Research paper

Study on the Mechanism of Action of MicroRNA-140-5p in the Treatment of Autism by Regulating the Nuclear Factor Kappa B Signaling Pathway

XIAO XIAO, RONG AI[1], Y. TIAN, NING MI[2], W. LIU, S. CHENG, NA QIAN AND X. ZHU*

Department of Pediatrics, The Affiliated Hospital of Guizhou Medical University, [1]Department of Pharmacology, Guizhou Medical University, Guizhou 550004, [2]Department of Cardiovascular Medicine, First People's Hospital of Qingzhen, Guiyang, Guizhou 551499, China

To study the effect and mechanism of microRNA-140-5p on inflammatory factors in autistic rats induced by valproate. 50 Sprague-dawley rats at 12.5 d of pregnancy were randomly selected and divided into groups. The valproate group (10 rats) was intraperitoneally injected with valproate (600 mg/kg); the valproate+microRNA-negetive control group was intraperitoneally injected with valproate (600 mg/kg) and microRNA-negetive control; valproate+microRNA-140-5p group, intraperitoneal injection of valproate (600 mg/kg) and microRNA-140-5p; valproate+microRNA-140-5p+phorbol 12-myristate 13-acetate group was intraperitoneally injected with valproate (600 mg/kg) and microRNA-140-5p and phorbol 12-myristate 13-acetate. The control group (10 rats) was intraperitoneally injected with the same amount of normal saline. The impact of injury, polymerase chain reaction and enzyme-linked immunosorbent assay methods to detect the impact of inflammation-related and nuclear factor kappa B-related genes and proteins expression. The results showed that valproate-induced autistic rats may cause death or malformation, microRNA-140-5p may alleviate the death or malformation caused by it and phorbol 12-myristate 13-acetate may reverse the therapeutic effect of microRNA-140-5p; valproate modeling will cause the weight loss of rats and the high expression of microRNA-140-5p can effectively alleviate the weight loss and the use of phorbol 12-myristate 13-acetate will cause weight loss; microRNA-140-5p can shorten the time of opening eyes for the first time in valproate mice and can improve the learning and memory ability of valproate rats and the pathological damage of brain tissue; microRNA-140-5p can also inhibit the activation of nuclear factor kappa B signal to achieve the effect of inhibiting the expression of inflammatory factors. MicroRNA-140-5p reduces the secretion of inflammatory factors in valproate-induced autistic rats by regulating the nuclear factor kappa B signaling pathway.

Key words: MicroRNA-140-5p, valproate, autism, nuclear factor kappa B

Autism is a serious neurodevelopmental disorder. The symptoms of autism are diverse and neurodevelopmental disorders include social communication difficulties, language communication difficulties, repetition, increased stereotyped behaviors and limited interests[1,2]. Children with autism often use primitive and atypical gestures to communicate with others, but they have special communicative functions for children with autism. The reason why children with autism use this method is that these behaviors can arouse people's attention and can greatly meet their communication needs. The main complaint in the first clinic of children with clinical autism is speech impairment, so speech recognition impairment has received widespread attention. As autistic children have their particularities in the development of cognition, thinking and social abilities, their language barriers have distinctive characteristics. And the differences between individuals are very large. Therefore, it is of great significance to find the specific markers of the disease and to understand its treatment mechanism[3,4].

***Address for correspondence**
E-mail: yofanowo@163.com

MicroRNA (miRNA) is a type of non-coding RNA molecule that exists widely in the biological world. Because it does not have an open reading frame, it cannot encode proteins. miRNA has a variety of biological functions and plays a vital role in cell growth, differentiation and embryonic development[5]. Research reports show that miRNAs are related to the occurrence of diseases, miRNAs play a regulatory function in diabetes, heart disease and human tumors[6,7] and miRNAs can also be used as molecular markers for disease diagnosis and treatment. MiR-140-5p has physiological and pathological regulatory effects. It has regulatory functions on cell energy metabolism and malignant transformation of tumor cells.

In this study, Valproate (VPA) rats were used as experimental subjects to explore its expression changes in VPA-induced autism models and to clarify the role of miR-140-5p in the secretion of inflammatory factors in VPA-induced autism models, in order to clarify VPA. The molecular mechanism of inflammation in the induced autism model and molecular targeted treatment of autism provide reference.

