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Title: A New Dipeptide H-MGL Partially Ameliorates Memory Impairment in an STZ-Induced Alzheimer Model in Male Rats

Authors: Sarieh Ghasempour[1], Nader Maghsoudi[2], Homa Manaheji[1,3], Rasoul Ghasemi[1,3], Ali Jaafari Suha[1], Jalal Zaringhalam[1,3], *

1. *Physiology Department, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*

2. *Neurobiology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*

3. *Neurophysiology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*

*Corresponding Author: Jalal Zaringhalam, Physiology Department, Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail address: jzaringhalam@yahoo.com

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that is identified by the gradual decline in memory and cognitive function. It is classified by the deposition of Aβ plaques, the build-up of intracellular neurofibrillary tangle and neuron loss. Neurotrophic factors play critical role in the treatment of Alzheimer's disease. However, the utilization of such neurotrophins has encountered certain difficulties and side effects. Novel technological advancements prioritize innovative dipeptides usage, which offer fewer side effects. The present study endeavors to analyze the compound hexamethylenediamide bis-(N-monosuccinyl-glutamyl-lysine) (Lab name: H-MGL), a newly discovered neurotrophin mimetic dipeptide, with the aim of alleviating memory impairment in an intracerebroventricular single dose streptozotocin (STZ)-induced Alzheimer model in rats. We arranged 4 groups consist of sham, groups receiving STZ and STZ+H-MGL (1 and 2mg/kg). The H-MGL was administered consecutively for 14 days following STZ injection subsequently, the Morris Water Maze test was performed. The findings suggest that administration of STZ caused significantly increment in mean escape latency and mean traveled distance in acquisition days. H-MGL at a dosage of 1mg/kg failed to yield any notable improvement in rats when compared to STZ. By contrast, a dosage of 2mg/kg of H-MGL led to a significant decrease in the latency to first platform crossing and frequency of platform crossings. Consequently, the aforementioned findings have engendered the notion that H-MGL partially ameliorate cognitive impairment so it may hold promise for having low side effects to alleviate cognitive deficits in Alzheimer's disease, or potentially decreases the symptoms associated with its progression.

Key words: Alzheimer Disease, Dipeptide, H-MGL, Escape latency, Latency to first

Introduction

Alzheimer's disease (AD) is the most common form of dementia, accounting for over half of the cases (1). Symptoms of AD consists cognitive and emotional disturbances that worsen over time (2). Specific diets, physical inactivity, midlife hypertension, depression, obesity, smoking, and low educational achievements are some risk factors of AD (3). There are three main histopathological features associated with AD: extracellular senile plaques (SPs) especially amyloid beta formation, intracellular neurofibrillary tangles (NFTs) and degeneration of basal forebrain cholinergic neurons. The cause of SPs is the amyloid peptide, whereas the principal cause of NFTs is the accumulation of tau, a microtubule-associated protein (4). The most affected regions of the brain are the cerebral cortex, entorhinal area, hippocampus, ventral striatum and basal forebrain (5). Senile plaques are depositions of a 40-42 amino acid peptide, derived proteolytically from amyloid precursor protein and it seems the amyloid beta formation is the main cause of AD (4). Microglia and astrocytes are activated around these depositions and produce inflammatory cytokines that contribute to the pathogenesis of AD (4).

Therapeutic approaches regarding cognitive decline and memory dysfunction in AD have targeted wide range of mechanisms. For example, aducanumab was approved by FDA, clears amyloid plaques from brain tissue, though its effectiveness in improving cognitive deficits has not been proven (6). Another strategy is the application of neurotrophic factors in affected patients. A structurally related family of neurotrophins consisted of NGF (Nerve Growth Factor), BDNF (Brain derived Neurotrophic Factor), neurotrophin 3 and 4/5, which are secreted by microglia and neurons, play an important role in the growth and survival of neurons and improvement of cognition, learning, and memory (7, 8). NGF can improve neural cell injuries by enhancing the NGF secretion and suppressing oxidative stress (9). Intranasal administration of hNGF-6

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(engineered NGF) to AD produces neuroprotective effects (10) and enhances cognition (11). Moreover, gathered evidence showed that BDNF level is decreased during dementia and neurodegenerative diseases such as AD (12), and BDNF polymorphism increases the vulnerability of the hippocampus-frontal cortex to AD pathology and cognitive decline (13).

