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Resveratrol mitigates Valproic Acid-Induced Autistic Features in mice via up-regulation of Nrf2/HO-1 signaling pathway

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Abstract

Introduction: A neurodevelopmental disease known as autism spectrum disorder (ASD) is linked to stereotypical behavior and poor social skills. Resveratrol (Res) has been shown to possess anti-inflammatory and antioxidant impacts. Objective: to demonstrate the underlying mechanisms and neuroprotective impact of Res in VPA-induced ASD Material & methods: Thirty male Swiss mice were split into three: control, VPA, and VPA+Res. Following neurobehavioral testing, the rats were sacrificed, and the cerebellar gene expression of Nrf2 and HO-1 was evaluated together with the measurements of MDA, SOD, TNF-α, IL-6, IL-10 and serotonin. GFAP and Caspase-3 cerebellar immunoreactions were performed. Results: While the VPA group's time of central crossing in OFT, percentage of alternation in the T maze test, Cerebellar SOD, Cerebellar IL-10, and Cerebellar gene expression of Nrf2 and HO-1 were dramatically lower than those of the control, the VPA 's number of crossing slots in OFT, rearing frequency in OFT, Cerebellar MDA, Cerebellar TNF-α, and Cerebellar IL-6 were all dramatically higher than those of the control group with impairments in social interaction. Res substantially reduced VPA-induced ASD. Conclusion: In addition to enhancing gliosis and up-regulating the Nrf2/HO-1 signaling pathway, Res also reduced VPA-induced ASD through anti-oxidant, anti-inflammatory and antiapoptotic pathways.

Keywords

- Autism
- Caspase-3
- GFAP
- HO-1
- Nrf2
- Resveratrol

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Introduction

A common neurodevelopmental illness, autism spectrum disorder (ASD) is typified by repetitive or stereotypical behaviors, as well as poor social interaction and communication skills.(1).One in 160 children globally has an ASD, according to a 2017 World Health Organization (WHO) report. ASDs significantly impair a person's ability to carry out daily tasks and engage in society, placing a heavy emotional and financial burden on those who are affected and their family.(2).

Oxidative stress (OS), mitochondrial dysfunction, and immunological dysregulation/inflammation all had an impact on the pathophysiology of ASD. While this is still somewhat contentious, more and more studies have hypothesized that OS and mitochondrial function may interact to influence the pathophysiology of ASD (3).

It is known that OS has a role in many neurodegenerative illnesses. Over the past ten years, there has also been mounting evidence that OS is altered in ASD and that the antioxidant system cannot restore equilibrium (4).

Autism has been linked to changes in serotonergic systems (5).Chronic inflammation is playing a bigger part in neurological conditions. Its function extends beyond particular neurotransmitter disorders; it also results in modifications to the brain's immunological, endocrine, oxidative/antioxidant, and neurotransmitter pathways (6).

A helpful model for examining autistic behavior and looking for novel treatments is the valproic acid (VPA) model (7).The cerebellum, and hippocampus undergo neuronal migration, myelination, synaptogenesis, and gliogenesis during the sensitive PND14 period. Giving VPA to animals with PND14, results in intrusions and neurodevelopmental regressions that produce behavioral impairment (8).

Nrf2 controls the expression of genes that code for detoxifying, anti-inflammatory, and antioxidant proteins as well as HO-1 (4).

Remarkably, the information that is now available suggests that Nrf2 is crucial for oxidative stress, mitochondrial malfunction, and immunological dysregulation/inflammation. Chronic neurological disorders may become more likely if Nrf2 activation is inhibited (9). Nrf2 separates from Keap1 in pathological circumstances and subsequently moves into the nucleus.Nrf2 will control the production of antioxidant proteins (10).

In order to control oxidative stress and neuroinflammation in the brain, the Nrf2 system must be functioning. Furthermore, there is growing evidence that Nrf2 may control metabolism and mitochondrial function. Thus, through the aforementioned mechanisms, Nrf2 activation reverses the pathophysiological and behavioral defects linked to ASD, fascinatingly (11).

Clinical interest in Nrf2 activator therapy for ASD is quite high. A number of well-known Nrf2 natural activators were crucial in controlling Nrf2 processes.(3) A naturally occurring polyphenolic, resveratrol (3,5,4-Trihydroxystilbene, or RSV) is found in grapes, red wine, berries, and nuts. It is created in response to attacks by chemicals, fungi, bacteria, and UV light. Since Res is an agonist of Nrf2, it may raise Nrf2 levels (12), which in turn may raise the production of antioxidant enzymes.

