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Ginsenoside Rg1 attenuates hippocampal damage and improves ASD-like behaviors in a VPA-induced rat model by modulating SIRT1 expression

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ABSTRACT

Objective: To evaluate the effects of different doses of ginsenoside Rg1 on the structural development of hippocampal neurons in rats and explore its potential underlying mechanisms.

Methods: Rats exhibiting ASD-like behaviors were induced by a single intraperitoneal injection of valproic acid (VPA, 600 mg/kg, 250 g/L) on day 12.5 of gestation in pregnant Wistar rats. The following experimental groups were utilized: saline group (NS group, n=3), autism spectrum disorder group (ASD group, n=3), high-dose ginsenoside Rg1 group (Rg1-H group, n=3), and low-dose ginsenoside Rg1 group (Rg1-L group, n=3). The rats in the Rg1-H and Rg1-L groups received daily intraperitoneal injections of ginsenoside Rg1 from Postnatal Day 21 (PND 21) to PND 35 (Rg1-H: 40 mg/kg; Rg1-L: 20 mg/kg). The rats in the NS and VPA groups received the same volume of saline (1 mL/kg) during the same period. Behavioral tests were conducted on day 35 to assess ASD-like behaviors, and hippocampal tissues were extracted for the detection of neuronal apoptosis, autophagy, and inflammatory biomarkers.

Results: Ginsenoside Rg1 normalized the levels of inflammatory response, apoptosis, and autophagy biomarkers in the hippocampus by downregulating SIRT1 expression, thereby improving social communication deficits and repetitive stereotyped behaviors.

Conclusions: This study demonstrated that ginsenoside Rg1 can ameliorate atypical behaviors in rats exhibiting ASD-like behaviors by ameliorating pathological morphology of the hippocampal, thereby suggesting its potential as a promising therapeutic agent for ASD.

KEY WORDS: ASD; autism; hippocampus; SIRT1; Neuroinflammation; neuroapoptosis; neuroautophagy

BACKGROUND

Autism spectrum disorder (ASD) represents a group of neurodevelopmental disorders characterized by two core symptoms: deficits in social communication and interaction; and restricted and repetitive patterns of behaviors, interests, and activities. In addition to these core symptoms, ASD is often accompanied by comorbidities such as attention deficit disorder, developmental delays, and emotional disorders. The prevalence of ASD has been rapidly increasing, particularly in recent years. A 2020 survey conducted in the United States among 8-year-old children in 11 states revealed an 8% increase in ASD incidence compared with that in 2018, whereby an incidence as high as 1 in 36 children was observed^{[1], [2]}. Although the pathogenesis of ASD is still unclear and heterogeneous, given the common phenotypic characteristics that are observed among different ASD cases, it can be inferred that there is a common biological basis for its pathogenesis, such as impairments in neurogenesis, excitatory/inhibitory balance, neurotransmitters, immune response, and functional connectivity in brain regions^{[3]-[5]}.

As the first target of the limbic system of the brain, the hippocampus is crucial for memory storage, learning, emotion, and spatial perception. Its neuronal dysfunction is closely related to various mental illnesses, such as depression, schizophrenia, and anxiety disorders^{[6], [7]}. In addition, the hippocampus is involved in social behavior, with social behavioral issues representing a core manifestation of ASD^[8]. The initial symptoms of ASD in children typically appear between 1 and 2 years of age, which coincides with the critical period of hippocampal development^[9]. An increasing number of studies have demonstrated that defects in hippocampal neurogenesis underlie the atypical behavioral phenotypes observed in ASD patients^[10]. Pagni et al. reported that, compared with the normal population, ASD patients exhibit reduced connection integrity and accelerated atrophy in the hippocampus, with significant impairment of neuronal function and memory deficits being observed. These memory deficits can lead to atypical social behaviors in ASD children, including social interaction difficulties, and may also be one of the reasons explaining the repetitive and stereotyped behaviors observed in ASD children^{[11]-[13]}. In recent years, neurobiochemical changes that have been demonstrated in the hippocampal tissues of ASD patients and rats exhibiting ASD-like behaviors have attracted considerable attention. Studies have demonstrated that the levels of biomarkers

related to apoptosis, autophagy, and inflammation in the hippocampal tissue of rats exhibiting ASD-like behaviors are abnormal and closely related to the behavioral deficits of these animals^{[3, [14]}. Therefore, we speculate that the normalization of the neurobiochemical indicators in the hippocampal tissue may be an effective strategy for treating ASD.

The silent Information Regulator protein (Sirtuin) family belongs to Class III histone deacetylases. Currently, seven members of the Sirtuin family have been identified in mammals, each participating in different biological pathways according to their distinct functions^{[15], [16]}. Among them, Sirtuin 1 (SIRT1), as the first member identified in mammals, has now become the most extensively studied protein within the family. Serving as a core regulatory hub, SIRT1 integrates inflammatory, oxidative stress, and mitochondrial homeostasis through its deacetylation function, while also participating in multiple biological processes such as immune responses and cell cycle (including differentiation and proliferation)^[17]. Research has found that SIRT1 is widely distributed in multiple brain regions, including the hippocampus, prefrontal cortex, and cerebellum, and exhibits high expression levels in the brain and spinal cord during embryonic development. It is closely associated with the developmental regulation and functional performance of the central nervous system^{[18], [19]}. For instance, SIRT1 can enhance synaptic plasticity and improve cognitive function by deacetylating key proteins such as ribosomal protein S6 kinase B-1 (RSK2) and the transcription factor cAMP-response element binding protein (CREB)^[20]. Additionally, it can activate the NF- κ B pathway, thereby increasing the resistance of nerve cells to inflammatory responses and exerting neuroprotective effects^[21]. Therefore, numerous studies have confirmed that SIRT1 is closely associated with a variety of central nervous system diseases. Although a direct link between SIRT1 and autism has not yet been reported, considering its potential role in regulating the balance between neural excitation and inhibition, SIRT1 may play a part in the pathogenesis of autism.

Ginsenoside Rg1 is one of the most effective components of ginseng saponins and exhibits excellent protective effects on the cardiovascular system, nervous system, and immune system^[22]. When acting on the central nervous system, this compound can cross the blood-brain barrier and enter brain tissues such as the hippocampus and lateral ventricles, thus exerting neuroprotective and neurotrophic effects on central

nervous system diseases^[23]. Studies have shown that in rats with Alzheimer's disease treated with ginsenoside Rg1, there is a significant reduction in A β plaques in the brain, decreased expression of caspase-3, and a significant reduction in the number of TUNEL-positive cells^{[24]-[26]}. In addition, the antiapoptotic effects of ginsenoside Rg1 have been reported in animal models of depression, Parkinson's disease, and cerebral ischemia-reperfusion^{[27]-[30]}. In studies on posttraumatic stress disorder (PTSD) models, ginsenoside Rg1 intervention was observed to inhibit the production of proinflammatory factors induced by lipopolysaccharide stimulation, increase the expression of hippocampal synaptic proteins, and alleviate and diminish both depressive-like and PTSD-like behaviors in a rat model^[31]. Furthermore, the pharmacological effects of ginsenoside Rg1 in regulating autophagy in central nervous system diseases have also been reported^[32]. These studies reveal the therapeutic potential of ginsenoside Rg1 for autism-like behaviors.

In this study, offspring rats with autism spectrum disorder (ASD) were induced by prenatal exposure to VPA and subsequently treated with ginsenoside Rg1 to evaluate the effects of different doses of ginsenoside Rg1 on hippocampal neuronal structures, as well as its possible mechanisms of action, in an attempt to provide new insights into the pharmacological treatment of ASD (Figure 1).

METHODS

1. Experimental Animals

SPF-grade healthy adult female and male Wistar rats weighing 200–220 g were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (qualification certificate number: SCXK [Liao]-2020-0001). The rats were acclimatized for one week under suitable conditions with free access to food and water. All of the animal experiments were conducted in strict accordance with the relevant regulations of the Animal Experimental Ethics Committee of Jiamusi University. Approval was obtained from the Medical Ethics Committee (ethics approval number: jmsukf-2021024).

2. Model Preparation and Experimental Grouping

When the Wistar rats achieved a weight of more than 220 g, they were paired at a female:male ratio of 2:1. The first day of pregnancy was designated as Embryonic Day 1 (ED1) upon detection of a vaginal plug. Pregnant female (N=10) rats were randomly divided into two groups and administered a single intraperitoneal injection

of either VPA (600 mg/kg, 250 g/L)(Sigma Institute of Biotechnology, SML0011) (N=7) or saline (1 mL/kg) (N=3) on ED12.5. The offspring of pregnant rats injected with VPA were recorded as VPA group, and the offspring of pregnant rats injected with NS were recorded as NS group. Pups born before 17:00 on each day were designated as Postnatal Day 1 (PND 1), and those born after 17:00 were designated as PND 0. The model is prepared in this study by exposure of rat fetuses to VPA on the 12.5th day of gestation, which can accurately mimic the core symptoms of ASD patients and demonstrate good reliability and validity. However, to independently verify that this method can stably induce core symptoms (namely, social deficits and repetitive stereotyped behaviors) under the specific conditions of this experiment and to provide an indisputable model foundation for subsequent research, this study conducted model identification on the offspring rats in the VPA group at 21 days of age by three-chamber social interaction test and hair-stroking experiments, selecting individuals with obvious core symptoms to participate in subsequent experiments. Since the neural circuits and behavioral capacities related to social interaction and environmental exploration in rodents do not reach maturity and stability until the third postnatal week (around 21 days of age), conducting identification at PND 21 is most appropriate^[33]. If a test rat remains immobile or asleep for more than 50% of the time in any given test, its behavioral data will be excluded and it will not participate in subsequent experiments. Using the behavioral data of the offspring rats in the NS group as a baseline, the study identified offspring rats in VPA group at PND 21 whose social interaction index and social novelty index in three-chamber social interaction test were lower than 1.5 standard deviations below the mean of the NS group, and whose grooming duration in hair-stroking experiments was higher than 1.5 standard deviations above the mean of the NS group, as individuals with pronounced core symptoms and were randomly divided into three groups: the Rg1-H group, the Rg1-L group, and the ASD group.

2.1 Three-Chamber Social Interaction Test

The apparatus used for this test is a transparent, open-top behavioral box (60×45×20 cm) divided into three equal chambers. Each chamber has a small, manipulable door (10×10 cm) allowing the pups to freely move between chambers. A similarly sized wire-mesh cage, capable of holding one rat, is placed in the corner of each of the left and right chambers. The test is divided into three phases:

1) Habituation Phase: The test pup is placed in the central chamber with the doors to the side chambers open, allowing it to freely explore all chambers for 10 minutes to become familiar with the environment.

2) Social Interaction Phase: After habituation, the test pup is returned to the central chamber with the side chamber doors closed. A Wistar rat from a different litter, of similar age and gender to the test pup, with no prior contact with the test rat, is randomly placed in the cage of either the left or right chamber, designated as "Stranger 1". The side chamber doors are opened, and the test pup is allowed to freely explore for 10 minutes. The area within 6 cm of the cage is considered the interaction zone. A contact behavior is considered to have occurred when both forelimbs of the test rat are within this zone and its head is facing the cage. The cumulative contact time (in seconds) with Stranger 1 and the empty cage is observed and recorded separately. The social interaction index is calculated as $(\text{Time with Stranger 1}) / (\text{Time with Stranger 1} + \text{Time with Empty Cage})$.

3) Social Novelty Preference Phase: After Phase 1, the test pup is again placed in the central chamber with the side chamber doors closed. A second Wistar rat from a different litter, of similar age and gender to the test pup, with no prior contact with the test rat, is placed in the empty cage of the other side chamber, designated as "Stranger 2". The side chamber doors are opened, and the test pup is allowed to freely explore for 10 minutes. The cumulative contact time (in seconds) with Stranger 1 and Stranger 2 is observed and recorded separately. The social novelty preference index is calculated as $(\text{Time with Stranger 2}) / (\text{Time with Stranger 1} + \text{Time with Stranger 2})$.

2.2 Hair-Stroking Experiments

The equipment used for the experiment was an open-top transparent behavioral chamber (45×45×35 cm³). Before the experiment begins, the test rat is placed inside the behavioral chamber and allowed to move freely for 5 minutes to familiarize and adapt to the environment. After the adaptation period, the rat is returned to the central area of the behavioral chamber and allowed to move freely for 10 minutes. During this time, the total duration (in seconds) of grooming behavior by the test rat is observed and recorded. Grooming behaviors include using the forelimbs or hindlimbs to comb and clean the face, abdomen, tail, and other parts of the body.

