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miR-155-5p is Involved in Regulating Autism-Like Behaviour, Pain Sensitivity and Inflammatory Response in Brain Tissue via the Wnt/β-Catenin Pathway in Valproic Acid Rat Model of Autism Spectrum Disorder

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JZ and YT conceived of the study. XY, LD, XZ, SX participated in its design and coordination. XW helped to draft the manuscript. All authors read and approved the final manuscript.

Key words
Autistic spectrum disorder, Wnt/β-catenin signalling pathway, miR-155-5p, Autism-like behaviour, Nociceptive sensitivity, Inflammatory response

ABSTRACT

Autistic spectrum disorder (ASD), also known as autism, is a relatively serious developmental disorder. It is of great significance for patients to understand the pathogenesis of the condition and implement treatment. In this study, a valproic acid (VPA) rat model of ASD (VPA rats) is constructed; miR-155-5p was overexpressed by lentivirus and rats were treated with ICG-001, an inhibitor of Wnt/β-catenin. The ASD behaviours were analysed in the VPA rats treated with and without ICG-001. The expression levels of brain tissue miR-155-5p, β-catenin, GSK-3β, cyclin-D1, c-myc and inflammatory factors are detected by ELISA and western blot. The results show that during a three-chamber sociability test, rats in the VPA group and miR-155-5p-mimics group had significantly decreased exploration time, longer dwell time in the middle compartment and reduced exploration time with the unfamiliar rat compared with the blank control group. The administration of ICG-001 significantly alleviated the above situation ($P < 0.05$). Similar results are observed in the open field test, self-grooming test and PWTL values, where ICG-001 administration significantly reduces the self-stroking time ($P < 0.05$). Compared with the blank control group, the relative expressions of β-catenin, GSK-3β, cyclin-D1, c-myc protein and inflammatory factors in rat brain tissues are increased in the VPA and miR-155-5p-mimics groups and decreased by ICG-001 treatment ($P < 0.05$). In summary, miR-155-5p affects the core clinical symptoms of VPA rats through the Wnt/β-catenin signalling pathway. Inhibition of miR-155-5p expression using ICG-001 can improve the core symptoms of social deficits, communication deficits, repetitive stereotypic behaviours and nociceptive thresholds in VPA rats, which provides an important basis for in-depth research on ASD clinical targeting.

INTRODUCTION

Autistic spectrum disorder (ASD), also known as autism, is a relatively serious developmental disorder. The clinical symptoms in children include mainly social communication disorders, narrow interest and stereotyped behaviour (Manoli and State, 2021). Due to changes in diagnostic criteria, improved performance in screening and diagnostic tools and increased public awareness, the incidence of ASD has been increasing annually worldwide

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in recent decades (Hirota and King, 2023). The number of children with ASD in China is also increasing sharply and the prognosis is poor. Most of these children lack self-reliance ability in adulthood, which brings heavy psychological and economic burdens to families and society (Zhou *et al*., 2020). The pathogenesis of ASD is complex, involving genetic, environmental, immune and other factors, and most existing studies focus on genetic factors (Willsey *et al*., 2022). At present, genome-wide association studies have found a variety of genes related to ASD; these genes are diverse and complex, which causes difficulties in further study of pathogenesis (Evans *et al*., 2022; Trost *et al*., 2022). Existing studies on the genetic pathways involved in the onset of ASD have not reached a consensus. There is still no effective treatment approach for ASD. It is thus urgent that the potential pathogenesis be clarified to explore the targets for effective treatment; an ideal animal model of ASD is an important means for doing so and deriving effective prevention measures.

At present, the research on ASD mainly depends on animal model and cell model. Successful and reliable experimental model is the cornerstone of ASD research. Up to now, the successfully constructed animal models of ASD include non-mammal model, prenatal exposure model of valproic acid (VPA), BTBR idiopathic mouse model, maternal immune activation model, rodent model based on ASD susceptibility gene and nonhuman primate model (Ornoy *et al*., 2019; Evatt *et al*., 2009). VPA is clinically used as an antiepileptic drug or emotional stabilizer, which can treat epilepsy, bipolar disorder and neuropathic pain (Christensen *et al*., 2013). It was found that the prevalence of neurodevelopmental disorders in children with a history of prenatal VPA exposure increased by 6-10 times, and ASD was the most common symptom of neurodevelopmental disorders in children with prenatal VPA exposure at the age of 6 (Moore *et al*., 2000). Studying the classical animal model of ASD is widely used to explore the potential therapeutic drugs of ASD. In terms of molecular mechanism, the researchers preliminarily explored the molecular mechanism of VPA inducing ASD in early embryonic development, and found that VPA exposure affected brain development and neural network maturity by regulating some signaling pathways, such as histone hyperacetylation, Wnt signaling pathway, ERK-p21 pathway and γ-aminobutyric acid level in the brain, which eventually led to ASD-like behavior phenotype in offspring. The VPA rodent model not only has some similarities with ASD patients in behavior and anatomy, but also shows intestinal flora imbalance similar to ASD patients, which makes the VPA rodent model one of the best models to study ASD (Rodier *et al*., 1997; Ingram *et al*., 2000).

