Effects of Retatrutide on Learning and Memory in Streptozotocin-Induced Diabetic Rats

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Abstract

Diabetes Mellitus (DM) is a rapidly increasing disease around the world. It is known that DM is associated with numerous complications which affect life quality by its debilitating nature. DM is associated with cognitive impairment and neurodegeneration, partly driven by neuroinflammation and disrupted neuronal signalling. Incretin-based treatments have recently been suggested to exert potential effects on the central nervous system in diabetic patients. However, the triple agonist of GIP/GLP-1/GCG Retatrutide's effects on cognition under diabetic conditions remain unexplored.

This study aims to reveal whether impaired cognitive performance, such as learning and memory, is ameliorated by Retatrutide treatment in diabetic rats, together with associated metabolic, inflammatory and histological changes.

Male Sprague–Dawley rats were allocated to four groups: control (C), streptozotocin-induced diabetic (STZ), streptozotocin-induced diabetic rats treated with Retatrutide (STZR), and sham rats treated with Retatrutide alone (R). DM was induced by streptozotocin injections. Spatial learning and memory were assessed using the Morris Water Maze and Passive Avoidance tests. Metabolic parameters were monitored, while neuroinflammatory markers (IL-1β, TNF-α), neurotrophic-related gene expression (BDNF, CREB, AKT), Tau protein levels, and histopathological changes in the cortex and hippocampus were evaluated using molecular, biochemical, and histological analyses.

Streptozotocin-induced diabetes resulted in persistent hyperglycaemia, total body weight loss, impaired learning and memory. Retatrutide treatment reduced blood glucose levels without achieving a full euglycaemia or preventing weight loss. Behavioural tests showed that Retatrutide treatment preserved spatial learning and short-term memory compared to untreated animals. These effects were accompanied by attenuation of neuroinflammatory responses, particularly reduced TNF-α levels, trends toward preserved neurotrophic-related

transcriptional profiles, and partial maintenance of cortical and hippocampal structural integrity. Retatrutide alone did not enhance cognitive performance beyond control levels.

These findings support the hypothesis that triple agonists may exert beneficial effects on cognitive performance under diabetic conditions. Retatrutide alleviates DM-associated cognitive impairment in streptozotocin-induced diabetic rats and is associated with reduced neural inflammatory burden and protected neuroanatomical structure. The observed cognitive benefits appear to extend beyond metabolic regulation alone. Further studies in models more closely reflecting type 2 diabetes are warranted to clarify the underlying mechanisms and translational relevance.

1. INTRODUCTION

Diabetes is a chronic metabolic disease characterised by persistent hyperglycaemia, which affects multiple organ systems and leads to various complications [1]. It has become a major global public health concern.

A large-scale meta-analysis including data from 1108 individuals reported that, as of 2022, approximately 828 million adults worldwide were living with diabetes, reflecting a dramatic rise in prevalence over recent decades [2]

Type 2 diabetes causes dysfunction in multiple organs, including the brain and nervous system. Cognitive impairments, particularly those involving learning and memory, are frequently observed as central nervous system complications. Studies have shown that diabetic patients have a 50% higher risk of developing Alzheimer's disease, vascular dementia, and other neuropsychological disorders compared to non-diabetic individuals [3]. Moreover, cognitive deficits related to diabetes occur not only in older adults but also in adolescents [1]. In this context, developing treatment strategies for cognitive impairments in prediabetic and diabetic adolescents is of great importance.

Glucagon-like peptide-1 (GLP-1) receptor agonists, also known as incretin mimetics, are widely used in the treatment of type 2 diabetes and obesity [4,5]. Glucose-dependent insulinotropic polypeptide (GIP) is another incretin hormone that modulates the secretion of insulin and glucagon (GCG). Preclinical studies have demonstrated that triple receptor agonists (RAs) activating all three receptors (GLP-1, GIP, and GCG) produce more pronounced weight loss than dual agonists targeting only two receptors [6]. Therefore, such agents are expected to become increasingly utilised. Retatrutide is a novel triple hormone agonist that simultaneously activates GIP, GLP-1, and GCG receptors. Both *in vivo* and *in vitro* studies in humans and animals are currently investigating its therapeutic potential for obesity and diabetes [6].

Animal studies have revealed that GLP-1 receptor agonists can cross the blood–brain barrier and distribute within various brain regions. Additionally, GLP-1 is endogenously synthesised in the nucleus of the solitary tract. Numerous preclinical studies have investigated the neuroprotective effects of GLP-1 analogues, demonstrating that they enhance neuronal survival and repair, promote mitochondrial biogenesis, and exert antioxidant and anti-inflammatory effects in the brain [7].

Proinflammatory cytokines also play a crucial role in diabetes-related cognitive decline. Tumour necrosis factor (TNF) released from microglia or perivascular macrophages activates the c-Jun N-terminal kinase (JNK) and inhibitor of κB alpha/nuclear factor κB (IκBα/NF-κB) signalling pathways, thereby amplifying neuroinflammatory responses throughout the brain parenchyma [8]. In diabetic patients, increased circulating levels of inflammatory markers such as C-reactive protein (CRP), interleukin-6 (IL-6), and TNF-α have been associated with poorer cognitive performance [9]. Similarly, elevated IL-1β levels have been observed in elderly patients with mild cognitive impairment compared with healthy controls [10]. Furthermore, a cross-sectional study involving 1712 diabetic patients found that chronic inflammatory diseases, such as rheumatoid arthritis and asthma, also constitute additional risk factors for cognitive decline in diabetes [11].

Brain-derived neurotrophic factor (BDNF) is a neuroprotective growth factor that supports neuronal proliferation, differentiation, and survival [12]. BDNF binds to the tropomyosin receptor kinase B (TrkB) receptor, promoting neuronal survival and plasticity through the activation of key intracellular signalling cascades, including the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), phospholipase C-γ (PLC-γ), and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways [13,14]. These signalling events ultimately enhance cAMP response element-binding protein (CREB) activation and regulate gene transcription in neurons. The BDNF/TrkB signalling axis thus plays an essential role in neuronal survival, memory formation, and antidepressant-like effects [15,16].

Streptozotocin (STZ) is a glucosamine–nitrosourea compound that selectively destroys pancreatic β-cells and is widely used to induce experimental diabetes models. In the present study, diabetes was induced using STZ at a dose of 60 mg/kg [17,18]. Although the streptozotocin-induced diabetes model primarily reflects severe insulin deficiency, it provides a robust and well-established platform for investigating diabetes-associated central nervous system alterations, particularly cognitive impairment and neuroinflammation. In this context, the present study was designed to evaluate whether retatrutide exerts direct or indirect neuroprotective effects on learning and memory that extend beyond its metabolic actions.

