latency (IL) of entrance into the dark room was recorded. The rat was subsequently removed from the PA apparatus to its home cage. The animals were tested regarding retention of the passive avoidance response one time, 24 h later. The rat was placed in the light room again with access to the dark room without shock for retention. The delay of entrance into the dark room from the light room was measured as the step-through latency (STL); the number of times the animal entered the room was measured as the error times (ET). If an animal did not enter the dark room within 300 s, the trial was terminated [28]. The absence of entry into the dark room or a longer duration in the light room indicated a positive response.

*Morris water maze test:* The test was conducted on PNDs 77-80 and consisted of two parts: spatial acquisition and the probe trial [29]. One

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**Figure 1.** Repetitive/stereotypic and exploratory activity & passive avoidance test for different groups. A. Stereotypic-like behavior scores; B. Exploratory activity; C. Mean step-through latency (STL); D. Mean error times in the passive avoidance test for model and control groups. \* $P < 0.05$  compared with the model group;  $\Delta P < 0.05$  compared with the control group; # $P < 0.05$  compared with the bumetanide group.

day prior to the spatial acquisition test, the rats were placed in an opaque temperature-controlled ( $26 \pm 1^\circ\text{C}$ ) tank with diameter of 100 cm, and allowed to swim freely for 2 min for adaptation (without the visible platform). The experiment was then started. The rat was placed in the desired start position in the maze, facing the tank wall. A computerized tracking program was started the moment that the animal was released. The timer was stopped when the animal reached the visible platform. The swimming distance, time spent (latency), and the track each rat took to reach the platform were measured. The standard trial limit was 2 min per trial. Animals that did not find the platform within the time limit were guided to the platform. After a short break, the animal was placed in the maze again, but at a new start location. Four trials were conducted each day until the fifth experimental day, when the plat-

form was removed. The rat was placed in a novel start position in the maze and removed after 90 s. The object of the probe trial was to determine whether or not the animal remembered where the platform was located. Indications of such memory include the number of platform-site crossovers and the time and distance spent in the target quadrant.

### Biochemistry examination

Following the completion of behavioral tests, approximately 5 ml of blood were collected from the ophthalmic vein of each rat. The serum was separated and stored in a freezer ( $-80^\circ\text{C}$ ) until analysis. The concentration of 5-hydroxytryptamine (5-HT), gamma-aminobutyric acid (GABA), and glutamic acid (Glu) in serum was measured using a high-performance liquid chromatography-tandem mass spectrometry method (HPLC-MS) [30].

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**Table 1.** Play behavior of the control, model, and bumetanide groups

Group	N	Frequency	Latency (min)	Mean duration (s)
Model group	10	0.57 ± 0.297	13.10 ± 1.165 $\Delta$ ,#	1.00 ± 0.488 $\Delta$ ,#
Control group	10	2.10 ± 0.348	8.40 ± 1.102*	2.70 ± 0.211*
Bumetanide group	10	1.40 ± 0.748	8.62 ± 1.608*	2.80 ± 0.697*

\*P<0.05 compared with the model group;  $\Delta$ P<0.05 compared with the control group;  
#P<0.05 compared with the bumetanide group.

**Table 2.** Social behavior in adulthood in the control, model, and bumetanide groups

Group	N	Frequency	Latency (s)	Mean duration (s)
Model group	10	6.29 ± 1.286	66.14 ± 14.126 $\Delta$ ,#	24.62 ± 11.769
Control group	10	8.20 ± 1.323	20.80 ± 4.136*,#	13.45 ± 4.276
Bumetanide group	10	12.10 ± 2.786	49.60 ± 12.007*, $\Delta$	7.74 ± 0.670

\*P<0.05 compared with the model group;  $\Delta$ P<0.05 compared with the control group;  
#P<0.05 compared with the bumetanide group.

## Pathology examination

After the blood was sampled, the entire brain was removed following continuous perfusion with 4% paraformaldehyde through the aorta; samples were then fixed in 4% paraformaldehyde for 7 d. Sections were selected from the frontal cortex (3 mm from bregma) and hippocampus (CA3 -3.80 mm), Nissl stained, and observed under an optical microscope. The average number of neurons was determined using a three high power field (10 $\times$ 40) and observed morphological changes were noted.