Res has been suggested as a potential treatment for ASD because of its anti-inflammatory and antioxidant impacts in a number of animal models of illness. There are currently no drugs for the

primary symptoms of ASD, and behavioral therapy is the mainstay of ASD treatment. On the other hand, pharmacological treatments are available for mental comorbidities that are often linked to ASD, including violent behaviors, seizures, and sleep difficulties. Because this molecule crosses the BBB, its impacts have also been recently investigated in a number of neurological illnesses (13).

These findings spark interest in researching the neuroprotective benefits of Res on VPA-induced ASD in mice as well as the potential underlying mechanism involving referral to the Nrf2/HO-1 pathway.

Materials and methods

Animals

Theodore the Bilharz Research Institute (Giza, Egypt) provided thirty male Swiss mice weighing 15–25 g, which were acclimated (5 mice/cage; two cages for each group).They were kept in environments with controlled temperature, humidity, With IRB NO:7/2025BIO13, the Faculty of Medicine's Ethics Committee at Menoufia University in Egypt gave its approval for the use and care of the animals

Experimental design

Mice were divided into three groups of ten at random.

(1) Control group: Starting with PND14, mice were given 1 mL of sodium carboxymethylcellulose solution intragastrically once daily for five weeks.

(2) Valproic acid-treated group (VPA): 400 mg/kg of sodium valproic acid (Sigma Aldrich, St. Louis, MO, USA) was administered subcutaneously once (14) on postnatal day (PND) 14 and 1 mL sodium carboxymethylcellulose solution was given intragastrically once daily starting on PND14 and continuing for five weeks.

(3) Valproic acid + Resveratrol group (VPA + Res): Mice received VPA on PND14 and Res (Sigma-Aldrich Co., Mo, USA), (20 mg/kg in 0.5 % sodium carboxymethylcellulose solution intra gastric) once a day beginning from PND14 and continued for five weeks (15)

24 hours following the conclusion of the intervention, the behavior of every mouse was assessed. Lastly, xylazine and ketamine anesthesia (10 and 35 mg/kg, respectively) were used to induce the euthanasia procedure. This was followed by brain dissection, cervical dislocation, and decapitation. The right half of the cerebellum was preserved for histological and immunohistochemical investigation using 10% buffered formalin, while the left half was split evenly for biochemical study and RT-PCR assay

Neurobehavioural Tests

Open field test: Mice were housed in a Plexiglas arena (30 x 20 cm) with 12 equal-sized squares for five minutes. Recorded were the number of crossing slots, rearing movements, and center crossing times (16).

T-Maze Spontaneous Alternation: This test assesses working memory-dependent exploratory behavior. For ten consecutive trials, mice were placed on the T-maze base and permitted to travel toward either the left or right arm. When all four paws were in one arm, it was considered an entrance (17).

Social Approach (Three-Chamber) Test: The test was administered in two 10-minute sessions. A mouse was positioned in the middle of a Plexiglass box that was separated into three interconnected

compartments during the first session. It was given the option to engage with an empty wire cup in one side chamber or a similar wire cup with an unknown mouse (stranger I) in the opposite chamber after five minutes of habituation. The mice were matched in terms of strain, age, and sex. The amount of time spent using each cup was tracked. In the second session, put a second control mouse (Stranger II) in the other side chamber of the same wire confinement cup. It should be identical in terms of strain, age, and sex. Measurements were made of the amount of time spent interacting with each cup (18).

Tissue Homogenate Preparation

Each weighted cerebellar tissue was homogenized separately using a tissue homogenizer for 15 minutes at 11,000 rpm. The supernatant was then collected and stored at -80°C for the test.

Cerebellar TNF-α (Cat.: MBS2507393, MyBioSource, Sandiego, CA, USA), cerebellar IL-6 (Cat.: MBS269892, MyBioSource, Sandiego, CA, USA), cerebellar IL-10 (IL-10: ERI3010-1, Assaypro LLC, Saint Charles, Missouri, USA) and cerebellar serotonin (Cat. No. MBS160104, MyBiosource, Sandiego, CA, USA) were measured using the ELISA Kit in accordance with the manufacturer's instructions.