The increasing prevalence of diabetes and obesity, particularly at younger ages, underscores the importance of understanding how antidiabetic drugs influence cognitive function. To date, no studies have examined the cognitive effects of Retatrutide, a novel triple GLP-1/GIP/GCG receptor agonist. Therefore, this study aimed to investigate the effects of Retatrutide on learning and memory performance in streptozotocin-induced diabetic rats.

2. MATERIALS AND METHODS

Ethical Approval

The study was approved by the Kütahya Health Sciences University (KHSU) Animal Experiments Local Ethics Committee (Decision No: 2024.01.06, Date: 30.01.2024). The project was supported by the KHSU Scientific Research Projects Coordination Unit.

Chemicals

Retatrutide (RET) was purchased from ALFAGEN Laboratory Supplies Co. (İzmir, Türkiye). Streptozotocin (STZ) was obtained from certified laboratory stocks. STZ was dissolved in citrate buffer, and Retatrutide was dissolved in DMSO; all animals in the control group received the corresponding vehicle in equal volume to eliminate solvent-related confounding.

Animals and Housing

In total, 36 male Sprague Dawley rats were used. Animals were housed at 24 ± 2°C, 55 ± 15% humidity, under a 12 h light/dark cycle, with *ad libitum* access to water and standard chow. All animal procedures adhered to national and international guidelines. Experiments were conducted at the KHSU Experimental Animal Breeding Research and Application Centre and at the Department of Medical Pharmacology.

Experimental Design

Rats were divided into four groups with stratified sampling conducted by Barnes' Maze: Control (C, n = 8); Streptozotocin (STZ, initial number n = 9, final number n = 5); STZ + Retatrutide (STZR, initial number n = 11, final number n = 6); Retatrutide (R, n = 8)

STZ was administered intraperitoneally at 60 mg/kg in citrate buffer; an additional intraperitoneal dose of STZ (30 mg/kg) was administered to animals whose blood glucose levels remained below 250 mg/dL. Retatrutide was administered subcutaneously at 0.015 mg/kg in DMSO between days 1 and 21, based on previously reported preclinical data for triple GIP/GLP-1/GCGR receptor agonists[6]. To equalise solvent exposure, corresponding vehicles (citrate buffer or DMSO) were administered to the remaining groups. Diabetes induction was confirmed on day 3 by blood glucose measurement. Total body weight and blood glucose levels were recorded at the beginning and at the end of the experiment.

Morris Water Maze (MWM) and Passive Avoidance Test (PA) were conducted between days 15-19 and between days 20-21, respectively.

At the end of the experiment, rats were weighed and sacrificed under ketamine (90 mg/kg)-xylazine (10 mg/kg) anaesthesia by exsanguination method. Brains were divided into two hemispheres; while one of them was placed into formalin and fixed for histological examination, the other was kept in -80°C for molecular examinations.

In some animals that exhibited marked locomotor limitations accompanied by apparent metabolic disruptions, behavioural test data were excluded from analysis as they were considered unreliable. Accordingly, animal numbers varied across behavioural analyses due to predefined exclusion criteria related to severe locomotor impairment; however, tissue samples from these animals were included in molecular analyses.

Barnes Maze

At the initiation of the study, Barnes' Maze (BM) was administered in order to determine the spatial learning levels of rats and to balance the learning performances between groups. The apparatus, which was designed in standard measures suitable for rats, includes 20 holes on the surface and contains one escape box under one of these.

Rats were placed into the middle of the apparatus for five consecutive days, and were allowed for 120 seconds to explore in a random order. The animals which found the escape box were kept in the box for 15 seconds to consolidate learning [19]. The escape latencies and learning curves acquired from these five days were assessed, and animals were distributed into four groups with the stratified random sampling method, depending on these data. Barnes' Maze was used to provide inter-group homogenisation and was not evaluated as an experimental result measurement.

Morris Water Maze (MWM)

The Morris Water Maze (MWM) tests were conducted in a water tank with a diameter of 130 cm and a depth of 60 cm. The tank was virtually divided into four equal quadrants, with a 10 cm diameter escape platform placed 1 cm below the water level in one quadrant. The platform was designed in black, and the water in the tank was coloured in black with biosuitable paint to make the platform invisible, and the ambient light was kept dim. The water temperature was maintained at 24±2 °C. The platform's position did not change during the entire experiment, and cues were placed outside the water tank at distances and heights visible to the rats to help them learn the location of the hidden platform.

The MWM test was used to assess spatial (hippocampus-dependent) learning and memory. The rats were trained for 5 days to locate the platform during a maximum of 120-second swimming trials. During each trial, each rat was placed into the tank from one of four directions (north, south, east, or west) in a random order to prevent them from memorising a fixed escape route and to encourage them to use spatial cues. If a rat had failed to find the platform within 120 seconds during the first trial, it was gently guided to the platform with the assistance of a stick. After each time rats found the platform, the rats were allowed to stay on the platform for 30 seconds to reinforce learning through spatial cues.

The performance of rats over the 5 days was assessed [20] based on their platform-finding time (escape latency) in the MWM. The experiments were recorded using a DMK 22AUC03 monochrome camera. At the end of the experiment, the recorded movement data were analysed using an image recording and processing program developed by faculty members and students at our university's Faculty of Engineering and Natural Sciences.

Passive Avoidance (PA)

The Passive Avoidance Test was conducted in an apparatus which includes one light and one dark box. While the light box has a steel grating which does not apply electrical shock, the dark box was organised to apply mild electrical shock from its grating. These two boxes were separated by a door which is controlled by the researcher.

During T1 (training session), the rat was placed into the light box and allowed for 60 seconds to explore, and at the 60th second door is opened. As the rat moves into the dark box; door is immediately closed, and a 0.5 mA electrical shock is given for 3 seconds. Following the electrical shock, the session is finalised.

In T2 (1 hour, short-term memory) and T3 (24 hours, medium-long term memory) sessions, the latencies of rats to pass to the dark box from the light box are evaluated. Cut-off value for latencies is determined as 300 seconds [21].