## Synaptic plasticity measurement

The expression of NR2A, NR2B and CaMK II in the brain was measured using Western blot. Samples of tissue from the frontal lobe were used to extract total protein. Samples were placed on a PVDF membrane after electrophoresis and skimmed with milk powder. The PVDF membrane was placed in the shaker with a NMDA receptor hybridization solution (1:100) under 4°C for the night, and then incubated in a hybridization solution with secondary antibodies (IgG) marked by peroxidase (1:4,000) for 2 h. The expression of NR2A, NR2B and CaMK II under electrochemical luminescence (ECL) was measured.

## Statistical analysis

Significant differences were determined by t-test or ANOVA for independent samples; in the case of nonhomogeneity, the Kruskal-Wallis

test was used. The confidence limit of P<0.05 was considered statistically significant.

## Results

Compared with the control group, the VPA rats (model group & bumetanide group) spent significantly more time engaging in repetitive/stereotypic-like behaviors, such as continuous biting, gnawing, or licking (P<0.05; Kruskal-Wallis; **Figure 1A**). Moreover, the exploratory activity was significantly lower with respect to hole-poking behaviors (P<0.05; Kruskal-Wallis; **Figure 1B**). The behaviors of the VPA rats in these two tests indicated two of the defining features of autism, which were used to calibrate the VPA model.

## Social cognition tests

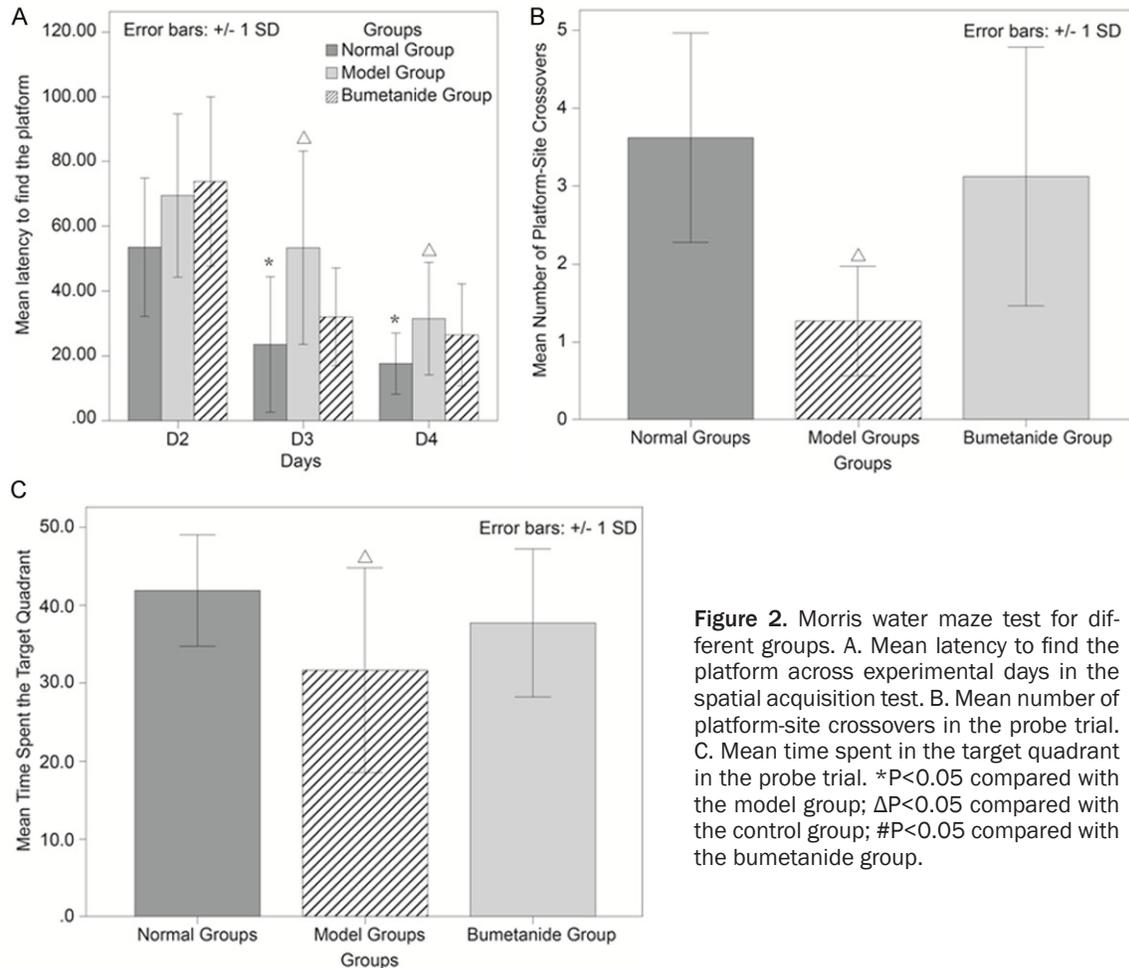
Compared with the control group, the adolescent model group showed a decreased latency to and duration of pinning during social behavior in prepubertal adolescence (**Table 1**). The bumetanide group showed significant improvement in social behavior (P<0.05, T-test) than model group. There was no difference in the frequency of pinning.