MATERIALS AND METHODS

Animal model preparation and grouping:

Healthy adult Sprague-Dawley (SD) rats in the reproductive cycle, weight: male 280-290 g, 10, female 240-250 g, 20. Raised for several days under the conditions of periodic light (07:00-19:00), constant temperature of 24° and constant humidity of 55 %. After adapting to the environment for 2 w, follow the method of Schneider[8] and others. At 17:00, the male to female ratio was 2:1. The vaginal smear was checked at 8:00 the next day. The sperm was found on the 1st d of pregnancy (E1); the pregnant rat is kept in another cage. Divide the pregnant rats into 5 groups immediately, At E12.5 d in the VPA group, pregnant rats in the model group were intraperitoneally injected with sodium valproate 600 mg/kg (sodium valproate powder was mixed with physiological saline to make a 250 mg/ml solution); pregnant rats in the VPA+miR-Negetive Control (NC) group were injected intraperitoneally with valproate sodium valproate 600 mg/kg (sodium valproate powder is mixed with physiological saline to make 250 mg/ml solution) and injected with miR-NC; pregnant rats in the VPA+miR-140-5p group were injected intraperitoneally with sodium valproate 600 mg/kg (valproic acid sodium powder was made into 250 mg/ml solution with physiological saline) and miR-140-5p was injected; pregnant rats in the VPA+miR-140-5p+Phorbol 12-Myristate 13-Acetate (PMA) group were injected intraperitoneally with sodium valproate 600 mg/kg (sodium valproate powder for physiological Saline was prepared as a 250 mg/ml solution) and injected with miR-140-5p and PMA; pregnant rats in the control group were injected with the same amount of normal saline intraperitoneally. The offspring born by the mother mouse in the model group are recorded as the sodium valproate group (VPA group, n=20); the offspring born by the mother mouse in the VPA+miR-NC group (VPA+miR-NC group, n=20); VPA+miR-140-5p pups from female rat (VPA+miR-140-5p group, n=20); VPA+miR-140-5p+PMA pups from female rat (VPA+miR-140-5p+PMA group, n=20); the offspring born by mother rat in the control group were recorded as the saline group (control group, n=20); the day of birth was recorded as the 1st d after birth (P1).

Comparison of the mortality and deformity rate of the five groups of offspring in 1 mo:

After the five groups of rats have grown up for 1 mo, observe their status and record the mortality and deformity rate of the offspring.

Comparison of the body weight of rats in each group:

Weigh the weights of rats in each group at P7, P14, P21, P56 and P90.

Eye-opening time measurement:

The rats in each group were observed to open their eyes at P12, P13, P14, P15 and P16. Scoring criteria: 0 points-both eyes are open; 1 point-1 eye is open; 2 points-both eyes are open.

Morris water maze test to detect the effect of miR-140-5p on the learning and memory ability of rats:

The Morris water maze experiment is divided into the 1st 5 d of positioning and navigation experiments and the 6th d of space exploration experiments. The water maze circular pool is divided into four quadrants and the escape platform is placed in one of the quadrants. A training experiment was carried out from d 1 to d 5 and the rats were trained once a day at a fixed time in the morning and afternoon. The rats were put into the water facing the pool wall and the time when the rat found the platform within 60 s and the swimming route before the platform were recorded. It was recorded as the escape latency and swimming distance of the rat. If the mouse cannot find the platform within 60 s, the escape latency is recorded as 60 s and the experimenter guides the mouse to the platform and stays for 20 s. Analyze the spatial learning ability of rat. After the

positioning and navigation experiment is over, the platform is removed on the 6th d and the rat are placed in the pool from any entry point and the time the rat stay in the target quadrant and the number of times they cross the platform are recorded[9,10].

Haemotoxylin and Eosin (HE) staining to observe the effect of miR-1400-5p on pathological damage in rats:

Rats are anesthetized and perfused into the heart; the brain tissue is removed, dehydrated, embedded and sliced, with a thickness of 7 μm. The sections were fixed with alcohol; stained with hematoxylin staining solution; differentiation with hydrochloric acid alcohol differentiation solution; ammonia returning to blue; staining with eosin staining solution; dehydrated with gradient alcohol and dried, mounted with neutral gum and covered with a cover glass. Observe the pathological damage of the brain tissue of each group of mice under a microscope at 10×20 times and take pictures to record[11,12].

