Title: The Effects of Galectin-1 on Classical Microglial Activation, Neuroinflammation and Neuronal Damage in Rats Exposed to Amyloid-β Peptide

Running Title: Gal-1 Skewed Microglial to M2 Phenotype

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ABSTRACT

Introduction: Microglia-mediated neuroinflammation is one of the most striking hallmarks of Alzheimer's disease (AD).Emerging evidence indicates that microglia can be polarized into proinflammatory M1 or anti-inflammatory M2 phenotypes, and excess activation of M1 microglia along with neuroinflammation are critically associated with the pathogenesis and progression of AD. The aim of the present study was to test the hypothesis that Galectin-1 (Gal-1), a member of endogenous β-galactoside-binding lectins family, may improve AD-related neuroinflammation and that the modulation of microglial polarization plays a role in this process.

Methods: Male Sprague-Dawley rats received a single intracerebroventricular injection of Amyloid-β_{1-42} peptide (Aβ) following 1, 5 or 10 μg Gal-1treatment for 14 days. Learning and memory abilities were estimated using the Morris water maze. Then, hippocampal samples were collected from rats, and the expression of M1 and M2 microglial markers was measured. The levels of inflammatory cytokines and the neuronal injury were also evaluated.

Results: At doses of 5 and 10 μg, Gal-1 exerted an effect on improving the Aβ-induced learning and memory impairments in rats ($P<0.05$, vs. Aβ group). Subsequently, Gal-1 efficaciously regulated microglial polarization, which evidenced by upregulating the expression of M2 microglial markers ($P<0.01$, vs. Aβ group) while downregulating M1 microglial markers ($P<0.05$, vs. Aβ group) and therefore contributed to decreasing the secretion of proinflammatory factors ($P<0.01$, vs. Aβ group) but increasing the production of anti-inflammatory factors ($P<0.01$, vs. Aβ group) as well as attenuating neuronal damage($P<0.01$, vs. Aβ group) mediated by Aβ.

Conclusions: Gal-1 may exert neuroprotective effects by shifting microglia from the M1 phenotype to the M2 phenotype, which is beneficial for alleviating neuroinflammation, and it might be a potential agent to prevent AD-like neurodegeneration.

Keywords: Alzheimer's disease, Neuroinflammation, Microglia, Galectin-1

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by extracellular β-amyloid (Aβ) deposits, neurofibrillary tangles and progressive memory loss. Although the factors contributing to AD progression are still unknown, accumulating evidence has revealed neuroinflammation has a prominent role in the pathogenesis of AD (Bolós et al., 2017). Microglia, the resident immune cell in the central nervous system, mediates neuroinflammation in the brain (Leng & Edison, 2021). In response to Aβ stimuli, it can rapidly transform from a normally "resting" state to an "activated" state upon exposure to Aβ (Wang et al., 2021). Interestingly, many studies have noted the neurotoxic nature of activated microglia, including neuronal degeneration, and subsequent contributions to neuronal loss (Shi et al., 2019), whereas other researchers claim that activated microglia are actually beneficial to neuronal myelin repair, and neuronal plasticity and prevent neural injury by secreting neurotrophic factors (Ennerfelt et al., 2022; Yao et al., 2017). The controversies regarding the neuroprotective and neurotoxic properties of microglia may depend on their different states of polarized activation. Microglia can polarized into classical (M1) or alternative (M2) activation states in different physiological or pathophysiological environments (Guo et al., 2022). Classically activated M1 microglia are characterized by the production of inflammatory cytokines and reactive oxygen species that contribute to neuronal damage; in contrast, alternatively activated M2 microglia release anti-inflammatory cytokines and neurotrophins that aid in neuronal repair and regenerative processes (Guo et al., 2022). A switch in the activated microglial phenotype from alternative microglia at the beginning of AD to classical microglia at an advanced stage has been reported in AD animal models (Frigerio et al., 2019). Meanwhile, an increasing number of studies have suggested that the excessive activation of M1 microglia results in an imbalance in M1/M2 microglial polarization that promotes the development of AD (Yang et al., 2022; Du et al., 2021).

Therefore, strategies modifying microglial activation and polarizing microglia into the M2 state appear to represent a reasonable alternative to attenuate neuronal damage in the brains of individuals with AD.