3. Intervention with Ginsenoside Rg1

Pups in the Rg1-H (N=3) and Rg1-L (N=3) groups received daily intraperitoneal injections of ginsenoside Rg1 (Chendu Ruifensi Institute of Biotechnology, R-015) (Rg1-H group: 40 mg/kg; Rg1-L group: 20 mg/kg; 10 mg/mL) beginning from PND 21 to PND 35 at 8:00 AM. Pups in the ASD groups (N=3) received saline injections (1 mL/kg) during the same time period. Three rats were randomly selected from the NS group to participate in subsequent experiments, and were injected with saline (1 mL/Kg) at the same time.

4. Efficacy Evaluation

This study evaluated the intervention effect of ginsenoside Rg1 on rats exhibiting ASD-like behaviors by examining the behavioral tests, hippocampal neuronal apoptosis, autophagy, and inflammation levels at PND35.

4.1 Behavioral Tests

4.1.1 Three-Chamber Social Interaction Test

The operation is the same as above.

4.1.2 Marble Burying Test

A white, opaque, covered box (45×25×25 cm) is lined with clean, odorless, and fresh bedding material to a thickness of approximately 5 cm, ensuring a suitable level of softness. Twenty steel marbles, each with a diameter of 1 cm, are arranged in a 4×5 grid pattern on the bedding material. The test rat is then placed in the box, and the lid is closed, allowing it to freely move around within the box for 20 minutes. During this time, the number of buried marbles is observed and recorded (a marble is considered buried if its exposed volume is equal to or less than 1/3 of its total volume).

4.2 Biochemical test

4.2.1 Brain Tissue Extraction

Following the completion of behavioral tests, the test rats are anesthetized with sodium pentobarbital (Sigma Institute of Biotechnology, 11715) and subjected to cardiac perfusion with saline followed by 4% paraformaldehyde solution. After perfusion, the whole brain is removed and immersed in a sterile centrifuge tube containing 4% paraformaldehyde solution. The tube is then placed in a 4°C refrigerator for 24 hours for fixation. Following fixation, the brain tissue undergoes dehydration, clearing, wax immersion, embedding, and sectioning. The sections are stored in a 4 °C refrigerator for subsequent HE staining, Nissl staining, TUNEL staining, and

immunofluorescence staining. The hippocampal tissue samples were isolated and used for western blot analysis and other analyses, washed in ice-cold phosphate-buffered saline (PBS) and stored at -80°C until used.

4.2.2 HE Staining

HE staining was performed with HE Staining Solution (Saiweier Institute of Biotechnology, Wuhan, China) according to the manufacturer's instructions and viewed under a microscope.

4.2.3 Nissl Staining

Nissl staining was performed with Nissl Staining Solution (Saiweier Institute of Biotechnology, Wuhan, China) according to the manufacturer's instructions.

4.2.4 TUNEL Staining

Brain sections were rinsed with PBS, treated with 0.3% H₂O₂ for 15 min and rinsed in PBS three times for 5 minutes each. The sections were incubated with C1086 TUNEL Staining Solution following the manufacturer's instructions (Saiweier Institute of Biotechnology, Wuhan, China). The sections were scanned and photographed with a TCS SP2 laser scanning confocal microscope to count positive cells.

4.2.5 Immunofluorescence Staining

Sequentially immerse the sections in eco-friendly dewaxing solution I for 10 minutes, eco-friendly dewaxing solution II for 10 minutes, eco-friendly dewaxing solution III for 10 minutes, absolute ethanol for 5 minutes, absolute ethanol II for 5 minutes, absolute ethanol III for 5 minutes, followed by rinsing with distilled water. Then proceed to antigen retrieval. During the retrieval process, prevent excessive evaporation of the buffer and avoid drying out the sections. After completion of retrieval, allow natural cooling. Place the slides in PBS (pH=7.4) and wash them on a decolorization shaker 3 times, 5 minutes each. After drying the sections by centrifuging, use a histochemical pen to draw a circle around the tissue. Add BSA (for primary antibodies derived from goats, use 10% donkey serum for blocking; for primary antibodies from other sources, use 3% BSA for blocking) and incubate for 30 minutes. Add the prepared

primary antibody and incubate the slides overnight at 4 °C in a humidified chamber. Place the slides in PBS (pH=7.4) and wash them on a decolorization shaker 3 times, 5 minutes each. Add the corresponding secondary antibody and incubate at room temperature in the dark for 50 minutes. Place the slides in PBS (pH=7.4) and wash them on a decolorization shaker 3 times, 5 minutes each. Add DAPI staining solution and incubate at room temperature in the dark for 10 minutes. Place the slides in PBS (pH=7.4) and wash them on a decolorization shaker 3 times, 5 minutes each. Then add autofluorescence quencher B solution for 5 minutes, followed by rinsing with running water for 10 minutes. Mount the slides using an antifluorescence quenching mounting medium, and finally acquire the images.

4.2.6 Western Blot

Wash the tissue with cold PBS for 1-2 times, cut the tissue into small pieces and placed in a grinding tube. Add three 3 mm grinding beads, and the volume of lysis solution is 10 times of the tissue was added, and the grinding program was set to grind the tissue. Take out the grinded grinding tube and put it on ice or 4 °C lysate for 30 minutes. Maintain constant agitation for 30 minutes in ice bath, use pipette to mix it up. Centrifuge at 12,000 rpm, 4 °C for 10 minutes, and collect the supernatant, which is the total protein solution. Taking some undenatured protein solution and measured by the BCA protein concentration measurement kit. The specific method is referred to the kit instructions. All the samples were loaded in SDS-8% polyacrylamide gel (pH = 8.3), and electrophoresis was done at 200 V for 30 minutes. After that, the samples are transferred to polyvinylidene difluoride membranes, which were blocked for 1 hour at room temperature in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST). Then, blots were incubated overnight at 4 °C with a primary antibody. TBST were used to wash membranes three times, and then, blots were incubated for 1 hour with secondary antibody. After rinsing, the membrane was treated with Chemiluminescent HRP Substrate and protein bands were detected with a chemiluminescence detector. Protein expression levels were measured using AIWBwell™ software.

5. Statistical Analysis

The statistical analysis was performed using SPSS 19.0 software. For data with a sample size of $N \geq 8$, normality assessment was first carried out using the Shapiro-Wilk

test. Data that conformed to a normal distribution were presented as mean $\pm$ standard deviation. For independent two-sample comparisons, a t-test was employed. For data with a sample size of $N < 8$ or data that did not conform to a normal distribution, they were presented as median and interquartile range (M, IQR). For independent two-sample comparisons, a Mann-Whitney U test was employed. For comparisons among multiple samples, a Kruskal-Wallis H test was used, followed by a Dunn's test for pairwise comparisons. A statistical significance was considered when the p-value was less than 0.05.

RESULTS

1. Rats prenatally exposed to VPA exhibit ASD-like behaviors

In this study, a total of seven pregnant rats were injected with VPA. Among them, two pregnant rats experienced miscarriage, and one died after VPA injection. Ultimately, only four pregnant rats successfully gave birth, with a litter size ranging from 3 to 5 pups per litter. In contrast, pregnant rats injected with NS showed no miscarriage or death, and their litter size ranged from 7 to 10 pups per litter. The aforementioned phenomena indicate that prenatal exposure to high doses of VPA in pregnant rats leads to a high miscarriage rate, a low litter size, and even death, which is consistent with previous research findings^[34]. The pregnant rats in the VPA group collectively gave birth to 14 pups, all of which participated in behavioral test at PND 21. Among them, three pups were in an inactive state during the test, and two pups failed to pass the model screening. Thus, the final effective sample size for the VPA group was $N = 9$ (Figure 2). The pregnant rats in the NS group collectively gave birth to 25 pups. Ten pups were randomly selected to participate in behavioral test at PND 21. Among them, two pups were in an inactive state, so their data were excluded. Therefore, the final effective sample size for the NS group was $N = 8$ (Figure 2). Subsequently, the pups in the VPA group were randomly divided into three groups: the Rg1-H group ($N = 3$), the Rg1-L group ($N = 3$), and the ASD group ($N = 3$).

2. Ginsenoside Rg1 treatment enhances social communication and reduces repetitive behaviors in rats exhibiting ASD-like behaviors in the three-chamber test and marble burying test

Behavior is the external manifestation of the central nervous system, and the level of neural development determines behavioral performance^{[35], [36]}. To investigate the

effect of ginsenoside Rg1 intervention on neuronal function in the hippocampal tissues of rats exhibiting ASD-like behaviors, we first tested the behaviors of the rats in each group. Given that deficits in social communication and interaction; and restricted and repetitive patterns of behaviors, interests, and activities are the core symptoms of ASD, rather than mere exploratory behaviors (such as interacting with unfamiliar conspecifics or novel objects), this study calculated a sociality index and a social novelty index while controlling for individual differences in activity levels. These indices were then used as behavioral indicators to evaluate drug efficacy. The results indicated that the sociability index (Cohen's $d = 0.736$) and social novelty index (Cohen's $d = 0.869$) significant decline, along with a notable increase in the duration of self-grooming behavior (Cohen's $d = 0.869$) in ASD group. However, after ginsenoside Rg1 intervention, these atypical behaviors were ameliorated in Rg1-H group (Figure 3). However, after receiving intervention with ginsenoside Rg1, the ASD-like behaviors in rats from the Rg1-H group showed improvement, whereas those in the Rg1-L group did not (Figure 3). The aforementioned phenomena suggest that the ameliorative effect of ginsenoside Rg1 on the behavioral symptoms of rats exhibiting ASD-like behaviors is dose-dependent, with higher doses of Rg1 capable of alleviating ASD-like behaviors, while lower doses fail to produce such effects.

3. Ginsenoside Rg1 treatment can alleviate pathological damage to the hippocampal tissues of rats exhibiting ASD-like behaviors

To visually observe the impact of ginsenoside Rg1 on hippocampal neurons in rats exhibiting ASD-like behaviors, HE staining and Nissl staining were performed on tissue samples from rats in each group. The results of HE staining revealed that (Figure 4a): In the ASD group, neurons in the hippocampal CA1, CA3, and DG regions of rats exhibited disordered and sparse arrangements. Some neurons displayed abnormal morphology, characterized by enlarged cell bodies appearing vacuolated, cytoplasm stained darkly, and nuclear dissolution. After intervention with ginsenoside Rg1, the arrangement of hippocampal neurons in the two drug-treated groups became relatively orderly, and the aforementioned abnormal changes were ameliorated. The results of Nissl staining (Figure 4b) further demonstrated that the number of Nissl bodies within neurons in various hippocampal regions of the ASD group decreased, and the staining became lighter, suggesting impaired protein synthesis function in neurons. Following intervention with ginsenoside Rg1, the number of Nissl bodies in the CA1 and DG

regions of rats in the Rg1-L group increased and showed statistical differences compared to the ASD group, while the number of Nissl bodies in the CA3 region increased but did not exhibit a statistical difference from the ASD group. In the Rg1-H group, there were no statistical differences in the number of Nissl bodies in all regions compared to the ASD group (Figure c).

4. Ginsenoside Rg1 treatment can regulate apoptotic, autophagic and inflammatory levels in the hippocampal tissue of rats exhibiting ASD-like behaviors

To further explore the mechanisms underlying the alleviation of pathological damage in the hippocampal tissues of rats exhibiting ASD-like behaviors as a result of ginsenoside Rg1 treatment, this study investigated biomarkers of apoptosis, autophagy and inflammation, via TUNEL staining (Figure 5a), immunofluorescence (Figure 5c, g, i), and Western blot analysis (Figure 5d, f, k, l). The results revealed that fluorescent signals from TUNEL-positive cells could be observed in the hippocampal tissue of rats exhibiting autistic-like behaviors (whereas fluorescent signals were almost undetectable in the other three groups, Figure 5a), with a decreased ratio of the antiapoptotic protein Bcl2 to the proapoptotic protein Bax (Figure 5c, d) and increased expression of the apoptotic marker known as active caspase-3 also being observed (Figure 5f). The levels of the autophagic biomarker LC3 was also increased (Figure 5g, k), with increased colocalization observed between neurons and LC3 (Figure 5i). Additionally, the level of the inflammatory biomarkers IL-6, TNF- α , and NF- κ B p65 were elevated (Figure 5L). Thereby suggesting elevated levels of apoptosis, autophagy and inflammation in the hippocampal tissue.