Neurotrophic factors and their precursors have diverse biological roles which are depending on their interactions with one of two receptors: tyrosine receptor kinase (Trk), and/or p75 neurotrophin receptor (p75NTR), a member of the tumor necrosis factor (TNF) receptor superfamily. Neurotrophins interacting with specific Trk receptors (NGF binding to TrkA, BDNF and NT4/5 binding to TrkB, and NT3 binding to TrkC) mediate survival and growth responses. Moreover, their interaction with p75NTR leads to the modulation of brain plasticity and apoptosis in the central nervous system(14). Although many advantages of neurotrophin factors have been reported, it has found only a limited application in clinical practice due to its poor stability in biological liquids and a number of disadvantages of nerve growth factors, including hyperalgesia, severe weight loss, and low blood-brain barrier permeability (15, 16).

Novel dipeptides and proteins are an influential resource in the creation of new and successful medicines (17). This new technology has been able to reduce to some extent the side effects caused by proteins such as neurotrophins. Dipeptides have advantages such as the ability to cross the blood-brain barrier, less toxicity, and higher bioavailability (16, 18). The active site of peptides and proteins that regulate and interact with the receptor molecule is composed of a specific number of amino acids, which is influenced by the balance between recognition accuracy and the speed at which the peptide receptor complex breaks apart. (18). Usually, an active site of regulatory peptides represents a peptide chain β turn. Four residues are involved in β turn which is in the middle of it. According to geometrical reasons, their side chains immerse most fully into the cavity of the

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receptor upon peptide-receptor interaction and therefore play a major role in the recognition of whole peptide by receptor. This is a theoretical basis for the dipeptide drug design (18).

Bis-(monosuccinyl-L-glutamyl-L-lysine) hexamethylenediamide(H-MGL), constructed on the basis of the β turn of its fourth loop, is neurotrophin mimetic with much smaller size. Because of expected mentioned criteria, it was selected for development as a potential neuroprotective drug. Given that dipeptides possess a capacity for imitating neurotrophic factors and conferring neuroprotective properties, it appears that they may hold considerable promise for ameliorating instances of inflammation, neurodegenerative ailments, and memory impairments. Research on these fronts, however, remains scarce. Therefore, in our study, we examined the effect of this dipeptide on spatial memory performance in Morris Water Mmaze (MWM) test in an STZ-induced Alzheimer model in rats.

Methods and Materials

Laboratory Animals

The animals were obtained from the Laboratory Animal Center at Shahid Beheshti University of Medical Sciences. Male Wistar rats weighing between 250 and 300 grams were accommodated in groups of two or three within Plexiglas enclosures. Throughout the course of the investigation, the animals were meticulously maintained within animal room wherein a consistent temperature of 25°C and relative humidity ranging from 60% to 70% were maintained. The light within the room was on from 8 a.m. until 8 p.m. The subjects were provided unrestricted access to water and food. All experimental procedures were granted approval by the ethics committee At the Shahid

Beheshti University of Medical Sciences (IR. SBMU.MSP.REC.1398.759). In compliance with the guidelines provided by the National Institutes of Health (NIH) on the Care and Use of Laboratory Animals, proper protocols were followed for the maintenance and welfare of laboratory animals during the study. The experimental subjects were randomly allocated to one of four groups: a sham group (which is surgery performed); a STZ group; a STZ + H-MGL 1mg/kg; a STZ+ H-MGL 2mg/kg; and an STZ + saline as STZ and H-MGL solution. Notably the findings of the saline group were omitted from the report, as it has been determined within our laboratory that saline did not produce any discernible impact on the rats. Each group consist of eight rats.

Chemicals

Streptozotocin (STZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hexamethylenediamide bis- (N-monosuccinyl- glutamyl-lysine) (Lab name: H-MGL) was designed and synthesized by Hamer Pharmaceutical Co. IR. Other reagents were also obtained from local commercial source.

