

Relaxin-2 protects synaptic integrity *via* hippocampal NLRP3-mTOR-autophagy pathways in an age-dependent Alzheimer-like model

DAOHE WANG
ZHIGANG ZHAO
YUMEI DING* 

Neurological Medicine Center
Gansu Wuwei Tumor Hospital
Wuwei, 733000, China

ABSTRACT

Alzheimer disease (AD) is associated with age-related synaptic degeneration, neuroinflammation, and impaired autophagy. The present study aimed to investigate the age-dependent neuroprotective effects of relaxin-2 (RLN2) in amyloid-beta 1-42 ($A\beta_{1-42}$)-induced AD-like pathology and hypothesised that RLN2 would provide greater therapeutic benefit in young than aged mice. Young (3–4 months) and aged (12–14 months) mice were intracerebrally (*i.c.*) injected with amyloid- β ($A\beta_{1-42}$) to develop an AD-like phenotype and treated with RLN2 or a control. Western blot analysis of hippocampal tissues was performed to assess the expression levels of ionised calcium-binding adaptor molecule 1 (Iba1) (microglial activation marker), NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome components, and mechanistic target of rapamycin (mTOR) pathway activity. Neuroinflammation was determined by interleukin-1 beta (IL-1 β) and tumour necrosis factor-alpha (TNF- α), while synaptic integrity was assessed by synaptophysin and PSD-95. The Morris Water Maze (MWM) and Novel Object Recognition (NOR) tests were used to measure cognitive and memory performance. Neuroinflammation markers IL-1 β and TNF- α were significantly elevated in $A\beta_{1-42}$ -injected mice, with aged mice showing a greater increase than young mice. RLN2 treatment markedly reduced IL-1 β and TNF- α levels in young mice, whereas the reduction was limited in aged mice. RLN2 treatment had a significant inhibitory effect on hippocampal NLRP3 signalling, reduced mTOR signalling, modulated autophagy-related protein expression, preserved synaptic proteins, and improved cognitive and memory performance (decrease in the MWM escape latency and increase in the NOR discrimination index). RLN2 was not effective in the aged mice, and there was persistent inflammatory activation, impaired autophagy, minimal improvement in synaptic recovery, and a moderate improvement in cognition. These findings suggest that RLN2 could be a potential therapeutic candidate for early-stage AD by targeting neuroinflammation, mTOR-mediated autophagy-related dysregulation, and synaptic loss, while also potentially providing a basis for age-based stratification of treatment responsiveness; however, further studies are

License



© 2026 Wang *et al.*

This article is distributed under the terms of the license Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License

Accepted September 1, 2026
Published online September 5, 2026

* Correspondence; e-mail: Forget20260330@outlook.com

required to determine its utility as a biomarker of treatment response or disease progression. Overall, RLN2 exerted neuroprotective effects in *i.c.* A β_{1-42} -induced AD-like pathology in an age-dependent manner.

Keywords: relaxin-2, synaptic integrity, microglia, NLRP3 inflammasome, autophagy

INTRODUCTION

Alzheimer disease (AD) is an age-related progressive neurodegenerative disease that is associated with cognitive deterioration, synaptic anomalies, and neuronal loss (1). Synaptic damage is among the first of the pathological processes in AD, and it is closely related to cognitive impairment rather than to the amyloid plaque burden alone (2). The growing body of evidence suggests that age-dependent neuroinflammatory responses and impaired cellular clearance systems that are dependent on age play a critical role in synaptic vulnerability during the initial phases of AD development (3).

The immune cells of the central nervous system are called microglia, and they have a dual and opposing role in maintaining synaptic homeostasis and regulating neuroinflammation (4). Within the framework of AD, chronic microglial stimulation results in an excessive response and inappropriate production of pro-inflammatory cytokines, in particular, through the activation of the NLRP3 inflammasome (5). Activation of NLRP3 facilitates the maturation and release of IL-1 β and TNF- α that enhance synaptic loss and cognitive decline (6). Notably, ageing increases microglial priming, making the aged brain more vulnerable to inflammation-induced neurotoxicity.

In line with neuroinflammation, the dysregulation of the mTOR signalling pathway has emerged as a core AD pathological feature (7). mTOR hyperactivation inhibits autophagy, a lysosome-dependent degradation pathway that is essential for the clearance of misfolded proteins and damaged organelles (8). Impaired autophagic flux contributes to the accumulation of A β , loss of synaptic proteins, and prolonged inflammatory signals. Emerging evidence indicates that there is a functional crosstalk between NLRP3 inflammasome activation and mTOR signalling, which places the NLRP3-mTOR-autophagy axis as a critical controller of synaptic integrity in AD (9).

RLN2 is an insulin-like hormone from the corpus luteum that relaxes pelvic ligaments and softens the uterus during pregnancy (10). In addition to these reproductive roles, RLN2 has lately become a major focus of interest due to its antioxidant, anti-inflammatory, cytoprotective, vasodilatory and antifibrotic effects, particularly in the cardiovascular and central nervous systems (11). The biological action of RLN2 is regulated by its binding to the relaxin family peptide receptor-1 (RXFP1), which is present in neurons and glial cells and is involved in the control of immune and glial functions in the brain (12). Research indicates that RLN2 may suppress vascular and cellular inflammation and reduce oxidative stress by increasing reduced glutathione levels (13). It suppresses the excessive activation of innate immune pathways, including the NLRP3 inflammasome, as part of its antifibrotic actions (14). Moreover, RLN2 has been shown to inhibit neuronal apoptosis *via* the extracellular signal-regulated kinase – neuronal nitric oxide synthase – nitric oxide (ERK-nNOS-NO) signalling pathway as well as to control intracellular homeostatic activities such as autophagy, which is important for neuronal survival and protein quality regula-

tion (15). The neuroprotective efficacy of RLN2 is also supported by evidence that it improves neurological recovery, minimises oxidative stress and oedema, inhibits pro-inflammatory cytokines, and induces a switch from pro-inflammatory M1 microglia to a neuroprotective M2 phenotype by regulating nuclear factor kappa B (NF- κ B) and signal transducer and activator of transcription 6 (STAT6) signalling pathways (16). Though the direct evidence of the role of RLN2 in AD is still scarce, all these data indicate that this peptide could have neuroprotective activity by controlling neuroinflammation, oxidative stress, and cellular homeostasis, which play a crucial role in neurodegeneration.

Mechanistically, RLN2 can mediate neuroinflammatory and neurodegenerative mechanisms by regulating the mTOR signalling pathway, one of the main controllers of autophagy and cellular metabolism, which also interacts with the activation of the inflammasomes. The interplay among NLRP3 signalling, mTOR activity, and autophagy is becoming more evident as an influential factor in the maintenance of synapses and neuronal survival during neurodegenerative diseases. Nevertheless, despite these encouraging findings, the possible neuroprotective effect of RLN2 in AD has not been well understood, particularly with regard to its capacity to preserve synaptic structure. Moreover, ageing, which is the most potent risk factor for AD, significantly changes the state of hippocampal activation, inflammasome signalling, and autophagic efficiency, which can affect the ability to respond to modulatory molecules like RLN2. Hence, investigating whether RLN2 can offer age-specific neuroprotection by regulating the NLRP3-mTOR-autophagy axis could provide significant insights into the processes that contribute to synaptic loss and its protection in AD-like diseases.

Therefore, this study aimed to investigate the age-dependent neuroprotective effects of RLN2 in A β_{1-42} -induced AD-like pathology, particularly through the modulation of the hippocampal NLRP3-mTOR-autophagy axis, neuroinflammation, synaptic integrity, and cognitive function. We hypothesised that RLN2 would exert greater neuroprotective effects in young mice than in aged mice by reducing neuroinflammation and improving autophagy, synaptic function, and cognitive performance.