Galectin-1 (Gal-1) is a member of the galectin family of endogenous β-galactoside-binding lectins (Ramírez et al., 2020). Gal-1 is found in the central nervous system and expressed by neurons and nonneuronal cells (Nio-Kobayashi & Itabashi, 2021). Gal-1 has been implicated in numerous fundamental cellular processes, including cell adhesion, proliferation, and interactions with mRNA splicing factors (Nio-Kobayashi & Itabashi, 2021). Recently, an increasing number of studies have supported the hypothesis that Gal-1 is involved in neuroinflammation processes. For example, Parikh and colleagues found that Gal-1 suppresses methamphetamine-induced neuroinflammation in human brain microvascular endothelial cells (Parikh et al., 2015). Similarly, Mari et al reported that a Gal-1 deficiency resulted in low anti-inflammatory factor expression in mice with experimental encephalomyelitis (Mari et al., 2016). More importantly, Li and coworkers found that Gal-1 may inhibit microglia-associated proinflammatory cytokine production (Li et al., 2020), suggesting that Gal-1 has a potential role in microglial activation. Therefore, we hypothesized that Gal-1 may improve AD pathology, restrain excess M1 microglial polarization and attenuate neuroinflammation. We firstly investigated the effect of exogenous Gal-1 on spatial learning and memory in a rat model of Aβ-mediated AD to assess this hypothesis. Furthermore, the effects of Gal-1 on M1/M2 microglial activation, inflammatory cytokine release and neuronal damage were measured using hippocampal samples.

2. Materials and Methods

Chemicals and reagents

Amyloid-β_{1-42} peptide, Galectin-1, and the 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Cresyl violet and RIPA lysis buffer were purchased from Beyotime Biotechnology (Shanghai, China). Antibodies against Ym1, CCL2, and β-actin were purchased from Abcam (Abcam, Cambridge, UK). IL-1β, TNF-α, IL-4 and IL-10 ELISA kits were obtained from Multisciences (Hangzhou, China). The protease inhibitor mixture, phosphatase inhibitor, BCA protein assay kit and enhanced chemiluminescence substrate kit were obtained from Pierce Biotechnology (Rockford, IL, USA). Guide cannulae were purchased from RWD Life Science (Shenzhen, China). All other chemicals used were of the highest grade commercially available.

Animals and surgery

Male Sprague-Dawley rats (8 weeks, 200-220 g) were purchased from Weitong Lihua Experimentary Animal Central (Beijing, China). Animals were housed in groups of 2 per cage at 22±1°C with a 12-hour light-dark schedule and free access to food and water at all times. After acclimation for 5 days, animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic apparatus (RWD Life Science). Guide cannulae for saline, Aβ or Gal-1 intracerebroventricular (i.c.v.) administration were placed over the right lateral ventricle (AP=1 mm, L=1.6 mm, 1 mm depth) (Dong et al., 2017). Following surgery, the rats were allowed to recover for 14 days, and 50 rats were randomly divided into 5 groups of 10 animals each: control, Aβ, Gal-1L, Gal-1M, and Gal-1H groups. The animal research protocols were reviewed and approved by the Animal Ethics Committee of Yunnan University (Approval No.YNU-19-014.). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Drug infusion

For the i.c.v. injection, of 5 μl of the appropriate solution was placed into an internal needle connected to a PE50 polyethylene tube. After unscrewing the cap, the needle was gently inserted into the guide cannula, and solution was slowly infused into the lateral ventricle over a period of 60 s. The injection needle remained inside the guide cannula for 1 min and then was slowly removed. The cap on the guide cannula was then placed, and the rat was returned to its home cage. All rats (except the control group) received 10 μg of aggregated Aβ only once, and control rats were administered the same volume of saline. Subsequently, rats received a single daily i.c.v. injection of Gal-1 at doses of 1 μg (Gal-1L group), 5 μg (Gal-1M group) or 10 μg (Gal-1H group) or an equivalent volume of saline (control and Aβ groups) for 14 days. The Aβ peptide was dissolved in HFIP and incubated for 1 h at room temperature to prepare aggregated Aβ. HFIP was evaporated, and the dried pellet was dissolved in sterile saline at a concentration of 2 μg/μl and then incubated at 37°C for 96 h to induce aggregation it before the injection. Gal-1 was dissolved in sterile saline at a series of concentrations before use.