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis:

In this section, we examined the expression of inflammation related genes and nuclear factor kappa B (NF-κB) related messenger ribonucleic acid (mRNA). The total RNA was isolated from rat brains using TRizol reagent (Invirtrogen), the tissue were converted complementary DNA (cDNA) by OneScript Reverse Transcriptase OneScript cDNA Synthesis Kit (Abm). 25 μl Dream Taq PCR Master Mix (Abm), 1.5 μl forward and reverse primer (Ribobio, Guangzhou), 2 μl cDNA and 20 μl water nuclease free in amplification reaction mixture (50 μl) and the PCR condition were as follows: 95° (2 min, a cycle), 95° (30 s), 58° (30 s), 72° (1 min), 35 cycles in total. Finally, 72° (10 min a cycle). β-actin served as the control[13,14].

Enzyme-Linked Immunosorbent Assay (ELISA) method for determination of cytokine levels:

The brain tissues of rats were collected and the levels of interleukin-6 (IL-6), IL-1β, Tumour Necrosis Factor alpha (TNFα), NF-κB (p65), p-IκBα and NFKB inhibitor α (IκBα) in rat tissues were determined according to the method steps of the ELISA kit instructions[15,16].

Statistical analysis:

All data are shown as mean standard±deviation. Graphpad 6.0 statistical software (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analyses. Student's t-test was performed to analyze difference between two groups. One-way analysis of variance was used to detect differences between more than two groups. And p value was <0.05 was considered significant.

RESULTS AND DISCUSSION

The control group died within 1 mo with 1 rat without deformity. The VPA group died within the same periods of time 7 and 4 were deformed. The lethality and teratogenicity of the miR-NC+VPA group were the same as that of the VPA group. There were 3 deaths in the miR-140-5p+VPA group and 2 malformations, which was significantly lower than the miR-NC+VPA group. 5 deaths and 3 malformations in the miR-140-5p+VPA+PMA group (Table 1), the above results showed that VPA-induced autistic rats may cause death or malformation, miR-140-5p may alleviate the death or malformation caused by it and PMA may reverse the therapeutic effect of miR-140-5p.

At P7, there was no significant difference in body weight between the groups ($p>0.05$); after P14, the differences between the groups gradually showed that the VPA group was significantly lower in weight compared with the control group at P14 ($p<0.05$), Compared with miR-NC+VPA, the weight of rats in the miR-140-5p+VPA group increased significantly ($p<0.05$), while the weight of rats in the miR-140-5p+VPA group in the miR-140-5p+VPA+PMA group There was no significant difference; at P21, the weight of rats in the VPA group was significantly lower than that of the control group ($p<0.01$). Compared with miR-NC+VPA, the weight of rats in the miR-140-5p+VPA group was significantly increased ($p<0.01$), while the miR-140-5p+VPA+PMA group miR-140-5p+VPA group rats weight decreased ($p<0.05$); at P56 and P90, the weight change trend of each group was the same as that of 21 d, of which 90 d. The difference between the groups was the most significant, as shown in Table 2.

At P12, P13 and P14, the score of eye-opening time in the VPA group was lower than that of the control group ($p<0.05$); the eye-opening time score of miR-140-5p+VPA was higher than that of the miR-NC+VPA group; while the comparison between the miR-140-5p+VPA+PMA group and the miR-140-5p+VPA group has significant differences at P12 and P13, and there is no significant difference between the two groups at P14; at P15, there is no significant difference between the other groups ($p>0.05$). At P16 and later, the mice in each group opened their eyes completely. As shown in Table 3.

TABLE 1: COMPARISON OF MORTALITY AND DEFORMITY RATES

Group (n=20)	Death (n)	Deformity (n)	Death rate (%)	Deformity rate (%)
Control	1	0	5	0
VPA	7	4	35	20
miR-NC+VPA	7	4	35	20
miR-140-5p+VPA	3	2	15	10
miR-140-5p+VPA+PMA	5	3	25	15