MicroRNA (miRNA) is a single-stranded non-coding regulatory RNA that is highly expressed in the central nervous system and affects brain function and development and neuronal plasticity, maturation and differentiation (Huang *et al*., 2021; Liu *et al*., 2022). Existing studies found the dysregulation of multiple miRNA expression to be closely related to the development of ASD. In this context, miR-155-5p is a microRNA with the most ASD- risk gene targets (Huang *et al*., 2021; Rastegari *et al*., 2023; Stott *et al*., 2023). The Wnt/β-catenin signalling pathway is a key pathway affecting brain development and where abnormal changes are detected in many ASD patients (Caracci *et al*., 2021). Previous studies have proposed that the wingless (Wnt)/β-catenin signaling pathway, which is critical for brain development and synaptic functions, is one of the convergent cellular processes altered in ASD (Gaeun *et al*., 2023). Exposure to VPA has also been shown to activate the Wnt and mTOR pathways. Correspondingly, downregulating canonical Wnt/β-catenin signaling ameliorated ASD-like behavioral phenotypes in VPA-exposed rats (Ming *et al*., 2022). VPA was also shown to increase the proliferation of neural progenitor cells by activating Wnt/β-catenin signaling during development (Park *et al*., 2021). And some studies have found that Wnt/β-catenin inhibitor-ICG-001 can improve the autism-like behavior and the morphological development of dendritic spines of hippocampal pyramidal neurons in rats (Wang *et al*., 2023). Studies show that the Wnt/β-catenin pathway is partially regulated by specific miRNAs in the development of children with ASD (Huang *et al*., 2021). Some studies have found miR-155-5p to be abnormally expressed in the peripheral blood and brain tissue of children with ASD (Huang *et al*., 2021). This study aimed to explore the possibility that miR-155-5p may participate in the development of ASD by activating the Wnt/β-catenin signalling pathway and provide new ideas for pathogenesis research and the clinical treatment of ASD.

MATERIALS AND METHODS

Materials

Tris base, valproic acid (VPA) was purchased from Sigma; ICG-001 (β-catenin/CBP inhibitor) was provided by Shanghai Xinyu Biotechnology Co., Ltd.; the lentivirus overexpressing miR-155-5p was purchased from Suzhou Genepharma Co.,Ltd. ; IL-1β, IL-6 and TNF-α ELISA kits were purchased from Shanghai Hengyuan Biotechnology Co., Ltd.; a lentiviral vector was purchased from Shanghai Hewu Biotechnology Co., Ltd.; a 10% SDS solution was purchased from Sinopharm Chemical Reagent Co., Ltd.; a protein marker was purchased from Shanghai Yisheng Biotechnology Co., Ltd.; β-catenin rabbit monoclonal

antibody, GSK-3β rabbit monoclonal antibody, cyclin-D1 rabbit monoclonal antibody, c-myc rabbit monoclonal antibody and goat anti-rabbit secondary antibody were purchased from Abcam (USA), and mouse anti-GAPDH monoclonal antibody and antibody diluent were purchased from Shanghai Yisheng Biotechnology Co., Ltd. Pro-light HRP chemiluminescence detection reagents and ECL chemiluminescence detection kits were purchased from Tiangen Biochemical Technology (Beijing) Co., Ltd. All other chemical reagents were of analytical grade.

Experimental animal preparation

Thirty female Sprague Dawley (SD) rats and 20 male SD rats in childbearing period with a body mass of 18 ~ 24 g were purchased from Zhengzhou University (Henan Experimental Animal Center; animal certificate number: SCXK [Yu] 2022-0001). The rats were kept in the SPF environment of the centre and the appearance, shape and actions of the rats were regularly observed. Adequate food and water were supplied. A constant temperature of 23℃ was maintained, relative humidity at 50%–60% and 12 h/12 h day/night illumination. After one week of routine adaptive housing, male and female SD rats were caged at a 1:2 ratio overnight. Female mouse vaginas were examined at 6:00 on day 2. If a vaginal plug was present, this was regarded as successful fertilisation (i.e. a pregnant rat) and recorded as 1 day of pregnancy.

Establishing and grouping the ASD model rats (Melancia et al., 2018)

At 12.5 days of pregnancy, the SD rats were randomly divided into two groups, i.e. the parental saline (NaCl) group and the parental VPA group. The rats in the latter group were injected with 600 mg/kg VPA, and the offspring were in the subsequent VPA group. The parental NaCl group was injected with the same volume of normal saline. The mothers of each litter nursed their juvenile off spring alone. To monitor the growth and development indicators, the study group was appropriately killed to ensure the same feeding environment, number and sex of each female rat (male juvenile rats were selected in this study). Male juvenile rats were randomly divided into five groups as follows: The control group (NaCl injected), the VPA group, the miR-155-5p-mimics group (miR-155-5p overexpression), the ICG-001 group (Wnt/β-catenin inhibitor [ICG-001]) and the miR-155-5p-mimics + ICG-001 group (miR-155-5p overexpression + ICG-001). Among them, the lateral ventricular brain of the rats in the miR-155-5p-mimics group and the miR-155-5p-mimics + ICG-001 group were injected once a week with lentivirus overexpressing miR-155-5p (3 * 10 ^ 7 TU). After 21 days, ICG-001 (5 mg/kg) was injected intraperitoneally for 7 consecutive days in the ICG-001 group and the miR-155-5p-mimics + ICG-001 group.