Morris water maze

The Morris water maze task was carried out from the 12th day to the 15th day after the Aβ injection. This procedure of the Morris water maze consisted of 3 days of learning and memory training and a probe trial on day 4 which similar to our previously described (Dong et al., 2013). Briefly, animals were trained in a circular tank located in a lit room with visual cues. The tank was 180 cm in diameter and 50 cm deep with nontoxic black-dyed water maintained at 21 ± 1°C. A black escape platform (10 cm in diameter) was submerged 1 cm beneath the water surface and fixed in the middle of the southwest quadrant. In each trial, each rat was randomly placed in water at one of the starting points (the midpoints of the four

quadrants in the tank except the platform quadrant) and was allowed a maximal time of 60 s to find the platform. If the animals failed to find the platform within this time, the experimenter led the rat to the platform and allowed it to remain on the platform for 15 s; the time was recorded as 60 s. Two training trials were conducted each day for 3 consecutive days. 24 h after the last training trial, the escape latency (time to reach the platform) was recorded firstly in the probe trail, and then the platform was removed, each rat was allowed to swim freely for 60 s inside the pool, and the time spent in the southwest quadrant (exploration time) was recorded with a computerized video system (Beijing Sunny Instruments Co. Ltd., China).

Sample preparation

One day after the last behavioral test, 7 animals in each group were deeply anesthetized by isoflurane inhalation (0.5ml/min) and decapitated. The brain was removed and dissected on ice, and hippocampi were collected for further analyses. The remaining 3 animals were perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde under deep anesthesia. Their brains were removed and fixed with 4% paraformaldehyde for 8 h at 4°C prior to placing them in PBS containing 10%, 20%, and finally 30% sucrose. After the brains had sunk in the last concentration of sucrose solution, 6 μm thick frozen sections were cut using a cryostat microtome (Leica, Germany) for morphological analyses.

Nissl staining

Nissl staining was done as described by Prisco with minor modification (Prisco et al., 2022). Briefly, brain sections were dried and soaked directly in chloroform for 15 min at room temperature. After washes with PBS, the sections were stained with a warm 0.05% cresyl violet solution for 20 s, rinsed with distilled water, and dehydrated in a series of graded ethanol solutions (70%, 90% and 100%). Tissue slices were cleared with xylene and then

cover slipped using mounting medium. Images were captured using an optical microscope (Leica, Germany). The integral optical density of the hippocampal CA1 region was estimated using Image-Pro software (Media Cybernetics Inc., Rockville, MD, USA) to evaluate neuronal damage. Twelve sections were selected randomly for analysis, and the mean optical density was recorded for each group.

Enzyme-linked Immunosorbent Assay (ELISA)

Hippocampal tissue (30 mg) was homogenized with a sonic dismembrator (ThermoFisher Scientific., Waltham, MA, USA) in 500 μl of deionized water and centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant was collected, and protein concentrations were determined using a BCA Protein Assay Kit. The concentrations of IL-1β, TNF-α, IL-4, and IL-10 in the supernatant were measured using ELISAs according to the manufacturer's protocol. Data are presented as pg per milligram of protein.

Western Blotting

According to our described before (Dong et al., 2017), hippocampal tissue was lysed with ice-cold RIPA buffer supplemented with a protease inhibitor cocktail and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected, and protein concentrations were determined using a BCA Protein Assay Kit. Proteins (20 μg) were loaded and separated on SDS–PAGE gels, and then transferred to PVDF membranes. Blots were blocked with 5% nonfat milk for 2 h at room temperature and then incubated overnight at 4°C with the following primary antibodies: anti-Ym1 (1:1000), anti-CCL2 (1:1000) and anti-β-actin (1:2000). After washing, the membranes were incubated with a goat horseradish peroxidase-conjugated secondary antibody (1:5000) at room temperature for 1 h, followed by detection using an enhanced chemiluminescence detection kit. The band density was quantified using Image-Pro software.

Statistical Analysis

Data are presented as the means ± SEM. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test with SPSS 18.0 software. P values < 0.05 were considered statistically significant.

3. Results

Effect of Gal-1 on learning and memory in AD rats

As shown in Fig. 1, animals that received Aβ alone showed a significantly increased escape latency but decreased exploration time in the probe test compared to control animals($P<0.01$). Although 1 μg of Gal-1 had no effect on the learning and memory impairment induced by Aβ, the 5 and 10 μg Gal-1 treatments significantly shortened the escape latency(both $P<0.01$, vs. Aβ group) and lengthened the exploration time (both $P<0.05$,vs. Aβ group). However, no significant differences were observed between the Gal-1M and Gal-1H groups.