Biochemical Analysis

Molecular Analyses: Phosphorylated Tau protein in the brain tissue of sacrificed rats was determined using Western blot, while inflammation parameters TNFα and IL-1β were assessed using an ELISA kit. BDNF, CREB, and AKT gene expression levels were determined through RT-PCR. Thus, neuroinflammation parameters and cognitive markers were analysed together. Below, the details of the experiment are provided.

RT-qPCR

RT-qPCR experiments were conducted to determine the gene expression levels of BDNF, CREB, and AKT. GAPDH is used as the housekeeping gene. RNA isolation was performed using an RNA isolation kit (Promega, USA) according to the manufacturer's instructions. RNA concentration and purity were assessed by measuring absorbance at 260 and 280 nm, and RNA integrity was further evaluated by agarose gel electrophoresis. Complementary DNA (cDNA) was synthesised from equal amounts of RNA using a cDNA synthesis kit (Promega, USA).

Quantitative PCR reactions were performed using the Applied Biosystems StepOnePlus Real-Time PCR system, with equal amounts of cDNA loaded for all samples. Melt curve analysis was conducted to verify amplification specificity. Relative gene expression levels were calculated using the ΔΔCt method, and fold changes were determined using the 2^−ΔΔCt formula. For statistical evaluation, ΔCt values were used. All experimental procedures were conducted at the KUYAM laboratory.

ELISA Experiments for measuring TNFα and IL-1β levels used immunoassay kits following the solid-phase sandwich ELISA (Enzyme-Linked Immuno-Sorbent Assay) principle. The kit procedure was followed, and measurements were taken at the relevant absorbance using a ThermoScan brand plate reader. All necessary equipment was available at the KUYAM laboratory.

Western Blotting Experiments were conducted to determine TAU protein levels. Tissue samples were separated using Trizol for total protein extraction. The proteins were kept at -80°C until the Western blot procedure. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay method. For the Western blot, the Bio-Rad Mini Protean system (Bio-Rad, USA) was used. Protein samples were separated using a 12% SDS-PAGE gel. Afterwards, they were transferred to a polyvinylidene fluoride (PVDF) membrane and incubated in a blocking buffer. Following this, the samples were incubated overnight at 4°C with the relevant antibodies. After washing steps, the samples were incubated with HRP-

conjugated secondary antibodies, and following further washes, the membrane was transferred to a film cassette. Chemiluminescent detection was performed using an ECL kit. The chemiluminescent images were obtained using the Bio-Rad ChemiDoc Image system (Bio-Rad, USA). Band intensities were evaluated densitometrically. All necessary equipment was available at the KUYAM laboratory.

Histological Assessment

The brain tissues were immediately placed in a 4% paraformaldehyde solution. The tissues were then embedded in paraffin blocks, from which 4-micron-thick sections were taken. To assess neuronal damage, the CA1 hippocampal region was stained with Hematoxylin & Eosin and Cresyl Violet.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). Sample size planning was guided by previous literature on the streptozotocin (STZ) model and anticipated effect sizes, with allowance for expected attrition during the experimental period. Accordingly, an a priori power analysis was conducted before study initiation, assuming an α level of 0.05 and 80% statistical power.

Normality of data distribution was assessed using the Shapiro–Wilk test. Parametric data are presented as mean ± standard error of the mean (SEM) and were analysed using one-way analysis of variance (ANOVA), followed by Tukey or Games–Howell post hoc tests as appropriate based on variance homogeneity. Non-parametric data are expressed as median with interquartile range (IQR) and were analysed using the Kruskal–Wallis test; when a significant overall effect was detected, post hoc pairwise comparisons were performed using the Mann–Whitney U test.

Performance in the MWM across training days was analysed using two-way repeated-measures ANOVA, with group as the between-subject factor and time as the within-subject factor. Bonferroni correction was applied for multiple comparisons. Statistical significance was set at $p < 0.05$ for all analyses. Statistical significance markings were arranged according to figure layout to avoid visual complexity; accordingly, the reference group may differ between figures since some comparisons were done between different groups.

3. Results

Body weight

The percentage change in body weight was calculated using measurements from Day 1 and just before the euthanasia. A one-way ANOVA revealed a significant difference among the groups ($F(3, 23) = 46.88$, $p < 0.001$). Post hoc Games–Howell analysis showed that both diabetic groups (STZ and STZR) exhibited a marked decrease in body weight compared with the control (C) group ($p = 0.006$ and $p = 0.003$, respectively) and the Retatrutide-treated (R) group ($p = 0.008$ and $p = 0.004$, respectively), with no significant difference between STZ and STZR ($p > 0.05$). Mean ± SEM percentage changes in body weight were C: +5.08 ± 0.49,

STZ: −17.71 ± 3.22, STZR: −17.32 ± 3.10, and R: +3.98 ± 0.40. These findings indicate that Retatrutide did not prevent diabetes-induced weight loss, as STZ and STZR showed comparable reductions; by contrast, Retatrutide alone (R) maintained body weight similar to controls. (Figure 1).

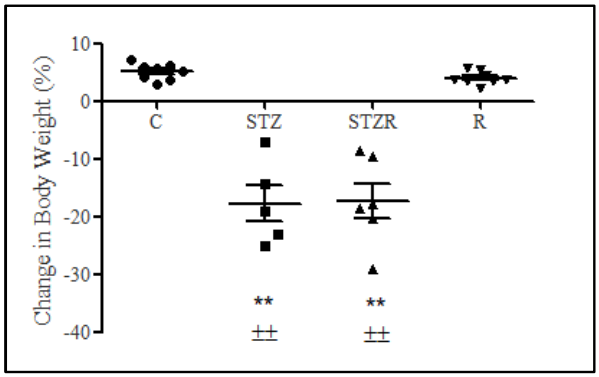

Figure 1. Percentage change in body weight across experimental groups. Data are presented as mean ± SEM (C: n = 8; STZ: n = 5; STZR: n = 6; R: n = 8). Statistical analysis was performed using one-way ANOVA followed by the Games–Howell post hoc test. Symbols indicate significant differences compared with the C (**p < 0.01), R(++p < 0.01). C: Control; STZ: Streptozotocin; STZR: STZ + Retatrutide; R: Retatrutide.

Blood glucose

Blood glucose levels were measured on Days 1 and 21 following diabetes induction to assess hyperglycaemia development and the effect of Retatrutide treatment. On Day 1, significant differences in blood glucose levels were observed among the experimental groups (Kruskal–Wallis H = 26.02, p < 0.001). Both STZ and STZR groups showed markedly elevated glucose levels compared with controls, indicating successful diabetes induction. No difference was detected between STZ and STZR groups, consistent with the absence of treatment at this time point. The R group exhibited slightly higher glucose levels than group C, although this difference was modest, and significantly lower levels than both diabetic groups (Fig. 2A).