Behavioural identification of autism in rats (Gürkan et al., 2023)

Autism-like behaviour was identified by a three-chamber sociability test, open field test and self-grooming test.

The tree chamber sociability test: the three-compartment social behaviour box is an open box divided into three independent chambers by two medical organic baffles. Beneath each baffle, there is a small door that can be controlled by a switch to facilitate the rats entering and leaving each room freely. There is an iron grid-like binding device in each corner of the left and right chambers to enclose the rats that have been put into it. Two days before the experiment, the rats that were to be tested were put into the behaviour room to familiarise them with the test environment and reduce experimental errors caused by environmental differences. When performing the experiment, it was typically carried out in a darker environment; the light in the behaviour box could be maintained at 40 lux, the ambient temperature was kept at 24℃ and the humidity was 55%. The experimenter avoided appearing in the line of sight of the rats, reduced noise, kept the ambient volume below 30 dB and remained silent. At the end of each rat experiment, 70% alcohol was used to remove the odour from the behaviour box and clean out faeces.

The experiment was divided into three stages:

(1) *The adaptation stage*: The experimental rats were placed in the central chamber of the behaviour box with their backs to the experimenter, and the small door of the left and right baffle was opened to enable them to move freely for 10 min and fully familiarise themselves with the environment. Social ability test was conducted after 24 h.

(2) *Social ability test*: After 10 min of free entry and exiting the behaviour box, the experimental rats were replaced into the central chamber, the small doors of the left and right baffles were closed and a rat of the same age (stranger 1) was randomly placed in the restraints of the left and right chambers, while the other was left empty; the doors of the left and right baffles were then opened to allow the rats to move freely for 10 min and recorded time spend in empty chambers and time spend for exploration. Social novelty test was conducted after 24 h.

(3) *Social novelty test*: After 10 min, the rats were replaced in the central chamber, the small doors of the left and right baffles were closed, stranger 1 was taken out and randomly put into any of the restraints, and then another rat of the same age (stranger 2) was placed in the other; the small doors of the left and right baffles were opened

to enable the rats to move freely for 10 min and recorded chamber residence time and touch and smell time. At the end of the experiment, the rats were put back into the cage and the behaviour box was cleaned. The time that the rats remained in each lateral chamber in the second and third stages and the period of olfactory communication with stranger 1 and stranger 2 was recorded by camera system and computer software. Open field test was conducted after 24 h.

Open field test

The open field behaviour box was used to detect spontaneous activity behaviour and to explore the anxiety that the rats experienced. The entire behaviour box comprise an open cube of 40 × 40 × 40 cm, and the pretreatment and experimental environments before conducting the experiment were the same as that of the social experiment. During the experiment, the rats were placed with their backs to the centre of the behaviour box; they were allowed to explore the environment freely for 10 min before the camera was switched on to record the total distance and total time, grooming, standing, running times and defecation of the rats in the behaviour box for 10 min. Before each experiment, the behaviour box was cleaned with 70% ethanol and left to dry for 2 ~ 3 min until no odour residue remained. Self-grooming test was conducted after 24 h.

Self-grooming test

The repetitive stereotyped behaviour of rats was detected using conventional feeding cages. The pretreatment and experimental environment before the experiment were the same as in the social experiment. During the experiment, the rats were placed in a clean and transparent feeding cage of 30 × 17 × 16 cm, given enough food and water and allowed to explore freely for 10 min. After 10 min, the camera was switched on, and the cumulative time of the rats in the 30 min washing their face, limbs, body and tail with their claws or mouth was recorded. The open area was cleaned with 70% ethanol before each experiment. Paw withdraw thermal latency (PWTL) test was conducted after 24 h.

Paw withdraw thermal latency determination (Gürkan et al., 2023)

The sensitivity of the animals to pain stimulation was illustrated by measuring the time required for a rat to withdraw its paw due to pain caused by plantar exposure to strong light. The PWTL of the hind limb was measured using a PL-200 Sting thermal imager. The room temperature was constantly maintained at 26℃. The intensity of the (infrared) light source was adjusted to 70%, and the maximum time of a single stimulation was 30 s. The light source was focused on the hind paw to start the test. When the rat withdrew its foot and developed a foot-licking reaction, the recorded response time from onset to foot withdrawal was taken as the PWTL value. Each rat was measured three times using the above procedure every 5 min, and the average was taken as the PWTL.

The expression of inflammatory factors (Huang et al., 2021)

Cryopreserved brain tissue was homogenised in PBS, centrifuged for 10 min at 10,000 r/min. The supernatant was used for detection of expression levels of IL-β, IL-6, and TNF-α using an enzyme linked immunosorbent assay (ELISA) kit, and its procedures were performed strictly according to the manufacturer's instructions.