Effect of Gal-1 on hippocampal neuronal injury in AD rats

We conducted Nissl staining to investigate neuronal injury in the hippocampus. As shown in Fig. 2. The results revealed pathological changes in hippocampal neurons of AD rats, such as decreased numbers of Nissl bodies, neuronal shrinkage and even disappearance in the hippocampal CA1 region, while these phenomena were alleviated by Gal-1 (5 and 10 μg) treatment compared to the Aβ group. Accordingly, these observations were also supported by the statistical analysis indicating that the mean optical density in the hippocampal CA1 region decreased in the Aβ group compared to that in the control group ($P<0.01$), and Gal-1 significantly reversed the Aβ-induced changes.

Effect of Gal-1 on microglial polarization in AD rats

We assessed the expression of the M1 marker CCL2 and the M2 marker Ym1 in the hippocampus to determine whether microglial activation was associated with the neuroprotective effect of Gal-1. As shown in Fig.3, the results of the western blotting analysis revealed significantly increased CCL-2 expression in the Aβ-treated group compared to control rats ($P<0.01$); conversely, Ym1 expression was significantly decreased in the Aβ-treated rats ($P<0.05$). Importantly, Gal-1 (5 and 10 μg) attenuated these changes that were effectively induced by Aβ. Thus, Gal-1 influenced microglial polarization.

Effect of Gal-1 on hippocampal cytokines level in AD rats

We used ELISAs to detect the pro- and anti-inflammatory cytokines concentrations in the hippocampus. As shown in Table 1, Compared with control group, IL-1β and TNF-α (proinflammatory factors) levels were significantly increased ($P<0.01$), but IL-4 and IL-10 (anti-inflammatory factors) levels were decreased ($P<0.01$) in AD animals, while these effects were attenuated by Gal-1 (5 and 10 μg) treatment.

4. Discussion

In this study, we used an AD model established in Sprague-Dawley rats treated with Aβ, a neurotoxin that activates microglia and induces an AD-like pathology. We investigated the effects of Gal-1 on microglial polarization, and the results indicated that Gal-1 attenuated Aβ-induced M1 microglial polarization. Subsequently, neurotoxicity related to excess M1 microglial activation, i.e., memory deficits, neuroinflammation, and neuron injury, were improved after treatment with Gal-1.

Microglial activation is a hallmark that occurs in the central nervous system during the AD neurodegenerative process. In the brains of individuals with AD, Aβ triggers M1 microglial

activation that exacerbates neuronal degeneration and death by releasing proinflammatory cytokines, whereas M2 microglial activation contributes to neuronal survival by generating neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and stimulating the clearance of amyloid plaques (Du et al., 2021; Yang et al., 2019; Wang et al., 2019). Therefore, strategies designed to reduce the overactivation of M1 microglia or promote microglial polarization towards the M2 phenotype would theoretically improve the outcome of neurodegenerative diseases.

The hippocampus is the most important structure in the pathophysiology of AD and is usually observed in clinical and experimental studies of this disease (Palmer & Good, 2011). We firstly assessed histopathological changes in the hippocampus of Aβ-induced AD animals. Nissl staining revealed the significant loss and shrinkage of neurons in AD rats compared with the control rats, similar to most previous reports (Zhao et al., 2019, Dai et al., 2018). However, neuronal damage mediated by Aβ was alleviated by Gal-1 (5 and 10 μg) treatment, indicating that Gal-1 exerted a repairing effect on damaged neurons. Therefore, we were not surprised to find that the learning and memory deficits were attenuated in Gal-1-treated rats, indicating the potential ability of Gal-1 to improve AD.

Then, we assessed the effects of Gal-1 on the trends in Aβ-induced microglial M1 and M2 activation. The increase in CCL2 expression but decrease in Ym1 expression induced by Aβ was significantly attenuated when the animals were treated with Gal-1. This result is similar to the findings reported by Suryawanshi et al., who observed that a Gal-1 treatment shifted proinflammatory T cells towards an anti-inflammatory T cell type (Suryawanshi et al., 2013). The present changes in the microglial activation status appeared to indicate that Gal-1 is involved in regulating microglial polarization in AD animals. Although the mechanism by which Gal-1 regulates microglial polarization was not explored in this study, previous

experiments have found that the NF-κB pathway plays a role in microglial M1/M2 polarization (Wang et al., 2021; Chen et al., 2017). At the same time, Toegel and colleagues reported that Gal-1 possesses the ability to regulate NF-κB signaling (Toegel et al., 2016). Perhaps we can speculate that NF-κB signaling is involved in the effect of Gal-1 on microglial polarizing, but further research is necessary to verify this hypothesis.