TABLE 2: WEIGHT COMPARISON BETWEEN GROUPS

	Control	VPA	miR-NC+VPA	miR-140-5p+VPA	miR-140-5p+VPA+PMA
P7	14.8±1.4	13.48±1.6	13.2±1.4	13.9±1.2	13.5±1.2
P14	28.5±1.9	21.1±1.3*	20.7±1.6	27.7±1.6$^{\#}$	25.7±1.3
P21	39.8±4.7	27.6±7. 1**	16.9±6.4	38.9±4.2$^{\#\#}$	27.7±4.1$^{\&}$
P56	178.1±7.4	167.3±8.1**	165.2±7.3	177.8±6.9$^{\#\#}$	160.6±5.9$^{\&\&}$
P90	302.7±15.2	284.5±12.7**	281.9±13.4	300.9±14.7$^{\#\#}$	284.5±13.2$^{\&\&}$

Note: *p<0.05 *vs.* control; **p<0.01 *vs.* con; $^{\#}$p<0.05 vs. miR-NC+VPA; $^{\#\#}$p<0.01 *vs.* miR-NC+VPA; $^{\&}$p<0.05 *vs.* miR-140-5p+VPA

TABLE 3: COMPARISON OF EYE OPENING IN EACH GROUP

	Control	VPA	miR-NC+VPA	miR-140-5p+VPA	miR-140-5p +VPA+PMA
P12	0.60±0.69	0.00±0.00*	0.00±0.00	0.40±0.43$^{\#}$	0.00±0.00$^{\&}$
P13	1.40±0.85	0.00±0.00**	0.00±0.00	1.20±0.92$^{\#\#}$	0.50±0.44$^{\&\&}$
P14	1.70±0.56	1.00±0.58*	1.00±0.47	1.50±0.46$^{\#}$	1.20±0.37
P15	2.00±0.00	1.70±0.45	1.80±0.56	2.00±0.00	1.90±0.32
P16	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00

Note: *p<0.05 *vs.* control; **p<0.01 *vs.* con; $^{\#}$p<0.05 *vs.* miR-NC+VPA; $^{\#\#}$p<0.01 *vs.* miR-NC+VPA; $^{\&}$p<0.05 *vs.* miR-140-5p+VPA

In the Morris water maze experiment, compared with the blank control group, the escape latency and swimming distance of the VPA group were significantly increased and the number of crossing platforms and the percentage of stay time in the target quadrant were significantly reduced ($p<0.05$ or $p<0.01$). Compared with the miR-NC+VPA group, the miR-140-5p+VPA group had significantly shorter escape latency and swimming distance and significantly increased the number of platform crossings and the percentage of stay time in the target quadrant; compared with the miR-140-5p+VPA group, miR-140-5p+VPA+PMA's escape latency and swimming distance are relatively prolonged and the number of crossing platforms and the percentage of stay time in the target quadrant are reduced; the above results indicate that the miR-140-5p+VPA group can improve the learning and memory ability of VPA rats, as shown in fig. 1.

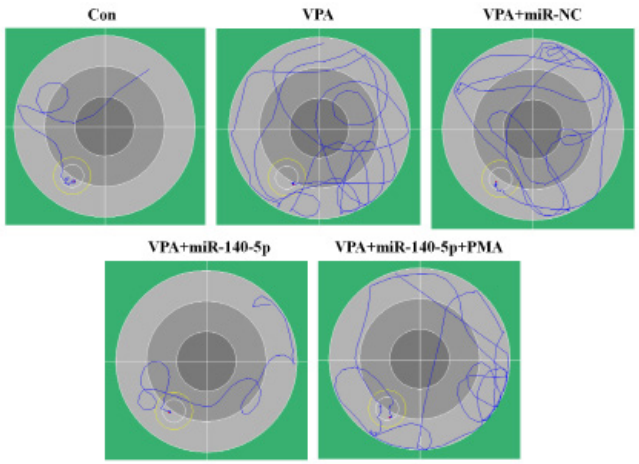

Fig. 1: Effects of miR-140-5p on the learning and memory function of VPA rats

The HE staining results shows (fig. 2) that in the blank control group, the cells in the Carbonic Anhydrase 3 (CA3) area of the hippocampus of rats are evenly colored and the brain tissue cells are clearly and densely distributed, uniformly arranged, full cell bodies, clear nuclear membranes, complete cell structures and clear outlines. Compared with the blank control group, the cells in the hippocampal CA3 area of the VPA group are disorderly arranged with large gaps, irregular, lighter coloration, cell membranes and nuclear membranes are not clear. Compared with miR-NC+VPA, the brain tissue cells in the miR-140-5p+VPA group are clearly and densely distributed, evenly arranged and the cell body is full, that is, miR-140-5p can improve the brain tissue damage of VPA rats, while miR-140-5p+VPA+PMA and miR-140-5p. Compared with miR-140-5p+VPA, the arrangement is relatively disordered and the gap is large, irregular and the coloring is lighter. The above results indicate that PMA may reverse the therapeutic effect of miR-140-5p on the brain tissue of VPA rats.