Calorimetric kits (Biodiagnostic Company, Dokki, Giza, Egypt) were used to assess cerebellar MDA and SOD in accordance with the manufacturer's instructions.

Quantitative RT-PCR

One piece of cerebellar tissue was taken from each rat and placed in a falcon tube, where it was kept at -80 °C for RNA extraction and the Nrf2 and HO-1 assay. A 7500 real-time PCR machine (Applied Biosystems, CA, United States) was used to identify Nrf2 and HO-1.The first step of PCR was the synthesis of complementary DNA using the QuantiTect Reverse Transcription Kit (205311; Qiagen, Applied Biosystems, USA), and then the second step of PCR (the real-time PCR step) after RNA was extracted from cerebellar cells using a direct—zol RNA miniprep kit (Cat. No. R2051; ZymoResearch, USA).The following primers were used for the Nrf2 gene:

(1) Forward primer: 5-GGTTGCCCACATTCCCAAATC-3

(2) Reverse primer: 5-CAAGTGACTGAAACGTAGCCG-3

The following primers were used for the HO-1 gene:

(1) Forward primer: 5-AGGTGCACATCCGTGCAGAG-3

(2) Reverse primer: 5-CTTCCAGGGCCGTATAGATATGGTA-3

The endogenous control was β actin. Ten microliters of SYBR Green (2× QuantiTect PCR Master Mix), three microliters of cDNA, one microliter of forward primer, one microliter of reverse primer, and five milliliters of RNase-free water were used in each PCR reaction, which was carried out in a final volume of 20 microliters. Denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 31 s were the next 55 cycles. The data was processed using the Applied Biosystems 7500 software version 2.0.1.Gene expression was measured relative to one another using the comparative Ct technique. The Nrf2 and HO-1 genes' melting curve and amplification plot.

Histopathological method:

The brain was embedded in paraffin wax after being preserved in formalin.Deparaffinized5-µm

slices were subjected to histopathologic alanalysis. These were then successively rehydrated using ethanol grades of 100%, 90%, and 70%.The dye Hematoxylin and Eosin was applied.

Immunohistological staining,

In order to limit endogenous peroxidase activity, 5-µm slices were blocked for 30 minutes in 0.1% H2 O2 after being rinsed with PBS. The sections then were incubated with the primary antibodies {Anti-GFAP [1:300, mouse monoclonal) and anti-Caspase-3,(Abcam, working dilution 1:500)

Statistical analysis

Following data collection and analysis, they were found to satisfy the parametric assumptions based on the results of the Shapiro-Wilk test. As a result, one-way ANOVA and post hoc Bonferroni's tests were applied to the data. The data was displayed using the mean ± SD .Significance was considered to exist when the p value was 0.05 or less.

Results

While the VPA group's time of central crossing in OFT, percentage of alternation in the T maze test, cerebellar SOD, cerebellar IL-10, cerebellar serotonin, and cerebellar gene expression of Nrf2 and HO-1 were substantially lower than those of the control, the VPA group's number of crossing slots in OFT, rearing frequency in OFT, cerebellar MDA, cerebellar TNF-α, and cerebellar IL-6 were all substantially higher than those of the control group. The VPA +Res group exhibited substantially higher levels of time of central crossing in OFT, percentage of alternation in T maze test, cerebellar SOD, cerebellar IL-10, cerebellar gene expression of Nrf2 and HO-1, and substantially lower numbers of crossing slots in OFT, rearing frequency in OFT, cerebellar MDA, cerebellar TNF-α, and cerebellar IL-6.Table (1).

Table (1): The measured OFT results, % of alternation in T maze test, cerebellarMDA,cerebellar SOD, cerebellar TNF-α, cerebellar IL-6, cerebellar IL-10,cerebellar serotonin, cerebellar Nrf2 gene expression and cerebellarHO-1 gene expression in all studied groups