Drug administration

STZ intracerebroventricular injection

The rats were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, injected intraperitoneally. To settle the animals after anesthesia, stereotaxic devices were utilized, and mouth and ear bars were embedded carefully and avoid heat loss (19). To uncover the bregma on the cranium, 2mm fine midline cut was made on the skull skin and the cranium was cleaned with 70% ethanol and tenderly shaved. For getting to the horizontal ventricles, burr gaps were made at 0.8 mm back to bregma, 1.5 mm lateral to midline and 3.5 mm underneath the surface of the skull

(Paxinos et al. 1980). A single dose of STZ (3 mg/kg) was injected bilaterally intracerebroventricularly right after surgery. STZ was dissolved down in sterile 0.9% saline and was utilized 2µl per infusion location.

Administration of H-MGL

H-MGL (1 or 2 mg/kg) was injected intraperitoneally for 14 successive days (Fig. 1), and the vehicle solution gotten the same volume of sterile 0.9% in solution group.

Behavioral assessment

Spatial learning and memory performance were evaluated by MWM test. A dark colored circular pool with 80 cm radius and 80 cm height was utilized for the MWM test. Water was filled to a depth of 35 cm that had been warmed to 21±2°C. The pool was separated into four virtual quadrants, and a stage (diameter: 10 cm) submerged 1.5 cm underneath the surface of the water in one of the quadrants served as a target place. This test was done through four successive days. On each of three acquisition test days, animals were released within the pool for 90 seconds and were given the opportunity to find the stage. Finding the platform, they were let to remain on it for 20 seconds and then returned to their home cage. For each trial, this handle was done. Every test day consisted of four trials, each from different release point. On day fourth, the probe test trial included picking up the stage and releasing the rats within the pool from the reverse quadrant to the target quadrant, letting them to swim for 60 seconds while their activity was recorded. Rats were assessed for sensorimotor performance after the probe test trial. This was fulfilled by setting the clearly visible stage covered by aluminum foil in a distinctive quadrant and conducting four trials from different release points. In acquisition days, mean escape latency and mean travel

distance and in probe day, duration spent in target and opposite of target zone, frequency platform crossing and latency to first platform crossing were analyzed. A computerized framework was utilized to prepare and analyze the recorded behaviors of the rats (Noldus Ethovision XT).

Experimental design

On the day zero, rats received intracerebroventricular STZ (3 ml/kg). A short time after surgery, H-MGL (1 and 2 mg/kg) were injected intraperitoneally for 14 consecutive days. On the day 14, the MWM test was started and conducted through four test days.

2-4 Statistical analysis

Data were analyzed by GraphPad Prism 6.07. Behavioral data were analyzed by two-way or one-way analysis of variance (ANOVA) followed by LSD post-hoc test. Values were reported as mean $\pm$ SEM and $p < 0.05$ was considered significant.

Results:

The effect of STZ and H-MGL on mean escape latency in MWM

In figure 2, mean escape latency was significantly different between the sham, STZ and STZ+H-MGL1 ($F_{(3,81)}$ =11.40 P<0.0001). For all three days of training, the mean escape latency was significantly greater in the STZ than the sham (P=0.03, P=0.001, and P=0.01, respectively). In addition, the mean escape latency was significantly greater in the STZ+H-MGL1 than the sham (P=0.02, P=0.0004, and P=0.001, respectively). H-MGL2 partially reduced escape latency on first

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day, however, on the second and third days, there is a significant difference between sham and STZ+H-MGL2 (P=0.006 and P=0.01, respectively) (Fig. 2).

The effect of STZ and H-MGL on mean travel distance in MWM

A significant difference in mean travel distance ($F_{(3,81)}$ =11.72 P<0. 0001) was also observed between sham and STZ for each test days (P=0.007, P=0.0001, and P=0.012, respectively) consistent with the results of mean escape latency. However, H-MGL could significantly reduce the increased travel distance in STZ group (P=0.008) on first test day. Again, travel distance on the second and third days was significantly higher than sham for STZ+H-MGL1 (P<0.01 and P<0.01, respectively) and STZ+H-MGL2 (P=0.005 and P=0.003, respectively) (Fig. 3).