Real-time PCR

The real-time PCR (RT-PCR) assay was carried out in the present study in line with previously reported methods (Liu *et al.*, 2022). Whole cerebral tissue sampling of RNA was accomplished with TRIzol (Invitrogen, San Francisco, CA, USA) as per the guidelines of the manufacturer. The RNA isolates were quantified utilizing a Nanodrop-2000 spectrophotometer. For the synthesis of cDNA templates from the total RNA, a Transcriptor First-Strand cDNA Synthesis kit (Vazyme RT kit, Nanjing, China) was utilized. A RT-PCR System (StepOnePlus; Vazyme, Nanjing, China) was exploited for the RT-PCR assessment using the Master Mix (ChamQ Universal SYBR). Normalization of target gene levels was accomplished against GAPDH. The mRNA expressions of genes were assessed by the $2^{-\Delta\Delta Ct}$ approach. Table I shows the details of the RT-PCR primer sequences.

Table I. Primer sequence.

Gene	Primer sequence (5'-3')
miR-155-5p	F GCTTCGGTTAATGCTAATCGTG
	R CAGAGCAGGGTCCGAGGTA
GAPDH	F GGAAGCTTGTCATCAATGGAAATC
	R TGATGACCCTTTTGGCTCCC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Western blot detection

Western blot was conducted following the prior procedure (Rastegari *et al.*, 2023). Whole cerebral tissue proteins were extracted using a RIPA lysate and the protein concentration was determined using a BCA protein concentration determination kit. Separation and concentration gels were prepared for sodium dodecyl

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at a dose of 30 μg protein, and trarsmembrane was conducted to a polyvinylidene difluoride (PVDF) membrane. Blocking was performed with 50 μg/L skimmed milk for 60 min at room temperature. The diluted primary antibody (1:1000 dilution) was incubated at 4℃ overnight, and the membrane was washed three times with a membrane washing buffer (TBST). The images were exposed using an ECL exposure solution in a gel imaging analyser, and the grey values of the target proteins and internal parameters were analysed using Image Lab 6.1 (v.) software. Based on the expression of β-actin, the expression of target protein was calculated.

Statistical analysis

Statistical comparisons between the experimental and control groups were made using the student's t-test generated by the SPSS Statistics 26 (v.) software. The results shown in all tables and figures represent the mean ± standard deviation (SD), with $P < 0.05$ indicating a statistical significance.

RESULTS

Behavioural identification of autism

Social ability of offspring rats

The three-chamber sociality test usually collects indicators such as the stay time of animals in social boxes, empty boxes and central boxes, and olfactory touch time. If the test animal stays in the empty box for a longer time, it means that the test animal has a low social interest in strange animals or has social obstacles. In addition, we can evaluate the social ability of test animals by observing the number of interactions with strange animals.

During the adaptation period, rats in the VPA group and the miR-155-5p-mimics group remained a longer time in the chamber with stranger 1 compared with those in the blank control group; olfactory touch time was significantly reduced, and the differences were statistically significant ($P < 0.05$). Compared with the miR-155-5p-mimics group, rats in the miR-155-5p-mimics + ICG-001 group had decreased residence time in the empty chamber and increased olfactory touch time, and the differences were statistically significant ($P < 0.05$; see Fig. 1 and Table II). During the social preference period, stranger 2 was placed in the empty chamber. Compared with the blank control group, rats in the VPA and miR-155-5p-mimics groups remained longer in the middle chamber and spent significantly less time in the chamber with stranger 2, with reduced olfactory touch time ($P < 0.05$). Compared with the miR-155-5p-mimics group, rats in the miR-155-5p-mimics + ICG-001 group had decreased residence time in the middle chamber and increased olfactory touch time with stranger 2 ($P < 0.05$; see Table III).

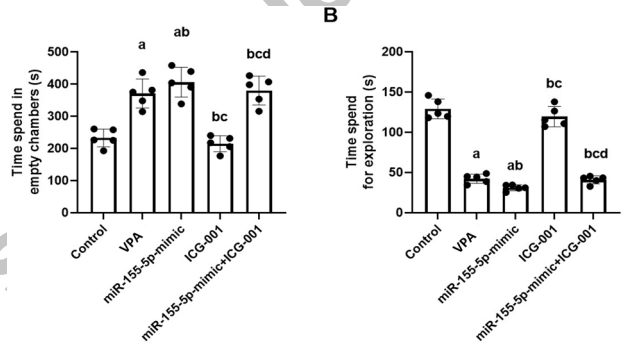

Fig. 1. Three-box social experiment of VPA rats in each group. A, The time spend in empty chambers for VPA rats in each group. B, The time spend on exploration for VPA rats in each group. Compared with the blank control group, aP<0.05; Compared with the ASD, bP < 0.05, Compared with the miR-155-5p-mimics group, cP < 0.05; Compared with the ICG-001 group, dP < 0.05.

Spontaneous activity and exploration behaviours of offspring rats

The number of cross grid of central grids simply refers to the number of animals entering and leaving the

Table II. Results of three chamber sociability test of ASD rats in each group in the adaptation stage ($\bar{x}$±s).