	Control group	**VPA group**	**VPA +Res group**
Number of crossing slots in OFT	131±8.9	62±7.9*	96±6.8*#
Time of central crossing in OFT	21±2.1	9±1.8*	14±2.1*#
Rearing frequency in OFT	31±3.4	59±2.14*	44±4.3*#
% of alternation in T maze test (%)	75±2.9	38±4.9*	59±3.8*#
Cerebellar MDA (nmol/ gm. Tissue)	2.9±0.11	18.9± 0.99*	9.2± 1.1*#
Cerebellar SOD (U/gm. Tissue)	4.2±0.3	1.1±0.29*	2.33±0.33*#
Cerebellar TNF-α (pg/ml)	95±5.9	211.9±7.98*	157±4.9*#
Cerebellar IL-6 (pg/mL)	131.8±3.98	340.9±8.97*	220.9±8.6*#
Cerebellar IL-10 (ng/mL)	9.88±0.32	5.89±0.14*	7.81±0.3*#
Cerebellar Serotonin(ng/ml)	135.9±7.9	68.9±4.18*	93.9±4.44*#
Cerebellar Nrf2 gene expression	1	0.39±0.05*	0.68±0.03*#
CerebellarHO-1 gene expression	1	0.41±0.02*	0.72±0.06*#

* Significant compared with control, # Significant compared with VPA.

In the first session, it was shown that the time spent by the subject mice in the empty chamber (399.8±11.4 vs. 140±10.4 s) was considerably less than that of the time spent by the subject mice in the control with stranger mice ($p<0.05$).However, mice in the VPA spent almost the same amount of time in the stranger and empty chambers (132±14.9 vs. 140.8±18.9 seconds), indicating that they did not prefer social proximity. The time spent with the stranger mouse was much longer ($p<0.05$) in the VPA+Res than the time spent in the empty chamber (290.8±10.9 vs. 150.8±18.9

seconds).In session two, the subject mice in the control spent 410±20.8 seconds with stranger II compared to 85.9±13.9 seconds with stranger I. This difference was statistically significant. However, mice in the VPA group spent almost the same amount of time in the strangers I and II chambers (160.8±12.8 vs. 167±10.1 seconds), indicating that they did not prefer social proximity. The amount of time spent with stranger II in the VPA+Res was much longer than that spent in the stranger I chamber (280±9.8 vs. 130±12.3 seconds).(Table 2)

Table 2: Three chamber test results in all studied groups

groups	Control		VPA		VPA+Res	
	Empty	Stranger	Empty	Stranger	empty	Stranger
Session I	140±10.4	399.8±11.4	132±14.9	140.8±18.9	150.8±18.9	290.8±10.9
	Stranger I	Stranger II	Stranger I	Stranger II	Stranger I	Stranger II
Session II	85.9±13.9	410±20.8	167±10.1	160.8±12.8	130±12.3	280±9.8

Histological results:

H&E:

Three layers made up the control group's cerebellar cortex: the inner granular cell layer, the middle Purkinje cell layer, and the outside molecular layer. These layers had a typical histological appearance. Purkinje cells appeared undersized, deformed with condensed chromatin, and encircled by vacuolated gaps, according to the VPA group. Cells were packed closely together to form the granular layer. Dispersed, strongly pigmented pyknoticcells were visible in the molecular layer. The VPA-Res group demonstrated improvement in the prior three layers' appearance. (Fig. 1)