EXPERIMENTAL

Materials and reagents

Synthetic A β_{1-42} was obtained from (Sigma-Aldrich/Merck Chemicon, USA, AG972, > 97 % purity). Recombinant human RLN2 was purchased from (Abcam, UK, ab63227, > 95 % purity). Primary antibodies against Iba1 (Abcam, ab178847, 1:1,000), NLRP3 (Proteintech, USA, 30109-1-AP, 1:3,000), ASC (Proteintech, 10500-1-AP, 1:2,000), p-mTOR (Proteintech, 67778-1-Ig, 1:1,000), p-p70S6K (Cell Signaling Technology, USA, 9234, 1:1,000), LC3 (Cell Signaling Technology, 2775, 1:1,000), p62 (Proteintech, 18420-1-AP, 1:1,000), synaptophysin (Proteintech, 17785-1-AP, 1:1,000), PSD-95 (Proteintech, 20665-1-AP, 1:2,000), and β -actin (Proteintech, 66009-1-Ig, 1:5,000) were used for Western blot analysis. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Proteintech, Cat. No. SA00001-2, 1:5,000) and HRP-conjugated goat anti-mouse IgG secondary antibody (Proteintech, Cat. No. SA00001-1, 1:5,000) were used as secondary antibodies for Western blot analysis. Mouse IL-1 β and TNF- α levels were measured using ELISA kits from Elabscience Biotechnology Inc., USA (Cat. Nos. E-EL-M0037 and E-EL-M0049, respectively), according to the manufacturer's instructions.

Animals and experimental design

In the current study, male C57BL/6J mice were used and kept under normal laboratory conditions (12 h light/dark cycle, regulated temperature and humidity) and allowed *ad libitum* access to food and water. Animals were divided into two age groups: young (3–4 months) and aged (12–14 months) mice. The study was approved by the Chengdu University of Traditional Chinese Medicine Laboratory Animal Ethics Committee (Ethics Number: CDUTCM-2609186).

The study involved a total of 36 mice, comprising 18 young mice and 18 aged mice. In each age group, animals were randomly allocated to six experimental groups ($n = 3$ mice per group): (i) Sham control, (ii) $A\beta_{1-42}$, (iii) $A\beta_{1-42} + RLN2$ (50 ng), (iv) $A\beta_{1-42} + RLN2$ (100 ng), (v) $A\beta_{1-42} + NLRP3$ inhibitor, and (vi) $A\beta_{1-42} + mTOR$ inhibitor.

The experimentation was approved and carried out in accordance with the provisions of national and international standards for the use and care of laboratory animals. Randomisation to groups was done, and both behavioural and biochemical studies were conducted by investigators who were not aware of the treatment conditions.

Preparation of $A\beta_{1-42}$ peptide and recombinant RLN2

Synthetic $A\beta_{1-42}$ was dissolved in pharmacological-grade phosphate-buffered saline (PBS) to attain a final concentration of 1 mg mL^{-1} and left to incubate at 37°C for a 72 h period to facilitate oligomer formation. Aliquots were frozen at -80°C and thawed just before intracerebral injection.

To prepare a stock solution, recombinant human RLN2 was purchased in lyophilised form and reconstituted in sterile PBS as per the instructions given by the manufacturer. The solution was gently swirled so that it could thoroughly dissolve and then filtered through a $0.22 \mu\text{m}$ sterile syringe filter. Aliquots were frozen at -80°C until required. Fresh working solutions containing the working solutions were made by diluting the stock solution with sterile PBS to the required concentration immediately before administration.

Intracerebroventricular injection of $A\beta_{1-42}$

Mice were anaesthetised using ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and placed in a stereotaxic apparatus. $A\beta_{1-42}$ ($3 \mu\text{g}$ in $3 \mu\text{L}$ PBS) was injected into the right lateral ventricle at the following coordinates with respect to bregma: anteroposterior -0.22 mm , mediolateral $+1.0 \text{ mm}$, dorsoventral -2.5 mm . Sham-operated mice were injected with an equal amount of PBS. The injections were administered at $0.5 \mu\text{L min}^{-1}$ and the needle was left in place for 5 min to prevent backflow.

RLN2 and pharmacological treatments

RLN2 was used intracerebroventricularly at the dose of 50 ng or 100 ng per mouse (17). The treatment was started 24 h after the injection of $A\beta_{1-42}$ and was administered daily throughout the experimental period. Vehicle injections were administered to the control animals. To identify pathway involvement, mice were treated with the selective NLRP3 inflammasome inhibitor (MCC950) or the mTOR pathway inhibitor (rapamycin) at doses

that had been previously tested to determine their effects. MCC950 was administered at a dose of 10 mg kg⁻¹ body mass (intraperitoneally), while rapamycin was administered at 5 mg kg⁻¹ body mass (intraperitoneally) (18, 19). For the combination treatment, RLN2 (100 ng) was combined with either of the inhibitors to determine whether there were additive or overlapping effects.

Behavioural assessments

Morris Water Maze (MWM). – The MWM was used to determine spatial learning and memory. The equipment comprised a round pool (120 cm diameter, 40–50 cm in height) that contained water (22 ± 1 °C) which had been made opaque using non-toxic white paint. In the centre of one of the quadrants, a transparent escape platform (10 cm diameter) was submerged 1 cm below the water surface and maintained in place during the entire acquisition phase. Mice were trained on five consecutive days with four trials per day. In every trial, there were four semi-random starting positions, with the mice facing the wall of the pool. The mice were released and given 60 s to find the hidden platform. If a mouse was unable to locate the platform in 60 s, it was gently guided to reach the platform and given 15 s to stay on the platform. The video-tracking system was used to record the escape time (latency to reach the platform), swimming speed, and path length. The probe trial was conducted 24 h following the last training session to determine the retention of memory. In this experiment, the platform was removed, and the mice were given the opportunity to swim freely within a period for 60 s. The time spent in the target quadrant, number of platform crossings, and search strategy were measured and analysed.

Novel object recognition (NOR)

The NOR test was used to measure recognition memory. The device was made up of a square open field arena (40 × 40 × 40 cm) with opaque walls. It was a three-stage test, which included the habituation, familiarisation, and test phases.

At the habituation stage, mice were given the freedom to roam freely in the empty arena, over the period for 5–10 min each day for two days. During the familiarisation phase, two identical objects were introduced into the arena, and every mouse was given 10 min to explore them. Exploration was considered when the nose was directed toward the object at a distance of ≤ 2 cm and/or when the object was touched. Sitting or climbing on the object was not considered exploratory behaviour.

One of the familiar objects was changed to a novel object, which was of different shape and texture yet of similar size after the 24-h retention period. Mice were once more given 5 min of free exploration. The time spent searching the novel and the usual things was documented. The recognition memory was measured in the form of the discrimination index (DI), which was:

$$DI = \frac{T_{\text{novel}} - T_{\text{familiar}}}{T_{\text{novel}} + T_{\text{familiar}}}$$

Between the trials, all of the apparatus and objects were washed with 70 % ethanol to remove olfactory cues. An experimenter who was blinded to the treatment groups performed behavioural testing.

Tissue collection and hippocampal homogenization

Mice were anaesthetised and euthanised after behavioural testing. Brains were quickly dissected, and the hippocampi were dissected on ice. The tissues were homogenised using ice-cold radioimmunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitors. Samples were centrifuged at 12,000 *g* for 20 min at 4 °C, with the supernatants collected. Protein concentration was determined using a bicinchoninic acid (BCA) assay.

Western blot analysis

Protein (30–40 µg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes after electrophoresis. Primary antibodies against Iba1, NLRP3, ASC, cleaved caspase-1, phospho-mTOR, phosphorylated p70 ribosomal S6 kinase (phospho-p70S6K), microtubule-associated protein 1 light chain 3 (LC3), sequestosome 1 (p62), synaptophysin, postsynaptic density protein 95 (PSD-95) and β -actin were then incubated with the membranes overnight at 4 °C after blocking. After incubation with HRP-conjugated secondary antibodies, protein bands were visualised using enhanced chemiluminescence with a UVP ChemiStudio Plus Imaging System (Analytik Jena GmbH, Germany) and analysed using VisionWorks software (Analytik Jena GmbH). Densitometric analysis was performed using ImageJ software (National Institutes of Health, USA).

Assessment of autophagy-related proteins

Autophagic activity was assessed by measuring the expression of autophagy-related proteins, including LC3 and p62, using Western blot analysis. The LC3-II/LC3-I ratio and p62 protein expression were analysed to evaluate changes in autophagy-related processes in the hippocampal tissues.

Measurement of IL-1 β and TNF- α by ELISA

The cytokines (IL-1 β and TNF- α) in the hippocampus were measured with the help of commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer's protocol. The 96-well plates, which had been coated with antibodies, were incubated at room temperature with equal amounts of protein samples. Biotinylated detection antibodies and HRP-conjugated streptavidin were added after washing. The development of colour was carried out with the help of tetramethylbenzidine substrate, and the absorbance was measured at the wavelength of 450 nm. Standard curves were used to determine the concentration of cytokines, and the results were expressed as pg mg⁻¹ protein.

Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical analyses were performed using one-way ANOVA for comparisons among multiple treatment groups within each age group and two-way ANOVA to assess the effects of age and treatment and their interaction (age

× treatment), followed by Tukey's multiple-comparisons test. Statistical analyses were performed using GraphPad Prism (version 11.0). Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

RLN2 suppresses hippocampal activation and NLRP3 inflammasome signalling in an age-dependent manner

Intracerebral injection of $A\beta_{1-42}$ to strong hippocampal activation and inflammasome maturation in young and aged mice, as indicated by high levels of Iba1, NLRP3, ASC, and cleaved caspase-1 (Fig. 1). The use of RLN2 led to a significant dose-dependent reduction

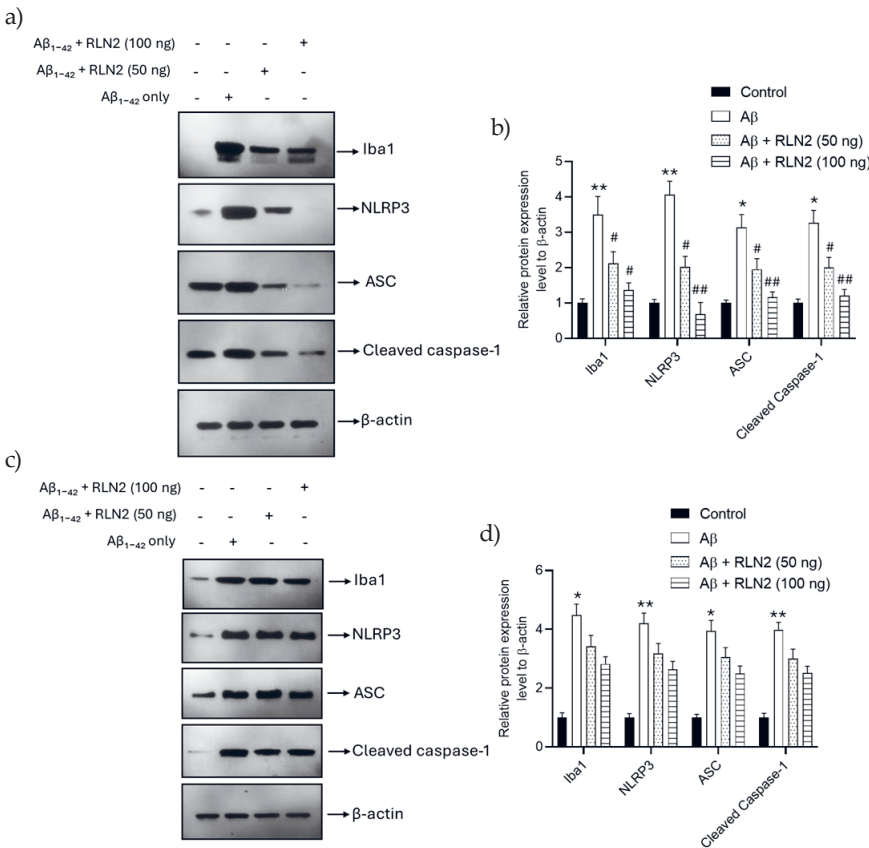


Fig. 1. Age-dependent suppression of hippocampal activation and NLRP3 inflammasome signalling by RLN2: a) representative Western blots of Iba1, NLRP3, ASC, and cleaved caspase-1 in the hippocampi in young mice; b) densitometric analysis of Iba1, NLRP3, ASC, and cleaved caspase-1 protein levels in young mice; c) representative Western blots of Iba1, NLRP3, ASC, and cleaved caspase-1 in hippocampi in aged mice; d) densitometric analysis of Iba1, NLRP3, ASC, and cleaved caspase-1 protein levels in aged mice. * $p < 0.05$ and ** $p < 0.01$ vs. control group; # $p < 0.05$ and ## $p < 0.01$ vs. $A\beta$ group.

in the expression of the Iba1 protein and the components of the NLRP3 inflammasomes in young mice, with the effect of the 100-ng dose being stronger than that of the 50 ng dose. Conversely, aged mice showed only partial attenuation of hippocampal activation and inflammasome signalling following RLN2 treatment, which suggests that the age-related attenuation in the responsiveness to RLN2-mediated anti-inflammatory effects was significant.

Hippocampal microglial activation and NLRP3 inflammasome signalling have now been identified as a key mediator of neuroinflammation and synaptic dysfunction in AD (20). Research has demonstrated that A β activates the NLRP3 inflammasome, which results in caspase-1 activation and the production of IL-1 β , which further worsens neuronal damage and accelerates cognitive impairment (21). Consistent with these reports, we found that there was strong upregulation of Iba1, NLRP3, ASC and cleaved caspase-1 after administration of A β_{1-42} in young and aged mice.

Particularly, our data build upon previous findings that show that RLN2 suppresses hippocampal activation and inflammasome signalling in, again, a distinct dose-dependent manner, particularly in young mice (16). Previous studies have indicated anti-inflammatory effects of RLN2 in peripheral immune cells and vascular tissues, including the inhibition of inflammasome-mediated cytokine production (22). Nevertheless, limited evidence has been available to support the idea of RLN2-regulated hippocampal inflammasome activity in Alzheimer's disease-like conditions. The findings of our study provide, to our knowledge, the first *in vivo* demonstration that RLN2 can regulate hippocampal NLRP3 signalling in the brain and that this ability is significantly reduced with age. The partial responsiveness of aged mice is in line with the notion of hippocampal priming during old age, whereby aged microglia show an increased basal inflammatory tone and a decreased response to anti-inflammatory cues (23). This observation may be one of the reasons why RLN2 is less effective in inhibiting inflammasome signalling in the aged brain.

In order to determine whether RLN2 alters hippocampal activation and inflammasome signalling under an age-regulated setting, we assessed the hippocampal expression of the microglial marker Iba1 and key components of the NLRP3 inflammasome.

RLN2 differentially regulates mTOR hyperactivation and autophagic-related protein expression in young and aged mice

Considering the established connection between inflammasome activation, mTOR signalling and autophagy, we then analysed the ability of RLN2 to modulate mTOR pathway activity and autophagy-related protein expression. The administration of A β_{1-42} , as shown in Fig. 2, caused considerable phosphorylation of mTOR and its downstream protein p70S6K in young and aged mice, accompanied by alterations in autophagy-related protein expression, as indicated by changes in the LC3-II/LC3-I ratio and p62 levels (Fig. 2). RLN2 treatment had a profound inhibitory effect on the hyperactivation of mTOR and modulated autophagy-related protein expression in young mice in a dose-dependent manner. Conversely, aged mice exhibited sustained mTOR activation and altered autophagy-related protein expression even after RLN2 administration, with slight changes in LC3 conversion and continued p62 accumulation. The results indicate that the process of ageing reduces the ability of RLN2 to normalise mTOR-autophagy signalling in response to A β_{1-42} .