The effect of STZ and H-MGL on memory retention

Figure 4 illustrates difference between groups receiving STZ and H-MGL on probe day. Based on the results, as indicated in Figure 4A, there is no significant difference between the sham compared with STZ and the H-MGL 1 and 2 in time spent in the target quadrant, though H-MGL2 shows a little nonsignificant elevation (Fig. 4A). However, as figure 4B illustrated, STZ significantly increased the time spent in opposite quadrant (P<0.05) and H-MGL2 could significantly reduce this increment compared to STZ (P=0.0005) (Fig. 4B).

The effect of STZ and H-MGL on frequency crossing and latency to first to platform in probe

As figure 5A demonstrated there is no significant difference between the sham compared with STZ and the H-MGL 1 and 2 in frequency of platform crossing in the target quadrant, though H-MGL2 shows a little nonsignificant elevation (Fig. 5A). However, as figure 5B illustrated, STZ significantly increased the latency to first crossing platform in the target quadrant (P=0.0002) and H-MGL2 could significantly reduce this increment compared to STZ (P=0.001) (Fig. 4B). H-MGL1 have shown no improving effect on the latency to first crossing platform and was significantly higher compared to sham (P=0.0003).

Figure 6 depicts a discernible heat map demonstrating a comparatively elevated degree of recall and superior memory with regard to locating the platform in sham and STZ+H-MGL2. The platform's location in the picture is denoted by the black circle. Each of the depicted images constitutes a layered depiction emanated from the experimental trial conducted by each respective group. The STZ predominantly navigate the incorrect zone, which is situated in opposition to the zone incorporating the platform's location. Moreover, this group exhibited a greater tendency towards thigmotaxis. In the context of the experiment, it was observed that the animals which were administered STZ in combination with H-MGL demonstrated a reduction in thigmotactic behavior. Additionally, these rats predominantly displayed swimming behavior in the target area (Z_1) and at the periphery of the platform location. The present behavior was perceptibly observed in the STZ+H-MGL2 subject group (Fig. 6).

The effect of STZ and H-MGL on somatosensory and visual activity

To make sure that STZ and H-MGL didn't harm animals' ability and motivation to see and move, we examined the latency to first of crossing platform (Fig 7A) and swim velocity (Fig. 7B) in visible test. The two-way ANOVA revealed no significant difference between groups for the latency to first of crossing platform (F (3,81) =1.082 p=3614) and for swim velocity (F (3,81) =1.017 p=0.389) and shows that treatment did not influence coordination of sensory-motor, inspiration, and vision of rats.

Discussion

In the present study, we have examined the possible protective role of H-MGL, a dipeptide, in a STZ-induced memory impairment as a model of AD. The induction of model was verified via a significant increase of the escape latency and distance traveled to find the hidden platform during the learning period of MWM task. Moreover, administration of STZ decreased the time spent in the target quadrant and the number of passing through the platform location, and increased the time spend in the opposite quadrant and the delay in reaching the platform location on probe day. Treatment with H-MGL ameliorated the STZ induced memory disturbances during acquisition and retrieval trials. However, H-MGL have demonstrated a degree of dose-dependency regarding its ameliorative effect on memory performance.

Consistent with our findings, prior investigations have demonstrated that the central administration of STZ induces cognitive dysfunction (20). The findings of our study demonstrated that memory impairment in acquisition phase was mitigated in a dosage-dependent manner through the administration of H-MGL. Lower dose (1mg/kg) of H-MGL resulted in similar outcomes to those observed in the AD model animals. This suggests that the dosage may not be optimal. In contrast to our findings, Zarzhetskii et al. reported that the administration of a dipeptide at a dosage of 1 mg/kg exhibited potential efficacy in the treatment of

brain injury subsequent to resuscitation (21). In this way, as we used a higher dosage of the dipeptide (2 mg/kg), we observed that the group receiving 2 mg/kg H-MGL demonstrated improved cognitive function by efficiently locating the invisible platform similar to the sham group. These outcomes suggest that the higher dosage of H-MGL dipeptide may be efficacious in alleviating cognitive deficits in AD-like conditions. However, Povarnina et al. have asserted that the utilization of dipeptide at a dosage of 2 mg/kg did not yield any discernible alterations in the acquisition test (22). It appears that identifying the optimal therapeutic dose while minimizing adverse effects of drug may be achieved through strategies aimed at determining the bioavailability of medications. Such measures can potentially mitigate patient discomfort and enhance treatment outcomes.