By Day 21, group differences remained significant (Kruskal–Wallis H = 19.86, p < 0.001). Blood glucose levels were significantly higher in the STZ and STZR groups compared to group C, confirming persistent hyperglycaemia. Notably, STZR animals showed a significant reduction in glucose levels relative to the STZ group, indicating the anti-hyperglycaemic effect of Retatrutide. No difference was observed between groups C and R. Overall, Retatrutide treatment significantly attenuated STZ-induced hyperglycaemia but did not fully normalise blood glucose levels (Fig. 2B).

A. **B.**

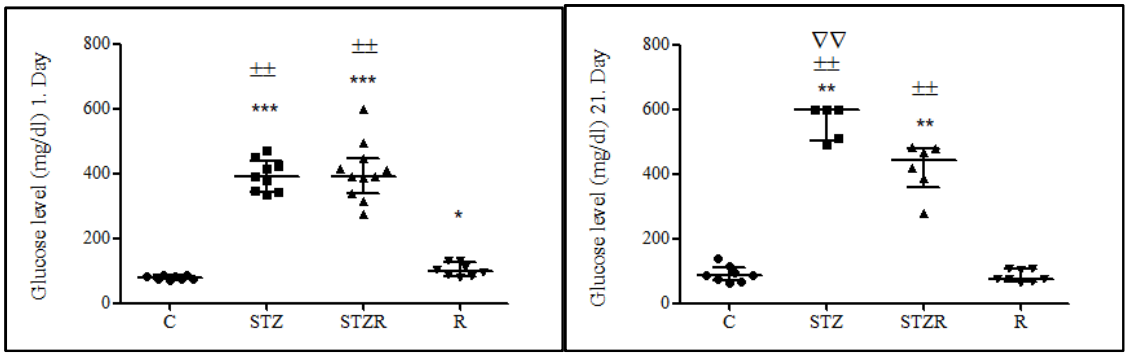

Figure 2. Blood glucose levels on Days 1 and 21 following diabetes induction. (A) Day 1 and (B) Day 21 measurements. Data are presented as median with interquartile range (IQR) (Day 1: C n = 8, STZ n = 9, STZR n = 11, R n = 8; Day 21: C n = 7, STZ n = 5, STZR n = 6, R n = 8). Statistical analysis was performed using the Kruskal–Wallis test followed by Mann–Whitney U tests for pairwise comparisons. Symbols indicate significant differences compared with the C (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), R (±$p < 0.05$, ±±$p < 0.01$), or STZ group (∇∇$p < 0.01$). C: Control; STZ: Streptozotocin; STZR: STZ + Retatrutide; R: Retatrutide.

Behavioural tests

Morris Water Maze

In the Morris Water Maze (MWM) test, which evaluates escape latencies, significant differences were observed among the experimental groups. Two-way repeated-measures ANOVA demonstrated a significant effect of training day, indicating progressive learning across days (main effect of day: $F(4,96) = 101.34$, $p < 0.001$). In addition, a significant main effect of group was detected, independent of training days (main effect of group: $F(3,24) = 11.13$, $p < 0.001$), with post hoc comparisons revealing that the STZ group exhibited significantly longer escape latencies than the other groups throughout the experiment (Figure 3).

Evaluation of learning curves revealed significant group-dependent differences in learning trajectories across training days (Pillai's Trace, $F(12,69) = 2.796$, $p = 0.004$). While learning curves differed significantly across groups, the STZ group displayed a distinct and impaired learning profile, whereas the STZR and R groups exhibited learning trajectories comparable to controls. To further characterise these differences, MWM performance was analysed on individual training days. One-way ANOVA revealed significant group differences on Day 1 ($F(3,24) = 7.90$, $p = 0.001$) and Day 5 ($F(3,24) = 15.31$, $p < 0.001$), with the STZ group consistently showing longer escape latencies compared with all other groups.

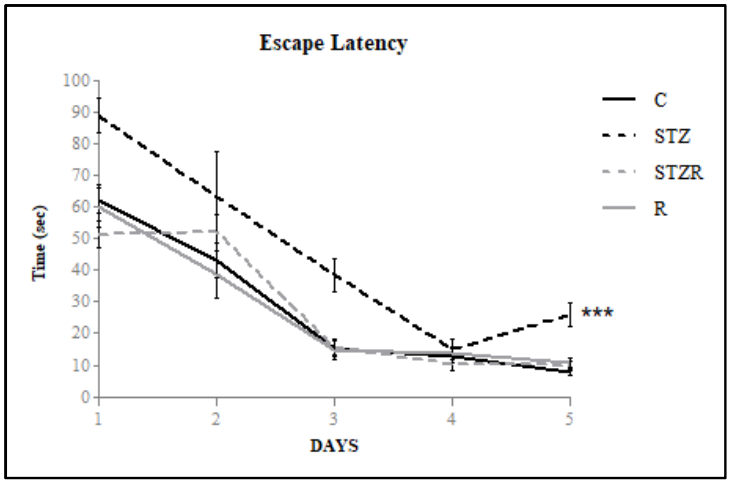

Figure 3. Escape latency in the Morris Water Maze (MWM) across training days. Data are presented as mean ± SEM (C: n = 8; STZ: n = 5; STZR: n = 6; R: n = 8). Statistical analyses were performed using two-way repeated-measures ANOVA followed by Bonferroni post hoc tests. Symbols indicate statistically significant differences in overall learning curves between the STZ group and the C, STZR, and R groups across training days (***$p < 0.001$). C: Control; STZ: Streptozotocin; STZR: STZ + Retatrutide; R: Retatrutide.

Passive Avoidance

In the PA test, the transition latency of moving into the dark box from the light box is measured and analysed for memory assessment. Accordingly, the data did not show a normal distribution, so non-parametric statistical methods were applied.

A significant difference was determined in the T2 (1 hour) time point between groups ($H(3) = 9.21$, $p = 0.027$; Figure 4A). In pairwise comparisons, it was shown that group STZ had shorter transition latencies compared to C ($U = 0.00$, $p = 0.002$) and R ($U = 7.00$, $p = 0.045$). Significant differences were not determined between the other groups ($p > 0.05$).