Abnormal mTOR signalling and autophagy dysfunction are typical characteristics of ageing and AD (24). The hyper-phosphorylation of mTOR inhibits autophagy-mediated degradation of A β and damaged cellular components and thus maintains neuroinflammation and loss of synapses (25). In line with previous reports, we did find significant mTOR hyperactivation and altered autophagy-related protein expression after exposure to A β_{1-42} in the two age groups. We found that RLN2 in young mice suppresses mTOR and modulates autophagy-related protein expression, as indicated by reduced p-mTOR and p-p70S6K levels, changes in the LC3-II/LC3-I ratio, and reduced p62 levels. These findings are consistent with those of previous studies that have shown that mTOR inhibition enhances autophagy and cognitive functions in AD models. Notably, our investigation is the first, to our knowledge, to associate RLN2 signalling with mTOR-autophagy regulation in the con-

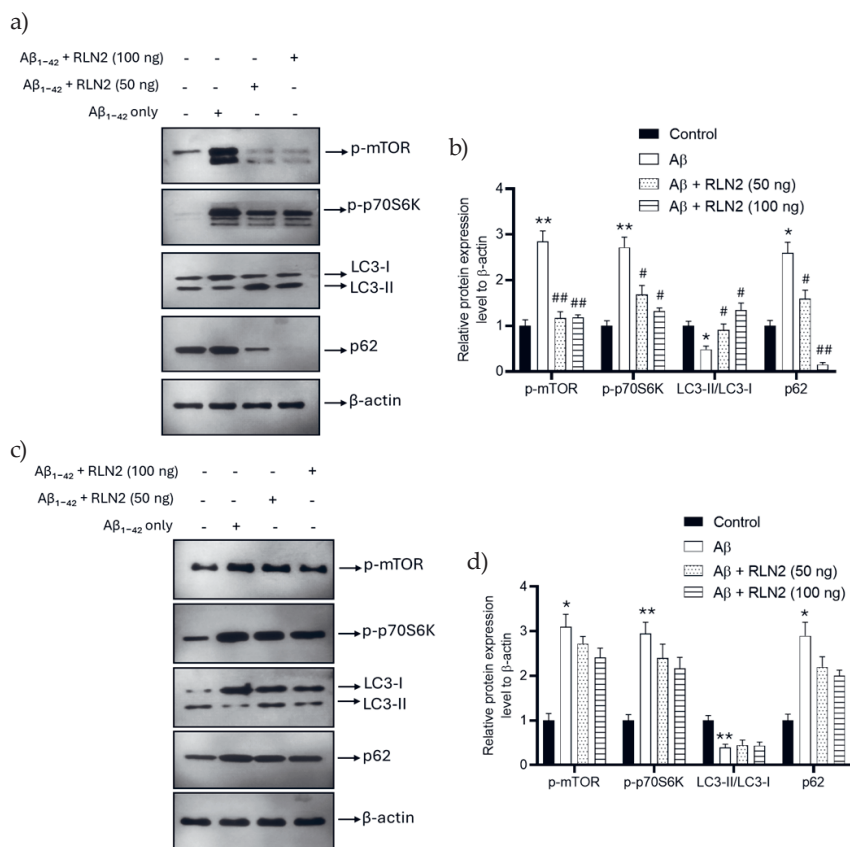


Fig. 2. RLN2 differentially modulates mTOR hyperactivation and autophagic flux in young *versus* aged A β_{1-42} -treated mice: a) representative Western blots of p-mTOR, p-p70S6K, LC3-I/II, and p62 in the hippocampi of young mice; b) densitometric analysis of p-mTOR, p-p70S6K, LC3-I/II, and p62 protein levels in young mice; c) representative Western blots of p-mTOR, p-p70S6K, LC3-I/II, and p62 in the hippocampi of aged mice; d) densitometric analysis of p-mTOR, p-p70S6K, LC3-I/II, and p62 protein levels in aged mice. * $p < 0.05$ and ** $p < 0.01$ vs. control group; # $p < 0.05$ and ## $p < 0.01$ vs. A β group.

text of Alzheimer-like pathology. Conversely, aged mice exhibited sustained mTOR activation and altered autophagy-related protein expression even after RLN2 administration. This finding aligns with previous data showing that the ageing process impairs lysosomal function and autophagic capacity, which makes autophagy-inducing interventions less efficient (26). Therefore, it is possible to state that ageing also places a mechanistic limit on the RLN2-mediated restoration of cellular homeostasis.

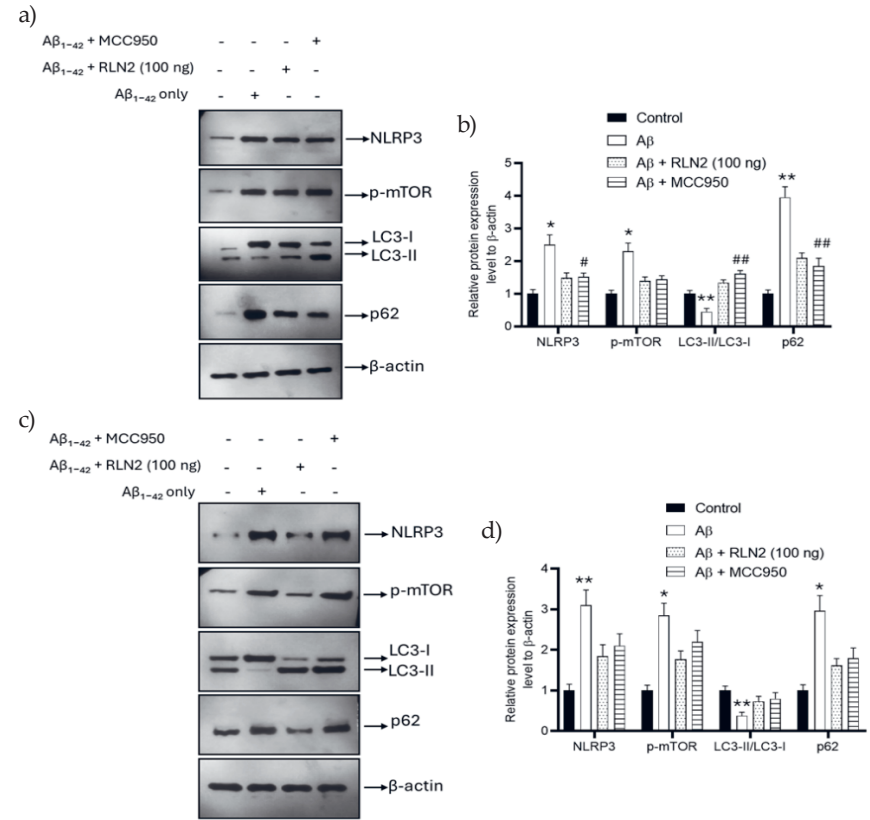
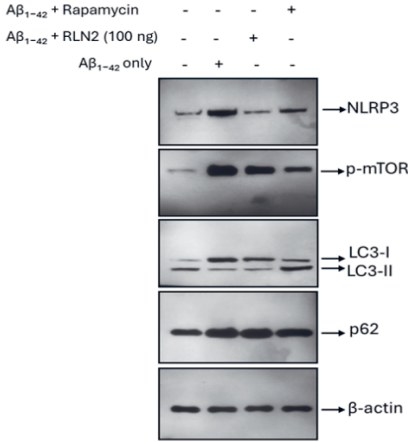
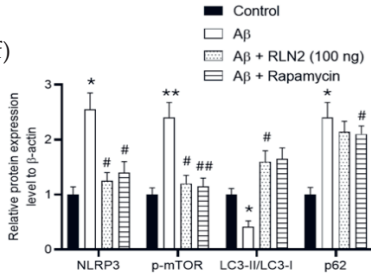


Fig. 3. Age-dependent effects of NLRP3 and mTOR inhibition on hippocampal autophagy in $A\beta_{1-42}$ -treated mice: a) representative Western blots of NLRP3, p-mTOR, LC3-II/LC3-I, and p62 in the hippocampi of young mice in the presence of NLRP3 inhibitor (MCC950); b) densitometric analysis of NLRP3, p-mTOR, LC3-II/LC3-I, and p62 protein levels in young mice following MCC950 treatment; c) representative Western blots of NLRP3, p-mTOR, LC3-II/LC3-I, and p62 in the hippocampi of aged mice in presence of NLRP3 inhibitor (MCC950); d) densitometric analysis of NLRP3, p-mTOR, LC3-II/LC3-I, and p62 protein levels in aged mice following MCC950 treatment; e) representative Western blots of NLRP3, p-mTOR, LC3-II/LC3-I, and p62 in the hippocampi of young mice in presence of mTOR inhibitor (rapamycin); f) densitometric analysis of NLRP3, p-mTOR, LC3-II/LC3-I, and p62 protein levels in young mice following rapamycin treatment; g) representative Western blots of NLRP3, p-mTOR, LC3-II/LC3-I, and p62 in the hippocampi of aged mice in presence mTOR inhibitor (rapamycin); h) densitometric analysis of NLRP3, p-mTOR, LC3-II/LC3-I, and p62 protein levels in aged mice following rapamycin treatment. * $p < 0.05$ and ** $p < 0.01$ vs. control group; # $p < 0.05$ and ## $p < 0.01$ vs. $A\beta$ group.