Group	n	Residence time in empty chamber (s)	Olfactory touch time (s)
Blank control group	5	232.53±24.90	129.05±10.98
ASD group	5	371.01±40.23^{a}	42.28±5.06^{a}
miR-155-5p-mimics group	5	405.98±41.49^{ab}	31.14±3.36^{ab}
ICG-001 group	5	214.48±22.16^{bc}	119.53±11.27^{bc}
miR-155-5p-mimics+ICG-001 group	5	380.17±40.09^{bcd}	40.96±4.48^{bcd}
F value		26.989	98.981
P value		<0.001	<0.001

Table III. Results of the social preference phase in the three chamber sociability test of ASD rats in each group ($\bar{x}$±s).

Group	n	Time spend in empty chambers (s)			Time spend for exploration (s)	
		Stranger 1	Middle chamber	Stranger 2	Stranger 1	Stranger 2
Blank control group	5	264.98±29.35	50.56±5.38	378.98±38.98	35.76±3.79	84.53±9.01
ASD group	5	165.58±18.87[a]	178.89±18.85[a]	178.89±18.85[a]	178.89±18.85[a]	36.68±4.09[a]
miR-155-5p-mimics group	5	120.14±13.54[ab]	210.58±22.47[ab]	128.81±13.54[ab]	10.02±1.17[ab]	22.39±2.61[ab]
ICG-001 group	5	256.17±30.18[abc]	47.41±5.59[abc]	373.17±40.22[abc]	34.98±4.45[abc]	78.20±9.47[abc]
miR-155-5p-mimics+ICG-001 group	5	160.64±18.84[bcd]	170.18±20.03[bcd]	160.72±18.46[bcd]	15.54±1.69[bcd]	33.37±4.09[bcd]
F value		38.574	75.352	106.083	120.131	124.058
P value		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Compared with the blank control group, [a]P<0.05; Compared with the ASD group, [b]P < 0.05, compared with the miR-155-5p-mimics group, [c]P < 0.05; compared with the ICG-001 group, [d]P < 0.05.

central grids, and a reduced number of cross grid was associated with a state of anxiety among the rats. The vertical score refers to where a pair of front feet of a rat were vacated or attached to the wall of the box and lowering claws after standing was upright once. The number of upright reflected exploratory behaviour and curiosity about the new environment.

Compared with the blank control group, the number of cross grid of central grids and the vertical score decreased in the VPA group, the miR-155-5p-mimics group and the miR-1555-5p-mimics + ICG-001 group and showed statistically significant differences (P < 0.05). Compared with the miR-155-5p-mimics group, the number of cross grid of central grids and the vertical score increased in the miR-155-5p-mimics + ICG-001 group and showed statistically significant differences (P < 0.05; see Fig. 2, Supplementary Table S 1).

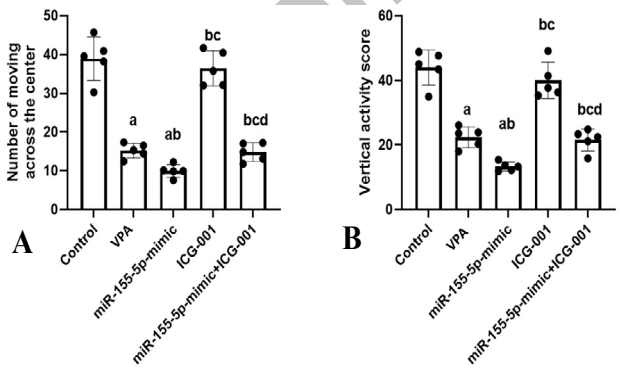

Fig. 2. Results of the mine field experiments in various groups of VPA rats. A, The number of moving across the center for VPA rats in each group. B, The vertical activity for VPA rats in each group. Compared with the blank control group, aP<0.05; Compared with the ASD, bP < 0.05, Compared with the miR-155-5p-mimics group, cP < 0.05; Compared with the ICG-001 group, dP < 0.05.

Stereotypical repetitive movements in VPA rats

Spent more time on self-grooming is usually detected in Self-grooming test. Spent more time on self-grooming can reflect the stereotyped repetitive behavior of animals, and the longer spent more time on self-grooming indicates that animals have stereotyped repetitive behavior.

Compared with the blank control group, the VPA, miR-155-5p-mimics and miR-155-5p-5-mimics + ICG-001 groups spent more time on self-grooming with statistically significant differences (P < 0.05). Compared with the miR-155-5p-mimics group, self-grooming time decreased in the miR-155-5p-mimics + ICG-001 group (see Fig. 3A, Supplementary Table S II).