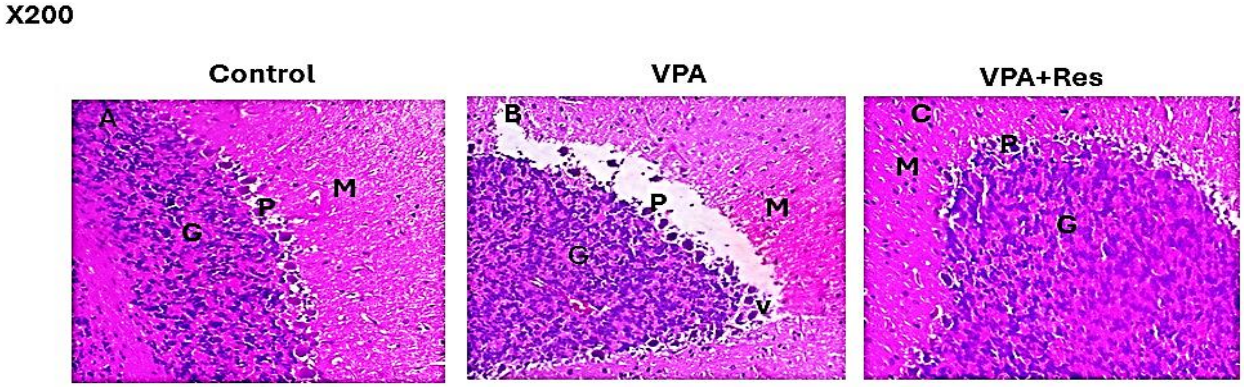

Fig. 1 : Rat cerebellar cortex H&E of all groups.The control group's cerebellar cortex had a normal histological appearance and was composed of three layers: the inner granular cell layer (G), the middle Purkinje cell layer (P), and the outer molecular layer (M).Purkinje cells (P) in the VPA group looked to be smaller, deformed, and encircled by vacuolated gaps due to condensed chromatin.The cells that made up the granular layer (G) were closely spaced.Dispersed, strongly pigmented pyknoticcells were visible in the molecular layer.The three layers showed amelioration in the VPA+Res group.

Immunohistochemical results:

When compared to the control, the VPA's percentage area of Caspase-3 increased substantially (53.5 ±0.31 vs. 3.4±0.21, $p<0.05$) in the Caspase-3 stain. However, as compared to VPA, the VPA+Res revealed a substantial drop in this proportion (13.2±0.04 vs. 53.5 ±0.31, $p<0.05$).(Fig.2: A-D).

Comparing VPA to control, the percentage area of GFAP increased substantially (59.5 ±0.22 vs.

2.9±0.01, p<0.05).Comparing this % to VPA, however, revealed a substantially drop in VPA+Res (14.2±0.04 vs. 59.5 ±0.22, p<0.05).(Fig.2: E-H).

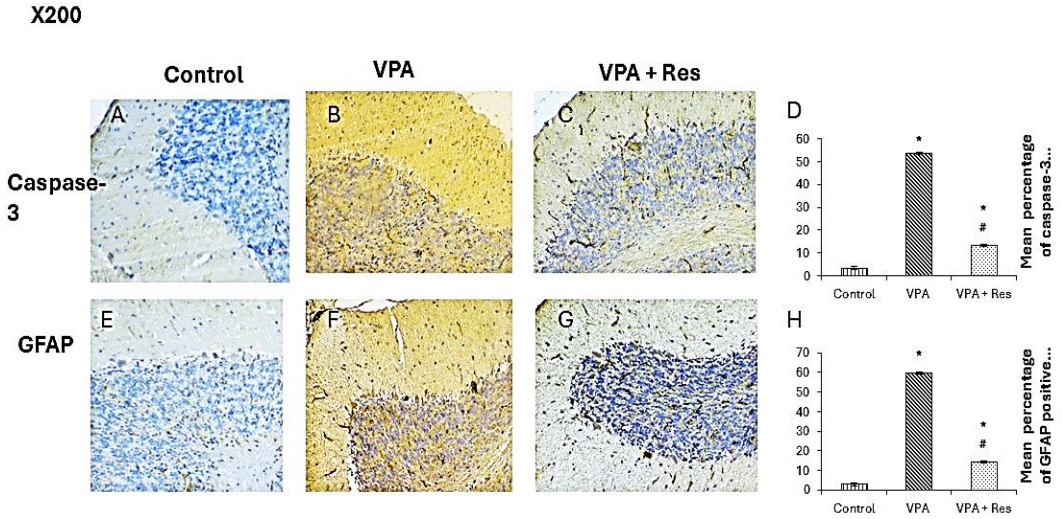

Fig (2): Representative micrographs of the different experimental groups showing a substantial upgrading of the Caspase-3 (A-D) and of the GFAP (E-F) immunoreaction in the VPA group and a significant declination of their percentage in the VPA+Res group. ×200 magnification.

Discussion

The first characteristic that distinguishes autism is a quantitative assessment of social interaction. The current study's findings showed that the VPA group's social interaction behavior was dramatically worse than that of the control. This outcome was in line with earlier published research [19]. Every autistic person exhibits repetitive behaviors [19].Comparing the VPA to the control, the current study showed a substantial decrease in the proportion of T-maze test alteration, suggesting repeated tendencies. This outcome was consistent with previous research [20].

Regarding the open field test, the current study's findings showed that the VPA 's behavior changed dramatically, as evidenced by the fact that the number of crossing slots and center entry times were substantially lower in the VPA than in the control. These findings concurred with previously published information [19].However, compared to the control, the number of rearings was noticeably higher. This outcome aligned with previous research [21].