In the T3 time point (24 hours), no significant statistical differences were found ($H(3) = 1.68$, $p = 0.642$; Figure 4B).

These findings indicate group-dependent differences in short-term memory performance.

A. **B.**

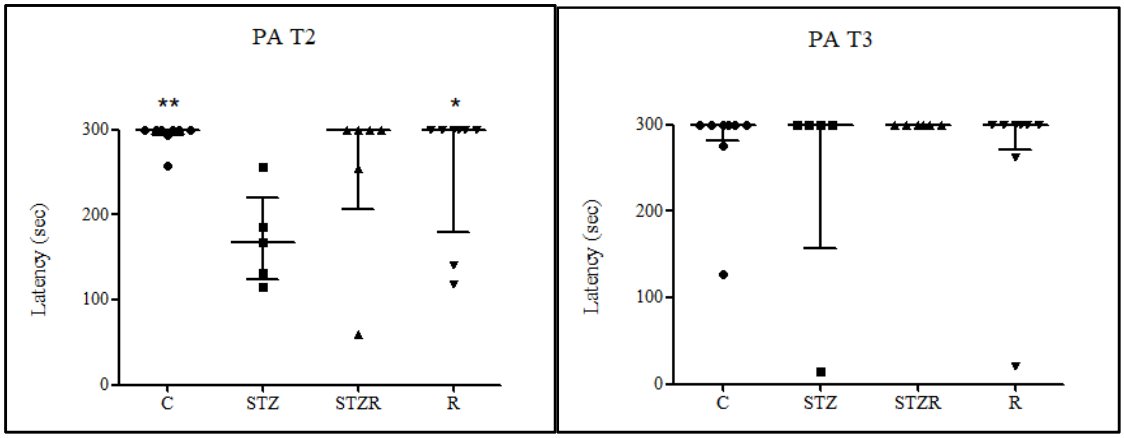

Figure 4. Latency to enter the dark compartment in the passive avoidance (PA) test. (A) Latency measured at T2 (1-h test, short-term memory) and (B) latency measured at T3 (24-h test, long-term memory). Data are presented as median with interquartile range (IQR) (C: n = 8; STZ: n = 5; STZR: n = 6; R: n = 8). Statistical analyses were performed using the Kruskal–Wallis test with Mann–Whitney U tests for pairwise comparisons. Symbols indicate significant differences compared with the STZ group (*$p < 0.05$, **$p < 0.01$). C: Control; STZ: Streptozotocin; STZR: STZ + Retatrutide; R: Retatrutide.

Biochemical Analysis

Molecular Analyses

RT-qPCR Experiments

A statistical difference was shown between groups in terms of comparing BDNF expression (Kruskal–Wallis H = 9.724, p = 0.021). The pairwise comparisons were done by the Mann-Whitney U test following the Kruskal-Wallis test, showed that BDNF levels in group R were significantly higher compared to groups C (p = 0.015), STZ (p = 0.003) and STZR(p = 0.043). On the contrary, no significant differences were determined between groups C, STZ and STZR ($p > 0.05$; Figure 5A).

Similarly, CREB expression differed significantly among groups (Kruskal–Wallis H = 10.573, p = 0.014). Post hoc analyses demonstrated that CREB expression was significantly increased in the R group compared with the C (p = 0.028) and STZ (p = 0.001) groups, whereas the difference between the R and STZR groups did not reach statistical significance ($p > 0.05$; Figure 5B).

In contrast, no significant difference was observed in AKT mRNA expression among the experimental groups (Kruskal–Wallis H = 5.947, p = 0.114; Figure 5C).

A. **B.** **C.**

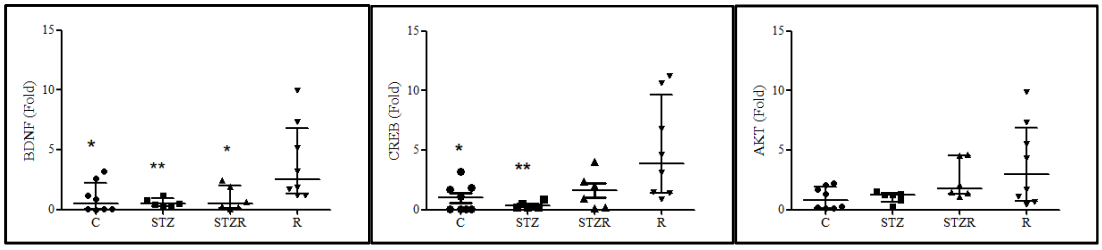

Figure 5. Relative gene expression levels of (A) BDNF, (B) CREB, and (C) AKT determined by RT-qPCR. Data are presented as median with interquartile range (IQR) (C: n = 8; STZ: n = 6; STZR: n = 6; R: n = 8). Statistical analyses were performed using the Kruskal–Wallis test with Mann–Whitney U tests for pairwise comparisons. Symbols indicate significant differences compared with the R group (*p < 0.05, **p < 0.01). C: Control; STZ: Streptozotocin; STZR: STZ + Retatrutide; R: Retatrutide.

ELISA Experiments

One-way ANOVA revealed significant differences in IL-1β levels among the experimental groups (F = 6.160, p = 0.004).

Post hoc Tukey HSD analysis demonstrated that IL-1β concentrations were significantly higher in the STZ group compared to the group C (p = 0.002) and the group R (p = 0.030), whereas no significant difference was observed between the groups STZ and STZR (p = 0.137) (Figure 6A).

TNF-α levels were significantly different among the experimental groups (F = 3.622, p = 0.027). Post hoc Tukey HSD analysis indicated that TNF-α concentrations were significantly higher in the group STZ compared to the group STZR (p = 0.030), whereas no statistically significant difference was detected between the groups STZ and C (p = 0.062) or between the groups STZ and R (p = 0.089) (Figure 6B).