In order to verify whether miR-140-5p can inhibit the expression of inflammatory factors and explore its relationship with the NF-κB signaling pathway, we used RT-PCR to detect the expression of related genes (fig. 3) and ELISA method to detect the expression of related proteins (Table 4). The results showed that compared with the control group, the expression of miR-140-5p in the brain of the VPA group was significantly reduced ($p<0.01$), miR-140-5p could significantly increase its expression ($p<0.01$) and PMA has no regulatory effect ($p>0.05$); at the same time, we detected the expression of inflammation-related factors including IL-6, IL-1β and TNF-α. The results showed that compared with the control group, the expression of IL-6, IL-1β, TNF-α were significantly increased ($p<0.01$); Compared with miR-NC+VPA, IL-6, IL-1β and TNF-α in the brains of rats in the miR-140-5p+VPA group were significantly reduced ($p<0.01$). The above results indicate that miR-140-5p can inhibit the expression of IL-6, IL-1β, TNF-α and other inflammatory factors. Compared with miR-140-5p+VPA, the expression of inflammatory factors has increased in miR-140-5p+VPA+PMA group. Therefore, we speculate that miR-140-5p inhibits the expression of inflammatory factors may be related to the NF-κB signaling pathway. The results showed that compared with the control group, the p65 and p-IκBα protein levels in the VPA group increased; miR-140-5p can significantly inhibit the p65 and p-IκBα protein levels (fig. 3 and Table 4); The protein expression levels of p65 and p-IκBα increased in the miR-140-5p+VPA+PMA group. NF-κB signal activator PMA increases the level of NF-κB signal activation and partially reverses the inhibitory effect of miR-140-5p on NF-κB signal. The above results indicated that miR-140-5p can inhibit the activation of NF-κB signal to achieve the effect of inhibiting the expression of inflammatory factors.

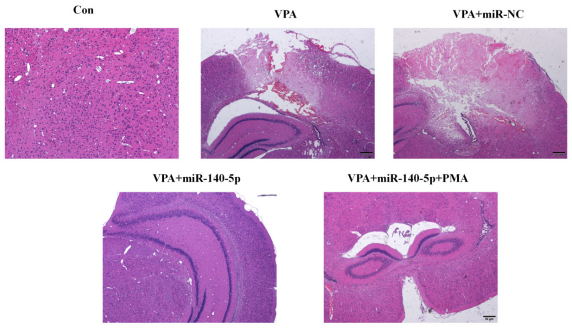

Fig. 2: HE staining to observe the effect of miR-140-5p on brain damage in autistic rats

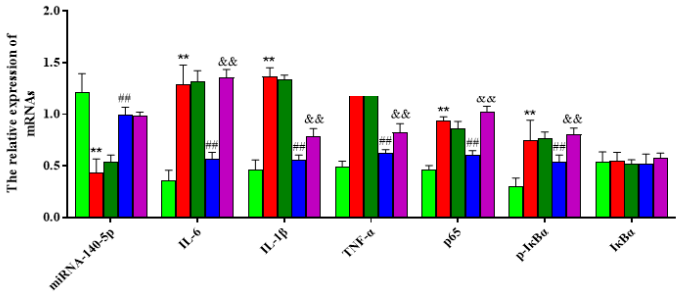

Fig. 3: The expression of IL-6, IL-1β, TNF-α, p65, p-IκBα and IκBα related genes were detected by RT-PCR
Note: *p<0.05 *vs.* con; **p<0.01 *vs.* con; #p<0.05 *vs.* miR-NC+VPA; ##p<0.01 *vs.* miR-NC+VPA; &p<0.05 *vs.* miR-140-5p+VPA, (▭) CON; (▭) VPA; (▭) MiR-NC-VPA; (▭) MiR-140-5p+VPA; (▭) MiR-140-5p+VPA+PMA

TABLE 4: VPA RATS TRANSFECTED WITH miR-140-5P, THE CONTENTS OF IL-6, IL-1B AND TNF-α IN THE BRAIN (pg/ml, x±s)