After treatment with ginsenoside Rg1, the Rg1-H group of rats showed a reduction in TUNEL-positive cells in the CA1 and CA3 regions (Figure 5a), along with an increased ratio of the anti-apoptotic protein Bcl2 to the pro-apoptotic protein Bax (Figures 5c, d), demonstrating statistical differences compared to the ASD group. In the DG region, although there was some recovery in the levels of these indicators, no statistical differences were observed compared to the ASD group. In contrast, the Rg1-L group exhibited no statistical differences in the levels of these indicators across the three regions (CA1, CA3, and DG) compared to the ASD group (Figures 5a, c, d). Regarding autophagy, the Rg1-H group of rats displayed enhanced LC3 fluorescence intensity and reduced co-localization intensity with neurons in the CA1 and CA3

regions, showing statistical differences compared to the ASD group. In the DG region, although both the LC3 fluorescence intensity and its co-localization intensity with neurons decreased, statistical differences were only observed in LC3 fluorescence intensity compared to the ASD group. The Rg1-L group of rats only showed a recovered co-localization intensity between neurons and LC3 in the CA1 region, with statistical differences compared to the ASD group, while no differences were noted in other aspects (Figures 5g, k, i). Furthermore, Western blot results indicated that the levels of inflammatory markers (IL-6, TNF- α , and Ac-NF- κ B P65) in the Rg1-H group of rats were reduced, showing statistical differences compared to the ASD group, whereas the levels of inflammatory markers in the Rg1-L group of rats, although decreased, showed no statistical differences compared to the ASD group (Figure 5L).

5. Ginsenoside Rg1 regulates inflammatory, apoptotic, and autophagic levels in the hippocampal tissues of rats exhibiting ASD-like behaviors by upregulating SIRT1

To further explore the neurophysiological mechanisms underlying ginsenoside Rg1-mediated enhancement of the stability and integrity of the hippocampal microenvironment in rats exhibiting ASD-like behaviors, this study examined SIRT1 protein levels via Western blot analysis. The results indicated that SIRT1 expression was decreased in the hippocampal tissues of rats exhibiting ASD-like behaviors. However, following ginsenoside Rg1 intervention, the expression level of SIRT1 in the Rg1-H group resumed an upward trend and showed statistical differences compared to the ASD group, whereas although the expression level of SIRT1 in the Rg1-L group increased to some extent, it did not exhibit statistical differences when compared to the ASD group (see Figure 6).

DISCUSSION

Various methods can be used to construct rat models^{[37]-[40]}, among which the offspring rat model induced by prenatal exposure to VPA can accurately mimic the core symptoms of ASD patients; moreover, this rat model exhibits neurophysiological similarities to autopsy reports of ASD patients, thereby demonstrating good reliability and validity. In this study, the method for preparing rats exhibiting ASD-like behaviors proposed by Schneider was utilized, and screened out rats exhibiting ASD-like behaviors that met the study's criteria through the three-chamber test and grooming test at PND 21^[41]. In this study, immediately after pregnant rats were injected with VPA,

symptoms such as severe abdominal contractions, lameness, and lying in a prostrate position were observed, and these symptoms were able to be spontaneously relieved. Pregnant rats injected with VPA exhibited high rates of abortion and a low litter size; additionally, few pregnant rats died after being injected with VPA. The offspring rats in the VPA group had short and deformed tails, and phenomena such as toe absence, polydactyly, toe deformities, and stiff hind limbs were observed. These characteristics may be related to the fact that exposure to a large amount of VPA during pregnancy can increase the incidence of neural tube defects and lead to fetal malformations^[42]. The results of model identification revealed that the rats in the VPA group exhibited obvious stereotypic behaviors and social deficits. These manifestations may be related to the fact that VPA (a histone deacetylase inhibitor) impairs the histone acetylation level of aldehyde dehydrogenase 1A1, thus leading to autism-like synaptic and behavioral deficits^{[43]-[45]}. All of the abovementioned phenomena conform to the identification criteria of the rats exhibiting ASD-like behaviors, thereby indicating the reliability and stability of the rats that exhibited ASD-like behaviors in this study, which provides a foundation for subsequent experiments.

Due to its various advantages (including few adverse reactions and high nutritional value), ginsenoside Rg1 has been widely investigated in models of central nervous system diseases because of its anti-inflammatory, antioxidant, and antiapoptotic effects^[20-23]. In addition to being influenced by the severity of the disease, the prognosis of ASD is also closely related to timely treatment. Research indicates that the ages of 2 to 3 years represent a critical window period for intervention in autism^[46]. In this study, drug intervention was initiated in the early developmental stage of rats exhibiting ASD-like behaviors to explore the early intervention effect of ginsenoside Rg1 on these rats. The dosage of ginsenoside Rg1 that was used in this study was determined based on the results of relevant literature and preliminary experiments. Liu demonstrated administered 40 mg/kg of ginsenoside Rg1 subcutaneously twice daily for 14 consecutive days to adult rats in a β -amyloid-induced Alzheimer's disease model. The results confirmed that this dosage could achieve an effective drug concentration in the hippocampus and exert biological effects within the central nervous system^[47]. Given that the intervention subjects in this study are juvenile rats, in this study, the protective effect of ginsenoside Rg1 on the hippocampal tissues of rats exhibiting ASD-like behaviors was explored by using intraperitoneal injections of a single dose

(40 mg/kg) of ginsenoside Rg1 for 14 consecutive days. Additionally, in a rat model of depression, Li observed that intervention with 20 mg/kg of ginsenoside Rg1 effectively improved depressive-like behaviors and suppressed neuroinflammation and mitochondrial dysfunction^[48]. Therefore, to explore the appropriate dosage of ginsenoside Rg1 in an autism rat model, this study set up two dosage levels for intervention, aiming to find a more suitable drug administration regimen for rats exhibiting ASD-like behaviors.

1. Impact of Ginsenoside Rg1 on the Core Symptoms of Rats Exhibiting ASD-like Behaviors

Behavior is the external manifestation of the central nervous system, and the level of neurological development determines behavioral performance^[34,35]. The hippocampal tissue plays crucial roles in memory, learning, and emotion^{[49], [50]}. However, in recent years, an increasing number of studies have demonstrated that defects in hippocampal neurogenesis are the basis for atypical behavioral phenotypes in ASD patients^[10]. Significant impairment of hippocampal neuronal function can lead to social behavioral abnormalities in ASD children, such as impairments in social communication skills and repetitive stereotyped behaviors^[11-14]. Therefore, when the protective effects of ginsenoside Rg1 on the hippocampal tissues of rats exhibiting ASD-like behaviors are explored, evaluations should be conducted from multiple aspects, including behavioral, pathological, morphological, and molecular biological perspectives.

This study revealed that pups in the ASD group exhibited severe deficits in social communication and social novelty. After intervention with ginsenoside Rg1, rats exhibiting ASD-like behaviors in Rg1-H group began to more frequently explore Stranger 1 during the social competence test phase instead of fearfully remaining in place around the empty cage, thereby indicating increased expression of social behavior. In the social novelty test phase, the model rats in Rg1-H group exhibited a certain degree of social novelty, which was initiated via the continuous exploration of novel objects and Stranger 2, rather than by rigidly remaining in place around familiar companions. This may be related to the phenomenon that ginsenoside Rg1 improved despair, anxiety-like and social avoidance behaviors^[51]. In the marble burying test, pups in the ASD group exhibited an increased number of buried marbles, thus indicating severe repetitive and stereotyped behaviors. After intervention with

ginsenoside Rg1, the repetitive and stereotyped behaviors of the rats in the Rg1-H group were significantly reduced, whereas those in the Rg1-L group were somewhat reduced but were not significantly different from those in the ASD group. In summary, ginsenoside Rg1 can improve social communication and interaction deficits, as well as repetitive and stereotyped behaviors, in rats exhibiting ASD-like behaviors, thus indicating that ginsenoside Rg1 is a potential drug for treating the core symptoms of ASD, but which exhibits a certain degree of dose-dependency, requiring the achievement of a specific drug concentration threshold to exert its therapeutic effects.

2. The Effect of Ginsenoside Rg1 on the Pathological Morphology of Hippocampal Tissues from Rats Exhibiting ASD-like Behaviors

In this study, HE and Nissl staining were used to conduct preliminary observations of the impact of ginsenoside Rg1 on structural and functional alterations of hippocampal neurons in rats exhibiting ASD-like behaviors. The results revealed that the hippocampal tissues of pups in the NS group exhibited almost no significant pathological changes, thereby suggesting that normal hippocampal tissue was present, with no atypical behavioral manifestations being observed. In contrast, the hippocampal tissues of pups in the ASD group exhibited sparse and disorganized cell arrangement; Most neurons exhibited enlarged cell bodies with a vacuolated appearance, deeply stained cytoplasm, and pyknosis and lysis of nuclei. And reduced numbers of Nissl bodies, thereby indicating severe pathological damage. These pathological changes were consistent with the atypical behavioral manifestations of these rats. After intervention with ginsenoside Rg1, the number of Nissl bodies increased in both intervention groups, which further suggested that ginsenoside Rg1 can effectively reduce pathological damage in the hippocampal tissues of rats exhibiting ASD-like behaviors. It is noteworthy that the histopathological results reflect the complexity of dose-response relationships. HE staining indicates that both intervention groups exhibit a positive trend in restoring overall tissue morphology and structural integrity, with no significant differences observed. However, Nissl staining, which more specifically marks the functional neuronal Nissl bodies, reveals that the 20 mg/kg dosage demonstrates a relative advantage in increasing the content of Nissl bodies within neurons, suggesting heightened synthetic activity. The inconsistency in these results may be due to errors in sample size, tissue collection, and other aspects; alternatively, it could be attributed to the fact that ginsenoside Rg1 may influence

different pathological aspects related to ASD through complex and multi-layered mechanisms. For instance, while a high dosage of ginsenoside Rg1 can moderately alleviate ASD-like abnormal behaviors and improve brain tissue pathological damage, its maintenance or promotion of basic synthetic functions within neurons (represented by Nissl bodies) may only be activated at lower dosages.

3. The Effect of Ginsenoside Rg1 on the Apoptosis of Hippocampal Neurons in Rats Exhibiting ASD-like Behaviors

This study employed TUNEL staining to label individual apoptotic nuclei, thereby allowing for a more accurate assessment of whether the apoptotic pathways in the hippocampal tissues of rats exhibiting ASD-like behaviors are abnormally activated. The results revealed that there is no significant TUNEL-positive cells were observed in the hippocampal tissues of pups in the NS group, thus suggesting that under normal conditions, the brain tissue is in a state of apoptosis inhibition. In contrast, a small number of TUNEL-positive cells was observed in various regions of the hippocampal tissues of pups in the ASD group, thereby indicating that prenatal exposure to VPA increased the percentage of apoptotic cells in the hippocampal tissues of offspring rats exhibiting ASD-like behaviors. This finding is consistent with the findings of previous extensive research^{[52], [53]}. After ginsenoside Rg1 intervention, the numbers of apoptotic cells in the hippocampal tissues of rats exhibiting ASD-like behaviors in Rg1-H group were significantly decreased, thus indicating that ginsenoside Rg1 can effectively reduce the percentage of apoptotic cells in the hippocampal tissues of rats exhibiting ASD-like behaviors. However, its therapeutic effects exhibit dose-dependency and brain region specificity. Furthermore, it is speculated that the abnormal initiation of neuronal apoptosis in the hippocampal tissues of rats exhibiting ASD-like behaviors leads to functional abnormalities. Ginsenoside Rg1 exerts neuroprotective effects at specific dosages by reducing the apoptosis rate in specific brain regions of the hippocampal tissues of rats exhibiting ASD-like behaviors, thereby improving their atypical behaviors.

Caspase-3 is a key downstream apoptotic execution protease involved in the caspase cascade reaction. When activated, caspase-3 can lead to condensation of the nucleoplasm, fragmentation of nuclear membranes and nucleoli, DNA cleavage, and mRNA decay, thus ultimately triggering apoptosis^{[54], [55]}. Therefore, the expression level of active caspase-3 can reflect the level of apoptosis in cells. Compared with that

in pups in the NS group, the expression of active caspase-3 in the hippocampal tissues of pups in the ASD group was significantly greater, thereby further verifying the occurrence of excessive apoptosis in the hippocampal tissues of rats exhibiting ASD-like behaviors. This finding is consistent with the findings of previous extensive animal experiments, cellular experiments, and clinical trials on ASD^{[56], [57]}.