A. **B.**

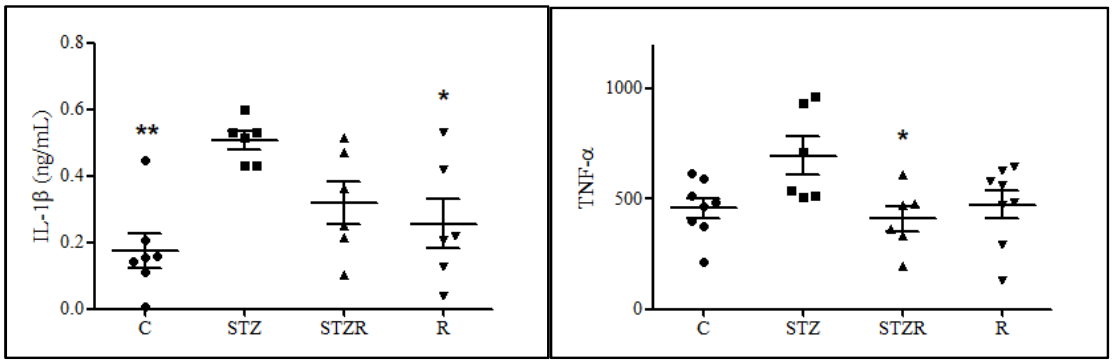

Figure 6. Brain cytokine levels measured by ELISA. (A) IL-1β and (B) TNF-α concentrations in brain tissue homogenates of experimental groups. Data are presented as mean ± SEM (IL-1β: C: n = 7; STZ: n = 6; STZR: n = 6; R: n = 6; TNF-α: C: n = 8; STZ: n =

6; STZR: n = 6; R: n = 8). Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc test. Symbols indicate statistically significant differences relative to the group STZ within each panel (*p < 0.05, **p < 0.01). C: Control; STZ: Streptozotocin; STZR: STZ + Retatrutide; R: Retatrutide.

Western Blot Experiments

Western blot analysis revealed a significant difference in Tau/β-actin levels among the experimental groups (Kruskal–Wallis, H = 8.07, p = 0.045). Post hoc Mann–Whitney U comparisons showed that Tau expression in the group C was significantly higher than in the group STZ (U = 3.0, p = 0.007), as illustrated in the quantitative analysis (Fig. 7A). No significant differences were detected between the group C and either the group STZR or R. Although Tau levels in the groups STZR and R exhibited a mild tendency upwards relative to the group STZ, these differences did not reach statistical significance. This pattern is consistent with both the scatter plot data and the representative blot images (Fig. 7B). Collectively, these results indicate that STZ-induced diabetes was associated with reduced total Tau protein levels, whereas Retatrutide treatment resulted in a partial, but statistically non-significant, restoration toward control values.

A. **B.**

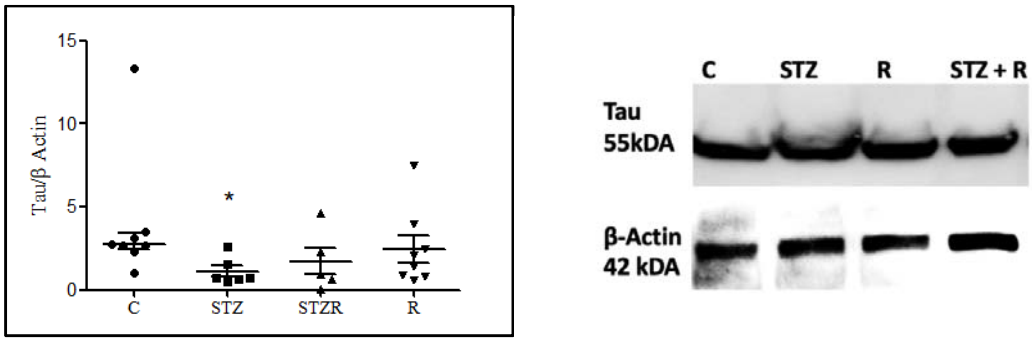

Figure 7. Brain Tau protein expression levels were determined by Western blot analysis. (A) Quantitative analysis of Tau/β-Actin ratios and (B) representative blot images of Tau (55 kDa) and β-Actin (42 kDa) in brain tissue homogenates of experimental groups. Data are presented as median with interquartile range (IQR) (C: n = 8; STZ: n = 6; STZR: n = 5; R: n = 8). Statistical analyses were performed using the Kruskal–Wallis test with Mann–Whitney U tests for pairwise comparisons. Symbols indicate statistically significant differences compared with the STZ group (*p < 0.05). C: Control; STZ: Streptozotocin; STZR: STZ + Retatrutide; R: Retatrutide.

Histological Assessment

Cortical Evaluation

Normal histological structure was observed in the brain samples from group C. In group STZ, neuron numbers seemed decreased and surrounded by wide vacuoles. There were dilatations in the perivascular zones. Neuron numbers in group STZR were observed to be higher compared to STZ. The number of vacuoles surrounding the neurons was decreased; however, perivascular dilatation was observed, similar in group STZR compared to STZ.

Hippocampal Evaluation

Normal histological structure was observed in group C.

In group STZ, the shrinkage in some pyramidal cells, and the pyknotic nuclei seen in CA1 and CA3 were shown.

Normal histological structure was observed in the CA1 region of groups STZR and RET. In the CA3 of group STZR pycnotic nuclei with some sporadic shrinkage in pyramidal cells, and disorganisation in the layered configuration of neurons. Neuronal disorganisation in the layered configuration in CA3 of group RET was observed with a similar appearance to group STZR.

Histopathological evaluation of the cerebral cortex and hippocampus revealed structural alterations in the STZ group compared to controls (Figure 8). In the cerebral cortex, the STZ group exhibited reduced neuronal density accompanied by prominent vacuolization and perivascular space enlargement. In contrast, cortical architecture in the group STZR showed partial preservation, with reduced vacuolization compared with the group STZ, while perivascular changes remained evident. The group R has displayed cortical morphology largely comparable to that of the group C.

In the hippocampus, control animals exhibited normal histological organisation in the CA1 and CA3 regions. The group STZ showed shrunken pyramidal neurons with pyknotic nuclei in both CA1 and CA3 regions. In the group STZR, normal histological appearance was observed in the CA1 region, whereas mild disorganisation of pyramidal neurons persisted in the CA3 region. Similarly, the group R has demonstrated preserved CA1 architecture, while neuronal arrangement in the CA3 region appeared comparable to that observed in the group STZR.