As stated above, neuroinflammation plays a key role in the pathological process of AD, and thus we analyzed the inflammatory changes in the hippocampus. In the current study, the levels of proinflammatory factors such as IL-1β and TNF-α were substantially increased by the Aβ infusion, while the secretion of anti-inflammatory factors, including IL-4 and IL-10, was suppressed. These results not only indicated that Aβ triggers neuroinflammation in AD animals but also supported the hypothesis that Aβ induces imbalanced M1/M2 activation pattern since proinflammatory and anti-inflammatory factors are another potential index of activated M1 and M2 microglial cells (Guo et al., 2022). Importantly, the secretion of proinflammatory cytokines was decreased and the release of anti-inflammatory cytokines was increased by the Gal-1 treatment. In this respect, our findings suggest that Gal-1 attenuates Aβ-initiated neuroinflammation by shifting the microglial M1 proinflammatory phenotype to the M2 anti-inflammatory phenotype.

Finally, we noted that no all Gal-1 concentrations were functional in the present study, the low dose Gal-1 did not protect against pathological changes mediated by Aβ. Although high Gal-1 levels were more efficient, the neuroprotective effects were not different between medium and high Gal-1 doses. The effects of Gal-1 on neuroinflammation exhibited a dose-response “plateau” at the concentrations used in the present study; therefore, a higher dose may not be more useful for alleviating neuronal damage. We also noticed that high dose Gal-1 treatment did not full reverse neuronal injury mediated by Aβ. It is well known, in addition

to trigger neuroinflammation, Aβ is toxic to neurons in multiple ways. It can disrupt cellular calcium balance, cause pore formation resulting in the loss of membrane potential, and destroy the cytoskeleton (Reiss et al., 2018), while the effect of Gal-1 mainly focused on immune regulation (Liu et al., 2012), which is probably the reason why Gal-1 cannot rescue neurons completely.

5. Conclusion

In summary, Gal-1 functions as a microglial modulator that is able to suppress microglial M1 polarization but favors M2 polarization, thereby attenuating excess inflammation and enhancing the neuroprotective effects of microglial cells. Although much more work is needed to further confirm the neuroprotective mechanisms of Gal-1 in AD, for example, the molecular mechanism by which Gal-1 regulates microglial polarization that was not investigated in the present study, the protective effects of Gal-1 on AD-associated neuroinflammation may help to provide the pharmacological basis of its future usage in preventing the progression of neurodegenerative disorders.

Ethical Considerations

Compliance with ethical guidelines

The animal research protocols were reviewed and approved by the Animal Ethics Committee of Yunnan University (Approval No.YNU-19-014.). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Authors' contributions

Shufei Li: investigation-acquisition of most of data, formal analysis; Yanmei Wang: investigation, formal analysis, writing-original draft; Jiali Li: methodology, investigation; Liuyuan Deng: methodology, investigation; Yilong Dong: study design, funding acquisition, supervision, writing - review & editing. All coauthors have discussed and critically revised the manuscript.

Conflict of interest

The authors declare no conflict of interest

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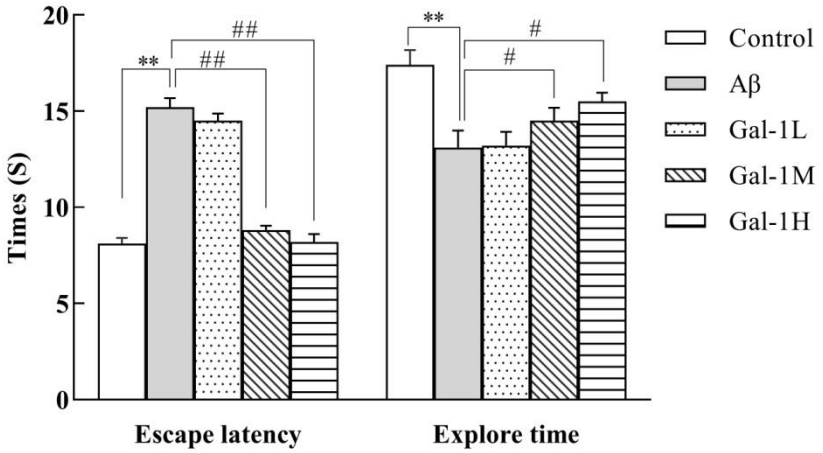

Figure 1: The escape latency and exploration time during the probe test session of the Morris water maze experiment.