Compared with the control group, the adult model group showed a longer latency to social behavior in adult (P<0.05, T-test) (**Table 2**). The bumetanide group showed shortened latency (P<0.05, T-test) than model group. There were no significant differences in the frequency and duration of social behaviors.

## Learning and memory tests

Based on the results of the passive avoidance test, the model group had a weaker memory compared with the control group after being confronted with injury, which manifested as a significantly shortened STL and increased ET in the test (P<0.05, T-test and Kruskal-Wallis) (**Figure 1C, 1D**). The bumetanide group per-

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**Figure 2.** Morris water maze test for different groups. A. Mean latency to find the platform across experimental days in the spatial acquisition test. B. Mean number of platform-site crossovers in the probe trial. C. Mean time spent in the target quadrant in the probe trial. \* $P < 0.05$  compared with the model group;  $\Delta P < 0.05$  compared with the control group; # $P < 0.05$  compared with the bumetanide group.

formed significantly better in the test than the model group ( $P < 0.05$ , T-test and Kruskal-Wallis).

**Figure 2A** shows the latency to find the platform in the Morris water maze test on different experimental days across groups in the spatial acquisition test. The model group took more time to find the platform than the control group. As the training progressed, the difference between the two groups became prominent and was significant on the 3<sup>rd</sup> ( $P < 0.01$ , T-test) and 4<sup>th</sup> ( $P < 0.05$ , T-test) experimental days. The bumetanide group showed a shorter latency than the model group ( $P < 0.05$ , T-test).

**Figure 2B, 2C** show the comparison between groups in the probe trial. The model group had significantly less platform-site crossovers compared with the control group (**Figure 2B**,  $P < 0.05$ , Kruskal-Wallis). The model group spent

less time in the target quadrant within the 90-s period compared with the control group (**Figure 2C**,  $P < 0.05$ , t-test). The bumetanide group showed great progress compared to the model group ( $P < 0.05$ , T-test and Kruskal-Wallis).