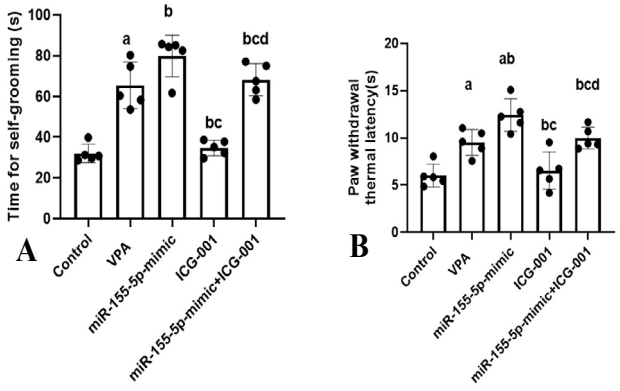

Fig. 3. The time for self-grooming (A), and the paw withdrawal thermal latency (B) for VPA rats in each group. Compared with the blank control group, aP < 0.05; compared with the ASD, bP < 0.05, compared with the miR-155-5p-mimics group, cP < 0.05; compared with the ICG-001 group, dP < 0.05.

PWTL measurement of the rats

Compared with the blank control group, the VPA, miR-155-5p-mimics and miR-155-5p-mimics + ICG-001

groups had increased PWTL values, indicating decreased sensitivity to pain stimulation, and the differences were statistically significant ($P < 0.05$). The PWTL values decreased in the miR-155-5p-mimics + ICG-001 group compared with the miR-155-5p-mimics group, indicating that the sensitivity to pain stimulation was significantly improved, and the differences were statistically significant ($P < 0.05$; Fig. 3B, Supplementry Table S III).

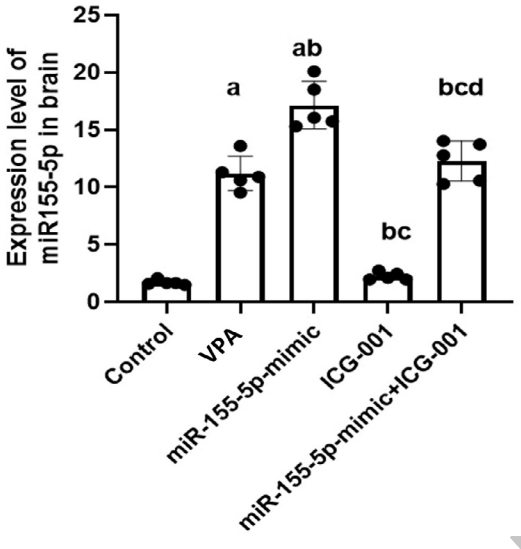

Fig. 4. The expression level of miR-155-5p in brain in each group. Compared with the blank control group, aP<0.05; Compared with the ASD, bP < 0.05, Compared with the miR-155-5p-mimics group, cP < 0.05; Compared with the ICG-001 group, dP < 0.05.

miR-155-5p expression in the rat brain

The miR-155-5p expression in the brain tissue of rats was increased in the VPA, miR-155-5p-mimics and miR-155-5p-mimics + ICG-001 groups compared with the blank control group, with statistically significant differences ($P < 0.05$). Compared with the VPA group, miR-155-5p expression in the brain tissue of rats was significantly increased in the miR-155-5p-mimics group ($P < 0.05$); miR-155-5p expression decreased significantly in the ICG-001 group compared with the VPA group ($P < 0.05$). Compared with the miR-155-5p-mimics group, miR-155-5p expression in the brain tissue of rats was decreased in the miR-155-5p-mimics + ICG-001 group ($P < 0.05$; see Fig. 4, Supplementry Table S IV).

Inflammatory factors in the rat brain

The expression of IL-β, IL-6 and TNF-α in the brain tissue of rats was increased in the VPA, the miR-155-5p-mimics and the miR-155-5p-mimics + ICG-001 groups, compared with the blank control group, and the differences were statistically significant ($P < 0.05$). The miR-155-5p-mimics + ICG-001 group reflected decreased IL-β, IL-6 and TNF-α expression in the brain tissue of rats ($P < 0.05$; see Table IV).

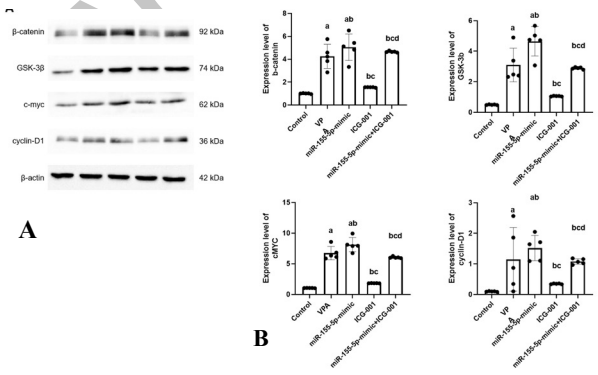

Fig. 5. Western blot analysis of indicated proteins. A, Representative images of western blot analysis. B, Statstics graphs of western blot results of indicated proteins. Compared with the blank control group, aP<0.05; Compared with the ASD, bP < 0.05, Compared with the miR-155-5p-mimics group, cP < 0.05; Compared with the ICG-001 group, dP < 0.05.

Table IV. Expression levels of inflammatory factors in the brain tissue of rats with ASD among groups ($\bar{x}$±s).