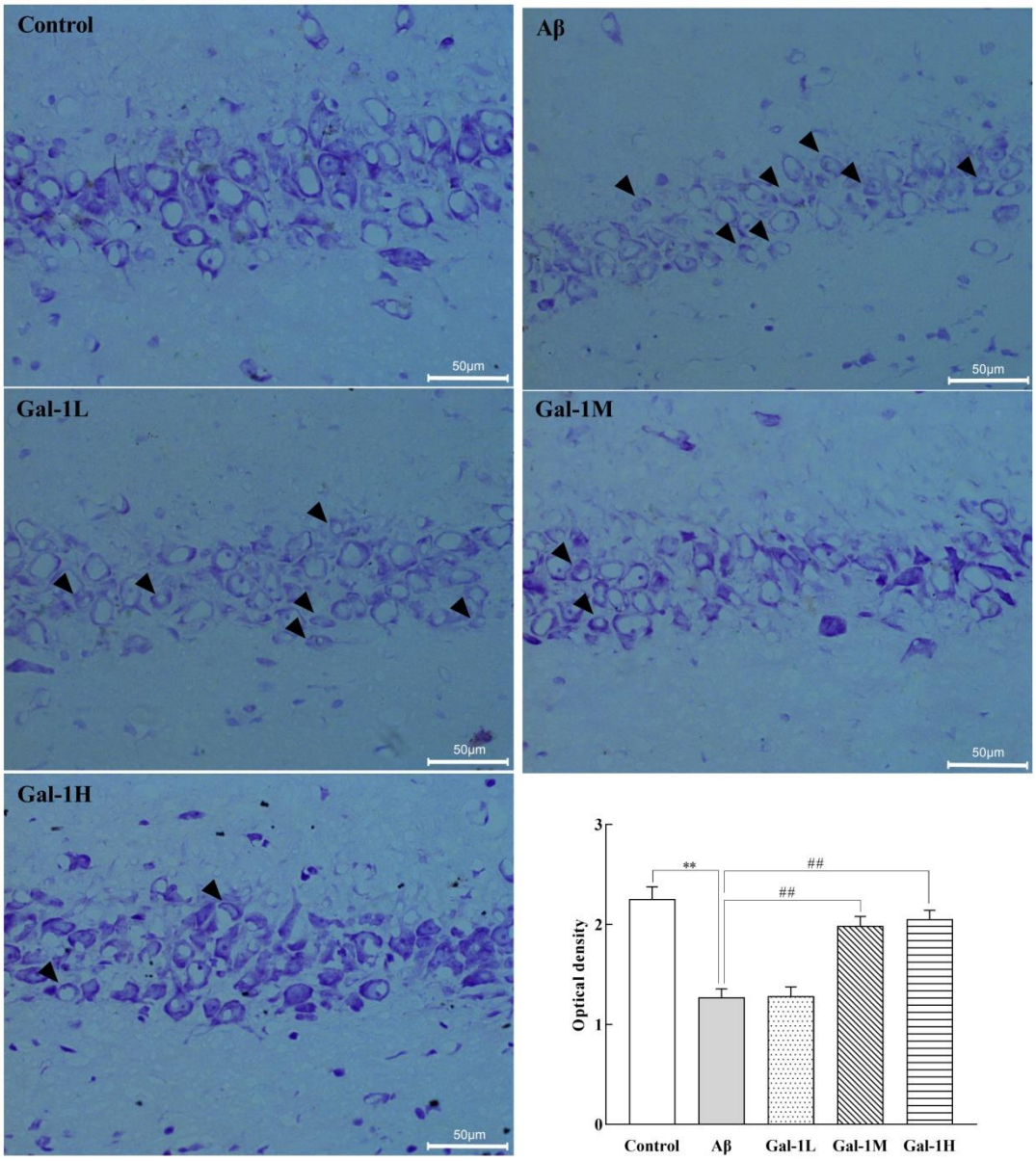

Figure 2: Neuronal injury in the hippocampal CA1 region was observed using Nissl staining.