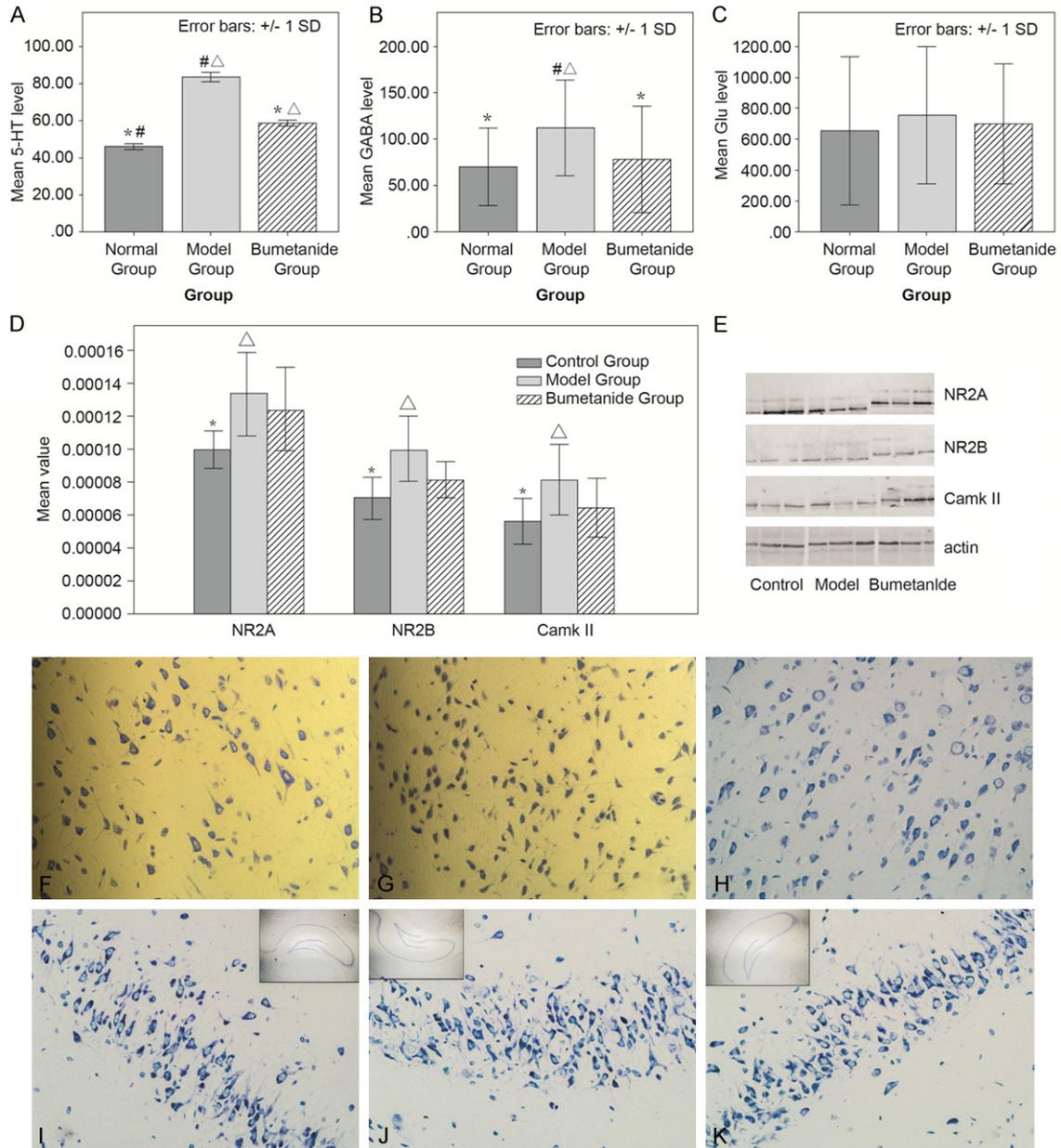
### Biochemistry examination

Concentrations of 5-HT, GABA, and Glu in serum across groups were measured using HPLC-MS (**Figure 3A-C**). Both model and bumetanide group had increased serum 5-HT and GABA compared with the control group ( $P < 0.05$ , ANOVA). The bumetanide group had lower 5-HT and GABA levels than the model group ( $P < 0.05$ , T-test). There were no differences in Glu levels between groups.

### Pathology examination

Pathology exams were completed on brain samples from each group (**Figure 3F-K**). Both

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**Figure 3.** Biochemistry examination, synaptic plasticity measurement & pathology examination for different groups. A-C. Concentrations of serum 5-HT, GABA, and Glu. A. Control group; B. Control group; C. Bumetanide group. \* $P < 0.05$  compared with the model group;  $\Delta P < 0.05$  compared with the control group; # $P < 0.05$  compared with the bumetanide group. D. Protein expression levels of NR2A, NR2B, and CaMK II for autism and control groups. \* $P < 0.05$  compared with the model group;  $\Delta P < 0.05$  compared with the control group; # $P < 0.05$  compared with the bumetanide group. E. Western blots of NR2A, NR2B, and CaMK II for the different groups. F-H. Pathology examination of the frontal lobe. F. Control group. Most neurons have an ovoid shape with normal volume, more synapses, and an even arrangement. G: Model group. Neurons are smaller in volume, with fewer synapses and a disordered arrangement. H. Bumetanide group. Appears similar to the model group. (Magnification: 400 $\times$ ). I-K. Pathology examination of the hippocampal region. I. Control group. Neurons are of normal volume, with more synapses and an even arrangement. J. Model group. Neurons are of smaller volume, with fewer synapses and disordered arrangement. K. Bumetanide group. Appears similar to the model group. (Magnification: 400 $\times$ ).

model and bumetanide group had a marked increase in neuron count in both the frontal

lobe and the hippocampal region compared with the control group ( $P < 0.05$ , ANOVA).

Morphological changes were also present, such as decreased volume, reduction of synapses, and disordered arrangement.