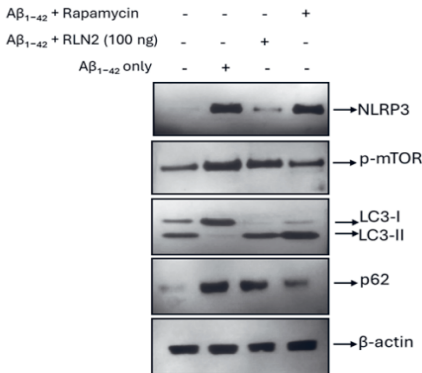
e)



f)



g)



h)

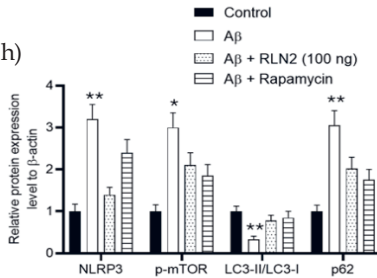


Fig. 3. Continued

Inhibition of NLRP3 or mTOR mimics RLN2-mediated restoration of hippocampal autophagy in young but not aged mice

To determine whether RLN2 acts through the NLRP3-mTOR-autophagy axis, pharmacological inhibitors of NLRP3 or mTOR were used to evaluate their effects in $A\beta_{1-42}$ -treated mice. In young mice, both NLRP3 and mTOR inhibition reduced NLRP3 and mTOR activation and modulated autophagy-related protein expression, as indicated by increased LC3-II/LC3-I ratios and decreased p62 levels (Fig. 3). Pharmacological inhibition of either NLRP3 or mTOR produced effects similar to those observed with RLN2 treatment in young mice, supporting the involvement of the NLRP3-mTOR-autophagy pathway in RLN2-mediated modulation of autophagy-related protein expression. By comparison, aged mice demonstrated only partial changes in autophagy-related protein expression after NLRP3 or mTOR inhibition, indicating that this signalling axis is less responsive in aged mice.

Young mice treated pharmacologically to inhibit NLRP3 or mTOR showed effects similar to those of RLN2 in suppressing inflammasome-related signalling and modulating autophagy-related protein expression. These results support the involvement of the NLRP3-mTOR-autophagy axis in RLN2-mediated effects. This is consistent with a growing body of literature that reveals reciprocal crosstalk between inflammasome activation and mTOR signalling, with NLRP3 activation promoting mTOR hyperactivation, which may further inhibit autophagy and enhance inflammation (27). However, in older mice, NLRP3 or mTOR inhibition had only partial effects on autophagy-related protein expression, which adds to the idea that age-related impairments in intracellular clearance systems constrain the efficacy of therapeutic interventions (28). This age-related resistance could be a manifestation of changes in lysosomal integrity, mitochondrial performance, or epigenetic regulation.

RLN2 preserves synaptic integrity preferentially in young $A\beta_{1-42}$ -treated mice

Since synaptic loss can be identified as a determining factor of cognitive deterioration in Alzheimer-like pathology, we examined the hippocampal expression of the presynaptic marker, synaptophysin, and the postsynaptic density protein, PSD-95. $A\beta_{1-42}$ suppressed the expression of synaptic proteins in both young and aged mice (Fig. 4). RLN2 treatment was effective in maintaining the levels of synaptophysin as well as PSD-95 in young mice, and the highest levels of protection were observed at the higher dose. However, with aged mice, RLN2 caused only a slight recovery of synaptic markers, consistent with the limited modulation of autophagy-related protein expression and persistent inflammatory signalling observed in the present study.

The best pathological correlate of cognitive impairment in AD is synaptic loss. In line with the previous reports, $A\beta_{1-42}$ caused a significant reduction in hippocampal synaptophysin and PSD-95 levels in young and aged mice (29). RLN2 preserved presynaptic and postsynaptic markers in young mice, which is consistent with previous data suggesting that suppression of neuroinflammation and modulation of autophagy-related processes may help preserve synaptic integrity (30).

Effect of RLN2 on neuroinflammatory cytokines (IL-1 β and TNF- α) in young and aged mice

A significant rise in both cytokines was observed following intracerebroventricular injection of $A\beta_{1-42}$ compared with sham control animals, which supported the induction of a neuroinflammatory state. In young mice, $A\beta_{1-42}$ administration significantly increased IL-1 β and TNF- α levels compared with sham controls (Fig. 5a,c). Similarly, $A\beta_{1-42}$ administration increased IL-1 β and TNF- α levels in aged mice compared with their respective sham controls (Fig. 5b,d).

RLN2 treatment brought about a significant anti-inflammatory effect on young mice. The IL-1 β and TNF- α levels were moderately suppressed with administration of RLN2 at a 50 ng dose, and a more pronounced effect was found with administration of 100 ng of RLN2, which reduced the levels of the two cytokines to levels closer to those of the sham controls. This implies that RLN2 effectively attenuated $A\beta_{1-42}$ -induced neuroinflammation in young mice.

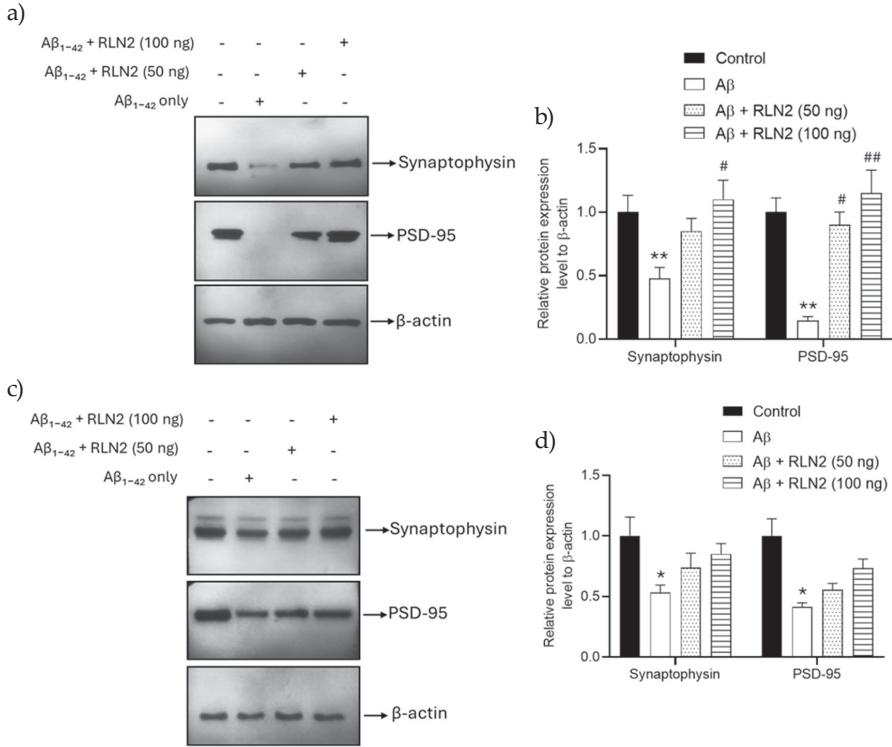


Fig. 4. RLN2 preserves synaptic integrity in an age-dependent manner following A β_{1-42} exposure: a) representative Western blots of synaptophysin and PSD-95 proteins in the hippocampi of young mice; b) densitometric analysis of synaptophysin and PSD-95 protein levels in young mice; c) representative Western blots of synaptophysin and PSD-95 proteins in the hippocampi of aged mice; d) densitometric analysis of synaptophysin and PSD-95 protein levels in aged mice. * $p < 0.05$ and ** $p < 0.01$ vs. control group; # $p < 0.05$ and ## $p < 0.01$ vs. A β group.

Conversely, aged mice responded partially to RLN2 treatment. Though both doses of RLN2 decreased IL-1 β and TNF- α concentrations relative to untreated A β_{1-42} -injected aged mice, the decreases in concentration were less pronounced, particularly at the 50-ng dose. At 100 ng RLN2, cytokine levels remained elevated relative to the respective sham controls, indicating incomplete suppression of A β_{1-42} -induced neuroinflammation in aged mice. Overall, these results indicate that RLN2 attenuated A β_{1-42} -induced increases in neuroinflammatory cytokine levels in both young and aged mice, with a more evident reduction observed at the 100-ng dose.