Neurotrophins have been demonstrated to be implicated in the regulation of microglial activity, which serves as a primary source of neurotrophin secretion and is susceptible to alterations in AD (23). In this regard, evidence has shown that BDNF has a potential role in hippocampus-dependent memory function, Tyler et al. have revealed that rats subjected to MWM testing exhibited a greater expression of mRNA encoding BDNF, with a difference of approximately 50%, than their counterparts who underwent the same assessment (24). In a study conducted in 2017, involving the CCI model, the observed decrease in levels of glutamate and BDNF was found to be associated with a significant decline in spatial memory and learning. (25). In addition, many researchers reported the direct relation between apoptosis process and AD (26, 27). Hasegava et al. declared neurotrophins has an important role in Preventing the advance of apoptosis (28). However, several unfavorable effects have been reported regarding application of neurotrophic factors. Hence, as dipeptide molecules potentially activates neurotrophic factor receptors, they can be used as a favorable substitute.

Although dipeptides have shown promising therapeutic potential and the fact that dipeptides could mimic neurotrophic factors and their neuroprotective effects in the treatment of neurodegenerative disease with limited side effects but a limited number of investigations have explored the therapeutic properties of the aforementioned substances (29). In addition, it has some limitation such as unwanted toxicity and how

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administration because of size and structure and it needs more studies. In accordance, Ostrovskaya et al. have demonstrated a neuroprotective effect of dipeptide in an in-vitro study (30). In this regard, other studies have indicated that dipeptides exhibit neuroprotective properties in various pathological conditions, including traumatic brain injury and hemorrhagic stroke models (31, 32). Povarnina et al. conducted a study that examined the efficacy of a dipeptide as a treatment for Parkinson's disease. The study indicated that treatment with a novel dipeptide known as Gk2 reduced haloperidol catalepsy (33). Belnik et al. illustrated the potential of dipeptide Noopept to ameliorate scopolamine-induced spatial memory deficits (34). Chang et al. demonstrated that the utilization of IF bioactive dipeptide resulted in a significant elevation of BDNF levels, accompanied by an amelioration of the memory impairment observed in AD (35). According to Piec et al., the muramyl dipeptide demonstrated a reduction in Aβ accumulation in AD (36). It appears that identifying the optimal therapeutic dose while minimizing adverse drug effects may be achieved through strategies aimed at determining the bioavailability of medications. Such measures can potentially mitigate patient discomfort and enhance treatment outcomes.

As previously stated, the occurrence of memory deficits in AD have been associated with reduced levels of BDNF at the synaptic cleft. In particular, the peptide Aβ has been shown to induce a reduction in the transcription of the neurotrophic factor BDNF, through its detrimental effect on the phosphorylation of CREB (37). There is no evidence showing if H-MGL dipeptide is able to perform as the same cascade or not. Neurotrophins such as BDNF and NGF are widely recognized for their therapeutic potential in the management of neurodegenerative disorders. However, their clinical application is often overshadowed by a number of adverse effects, including hyperalgesia, severe weight loss and impermeability of the blood-brain barrier (BBB)(16). Peptide drugs have clear advantages over non-peptide drugs, specially low toxicity and greater permeability(16). Regulatory peptides and proteins represent a vital resource for designing novel and efficacious pharmaceutical agents. (3). The binding sites of regulatory peptides and proteins engage in interactions with receptors that are comprised of a restricted quantity of amino acids, conferring a critical role in the discernment and precision of receptor recognition. Currently, a multitude of

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investigations are exploring the properties of dipeptides across various fields of study, such as neurology and diabetes. (38).