### *Synaptic plasticity measurement*

Expression levels of NR2A, NR2B, and CaMK II were greater in both model and bumetanide group than in the control group ( $P < 0.05$ , ANOVA; **Figure 3D, 3E**).

## **Discussion**

### *Cognitive features of autism rats*

Features of social cognition and communication. Based on the different behavioral characteristics of autism patients at different ages, tests were conducted on PNDs 35 and 75 in the rat model. Rats at PND 35 correspond to approximately 2-5 year-old children, and autistic symptoms become prominent during this period. Most children with autism insulate themselves from other peers, seldom interact, and concentrate on meaningless stuffs. The VPA rats exhibited similar obstacles in our experiments. A longer latency and lower frequency for pinning were measured. Rats at PND 75 correspond to approximately 12-15-year-old juveniles, during which time autistic symptoms appear to be relatively stable. Many autistic children may have more social interest and curtain skills; however, it is difficult for them to establish and maintain interpersonal relationships. We assessed the social behavior of PND 75 VPA rats and identified an increased interaction with strange rats, including licking, sniffing, chasing, or lying on a strange rat; however, the latency of these behavior remained significantly longer than the control rats. Both tests indicated that the model rats exhibited difficulties in social cognition and behavior, which is apparent during childhood and may be long-standing [2].

Features of learning and memory. The passive avoidance test is based on a rat's nature of skototaxis. By inducing noxious stimulations (electrical shock) to the rats that entered the dark box, an episodic memory was formed and was detected the second day. Compared with the control rats, a longer step-through latency and more error times were exhibited by the VPA rats, which indicated episodic memory disorders, consistent with clinical reports. Children

with autism often have difficulties in free recall for self-experienced events [31]. The Morris water maze test is a classic approach used to test the learning and memory of rats, which may be used to investigate the process and memory of spatial cognitive processing in a comprehensive manner. In the spatial acquisition test, the VPA rats spent more time finding the platform compared with the control rats on each trial. Furthermore, there was also a significant decrease in the time spent for both the VPA and control rats as the trials progressed; however, the progress of the VPA rats was not as rapid as the control rats. In the probe trial, the VPA rats exhibited inferiority compared with the control rats in the number of platform-site crossovers and the time and distance spent in the target quadrant. The Morris water maze test indicated spatial cognition and memory impairment in the VPA rats. Furthermore, in the first trial each day, the VPA rats exhibited a more substantial difference compared with the normal rats in finding the platform; however, the difference gradually diminished with subsequent trials, which demonstrates a superiority for cued recall of self-experienced events [31].

### *Potential factors involved in cognitive obstacles with autism*

Autistic individuals often exhibit narrowed interests and repetitive/stereotyped behaviors. Our experiment confirmed similar characteristics of these behaviors in the VPA rats. Individuals with these abnormal behaviors have less opportunity to discover new things and communicate with other individuals, which hinders the development of knowledge and skills and aggravates cognitive defects.

As a pervasive developmental disease, autism is associated with various brain abnormalities at both general and cellular levels. Our experiment further confirmed the cellular abnormalities in the VPA rat model in the frontal lobe and the hippocampus. The prefrontal cortex and the hippocampus are critical for cognition. Recent theories have focused on an 'orbitofrontal-amygdala circuit' and a 'dorsolateral prefrontal cortex-hippocampus circuit' [32, 33], considering that defects in these circuits result in the types of behavioral and cognitive dysfunctions seen in autism. The 'orbitofrontal-amygdala circuit' mainly concerns the regula-

tion of social cognition, whereas the 'dorsolateral prefrontal cortex-hippocampus circuit' plays an important role in the formation of memory. Abnormalities in the structure and function of these circuits may result in defects and imbalances that lead to the behavioral and cognitive impairments associated with autism [34].