RLN2 improves cognitive function in an age-dependent manner

Molecular results were closely correlated with behavioural results. RLN2 had a marked positive effect on spatial learning, memory retention, and recognition memory in young mice but produced only slight cognitive enhancement in aged mice. Such find-

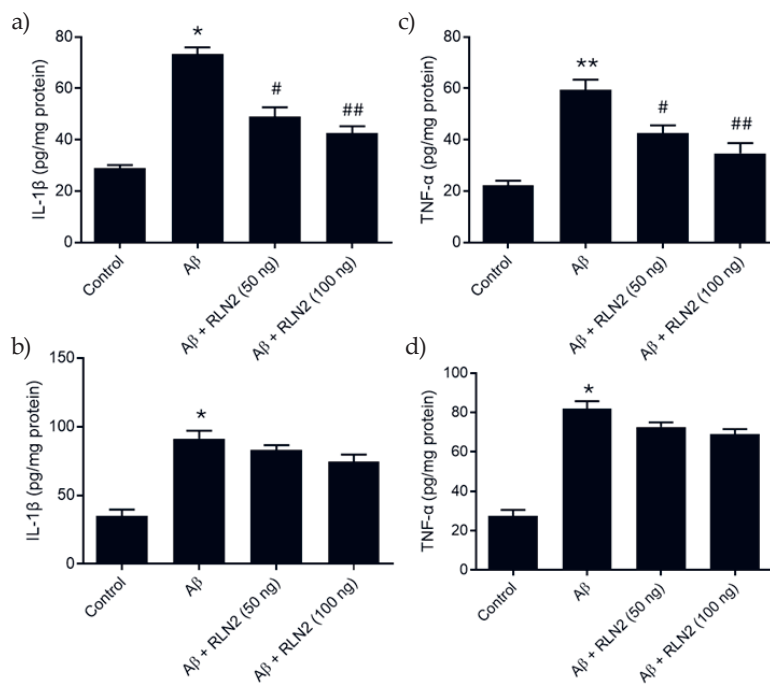


Fig. 5. Measurement of neuroinflammatory cytokines in hippocampal tissue. Hippocampal levels of IL-1 β and TNF- α were quantified using ELISA in young and aged mice across different experimental groups. Levels of: a) IL-1 β and b) TNF- α in the young mice, and levels of c) IL-1 β and d) TNF- α in aged mice. * p < 0.05 and ** p < 0.01 vs. control group; # p < 0.05 and ## p < 0.01 vs. A β group.

ings can be explained by clinical and preclinical evidence that inflammation- or proteo- stasis-based therapeutic interventions become increasingly effective during the early stages of disease progression. Together, our results provide evidence to support a model whereby RLN2 has neuroprotective properties by suppressing hippocampal NLRP3 and modulating mTOR signalling, modulating autophagy-related protein expression, and maintaining synaptic integrity, leading to improved cognitive functioning. These effects are however, very age-dependent, with the efficacy being greatly reduced in the brains of the aged mice.

Spatial learning and memory were measured using the Morris Water Maze in order to evaluate the functional relevance of RLN2-mediated molecular and synaptic actions. As compared to the controls, the A β_{1-42} -treated mice at both ages showed a significantly higher escape latency in the acquisition trials and a shorter duration spent in the target quadrant in the probe trials (Fig. 6). The efficacy of RLN2 treatment in enhancing learning and memory retention was more evident in young mice, as demonstrated by the reduction of the escape latency and increased target quadrant occupancy. Conversely, aged mice showed moderate behavioural improvements after RLN2 treatment, indicating a decrease in cognitive responsiveness as age advances.

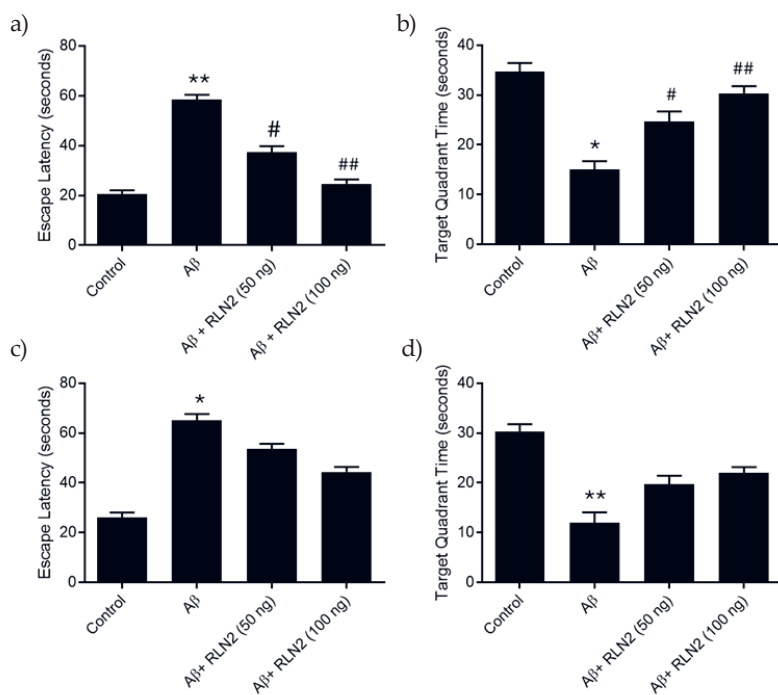


Fig. 6. RLN2 improves spatial learning and memory preferentially in young $A\beta_{1-42}$ -treated mice: a) escape latency during acquisition trials in young mice; b) time spent in the target quadrant in young mice; c) escape latency during acquisition trials in aged mice; d) time spent in the target quadrant in aged mice. * $p < 0.05$ and ** $p < 0.01$ vs. control group; # $p < 0.05$ and ## $p < 0.01$ vs. $A\beta$ group.

The recognition memory was also tested using the Novel Object Recognition test. Recognition memory performance in young and aged mice was greatly affected by $A\beta_{1-42}$, as indicated by the decreased exploration of the novel object as well as decreased discrimination indices (Fig. 7). Treatment with RLN2 substantially improved recognition memory in young mice in a dose-dependent manner but not in aged mice. Such results also support the presence of an age-dependent therapeutic window of RLN2-mediated cognitive protection.

Proposed model of age-dependent neuroprotection by RLN2

On the basis of the results of the experiment, a schematic diagram of the age-dependent neuroprotective effects of RLN2 is proposed (Fig. 8). $A\beta_{1-42}$ stimulates hippocampal NLRP3 inflammasome signalling and mTOR hyperactivation, which are associated with alterations in autophagy-related protein expression, synaptic degeneration, and cognitive impairment. RLN2 suppresses NLRP3-mTOR signalling, which is associated with modulation of autophagy-related protein expression in young mice and, consequently, preservation of synaptic integrity and cognitive ability. Persistent inflammatory stimulation and altered autophagy-related protein expression in aged mice may restrict the efficacy of

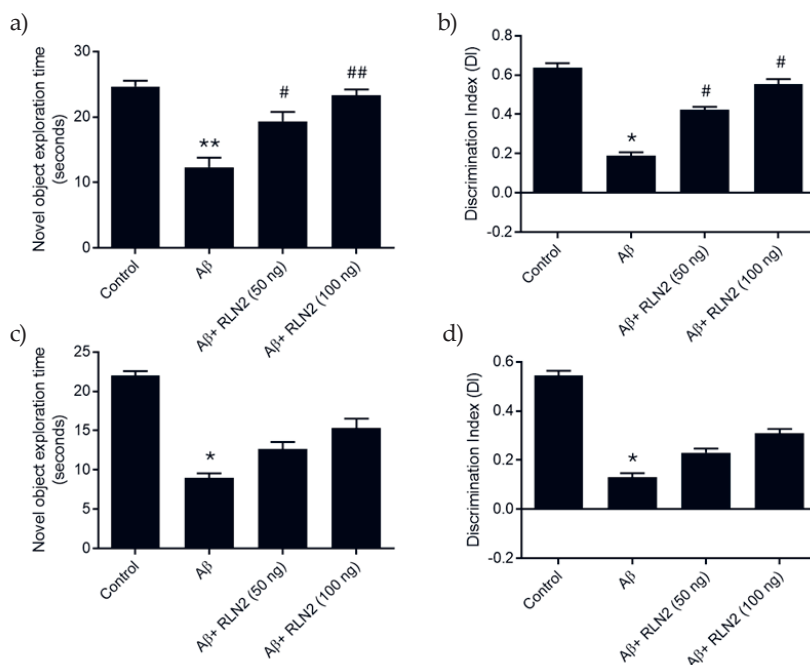


Fig. 7. RLN2 enhances recognition memory preferentially in young $A\beta_{1-42}$ -treated mice: a) exploration of the novel object in young mice; b) discrimination index in young mice; c) exploration of the novel object in aged mice; d) discrimination index in aged mice. * $p < 0.05$ and ** $p < 0.01$ vs. control group; # $p < 0.05$ and ## $p < 0.01$ vs. $A\beta$ group.