After ginsenoside Rg1 intervention with 40 mg/kg, the expression level of active caspase-3 in the hippocampal tissues of rats exhibiting ASD-like behaviors was decreased, which further verifies that a certain dosage of ginsenoside Rg1 exerts neuroprotective effects via its antiapoptotic activity. The antiapoptotic protein Bcl-2 and the proapoptotic protein Bax are closely related and functionally coordinated pairs of apoptosis regulatory factors. The ratio of Bcl-2/Bax determines whether apoptosis occurs in cells^[58]. When stimulated by apoptotic signals such as ischemia-hypoxia, increased intracellular calcium ions, and increased reactive oxygen species, the Bcl-2/Bax ratio decreases, thus resulting in an increase in antiapoptotic capacity and leading to apoptosis^{[59]-[61]}. Compared with that in the NS group, the Bcl-2/Bax ratio in the hippocampal tissues of pups in the ASD group was lower, which is consistent with the results of TUNEL staining and Western blot analysis. These findings suggest that the imbalance in the Bcl-2/Bax protein ratio in the hippocampal tissues of rats exhibiting ASD-like behaviors may be one of the mechanisms underlying the patterns of abnormal apoptosis observed in these rats. After ginsenoside Rg1 intervention, the Bcl-2/Bax ratio in the hippocampal tissues of rats exhibiting ASD-like behaviors in Rg1-H group was increased. These findings indicate that reductions in the apoptosis rate and active caspase-3 levels in specific regions of the hippocampal tissues of rats exhibiting ASD-like behaviors induced by ginsenoside Rg1 treatment may be related to the increase in the Bcl-2/Bax ratio.

4. Effects of Ginsenoside Rg1 on Autophagy in Hippocampal Neurons of Rats Exhibiting ASD-like Behaviors

LC3 is a key protein involved in the occurrence of autophagy. During the formation of autophagosomes, the precursor of LC3 is initially cleaved by Atg4 to form soluble LC3I, which is further modified by Atg3 to form the membrane-bound form known as LC3II. When autophagy is initiated, LC3II localizes to the inner and outer surfaces of the autophagosome membrane, and its level is proportional to the number of autophagosomes, thereby reflecting the level of autophagy in cells (to a certain

extent)^[62]. Previous studies have demonstrated that ginsenoside Rg1 can protect neural cells by regulating the level of autophagy in the brain tissues of model rats ^{[63], [64]}. This study found that the co-localization of LC3 and NeuN-positive cells in the CA1, CA3, and DG regions of the hippocampal tissue was significantly enhanced, along with an observed increase in LC3-II protein levels in ASD group. This result suggests that VPA exposure leads to a blockade of neuronal autophagic flux, resulting in the abnormal accumulation of autophagosomes within cells due to their ineffective clearance after formation.

This study demonstrates that intervention with 40 mg/kg of ginsenoside Rg1 can reduce LC3II protein expression levels in the CA1 and CA3 regions of the hippocampal tissue in autistic rats, while enhancing the co-localization of NeuN cells and LC3. However, this phenomenon was not observed in the DG region, consistent with other findings indicating that ginsenoside Rg1 exerts pharmacological effects only in specific brain regions. Interestingly, we found that intervention with a low dose of ginsenoside Rg1(20 mg/kg) appears to attenuate the co-localization of NeuN cells and LC3, although LC3 expression levels remained unchanged. This may be because, despite the difficulty of low-dose ginsenoside Rg1 in exerting anti-autophagic effects, it can enhance the clearance capacity of neurons. This intriguing phenomenon may correspond to the ability of low-dose ginsenoside Rg1 to maintain and promote basic synthetic functions within neurons, as exemplified by Nissl bodies. Thus, it can be inferred that a certain dose of ginsenoside Rg1 can regulate autophagy levels in specific regions of the hippocampus in autistic rats to a certain extent, thereby exerting neuroprotective effects.

5. Effects of Ginsenoside Rg1 on Inflammation in Hippocampal Neurons of Rats Exhibiting ASD-like Behaviors

The pathophysiological mechanisms of ASD remain unclear; however, numerous studies have demonstrated that neuroinflammation is closely related to the etiology of ASD. High levels of proinflammatory cytokines (including TNF- α , IL-6, and INF- γ , among others) are key signaling molecules of immune activation and are expressed in the brain, cerebrospinal fluid, and serum of ASD patients, as well as in model rats, thereby suggesting that neuroinflammation is involved in the occurrence and development of ASD^{[65]-[67]}. One study reported that after anti-inflammatory treatment (such as rapamycin and luteolin treatment) was administered to patients with autism

spectrum disorder (ASD), their core symptoms improved^{[68], [69]}. The current study revealed that prenatal exposure to VPA induced an increase in the expression of TNF- α and IL-6 in the hippocampal tissues of offspring rats exhibiting ASD-like behaviors, thereby suggesting a significant neuroinflammatory response. The elevated inflammatory levels may be related to the activation of the NF- κ B inflammatory pathway, which is consistent with previous autopsy reports on the brain tissues of ASD patients^[70]. This study revealed that the protein expression of NF- κ B p65, IL-6, and TNF- α in the hippocampal tissues of pups in the ASD group was significantly increased, thus demonstrating the activation of the NF- κ B inflammatory pathway and an increase in the expression of proinflammatory factors in the ASD group pups, with evident neuroinflammatory responses being observed in the hippocampal tissue. This finding is consistent with those of previous studies. For example, in a rat model of Huntington's disease, ginsenoside Rg1 was shown to counteract the release of inflammatory factors by inhibiting the activation of the NF- κ B pathway, thereby improving the loss of striatal neurons and ultrastructural damage and exerting a therapeutic effect on the disease^[71]. The anti-inflammatory effects of ginsenoside Rg1 have also been reported in other disease models^{[17, [72]}. In this study, after with a high dose of ginsenoside Rg1 intervention was implemented in rats exhibiting ASD-like behaviors, the protein expression of the proinflammatory factors IL-6 and TNF- α in the hippocampal tissue decreased, thus confirming the anti-inflammatory effect of ginsenoside Rg1 in these types of rats. Further research revealed that the reduction in the inflammatory response in the hippocampal tissues of rats exhibiting ASD-like behaviors elicited by ginsenoside Rg1 may be related to the inhibitory effects of ginsenoside Rg1 on NF- κ B p65 release, and this effect exhibits dose-dependency. The abovementioned findings suggest that anti-inflammatory treatment can effectively alleviate abnormal behaviors in rats exhibiting ASD-like behaviors and is a promising potential option for the treatment of ASD. Most of the previous studies on anti-inflammatory treatments for ASD have involved animal experiments, whereas few studies have focused on interventions for humans with autism from an inflammation-based perspective. More clinical trials are needed to confirm the inflammation-based theory regarding human autism in the future.

6. Mechanisms by which ginsenoside Rg1 Exerts Anti-inflammatory and Antiapoptotic Effects

Sirt1 belongs to the SIRT family of nicotinamide adenine dinucleotide-dependent deacetylases. It can regulate the acetylation or deacetylation of downstream proteins or genes. In addition to deacetylating genomic proteins and their protein products, SIRT1 may also act on important cytokines, thereby regulating biological processes such as the inflammatory response, oxidative stress, autophagy, and apoptosis. Additionally, SIRT1 is associated with a variety of central nervous system diseases^[73], ^[74]. As agonists of SIRT1, ginsenosides can either directly or indirectly regulate SIRT1. To further explore the mechanism by which ginsenoside Rg1 regulates the inflammatory response, neuronal apoptosis and autophagy in the hippocampal tissues of rats exhibiting ASD-like behaviors, this study detected the expression level of SIRT1 in the hippocampal tissues of these types of rats. The results revealed that the level of SIRT1 in the hippocampal tissues of rats exhibiting ASD-like behaviors was lower than that in the NS group. After intervention with ginsenoside Rg1, the level of SIRT1 in the hippocampal tissues of rats exhibiting ASD-like behaviors was increased. The results revealed that the SIRT1 level in the hippocampal tissue of autistic rats was lower than that in the NS (normal saline) group. However, this regulatory effect exhibited strict dose-dependency, which is that an elevation in SIRT1 levels was only observed in the Rg1-H group (40 mg/kg), while the changes in the Rg1-L group (20 mg/kg) were not statistically significant. This indicates that a certain dosage threshold of Rg1 must be reached to achieve its upregulatory effect on SIRT1 in the hippocampus of rats exhibiting ASD-like behaviors.

Furthermore, SIRT1 may be an important key node through which ginsenoside Rg1 improves the levels of neuroinflammation, neuronal apoptosis and autophagy in the hippocampal tissues of rats exhibiting ASD-like behaviors, thereby exerting a neuroprotective effect and ameliorating the atypical behaviors of these types of rats. The mechanism by which SIRT1 exerts its anti-inflammatory effect may be related to its binding to lysine at position 310 of NF- κ B (RelA/p65), which deacetylates the lysine residue, directly inhibits the transcriptional activity of NF- κ B, and ultimately suppresses the expression of downstream genes^[75]. Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) is an energy sensor that regulates cellular metabolism and stress responses by sensing changes in the intracellular AMP/ATP ratio. In the process of apoptosis, AMPK can inhibit apoptosis by regulating energy metabolism, suppressing the proapoptotic signaling pathway, and activating the cell

protection mechanism. Studies have shown that SIRT1 directly activates AMPK by deacetylating LKB1 and can also indirectly activate AMPK by regulating the level of NAD⁺ to inhibit apoptosis^[76]. SIRT1 may reduce the level of apoptosis in the hippocampal tissues of rats exhibiting ASD-like behaviors by upregulating AMPK. SIRT1 is generally regarded as being a positive regulator of autophagy, which contradicts the results of this study. However, some studies have also demonstrated that SIRT1 exerts dual effects on autophagy. Under certain circumstances, SIRT1 can also inhibit autophagy by activating mTORC1. In addition, autophagy is typically activated under conditions of high energy demand or energy deficiency. SIRT1 can also reduce the cellular demand for autophagy by regulating energy metabolism (such as promoting fatty acid oxidation and mitochondrial function), thereby inhibiting autophagy. However, whether ginsenoside Rg1 affects the SIRT1 level in the hippocampal tissues of rats exhibiting ASD-like behaviors via direct or indirect effects still requires further investigation.

CONCLUSIONS

This study confirms that ginsenoside Rg1 alleviates neuroinflammation, inhibits cell apoptosis, and regulates autophagy processes by upregulating SIRT1 expression in hippocampal tissue, ultimately exerting neuroprotective effects and thereby improving social communication deficits and repetitive stereotyped behaviors in rats exhibiting ASD-like behaviors induced by prenatal VPA exposure. Notably, its protective effects exhibit dose-dependency, meaning that the mechanisms of action vary across different dosages, which is that the high dose (40 mg/kg) exerts broad effects in anti-inflammation and anti-apoptosis, further positively influencing ASD-like behaviors; whereas the low dose (20 mg/kg) only demonstrates effects in promoting neuronal synthesis and clearance capacity, with limited behavioral improvements. Additionally, the efficacy of ginsenoside Rg1 varies across different brain regions. This study primarily observed the CA1, CA3, and DG regions, revealing that CA1 and CA3 may be the primary targets for the pharmacological effects of ginsenoside Rg1. Future studies could include more brain regions, such as the hippocampal CA2 region and cerebral cortex, to comprehensively evaluate its pharmacological characteristics. In summary, ginsenoside Rg1 improves the pathological state of hippocampal tissue in

autistic rats by regulating multiple neural pathways mediated by SIRT1, suggesting that SIRT1 may be a potential therapeutic target for ASD. However, further research is still needed in terms of dose optimization, brain region specificity, and clinical translation to further clarify its application prospects.