Group	n	IL-1β (ng/g)	IL-6 (pg/g)	TNF-α (ng/g)
Blank control group	5	20.86±3.05	11.96±1.75	60.35±7.05
ASD group	5	43.36±4.49[a]	25.46±2.68[a]	81.06±8.89[a]
miR-155-5p-mimics group	5	54.51±6.03[ab]	33.04±3.54[ab]	95.56±9.74[ab]
ICG-001 group	5	21.18±2.27[abc]	12.06±1.19[abc]	62.28±6.67[abc]
miR-155-5p-mimics+ICG-001 group	5	45.07±4.68[bcd]	26.68±3.07[bcd]	82.39±8.87[bcd]
F value		46.560	51.084	11.664
P value		<0.001	<0.001	<0.001

IL-1β, interleukin-1β; IL-6, Interleukin-6; TNF-α: tumor necrosis factor -α. Compared with the blank control group, [a]$P<0.05$; Compared with the ASD group [b]$P < 0.05$, Compared with the miR-155-5p-mimics group, [c]$P < 0.05$; Compared with the ICG-001 group, [d]$P < 0.05$.

Expression of β-catenin, GSK-3 β, cyclin-D1 and c-myc proteins in the brain

The relative expression of β-catenin, GSK-3β, cyclin-D1 and c-myc in the rat brain tissue samples were increased in the VPA, miR-155-5p-mimics and the miR-155-5p-mimics + ICG-001 groups, compared with the blank control group, and the differences were statistically significant ($P < 0.05$). Compared with the miR-155-5p-mimics group, the relative expression of β-catenin, GSK-3β, cyclin-D1 and c-myc in the rat brain tissue were decreased in the miR-155-5p-mimics + ICG-001 group ($P < 0.05$). A representative image of the western blot is shown in Figure 5 and Supplementry Table S V.

DISCUSSION

The pathogenesis of ASD is still unclear and has become a focus of research at home and abroad (Antaki *et al*., 2022; Bjørk *et al*., 2022; Solmi *et al*., 2022). In preclinical studies, the VPA rat model is commonly used to mimic an animal model of ASD. In this study, rats were used to establish the ASD model; using rats has the advantages of low cost, a short gestation period and a large litter size. Additionally, the technical conditions for the observation of rat behaviour, learning and memory are relatively mature. Rats are neuroanatomically, biochemically, electrophysiologically and genetically similar to humans, which facilitates the study of biological mechanisms. However, human physiological and behavioural characteristics are more abundant, biological characteristics are quite different, and some ASD characteristic signalling pathways are not the same. Therefore, it is of great significance to continue developing more suitable ASD biological models.

miRNA in brain is an important regulator of brain function. Human brain development is an extremely complicated process, and the interaction of genetic and non-genetic factors affects brain development (Bjørk *et al*., 2022). Therefore, it is very important to grasp the related interference factors, especially the factors regulating gene expression, at the critical stage of brain development to understand the evolution of human cognitive function. Transcriptional regulation has been proved in the process of brain development, but gene regulation at the post-translation level is equally important, and miRNA is the main regulator after translation (Solmi *et al*., 2022). Studies have shown that mir-155-5p may be one of the characteristic regulators of ASD (Huang *et al*., 2021; Rastegari *et al*., 2023). As one of the typical representatives of the miRNA family, miR-155-5p is involved in regulating pathological processes such as tumour cell differentiation and proliferation, and also plays an important role in the process of immune inflammation and microbial infection (Juźwik *et al*., 2019; Ji *et al*., 2022). Studies have shown that by inhibiting the expression of miR-155-5p in the prefrontal cortex of autistic mice, the social behavior disorder of VPA autistic mice can be improved and the ability to explore new things independently can be improved. This study established a VPA rat model and a miR-155-5p overexpression rat model and found that miR-155-5p was overexpressed in both rat models, and both reflected significant ASD-like features. Inhibiting the expression of miR-155-5p could effectively improve the ASD-like features of VPA rats.

Classical Wnt/β-catenin signalling pathway plays a very important role in the process of autism. Studies have shown that there is abnormal expression of Wnt/β-catenin signalling pathway in cerebellum of autistic patients. The Wnt/β-catenin signalling pathway is abnormally expressed in ASD patients (Zhang and Wang, 2020; Clevers adn Nusse, 2012; Mayer *et al*., 2020; Gazestani *et al*., 2019). To further determine the specific pathway by which miR-155-5p affects ASD-like characteristics in rats, we modulated the expression of miR-155-5p while regulating Wnt/β-catenin signalling. Pathway-related proteins were detected, and the results showed that compared with the control group, the two rat models overexpressing miR-155-5p not only indicated meaningfully increased ASD-like features but also significantly up-regulated the expression of proteins related to the Wnt/β-catenin signalling pathway, indicating that miR-155-5p and the Wnt/β-catenin signalling pathway significantly correlated with affecting autistic behaviour.

We selected ICG-001, a Wnt/β-catenin inhibitor, to down-regulate the expression of related proteins by inhibiting the Wnt/β-catenin pathway disease and observed the VPA rats and overexpressed miR-155-5p rats in terms of the variations in ASD-like features of the model. The results showed that after ICG-001 inhibited the Wnt/β-catenin signalling pathway, the expression of related proteins was down-regulated, and the ASD-like features were significantly improved.