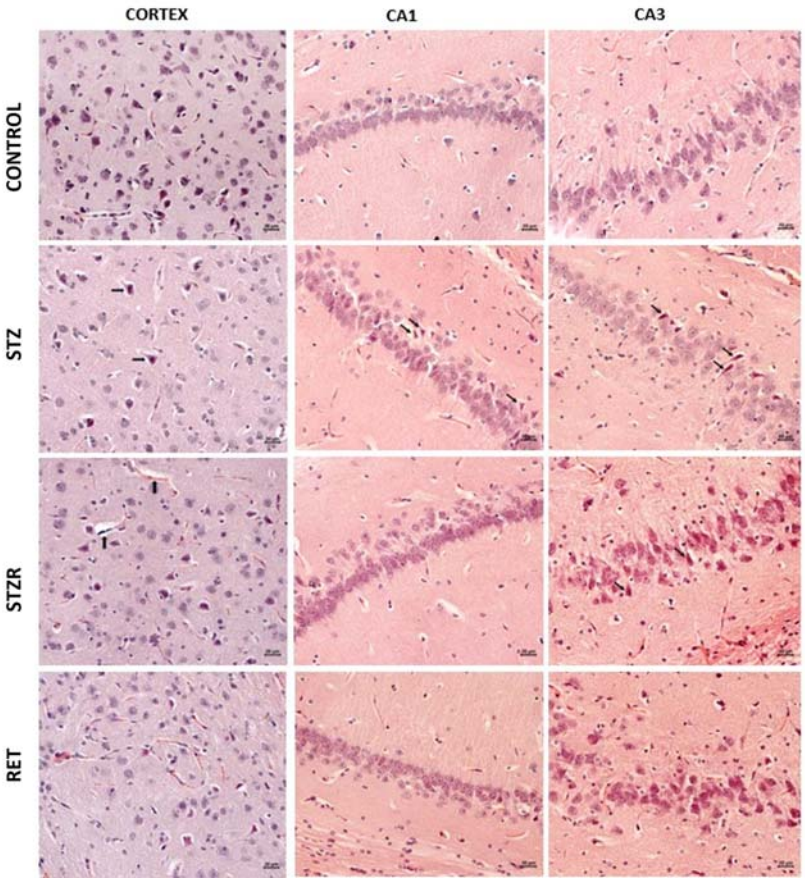

Figure 8. Representative histological images of the cerebral cortex and hippocampus following experimental treatments. Heamatoxylin and eosin (H&E)–stained sections show the cerebral cortex and hippocampal CA1 and CA3 regions in the control (C), streptozotocin (STZ), streptozotocin + retatrutide (STZR), and retatrutide (R) groups. Arrows indicate key histopathological features observed across groups: right arrows denote vacuolized neurons, upward arrows indicate perivascular space enlargement, and cross arrows indicate shrunken pyramidal neurons with pyknotic nuclei. C: Control; STZ: Streptozotocin; STZR: STZ + Retatrutide; R: Retatrutide.

4. DISCUSSION

In this study, the effects of Retatrutide on metabolic parameters and cognitive performance in diabetic and non-diabetic rats were evaluated from both physiological and behavioural perspectives. The findings demonstrated that streptozotocin (STZ)-induced diabetes was associated with marked metabolic disturbances and cognitive impairment, whereas Retatrutide treatment alleviated a substantial proportion of these alterations. As a novel GIP/GLP-1/Glucagon triple receptor agonist, Retatrutide has recently gained significant attention for its superior efficacy in metabolic disorders; however, its potential to counteract diabetes-associated cognitive decline represents an emerging frontier in neuropharmacology [22]. The insulin-deficient STZ model (T1DM-like) was selected to investigate diabetes-

induced neurocognitive impairment, focusing on mechanisms arising from hyperglycaemia and neuroinflammation rather than on a specific diabetes subtype [23,24].

The pronounced body weight loss and persistent hyperglycaemia observed in the STZ group are consistent with the severe insulin deficiency and catabolic state characteristic of this model. Retatrutide treatment did not prevent body weight loss, which may be explained by the dominant insulin deficiency and enhanced lipolysis and proteolysis masking potential effects on weight regulation. Although GLP-1/triple agonists show metabolic benefits [4,6], their effects on weight loss remain limited in insulin-deficient STZ models [25].

In contrast, blood glucose analyses revealed that Retatrutide significantly improved glycaemic control. While hyperglycaemia persisted throughout the experiment in the STZ group, a significant reduction in blood glucose levels was observed on Day 21 in the STZR group. The lack of complete glycaemic normalisation is consistent with the progressive β-cell destruction induced by STZ [17]. These findings indicate that Retatrutide may alleviate glycaemic burden even under severe diabetic conditions, although it does not fully restore normoglycaemia.

Behavioural assessments demonstrated that diabetes negatively affected cognitive function, whereas Retatrutide treatment mitigated these impairments. In the Morris Water Maze (MWM), spatial learning was markedly disrupted in the STZ group, as reflected by prolonged escape latencies, while the performance of the STZR and R groups remained comparable to that of controls. This pattern suggests that diabetes-related learning impairment can be attenuated by Retatrutide treatment. Importantly, Retatrutide did not enhance learning beyond control levels but rather preserved learning trajectories similar to those observed in non-diabetic animals.

Findings from the Passive Avoidance (PA) task supported the MWM results. At the T2 time point, representing short-term memory, transition latencies were significantly reduced in the STZ group, consistent with diabetes-associated short-term memory impairment. In contrast, preservation of transition latencies in the Retatrutide-treated groups supports a protective effect on learning and short-term memory performance.

At the T3 time point, reflecting longer-term memory, no statistically significant differences were detected among groups. Although latencies in the STZ group tended to be shorter, suggesting weakened memory retention, this pattern aligns with previous evidence indicating that STZ-induced cognitive deficits are more pronounced during early and short-term memory phases and may exhibit greater variability in long-term memory processes. Taken together, these findings indicate that diabetes selectively impairs short-term avoidance-related memory, which is partially preserved by Retatrutide treatment.

Overall, results from both the MWM and PA tests demonstrate that diabetes adversely affects learning and memory, while Retatrutide treatment significantly alleviates these deficits at the behavioural level. These findings are consistent with previous studies reporting potential cognitive benefits of GLP-1-related and multi-incretin agonists [7,26–31]. Triple-receptor agonism proposed to provide superior neuroprotection over mono-therapy, likely through synergistic GIP/GLP-1/Glucagon effects on synaptic plasticity [32].

Cognitive improvement cannot be fully attributed to metabolic recovery, as body weight dissociation tends to suggest direct central effects. Recent pharmacokinetic insights suggest that multi-incretin agonists may cross the blood-brain barrier and directly influence neuronal receptors and pathways, providing neuroprotection independent of peripheral glucose levels [33]. This interpretation is further supported when inflammatory responses, cellular survival pathways, and neuroanatomical integrity are considered collectively.