ABBREVIATIONS

ASD	Autism Spectrum Disorder
AMPK	adenosine 5'-monophosphate-activated protein kinase
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma-2
Caspase	cysteiny l aspartate specific proteinase
DAPI	4'6-Diamidino-2'-phenylindole
ED1	Embryonic Day 1
HE	Hematoxylin eosin
IL-6	interleukin 6
INF- γ	Tumor Necrosis Factor- γ
LC3	Microtubule-associated protein 1 light chain 3
NF- κ B	nuclear factor kappa-B
PTSD	post-traumatic stress disorder
PND1	Postnatal Day 1
TNF- α	Tumor Necrosis Factor- α
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP-X nick end labeling
VPA	sodium valproate

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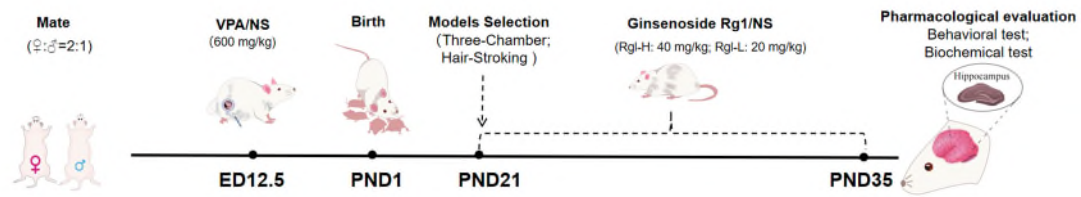
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(A)



(B)

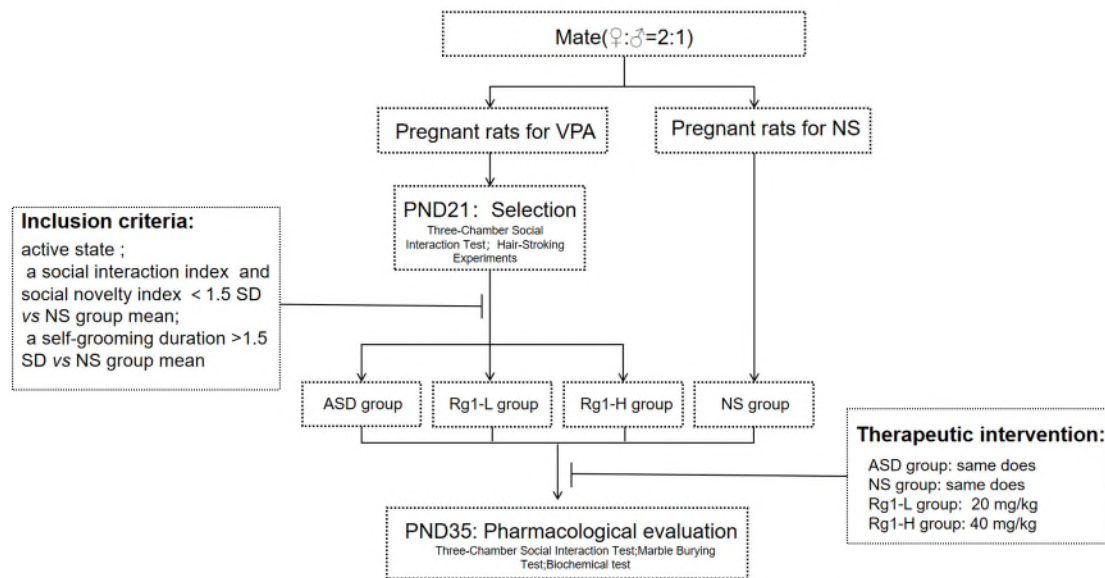


Fig.1 Schematic representation of the experimental design for administration of ginsenoside Rg1 for 21 days in rats exhibiting ASD-like behaviors.(A) The treatment timeline illustrates the schedule of VPA exposure, Rg1 administration, and subsequent behavioral or molecular analyses; (B) The flowchart summarizes the grouping of animals, intervention protocols, and key outcome measures assessed in the study.

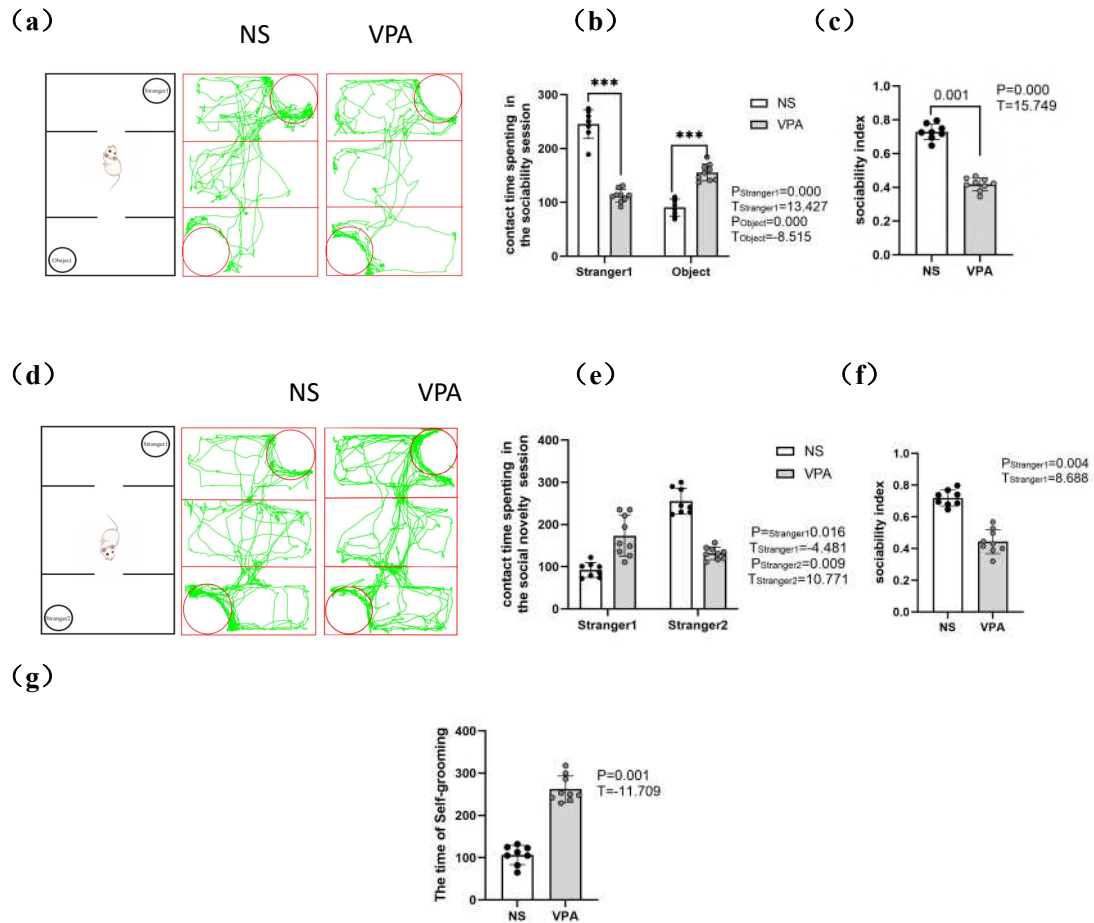


Fig.2 (a)Schematic representation and records of motion, (b)contact time spending, (c) sociability index, of the three-chamber test in the sociability session.(d)Schematic representation and records of motion, (e)contact time spending, (f)social novelty index, of the three-chamber test in the social novelty session.(g) the time of self-grooming (10 min). Data are mean and standard error of the mean (SEM). Statistical analysis was performed using the T test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; for $n = 8$ or 9 animals in each group.

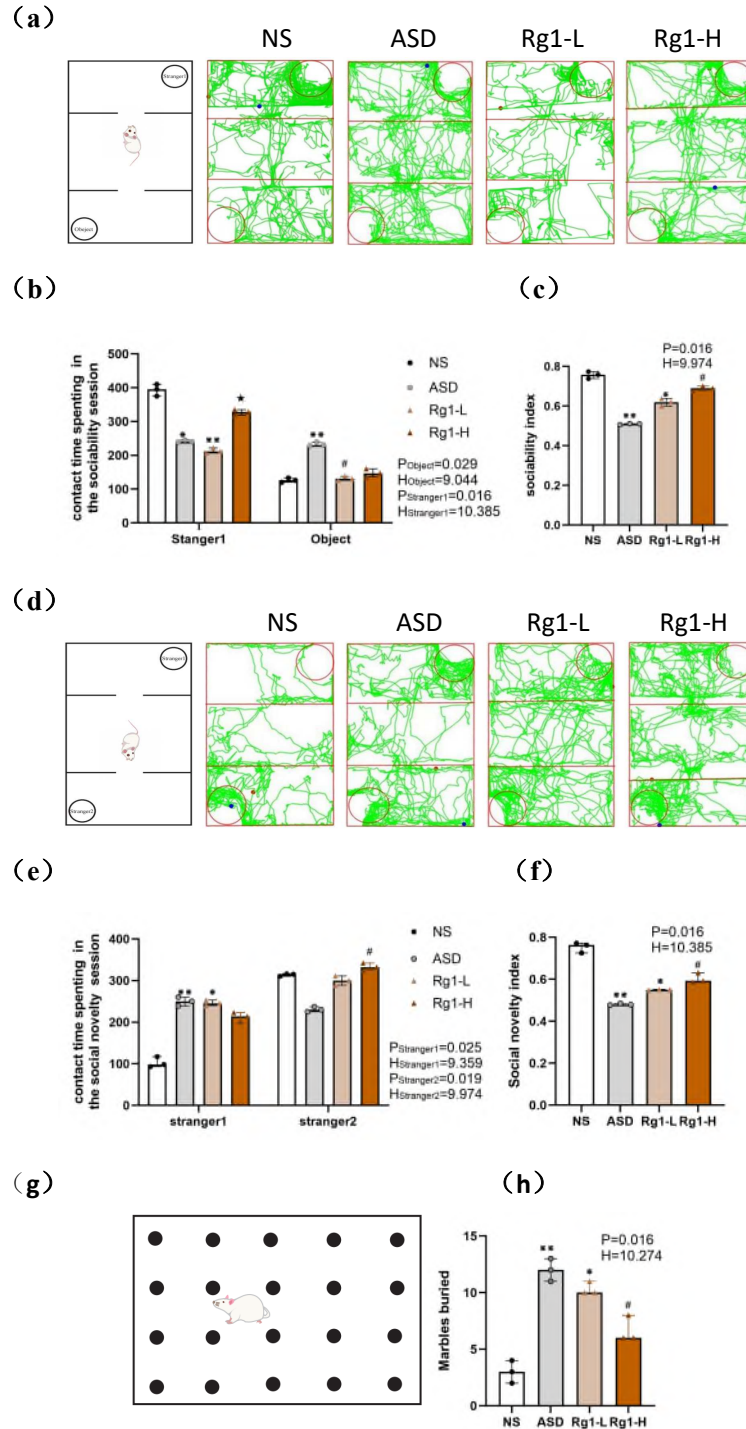


Fig.3 (a)Schematic representation and records of motion, (b)contact time spending, (C) sociability index, of the three-chamber test in the sociability session.(d)Schematic representation and records of motion, (e)contact time spending, (f)social novelty index, of the three-chamber test in the social novelty session.(g)Schematic representation, (h) numbers of buried marbles of the marble burying test (20 min). Data are presented as median with interquartile range (IQR). Statistical significance among multiple groups was determined by Kruskal-Wallis H test followed by post-hoc comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs NS group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs ASD group; ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$ vs Rg1-L group; for $n = 3$ animals in each group.