CONCLUSION

The overexpression of miR-155-5p led to the aggravation of ASD-like symptoms in rats, and this effect was significantly related to the Wnt/β-catenin signalling pathway. These results suggest that miR-155-5p may be a new target of ASD, and our results also show that miR-155-5p can regulate Wnt/β-catenin signaling pathway. We will continue to explore the relationship between miR-155-5p and Wnt/β-catenin signaling pathway. In addition, these results suggest that inhibitors of Wnt/β-catenin

signaling pathway may be a new strategy to treat ASD. These results provide a new idea for clinical treatment of ASD. These results have practical significance for further in-depth research on the pathogenesis and targeted therapy of ASD.

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Ethical statement

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of The Fifth Affiliated Hospital of Zhengzhou University (KY2020015).

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20230802054154

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

miR-155-5p is Involved in Regulating Autism-Like Behaviour, Pain Sensitivity and Inflammatory Response in Brain Tissue via the Wnt/β-Catenin Pathway in Valproic Acid Rat Model of Autism Spectrum Disorder

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Supplementary Table S I. Results of the open field test among groups of ASD rats ($\bar{x}$±s).

Group	n	No. of cross grid	Vertical score
Blank control group	5	38.94±5.02	43.98±4.90
ASD group	5	15.23±1.69[a]	22.39±2.87[a]
miR-155-5p-mimics group	5	9.98±1.47[ab]	13.32±1.26[ab]
ICG-001 group	5	36.43±4.06[bc]	39.98±5.03[bc]
miR-155-5p-mimics+ ICG-001 group	5	14.82±2.14[bcd]	21.56±3.07[bcd]
F value		121.135	64.742
P value		<0.001	<0.001

Compared with the blank control group, [a]$P<0.05$; Compared with the ASD group, [b]$P < 0.05$, Compared with miR-155-5p-mimics group, [c]$P < 0.05$; Compared with the ICG-001 group, [d]$P < 0.05$.

Supplementary Table S II. Results of self-grooming test among groups of ASD rats ($\bar{x}$±s).

Group	n	Self-grooming time (s)
Blank control group	5	32.08±4.01
ASD group	5	65.47±10.23[a]
miR-155-5p-mimics group	5	79.89±9.16[b]
ICG-001 group	5	34.75±3.38[bc]
miR-155-5p-mimics+ICG-001 group	5	68.24±7.05[bcd]
F value		30.195
P value		< 0.001

For statistical details see, Supplementary Table S I.

Supplementary Table SIII. Comparison of pain sensitivity among groups of ASD rats ($\bar{x}$±s).

Group	n	PWTL (s)
Blank control group	5	6.01±1.08
ASD group	5	9.51±1.23[a]
miR-155-5p-mimics group	5	12.42±1.54[ab]
ICG-001 group	5	6.53±1.75[bc]
miR-155-5p-mimics+ICG-001 group	5	9.98±1.04[bcd]
F value		21.060
P value		< 0.001

For statistical details see, Supplementary Table S I.

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0030-9923/2024/0001-0001 $ 9.00/0

Supplementary Table S IV. miR-155-5p expression in brain tissues of rats with ASD among groups ($\bar{x}$±s).

Group	n	miR-155-5p
Blank control group	5	1.69±0.20
ASD group	5	11.19±1.34[a]
miR-155-5p-mimics group	5	17.14±1.85[ab]
ICG-001 group	5	2.25±0.31[bc]
miR-155-5p-mimics+ICG-001 group	5	12.28±1.57[bcd]
F value		98.079
P value		< 0.001

For statistical details see, Supplementary Table S I.

Supplementary Table S V. Expression levels of β-catenin, GSK-3β, cyclin-D1, and c-myc in the brain tissue of rats with ASD in various groups ($\bar{x}$±s).

Group	n	β-catenin	GSK-3β	cyclin-D1	c-myc
The blank control group	5	1.01±0.03	0.50±0.02	0.10±0.01	1.05±0.02
The ASD group	5	4.25±0.95[a]	3.10±0.98[a]	1.15±0.93[a]	6.75±0.97[a]
The miR-155-5p-mimics group	5	5.05±1.03[ab]	4.65±0.86[ab]	1.52±0.37[ab]	8.13±1.03[ab]
The ICG-001 group	5	1.55±0.02[bc]	1.06±0.02[bc]	0.35±0.01[bc]	1.83±0.02[bc]
The miR-155-5p-mimics+ICG-001 group	5	4.65±0.06[bcd]	2.89±0.06[bcd]	1.08±0.08[bcd]	6.05±0.12[bcd]
F value		146.56	151.08	211.66	235.80
P value		<0.001	<0.001	<0.001	<0.001

β-catenin, β-phosphoprotein; GSK-3β: glycogen synthase kinase-3; Cyclin-d1: cyclin D1; c-myc: Cyto-myelomatosis virus oncogene. For statistical details see, Supplementary Table S I.