Suppression of neuroinflammation appears to be a key component underlying the cognitive effects of Retatrutide. The tendency toward reduced IL-1β and TNF-α levels in the STZR group, compared with the marked elevations observed in the STZ group, with TNF-α reduction reaching statistical significance, supports the role of neuroinflammation in diabetes-related cognitive impairment. IL-1β is known to disrupt long-term potentiation and synaptic transmission [34] while TNF-α alters postsynaptic AMPA receptor regulation [35], thereby impairing synaptic homeostasis. Previous studies reporting that GLP-1-based agents suppress microglial activation, inhibit NF-κB signalling, and reduce mitochondrial stress [36], further support the notion that Retatrutide may alleviate inflammatory burden, particularly through modulation of TNF-α. Recent research demonstrates that Retatrutide (a triple agonist) significantly suppresses microglial nitrite production, TNF-α release, and COX2 expression in LPS-activated microglia, outperforming single GLP-1R agonists like exendin-4 [37].

Cellular survival pathways closely linked to neuroinflammation also appear to be affected in the diabetic brain. Although no statistically significant differences were detected in AKT mRNA expression among groups, the downward trend observed in the STZ group may reflect impairment within the IRS-1/PI3K/AKT signalling axis, which has been associated with GSK-3β activation under diabetic conditions [38]. The modest upward trend in AKT mRNA expression in the STZR group may reflect partial preservation of transcriptional capacity; however, functional activation of AKT signalling is regulated primarily at the post-translational level and cannot be inferred without phosphorylation-based protein analyses.

When inflammation-related cellular dysfunction is considered, alterations in neurotrophic signalling pathways become more apparent. Although BDNF and CREB expression levels did not differ significantly in the STZ group, reduced median values suggest suppression of neurotrophic responses in the diabetic environment. This suppression appeared attenuated in the STZR group, while BDNF and CREB expressions were markedly increased in the R group. This response profile is consistent with evidence indicating that incretin-based agonists enhance BDNF transcription through CREB activation via the cAMP/PKA pathway [30,31,39]. Recent investigations confirm that multi-receptor agonism (tirzepatide) significantly upregulates the BDNF-TrkB axis through CREB activation, providing robust protection against neurodegeneration [40]. Partial restoration of the BDNF–CREB axis may therefore represent a subsidiary mechanism supporting behavioural cognitive recovery.

Consistent with these molecular findings, structural alterations related to neuronal integrity were confirmed by Western blot analysis. As detailed in the methodology, total Tau protein levels were assessed to evaluate cytoskeletal stability. The significant reduction in total Tau levels observed in the STZ group reflects compromised microtubule stability and extensive neuronal loss rather than pathological Tau hyperphosphorylation[41]. Since Tau is a critical microtubule-associated protein stabilising the neuronal cytoskeleton, its depletion indicates

severe cytoskeletal destabilisation and axonal degeneration in the diabetic brain [42]. In chronic diabetic state, total Tau depletion increasingly recognised as a potential biomarker associated with advanced neuronal loss and cytoskeletal collapse [25]. Although Tau levels were not fully restored to control baseline in Retatrutide-treated groups, the levels were notably higher compared to the STZ group, suggesting partial preservation of structural integrity. These molecular observations were corroborated by histopathological examination, which provided direct anatomical evidence of neuroprotection. The STZ group exhibited characteristic features of diabetic encephalopathy, including severe neuronal degeneration, pyknotic nuclei, cytoplasmic vacuolization, and disorganisation of the pyramidal cell layers in the hippocampus and cerebral cortex [43]. These morphological changes are often attributed to chronic hyperglycaemia-induced oxidative stress and neuroinflammation. Conversely, Retatrutide administration remarkably attenuated these degenerative features. The STZR group displayed a relatively preserved cytoarchitecture with reduced neuronal loss and fewer signs of vacuolization compared to untreated diabetic rats. This structural preservation implies that the anti-inflammatory actions of Retatrutide translate into tangible tissue-level protection, maintaining the neural circuits required for cognitive function.

Retatrutide's cognitive benefits may be interpreted to be reflecting multifaceted neuroprotection beyond glycaemic control: neuroinflammation suppression, BDNF–CREB preservation, and hippocampal cytoarchitecture maintenance. These convergent effects support the hypothesis that multi-incretin agonists may exert central benefits beyond metabolic regulation.

Nevertheless, the STZ model used in this study is characterised by severe insulin deficiency and may not fully reflect the complex pathophysiology of type 2 diabetes mellitus. Retatrutide effects were evaluated over a limited treatment period and within selected molecular pathways, and histopathological assessment was primarily qualitative. Therefore, these findings should be validated in models more closely resembling type 2 diabetes mellitus and extended through comprehensive molecular, functional, and clinical investigations.

5. CONCLUSION

This study investigated Retatrutide’s (a triple agonist of GIP/GLP-1/GCG) effects on cognitive performance and neuroinflammation in streptozotocin-induced diabetic rats in a structured manner, exploring behavioural, molecular and histological outcomes. This study is one of the first studies to suggest that triple agonists, especially Retatrutide, exhibit neuroprotective and anti-inflammatory effects in the diabetic brain. It was observed that the animals which received Retatrutide treatment in the diabetic state, had better performance on spatial learning, short-term memory, attenuated neuroinflammatory markers and preserved hippocampal-cortical structure compared to the untreated diabetic rats. These convergent changes suggest that Retatrutide’s effects on cognition may involve mechanisms extending beyond peripheral metabolic regulation, potentially including modulation of inflammatory and neurotrophic-related pathways. However, as the streptozotocin model primarily reflects severe insulin deficiency, the findings may not fully capture the complexity of type 2 diabetes–related cognitive dysfunction. Future studies should be conducted including models more representative of isolated type 2 diabetes, further molecular investigations and different

behavioural paradigms, in order to deepen the comprehension of Retatrutide's effects on cognitive performance.

6. CONFLICT OF INTEREST

The authors declare that they have no competing interests.

7. FUNDING

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8. ACKNOWLEDGEMENTS

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9. DECLARATION OF GENERATIVE-AI OR AI-ASSISTED TECHNOLOGIES USED IN THE STUDY

During the process of writing this article ChatGPT, OpenAI was used merely for grammatical corrections and refining the sentences for fluency. AI has not been involved in any processes of acquiring, creating, interpreting, visualising or expressing the data. All authors have revised the final version of the paper and verified the manuscript to ensure its accuracy and factuality. All authors take the full responsibility for the content of this publication

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