Experiments have confirmed multiple neurotransmitter disturbances of VPA exposure in rats. Our experiment has also confirmed that the concentration of 5-HT and GABA are markedly elevated in the rat model. 5-HT and its receptors are an important neurotransmitter system, regulating central nervous system functions, such as emotion, cognition, learning, and memory. 5-HT neurons connect extensively through the paracrine to the cerebral cortex, the hippocampus, the amygdala, and other cognitive-related regions; they regulate activities of glutamatergic, cholinergic, and GABAergic neurons to participate in learning and memory formation [35]. The 5-HT transporter (SERT) and the 5-HT receptor (A1/A2) both play important roles in regulating activity of 5-HT neurotransmission in the brain. Studies have shown that there are dysfunctions of the SERT and 5-HT (1A) receptors in the cerebral cortex and the hippocampus of individuals with autism [36]. There is also a SERT gene polymorphism [37]. Both of these dysregulations may cause dysfunctions of the 5-HT neuron and lead to cognitive impairment in individuals with autism. On the other hand, GABA is an important inhibitory neurotransmitter in the central nervous system. Studies have suggested elevated serum levels of GABA in some autistic individuals, but GABA receptor levels in the brain were reduced, suggesting increased vicarious GABA release from the presynaptic membrane [38]. GABAergic neurons have important effects on learning and memory. Neural plasticity is the basis of learning and memory and includes long-term synaptic transmission, such as long-term potentiation (LTP) and long-term depression (LTD). As the most important structure in the brain concerning learning and memory, the hippocampus continuously exhibits spontaneous electrical activity in the form of an areatus delta rhythm and can induce LTP. The GABA transporter directly affects LTP induced by the delta rhythm, and also affects the spontaneous electrical activity of the hippocampus, both of which

can affect the completion of learning and memory [39]. Other studies suggest that the excitation or inhibition of transformation of GABAergic neurons plays an important role in the development of the nervous system. Suppressed GABAergic inhibition and an imbalance of the transformation can promote the occurrence of autism [40].

Synaptic plasticity is the molecular basis of cognition and memory and manifests itself as LTP and LTD. Key proteins and receptors are crucial to the regulation of synaptic structure and functional plasticity. In our experiment, we found a surprisingly selective enhancement of protein expression of the NMDA receptor subunits NR2A and NR2B, as well as the closely linked kinase CaMK II in the VPA model group. A larger increase in the amplitude of the synaptic response was also recorded in Rinaldi's study [41], which demonstrated an increased postsynaptic LTP in the VPA rat model. The NMDAR is the synaptic coincidence detector essential for controlling synaptic plasticity [42] and memory formation [43, 44]. Increased levels of NMDA receptors may make the neocortex more vulnerable to insult and neurotoxicity as development proceeds [45]. Further, increased NMDA receptor expression can control synaptic plasticity by regulating postsynaptic AMPA receptors [46]. An increased ratio of NR2A:NR2B can also compress LTD range and constrain long-term memory [47]. CaMK II is a key messenger mediating the postsynaptic form of LTP by enhancing the insertion of AMPA receptors into the postsynaptic density [48]. CaMK II (known as the "molecular switch of memory") is involved in the induction and early maintenance of LTP, can enhance the channel function of the postsynaptic membrane, and regulates the synthesis and release of neurotransmitters [49]. Increased CaMK II level also amplifies the postsynaptic form of synaptic plasticity and effects learning and memory.

*Bumetanide may have potential long-term effects in the treatment of autism*

In our experiment, following treatment with bumetanide for 15 days, ameliorations of social and cognitive deficits were identified in the VPA rats; these animals performed better in both social cognition tests and learning and memory tests, even though most of these tests

were conducted a long time after bumetanide injection. These findings suggest that bumetanide may have long-term effects in the treatment of autism, which thus requires further investigation in humans. Bumetanide treatment markedly decreased serum levels of GABA in the VPA rats, and GABA<sub>A</sub> receptor is widely distributed in the central nervous system and consists of Cl<sup>-</sup> coupled ionotropic receptors. Therefore, it may be the direct acting site of bumetanide. Moreover, evidence indicates that bumetanide may induce morphological modifications in the brain, such as an enhancement cell proliferation and dendritic development of newborn dentate gyrus cells [50]. However, morphological changes were not assessed in the current study.

### Conclusions

(1) The VPA rat model of autism is characterized by atypical social cognition and communication disorders, as well as learning and memory obstacles. (2) Increased levels of 5-HT and GABA, neuropathological changes, and enhanced protein expression levels of NR2A, NR2B, and CaMK II were identified in VPA rats, which may represent the factors involved in producing the cognitive dysfunction of autism. (3) Bumetanide may have long-term effects for the amelioration of cognitive dysfunction in VPA rats. The mechanism may involve the inhibition of the NKCC1 transporter, which may reduce the concentration of Cl<sup>-</sup> within the neuron and regulate GABAergic function.

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### Disclosure of conflict of interest

None.

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