RLN2, establishing an age-sensitive therapeutic window of opportunity. This model underscores RLN2's potential as an early-stage therapeutic agent and as a guide for age-stratified intervention in Alzheimer's disease.

Limitations and future directions

Although the present research offers mechanistic insights, the small sample size of each group and the application of an acute $A\beta_{1-42}$ model are limitations, as they might not fully recapitulate chronic disease development. Additionally, NLRP3, mTOR, and autophagy-related proteins were assessed in whole-hippocampus homogenates; therefore, these changes cannot be attributed specifically to a single cell type and may reflect contributions from multiple hippocampal cell types. Future studies using cell type-specific isolation or co-localisation approaches are warranted to confirm cell-type-specific involvement. Further research should examine long-term RLN2 administration, receptor-specific signalling, sex-dependent effects, and validation in transgenic AD models. Moreover, it would be important to determine whether RLN2 can be combined synergistically with lifestyle or metabolic interventions that promote autophagy to increase its efficacy in ageing.

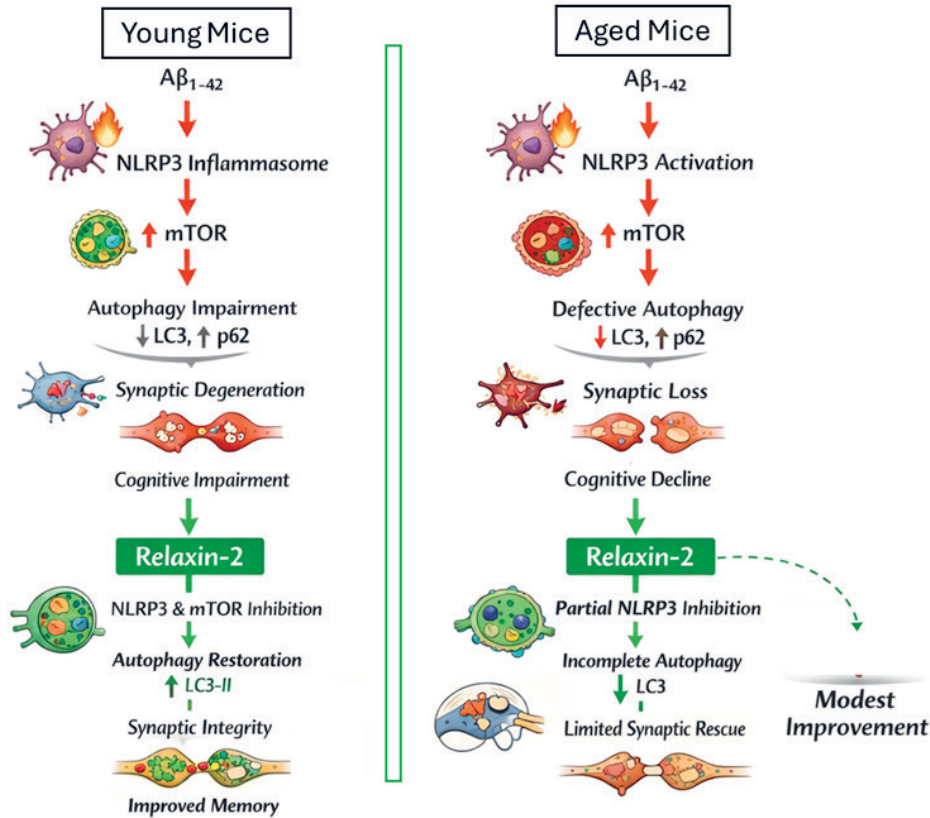


Fig. 8. Schematic model illustrating the age-dependent neuroprotective mechanism of RLN2 in Alzheimer-like pathology. Proposed model showing that Aβ₁₋₄₂ causes hippocampal NLRP3 inflammasome and mTOR activation, resulting in alterations in autophagy-related protein expression, synaptic degeneration, and cognitive deficits. RLN2-mediated inhibition of NLRP3-mTOR signalling is associated with modulation of autophagy-related protein expression in young mice, leading to the maintenance of synaptic integrity and cognitive performance. Persistent inflammatory stimulation and altered autophagy-related protein expression in aged mice may limit the efficacy of RLN2, which characterises an age-sensitive therapeutic window.

CONCLUSIONS

This paper indicates that RLN2 is neuroprotective against Aβ₁₋₄₂-induced pathological alterations associated with Alzheimer's disease in a distinct age-dependent fashion. Hippocampal NLRP3 inflammasome activation, mTOR signalling, autophagy-related protein expression, synaptic integrity, and cognitive performance are favourably modulated by RLN2 in young mice, whereas these effects are significantly reduced in aged mice. These results identify the hippocampal NLRP3-mTOR-autophagy axis as a potential mediator of RLN2 effects and emphasise the importance of ageing as a major factor in determining therapeutic efficacy. Together, the findings establish an age-sensitive thera-

peutic window and support the potential of RLN2 as an early-stage intervention in Alzheimer's disease, highlighting its potential as a therapeutic agent to target neuro-inflammation, modulate autophagy-related processes, and preserve synaptic function. Additionally, the age-dependent efficacy of RLN2 suggests the possibility of age-based stratification of therapeutic responses, although further studies are required to determine whether RLN2 could serve as a biomarker for patient stratification or therapeutic response monitoring.

Statement of ethics. – The authors have no ethical conflicts to disclose.

Conflict of interest. – The authors have no conflicts of interest to declare.

Author's contribution. – Conceptualisation, D.W.; study design, D.W.; methodology, D.W. and Z.Z.; statistical analysis, Y.D.; writing, original draft preparation, D.W.; writing, review and editing, Y.D.; supervision, Y.D. All authors have read and agreed to the published version of the manuscript.

REFERENCES

1. K. H. M. Chow and T. Abel, Neurodevelopmental origins of age-related neurodegenerative diseases, *EBioMedicine* **124** (2026) Article ID 106151 (12 pages); <https://doi.org/10.1016/j.ebiom.2026.106151>
2. E. M. Planalp, R. E. Langhough, E. M. Jonaitis, C. A. Van Hulle, R. E. Wilson, G. Kollmorgen, C. Quijano-Rubio, O. C. Okonkwo, B. B. Bendlin, S. Asthana, K. Blennow, H. Zetterberg and S. C. Johnson, Synaptic markers are associated with cognitive decline after accounting for amyloid burden among an at-risk Alzheimer's disease cohort, *Sci. Rep.* **15**(1) (2025) Article ID 19399 (14 pages); <https://doi.org/10.1038/s41598-025-04319-3>
3. S. E. J. Chee and E. Solito, The impact of ageing on the CNS immune response in Alzheimer's disease, *Front. Immunol.* **12** (2021) Article ID 738511 (15 pages); <https://doi.org/10.3389/fimmu.2021.738511>
4. G. Yang, X. Xu, W. Gao, X. Wang, Y. Zhao and Y. Xu, Microglia-orchestrated neuroinflammation and synaptic remodeling: Roles of pro-inflammatory cytokines and receptors in neurodegeneration, *Front. Cell. Neurosci.* **19** (2025) Article ID 1700692 (19 pages); <https://doi.org/10.3389/fn-cel.2025.1700692>
5. S. E. Corcoran, R. Halai and M. A. Cooper, Pharmacological inhibition of the Nod-like receptor family pyrin domain containing 3 inflammasome with MCC950, *Pharmacol. Rev.* **73**(3) (2021) 968–1000; <https://doi.org/10.1124/pharmrev.120.000171>
6. N. Demirtas, B. S. Mazlumoglu and S. S. Palabiyik Yucelik, Role of NLRP3 inflammasomes in neurodegenerative diseases, *Eurasian J. Med.* **55**(1) (2023) 98–105; <https://doi.org/10.5152/eurasian-jmed.2023.23349>
7. J. Zerdka, P. Brasse, M. Piszka, E. Kwapien, M. Kubicka, J. Bartkowski, J. Banach, H. Dacyl and A. Owczarska, The mechanistic target of rapamycin (mTOR) pathway as a target of anti-aging therapies: The role of rapamycin and its analogs in the regulation of cellular processes and their impact on longevity, *Cureus* **17**(12) (2025) Article ID e98514; <https://doi.org/10.7759/cureus.98514>
8. J. M. Carosi, C. Fourrier, J. Bensalem and T. J. Sargeant, The mTOR-lysosome axis at the centre of ageing, *FEBS Open Bio* **12**(4) (2022) 739–757; <https://doi.org/10.1002/2211-5463.13347>
9. J. Q. Lu, H. Q. Wang, M. R. Fang, X. M. Ye and K. Y. Song, Autophagy-NLRP3 inflammasome crosstalk in microglia: A therapeutic target for multiple sclerosis, *Inflammation* **49**(1) (2026) Article ID 23 (20 pages); <https://doi.org/10.1007/s10753-025-02355-9>