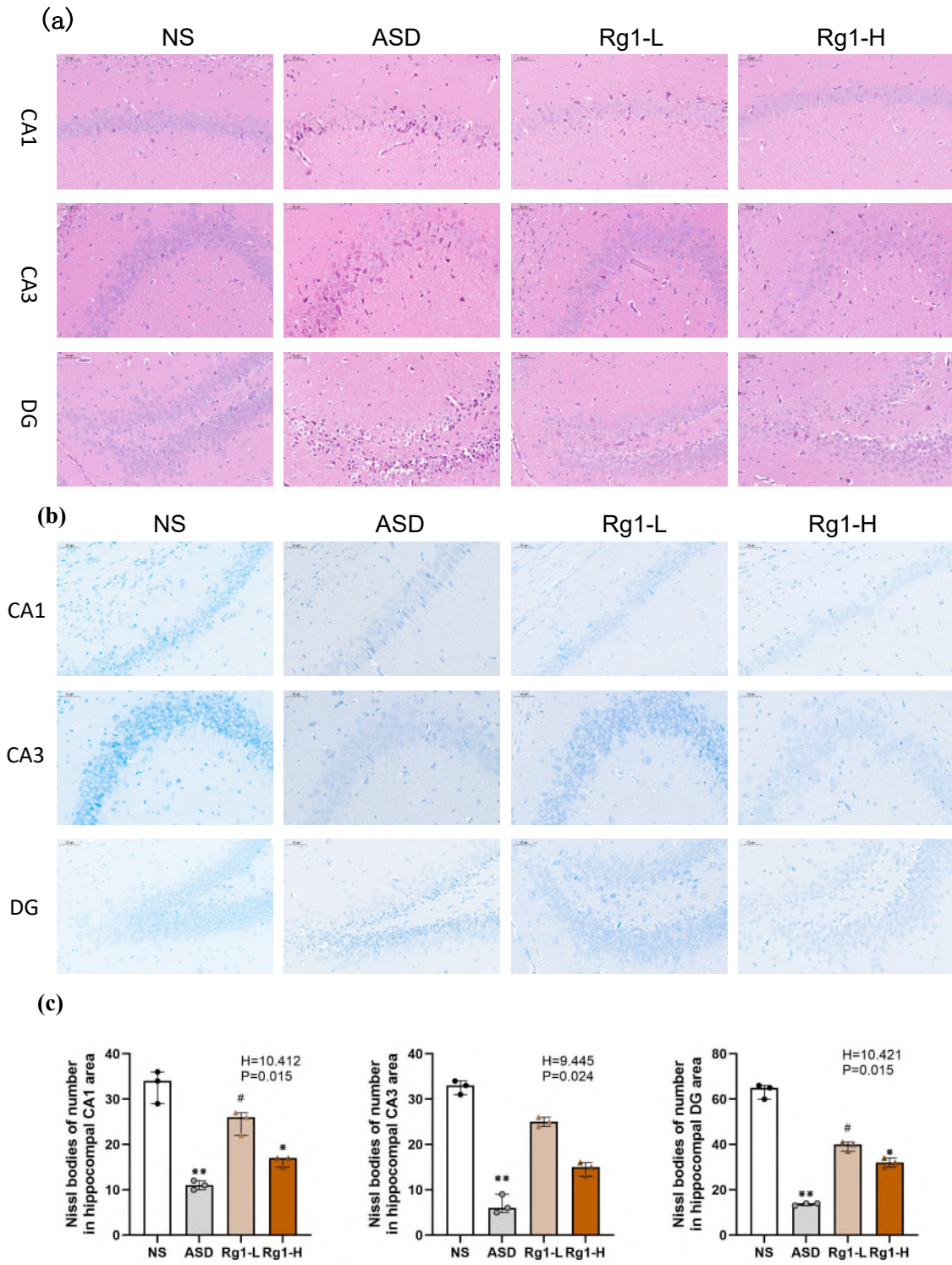
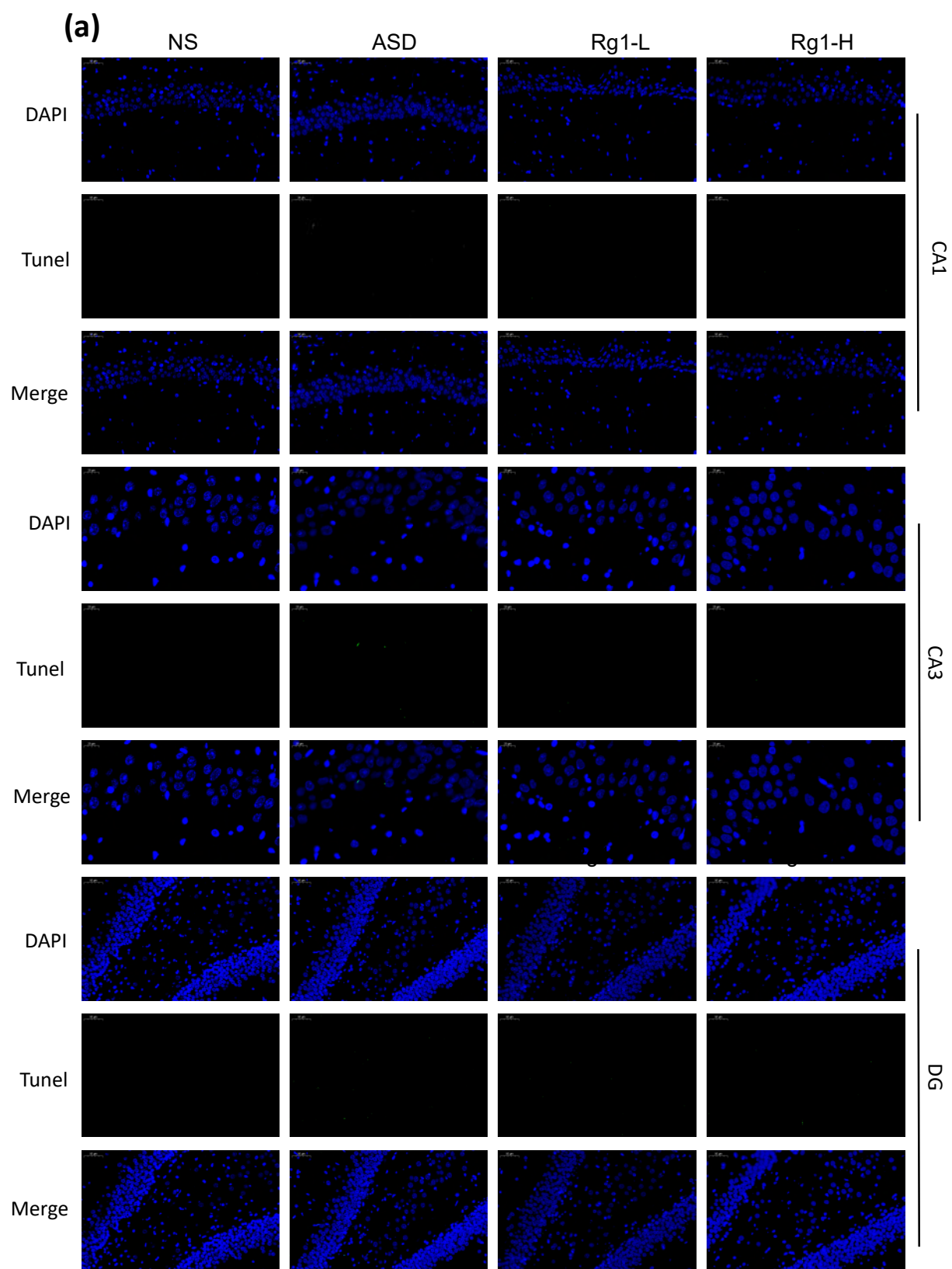
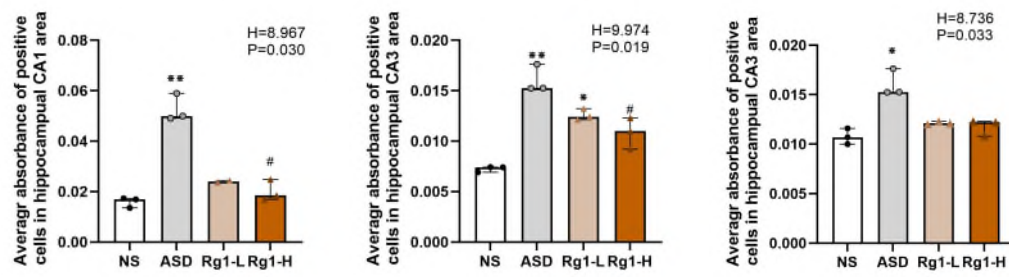


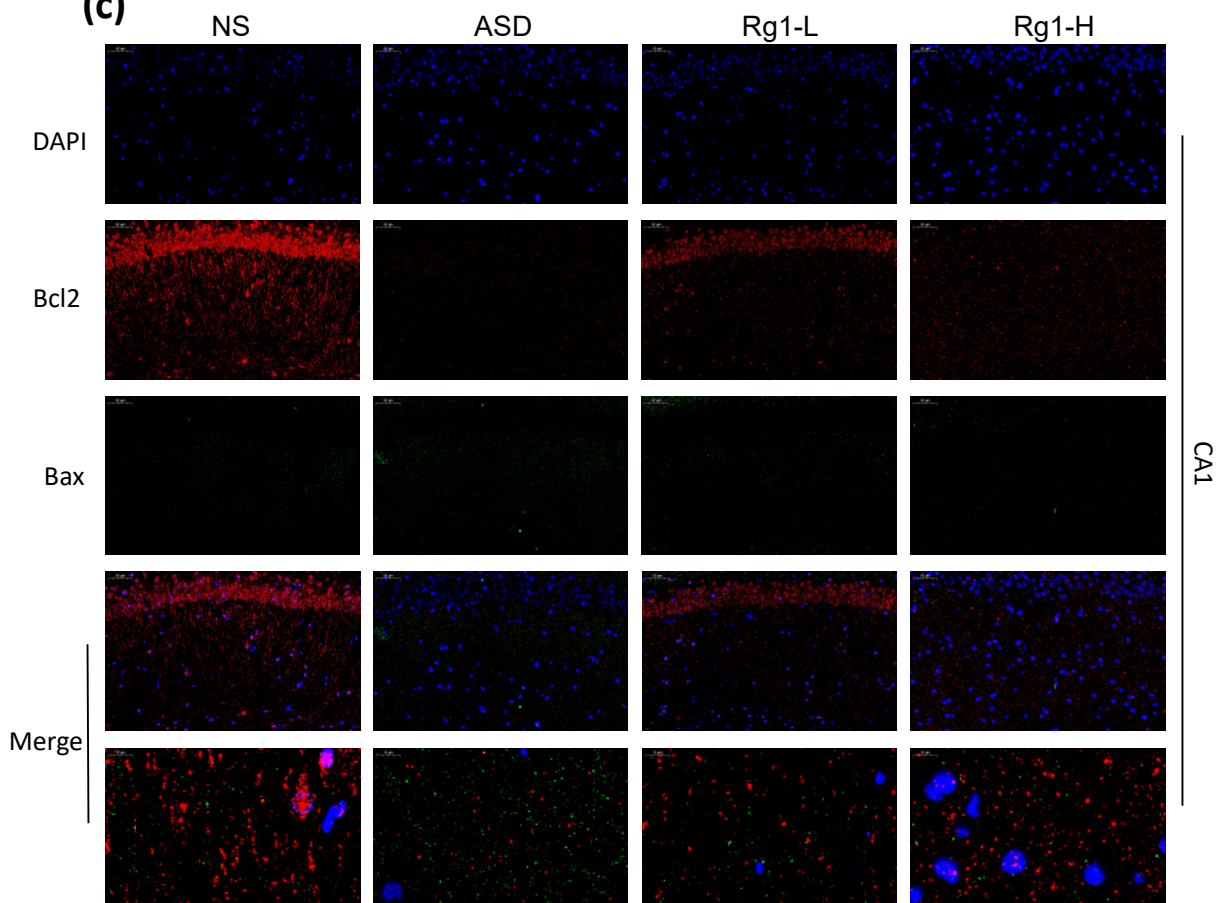
Fig.4 (a) HE staining, (b)Nissl staining, (c)numbers of nissl bodies of hippocampal tissue. The scale bar represents 50 μ m. Data are presented as median with interquartile range (IQR). Statistical significance among multiple groups was determined by Kruskal-Wallis H test followed by post-hoc comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. NS group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. ASD group; for $n = 3$ animals in each group.