However, the VPA + Res group shown a notable improvement in all disruptive behaviors. These findings concurred with those that had been previously published (22,23).This could be attributed to the antioxidant and anti-inflammatory impacts of Res (24).

Inflammatory and oxidative stress indicators were evaluated in order to determine the underlying mechanism of Res's ameliorative action. In the present investigation, the VPA group's brain MDA level was much higher than the control 's, whereas SOD was significantly lower. These findings concurred with those of earlier published research [25]. SOD enhances the anti-oxidative stress system, which helps with neuroprotection in autism [26].

Res dramatically ameliorated OS induced by VPA and this is in accordance with previous study (27). Res is thought to have antioxidant properties since it scavenges ROS. Additionally, via activating the Nrf2, Res raises glutathione levels and affects its metabolism (28)

Numerous studies have demonstrated immunological dysregulation and inflammation in both the brain and the periphery of people with ASD.(29)In the present investigation, the VPA group's inflammatory markers were noticeably higher than those of the control group with dramatically decreased IL-10.Similar outcomes have been documented before [30].Both the demyelination in the cerebellum and the autistic-like behaviors found in this study are caused by elevated IL-6.The length, shape, and distribution pattern of dendritic spines are abnormally altered when IL-6 levels are elevated because they alter both excitatory and inhibitory synaptic structures. Moreover, synaptic communication is disrupted [31].

Res reduced the inflammatory process that VPA caused, which is consistent with earlier research (27). Bakheet et al. [32] reported that transcription factors associated to T helper cells were repressedby Res. Additionally, previous study reported that Res therapy affected the levels of TLR4 and NF-kB mRNA and protein expression in brain tissue [33].Res inhibits this molecular pathway and lowers the levels of pro-inflammatory cytokines.[33)

Our histopathological results of the cerebellum validated our results and this goes in line with previous study (34), however Res dramatically ameliorated VPA induced cerebellar histopathological changes

Serotonin levels in the VPA group's cerebellum were lower. High levels of serotonin (5HT) in the whole blood during the early stages of brain development may account for this. It can enter the developing fetus's brain and cause the loss of 5-HT terminals through a negative feedback mechanism, which results in low intra-cerebral 5-HT concentrations in people with ASD. Throughout later development, this loss of 5-HT innervations continues. Consequently, autism symptoms manifest (35). However, Res was able to raise the cerebellum's serotonin levels. This outcome was in line with previous research (36,37).

GFAP immunoreaction was dramatically higher in the cerebellum of the VPA compared to control. Astrocytic dysfunction and GFAP expression decreased neuronal survival and were linked to adverse conditions in the CNS (38). After reactive activation, astrocytes release cytokines, according to Ramesh et al., which intensifies the inflammatory response and further damages the brain (39).

Nonetheless, the VPA+Res group significantly reduced the GFAP cerebellar immunoreaction, which is consistent with earlier research (15).

Through a variety of genes, Nrf2, a crucial transcription factor, controls detoxification and the antioxidant effect. After being oxidized, Nrf2 becomes active, moves into the nucleus, attaches itself to the ARE, and then increases the expression of its downstream antioxidant genesHO-1 (40).

VPA downregulated Nrf2/HO-1 singling pathway and this agreed with previous study (40). However Res up-regulated cerebellar Nrf2/HO-1 signaling pathway and this agreed with previous study (41).

Being an agonist of Nrf2, Res raises Nrf2 levels and cause it to translocate into the nucleus, activating genes containing AREs (12)

Furthermore, oxidative stress is essential for cell apoptosis.(42) .Our findings showed a markedly increased Caspase-3 cerebellar immunoreaction in comparison to the control, which is consistent with a prior study that showed exposure to VPA led to increased expression of apoptotic markers observed in the neuroepithelium (42).However, in contrast to the VPA group, Res significantly reduced the Caspase-3 cerebellar immunoreaction, which is consistent with other research (43), who reported that rather than controlling the amounts of apoptotic proteins in general, Res had an anti-apoptotic effect via inhibiting oxidative stress-induced apoptosis (43).

Conclusion

In addition to enhancing gliosis and up-regulating the Nrf2/HO-1 signaling pathway, Res also reduced VPA-induced ASD through anti-oxidant, anti-inflammatory and antiapoptotic pathways.

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