Group	Control	VPA	miR-NC+VPA	miR-140-5p+VPA	miR-140-5p+VPA+PMA
IL-6	26.84±2.17	35.76±2.03*	36.89±2.28	29.54±2.30$^{\#}$	38.61±2.08$^{\&}$
IL-1β	13.95±1.18	22.81±1.24*	23.53±1.85	17.38±1.42	20.95±1.32
TNF-α	5.15±0.40	63.21±3.27**	62.98±4.46	12.45±1.23$^{\#\#}$	28.45±2.19$^{\&}$
p65	32.12±3.27	51.23±5.34*	56.29±4.32	40.27±3.28$^{\#}$	52.29±5.62$^{\&}$
p-IκBα	18.27±2.34	45.39±4.69*	43.58±6.42	33.59±3.57$^{\#}$	50.27±6.47$^{\&}$
IκBα	46.28±5.37	47.24±8.32	45.38±8.58	46.27±6.74	47.29±6.42

Note: *p<0.05 *vs.* con; **p<0.01 *vs.* con; $^{\#}$p<0.05 *vs.* miR-NC+VPA; $^{\#\#}$p<0.01 *vs.* miR-NC+VPA; $^{\&}$p<0.05 *vs.* miR-140-5p+VPA

Mothers who take VPA in early pregnancy to treat psychosis or anti-epilepsy have a very high risk of autism in their offspring[17]. Simple injection of pregnant mice, VPA can cause behavioral changes in offspring and maternal exposure to VPA will reduce autism candidates gene expression[18]. Electrophysiological studies have shown that VPA offspring exhibit abnormal microcircuit connections, which may be related to the long-term functional pathway damage in autistic patients shown by magnetic resonance imaging studies. Therefore, this article uses pregnant SD rats to establish a VPA autism model for follow-up research[19,20]. In order to clarify the molecular mechanism of inflammation in the induced autism model and molecular targeted treatment of autism provide reference.

miRNA has many biological functions and plays an important role in human embryo development, tissue function maintenance and tissue aging[21]. miRNA affects tissue function by regulating cell growth, differentiation, movement and apoptosis[22-25]. At present, the regulation of miRNAs has been found in the development of human malignant tumors, nerve damage, diabetes and other diseases[26-28]. The expression of miRNAs changes during the progression of the disease, and can promote or inhibit the occurrence of diseases by influencing the function of cells. miR-140-5p is a miRNA molecule that is currently found to be related to the occurrence of human diseases[29]. miR-140-5p is related to inflammation, tissue repair[30,31] and is involved in the progression of a variety of malignant solid tumors, arthritis, intervertebral disc inflammation, etc. Therefore, this article is based on miR-140-5p is a key indicator to examine its relationship with the treatment of autism. This experiment showed that the expression level of miR-140-5p decreased in VPA-treated autistic rats and upregulation of miR-140-5p could inhibit the secretion of inflammatory factors in VPA-treated autistic rats. miR-140-5p may have inhibit inflammation.

This experiment further explored the mechanism of miR-140-5p and found that up-regulation of miR-140-5p can reduce the expression levels of p65 and p-IκBα proteins in VPA-induced rats. Both p65 and p-IκBα are the key factors of NF-κB signal transduction. The higher the expression level of both, the higher the activation degree of NF-κB signal. NF-κB is highly conserved in evolution and it mostly exists as a dimer in human tissues. p65 is an indispensable subtype of NF-κB to function. NF-κB is a transcriptional regulator ubiquitous in the human body, which participates in the processes of immunity and inflammation. Previous studies have shown that NF-κB is negatively regulated by miR-140-5p and is involved in the occurrence of inflammatory injury of the intervertebral disc. The mechanism of miR-140-5p is related to NF-κB. The results of this experiment show that NF-κB activator can reverse the inhibitory effect of miR-140-5p on the secretion of inflammatory factors in VPA-induced autism rats, suggesting that miR-140-5p regulates VPA-induced inflammatory factors in autistic rats secretion is related to NF-κB signaling.

In conclusion, miR-140-5p reduces the secretion of inflammatory factors in VPA-induced autistic rats by regulating the NF-κB signaling pathway.

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Declaration of interest:

The authors report no conflicts of interest.

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