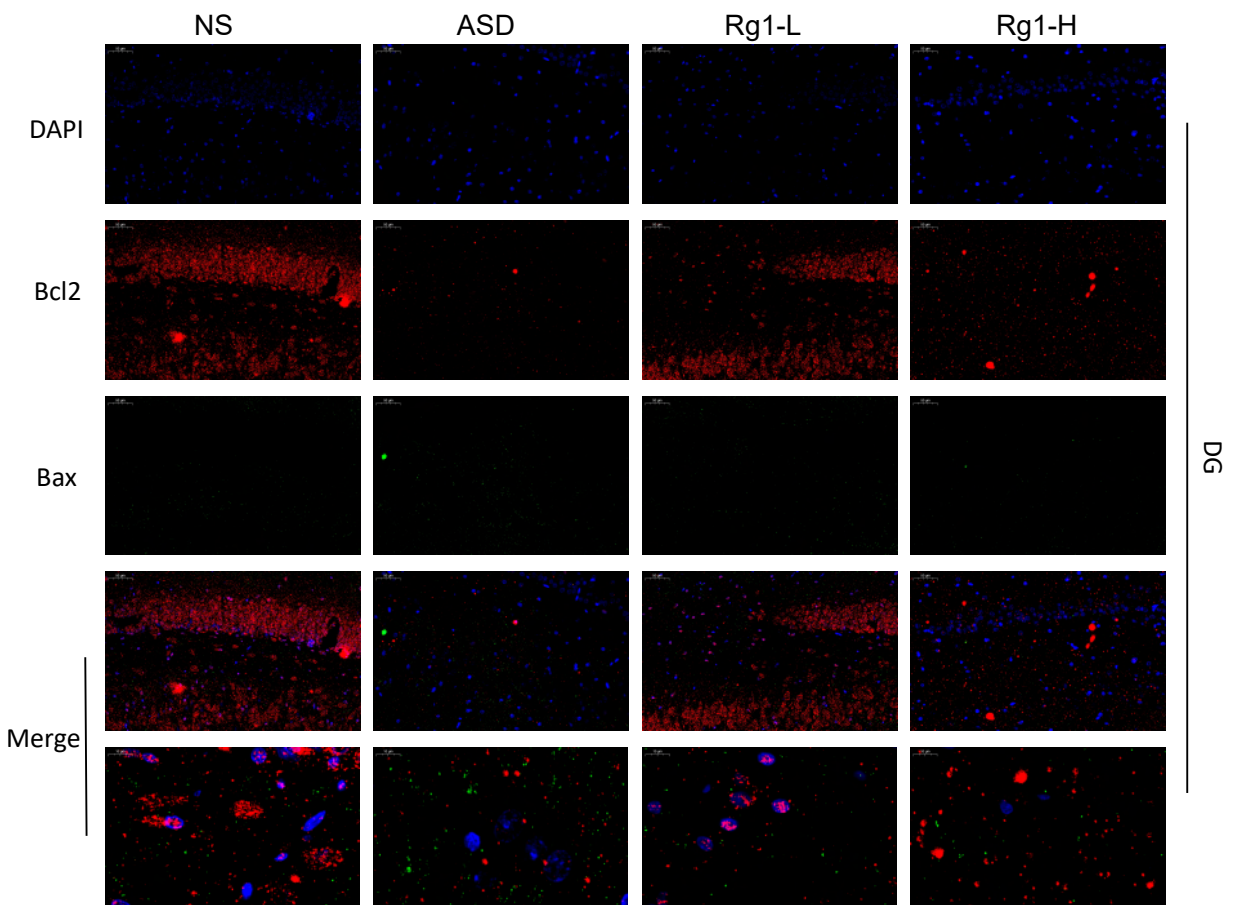
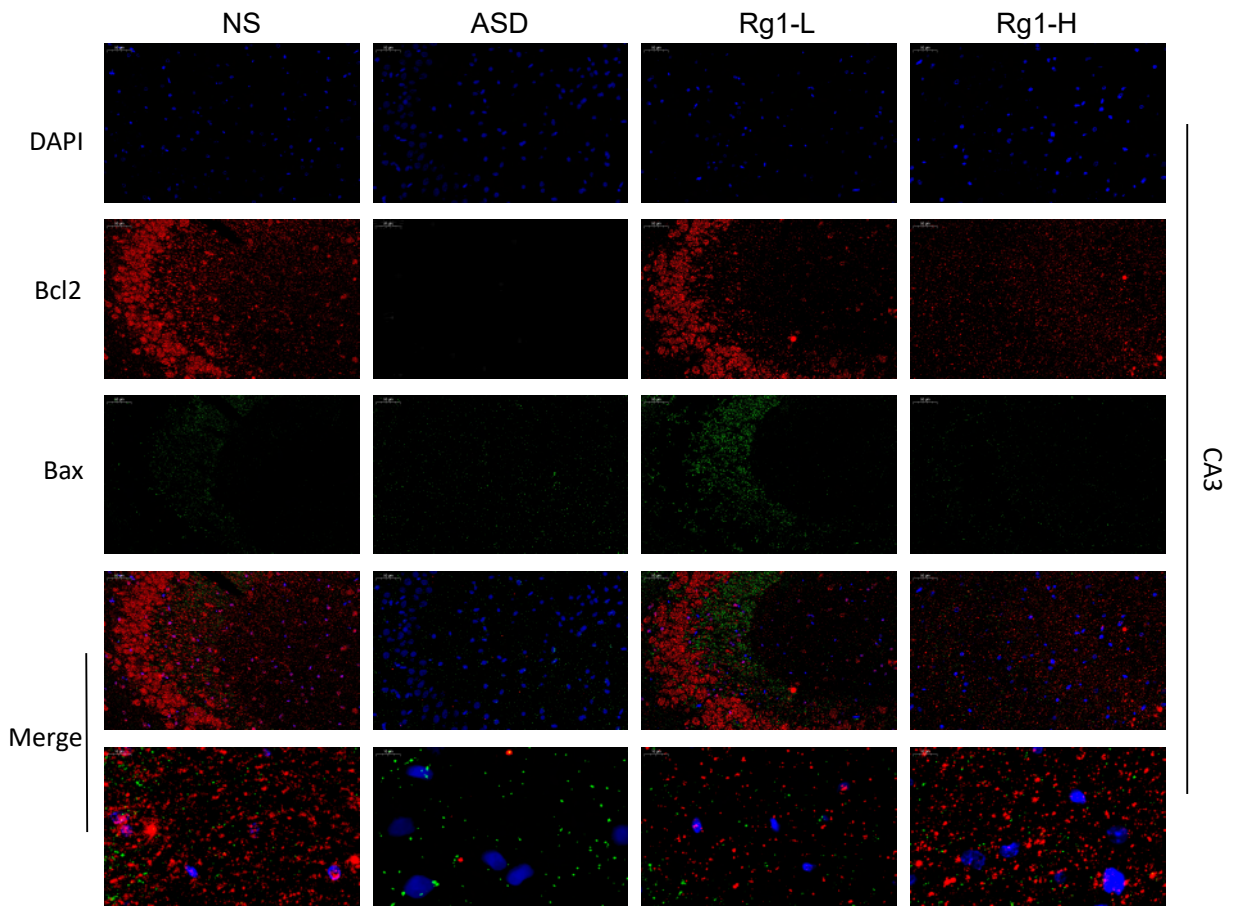


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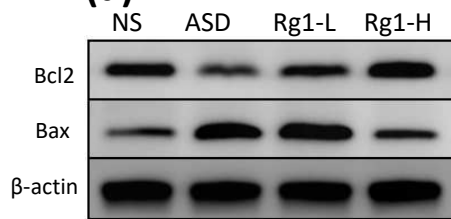


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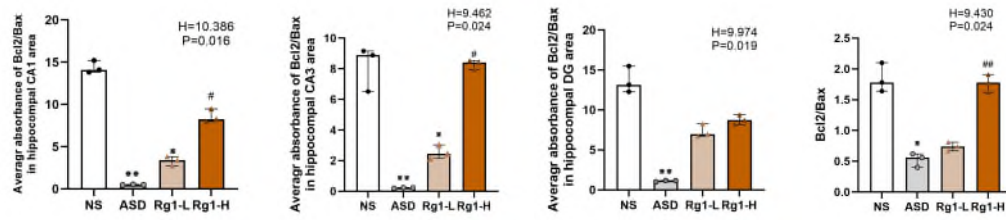




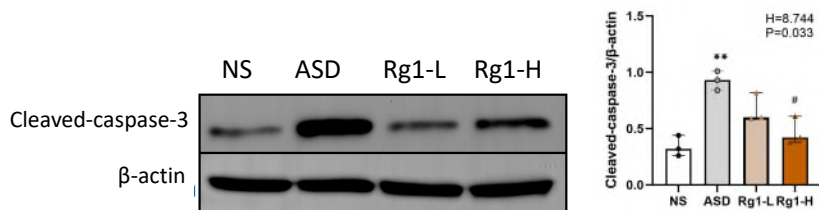
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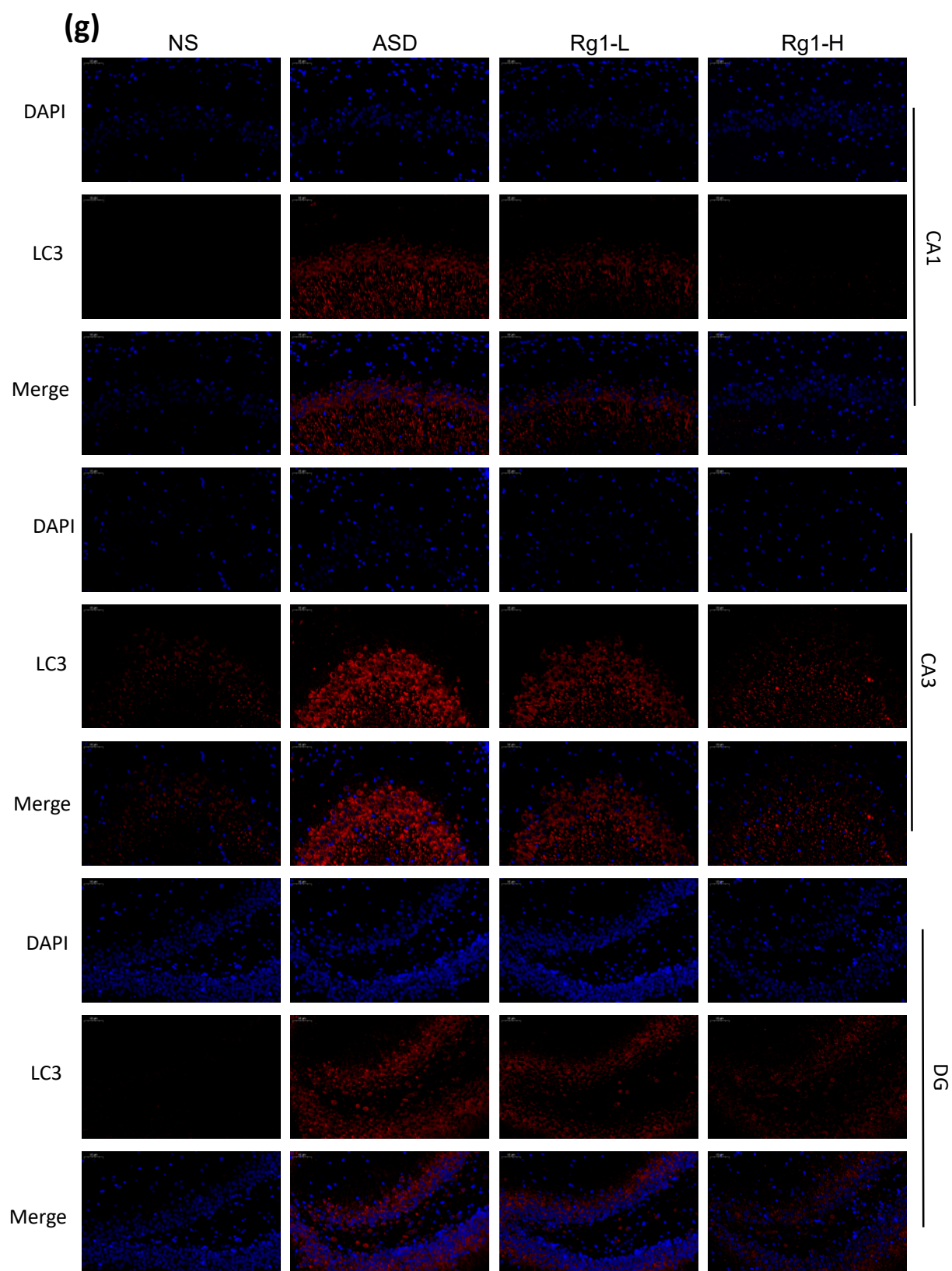


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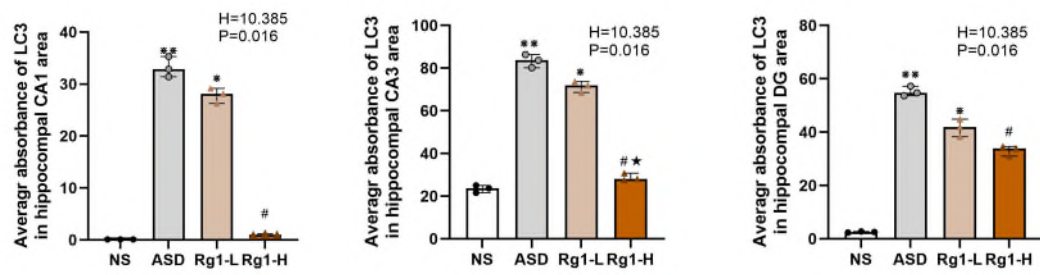