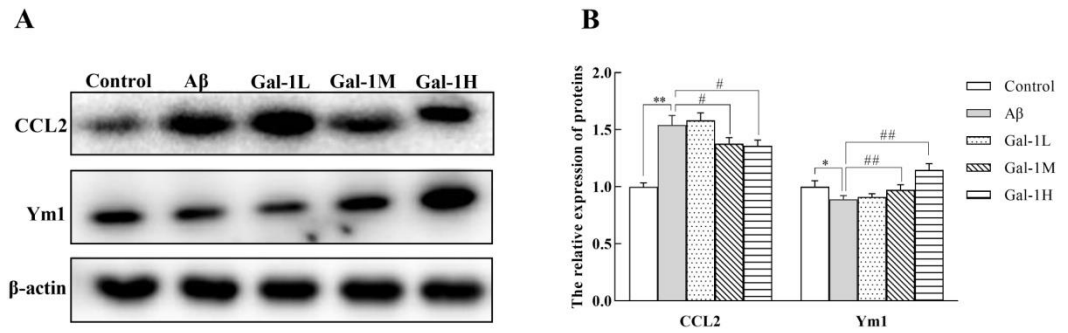

Figure 3: Effects of Gal-1 on microglia polarization induced by Aβ.

Figure legends

Figure 1. Gal-1 treatment ameliorated cognitive deficit induced by Aβ. **P<0.01 compared with the control group; #P<0.05 and ##P<0.01 compared with the Aβ group.

Abbreviation: Aβ: amyloid-β_{1-42} peptide (10 μg); Gal-1L: amyloid-β_{1-42} peptide (10 μg)+Galectin-1(1 μg); Gal-1M: amyloid-β_{1-42} peptide (10 μg)+Galectin-1(5 μg); Gal-1H: amyloid-β_{1-42} peptide (10 μg)+Galectin-1(10 μg)

Figure 2. Gal-1 treatment attenuated cell damage, as partially shown by the increase in Nissl bodies in the cytoplasm but decrease neuronal shrinkage (×400). **P<0.01 compared with the control group; ##P<0.01 compared with the Aβ group. The arrows show the neuronal shrinkage.

Abbreviation: Aβ: amyloid-β_{1-42} peptide (10 μg); Gal-1L: amyloid-β_{1-42} peptide (10 μg)+Galectin-1(1 μg); Gal-1M: amyloid-β_{1-42} peptide (10 μg)+Galectin-1(5 μg); Gal-1H: amyloid-β_{1-42} peptide (10 μg)+Galectin-1(10 μg)

Figure 3. Gal-1 treatment attenuated the upregulation of CCL2 and the downregulation of Ym1 induced by Aβ. A. M1/M2 microglial marker proteins were assessed using western blotting. B. Quantitative analysis of protein levels using densitometry. The western blotting data were normalized by setting the value of the control group to 1. *P<0.05 and **P<0.01 compared with the control group; #P<0.05 and ##P<0.01 compared with the Aβ group.

Abbreviation: Aβ: amyloid-β_{1-42} peptide (10 μg); Gal-1L: amyloid-β_{1-42} peptide (10 μg)+Galectin-1(1 μg); Gal-1M: amyloid-β_{1-42} peptide (10 μg)+Galectin-1(5 μg); Gal-1H: amyloid-β_{1-42} peptide (10 μg)+Galectin-1(10 μg)

Table 1. The cytokines contents in hippocampus (pg/mg protein).

	control	Aβ	Gal-1L	Gal-1M	Gal-1H
IL-1β	36.85±2.59	47.35±2.57**	46.29±3.06	43.29±2.24##	42.32±1.84##
TNF-α	3.67±0.31	4.59±0.43**	4.29±0.41	4.02±0.16 #	3.98±0.21##
IL-4	53.35±2.14	39.98±2.75**	42.18±2.24	43.73±2.98 #	46.56±2.12##
IL-10	231.72±7.44	189.31±10.59**	199.53±8.48	212.13±9.74 ##	221.09±8.57##

The cytokines have been measured by ELISA. **P<0.01，vs. control group；#P<0.05 and ##P <0.01，vs. Aβ group.