10. A. Silvano, O. Ammar, A. Parenti, N. Strambi, V. Seravalli, D. Bani and M. Di Tommaso, Relationship between relaxin-2 levels in serum and mode of conception in twin gestations: A prospective cohort study, *J. Obstet. Gynaecol. Res.* **51** (2025) Article ID e16190 (7 pages); <https://doi.org/10.1111/jog.16190>
11. N. Almeida-Pinto, T. B. Dschietzig, C. Bras-Silva and R. Adao, Cardiovascular effects of relaxin-2: Therapeutic potential and future perspectives, *Clin. Res. Cardiol.* **113**(8) (2024) 1137–1150; <https://doi.org/10.1007/s00392-023-02305-1>
12. A. Knezic, S. H. T. Truong, C. E. Moore, C. M. O. Barker, M. A. Hossain, C. S. Samuel and B. R. S. Broughton, The simultaneous targeting of RXFP1 and RXFP3 improves motor function and reduces brain inflammation, apoptosis and glial scarring following photothrombotic stroke in male mice, *Biochem. Pharmacol.* **243** (2026) Article ID (117523 (12 pages); <https://doi.org/10.1016/j.bcp.2025.117523>
13. S. Nistri, C. Fiorillo, M. Becatti and D. Bani, Human relaxin-2 (Serelaxin) attenuates oxidative stress in cardiac muscle cells exposed in vitro to hypoxia-reoxygenation. Evidence for the involvement of reduced glutathione up-regulation, *Antioxidants (Basel)* **9**(9) (2020) Article ID 774; <https://doi.org/10.3390/antiox9090774>
14. F. Tapia Caceres, T. A. Gaspari, M. A. Hossain and C. S. Samuel, Relaxin inhibits the cardiac myofibroblast NLRP3 inflammasome as part of its anti-fibrotic actions via the angiotensin type 2 and ATP (P2X7) receptors, *Int. J. Mol. Sci.* **23**(13) (2022) Article ID 7074 (22 pages); <https://doi.org/10.3390/ijms23137074>
15. J. Liu, Y. Cai, K. U. Rahman, Q. Zhou, G. Liu, H. Kang, M. Li, S. Zhang, G. Wang, W. Feng, X. Zhang, G. Zhang, Y. Song and P. Li, Rh-relaxin-2 attenuates oxidative stress and neuronal apoptosis via ERK-nNOS-NO pathway after germinal matrix hemorrhage in rats, *Fluids Barriers CNS* **22**(1) (2025) Article ID 8 (13 pages); <https://doi.org/10.1186/s12987-024-00616-7>
16. J. H. Wang, H. B. Sheng and J. K. Li, Relaxin-2 ameliorates spinal cord injury by inhibiting microglia activation, *Kaohsiung J. Med. Sci.* **41**(9) (2025) Article ID e70041 (9 pages); <https://doi.org/10.1002/kjm2.70041>
17. A. J. S. Summerlee, D. J. Hornsby and D. G. Ramsey, The dipsogenic effects of rat relaxin: The effect of photoperiod and the potential role of relaxin on drinking in pregnancy, *Endocrinology* **139**(5) (1998) 2322–2328; <https://doi.org/10.1210/endo.139.5.5966>
18. Y. Fan, L. Du, Q. Fu, Z. Zhou, J. Zhang, G. Li and J. Wu, Inhibiting the NLRP3 inflammasome with MCC950 ameliorates isoflurane-induced pyroptosis and cognitive impairment in aged mice, *Front. Cell. Neurosci.* **12** (2018) Article ID 426 (9 pages); <https://doi.org/10.3389/fncel.2018.00426>
19. A. Caccamo, S. Majumder, A. Richardson, R. Strong and S. Oddo, Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and Tau: Effects on cognitive impairments, *J. Biol. Chem.* **285** (2010) 13107–13120; <https://doi.org/10.1074/jbc.M110.100420>
20. T. Liang, Y. Zhang, S. Wu, Q. Chen and L. Wang, The role of NLRP3 inflammasome in Alzheimer's disease and potential therapeutic targets, *Front. Pharmacol.* **13** (2022) Article ID 845185 (21 pages); <https://doi.org/10.3389/fphar.2022.845185>
21. G. Sita, A. Graziosi, P. Hrelia and F. Morroni, NLRP3 and infections: beta-amyloid in inflammasome beyond neurodegeneration, *Int. J. Mol. Sci.* **22**(13) (2021) Article ID 6984 (14 pages); <https://doi.org/10.3390/ijms22136984>
22. W. Broszkiewicz and K. Dominska, The role of relaxins in blood cell modulation: interactions with relaxin family peptide receptor 1 (RXFP1) and glucocorticoid receptor (GR), *Clin. Sci. (Lond)* **139**(21) (2025) 1431–1450; <https://doi.org/10.1042/CS20256619>
23. K. M. Garner, R. Amin, R. W. Johnson, E. J. Scarlett and M. D. Burton, Microglia priming by interleukin-6 signaling is enhanced in aged mice, *J. Neuroimmunol.* **324** (2018) 90–99; <https://doi.org/10.1016/j.jneuroim.2018.09.002>

24. S. Davoody, A. Asgari Taei, P. Khodabakhsh and L. Dargahi, mTOR signaling and Alzheimer's disease: what we know and where we are?, *CNS Neurosci. Ther.* **30**(4) (2024) Article ID (e14463 (17 pages); <https://doi.org/10.1111/cns.14463>)
25. A. Subramanian, T. Tamilanban, A. Alsayari, G. Ramachawolran, L. S. Wong, M. Sekar, S. H. Gan, V. Subramanian, S. V. Chinni, N. N. Izzati Mat Rani, N. Suryadevara and S. Wahab, Trilateral association of autophagy, mTOR and Alzheimer's disease: potential pathway in the development for Alzheimer's disease therapy, *Front. Pharmacol.* **13** (2022) Article ID 1094351 (15 pages); <https://doi.org/10.3389/fphar.2022.1094351>
26. S. Ghosh Chowdhury, R. Ray and P. Karmakar, Relating aging and autophagy: A new perspective towards the welfare of human health, *EXCLI J.* **22** (2023) 732–748; <https://doi.org/10.17179/excli2023-6300>
27. S. Lv, H. Liu and H. Wang, The interplay between autophagy and NLRP3 inflammasome in ischemia/reperfusion injury, *Int. J. Mol. Sci.* **22**(16) (2021) Article ID 8773 (14 pages); <https://doi.org/10.3390/ijms22168773>
28. R. Tenchov, J. M. Sasso, X. Wang and Q. A. Zhou, Aging hallmarks and progression and age-related diseases: A landscape view of research advancement, *ACS Chem. Neurosci.* **15**(1) (2024) 1–30; <https://doi.org/10.1021/acscchemneuro.3c00531>
29. A. Azargoonjahromi, The duality of amyloid-beta: Its role in normal and Alzheimer's disease states, *Mol. Brain* **17** (2024) Article ID 44 (24 pages); <https://doi.org/10.1186/s13041-024-01118-1>
30. I. Bai, C. Keyser, Z. Zhang, B. Rosolia, J. Y. Hwang, R. S. Zukin and J. Yan, Epigenetic regulation of autophagy in neuroinflammation and synaptic plasticity, *Front. Immunol.* **15** (2024) Article ID 1322842 (13 pages); <https://doi.org/10.3389/fimmu.2024.1322842>