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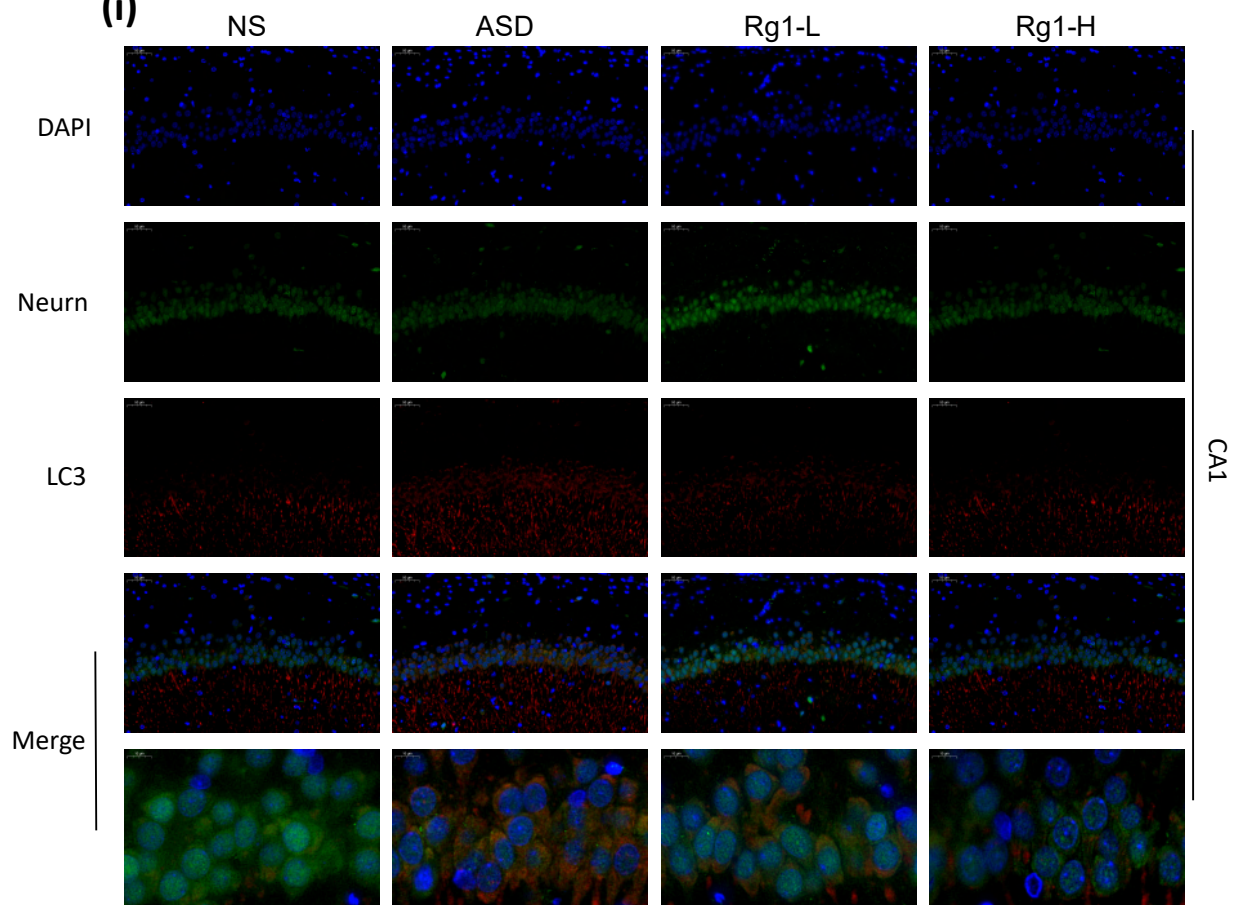


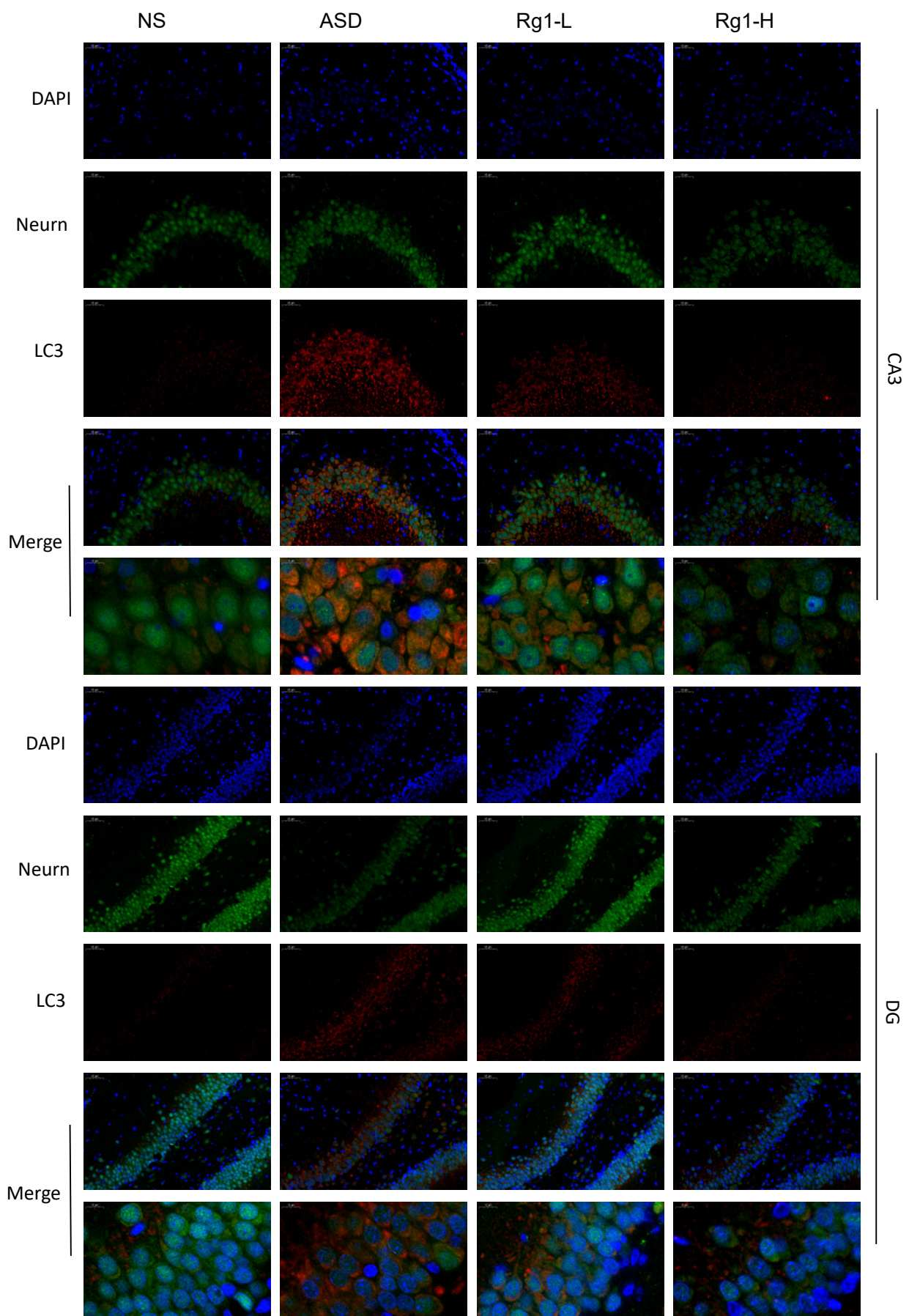


(h)

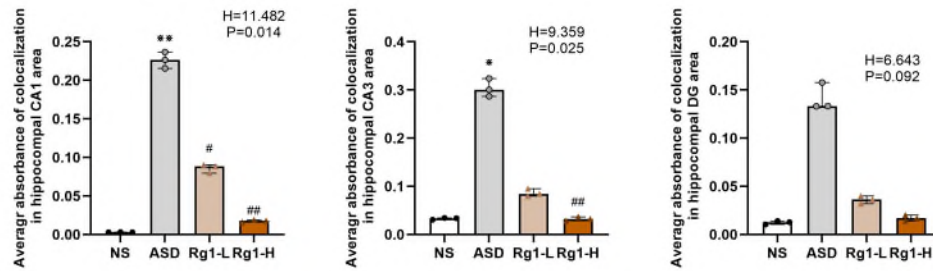


(i)

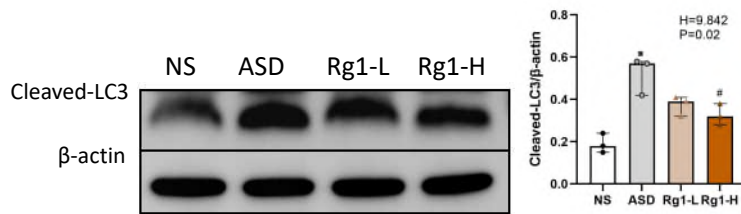




(j)



(k)



(L)

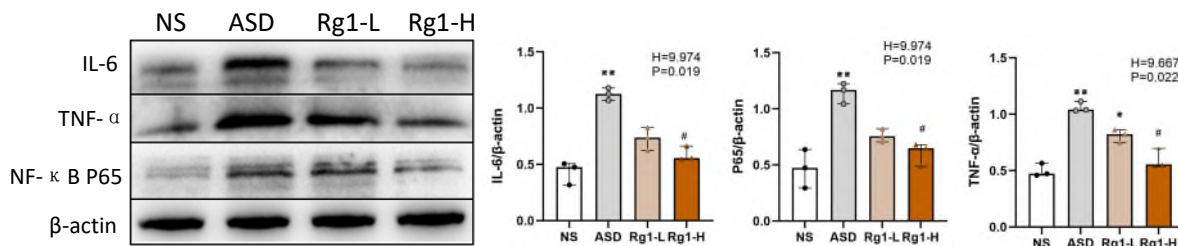


Fig.5 (a) TUNEL staining of hippocampal. (c) Bcl2/Bax, (g) LC3, (i) colocalization of Neuron and LC3, for immunofluorescence detection in hippocampal. (b) TUNEL positive cells, (h) Bcl2/Bax, (j) LC3, (h) colocalization of Neuron and LC3, for average absorbance in hippocampal. (d) Bcl2, Bax, (f) Cleaved-caspase-3, (k) Cleaved-LC3, (L) IL-6, TNF- α , NF- κ B P65 for Western blot. (c) Immunofluorescence detection of Bcl2 and Bax in hippocampal regions, and Merge means overlay of DAPI, Bcl2, and Bax channels (first row: the scale bar represents 50 μ m; second row: the scale bar represents 10 μ m). (i) Colocalization of Neuron and LC3 in hippocampal regions, and Merge means overlay of DAPI, Neuron, and LC3 channels (first row: the scale bar represents 50 μ m; second row: the scale bar represents 10 μ m). Data are presented as median with interquartile range (IQR). Statistical significance among multiple groups was determined by Kruskal-Wallis H test followed by post-hoc comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs NS group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs ASD group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Rg1-L group; for $n = 3$ animals in each group.

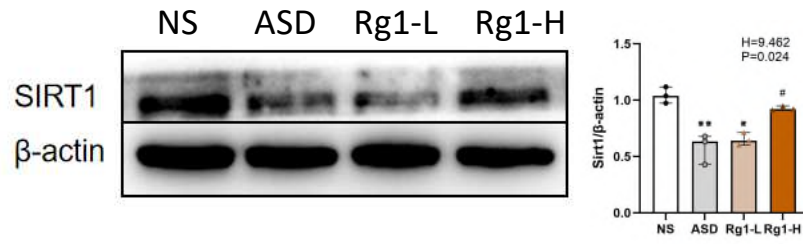


Fig.6 Western blot detection of SIRT 1 in hippocampal. Data are presented as median with interquartile range (IQR). Statistical significance among multiple groups was determined by Kruskal-Wallis H test followed by post-hoc comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs NS group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs ASD group; for $n = 3$ animals in each group.