Conclusion

In summary, the utilization of H-MGL as an analog of neurotrophins offers several notable advantages, including its favorable bioavailability, potent activity, non-toxic effects, minimal adverse reactions, and capacity to cross the blood-brain barrier. Consequently, it may serve as a promising therapeutic alternative in the future or act as a viable intervention to attenuate the advancement of neurodegenerative disorders. Given the dose-dependent nature of the obtained findings, further investigation involving various dosages is deemed imperative for the purpose of assessing its therapeutic prospects.

Acknowledgment

Conflict of interest

The authors of this manuscript declare that they do not have any conflicts of interest to disclose.

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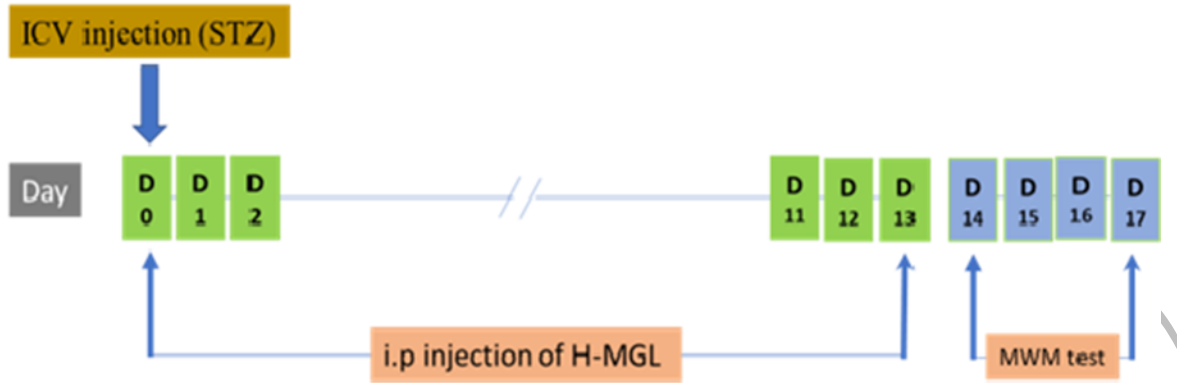
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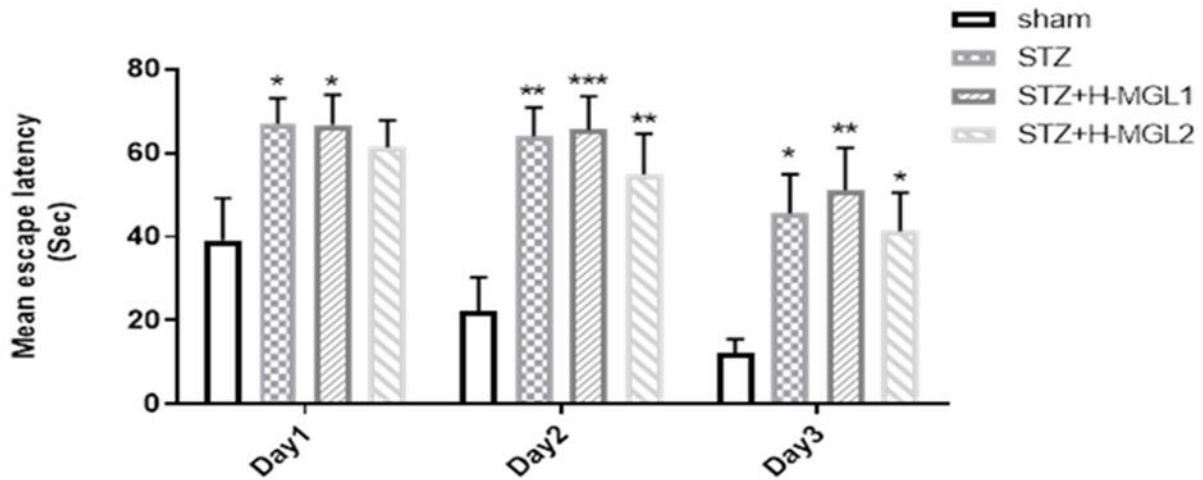
Figure 1. Experimental procedures diagram

Figure 2. H-MGL attenuated increased mean escape latency in MWM during training days following STZ injection. All values are represented as mean ± SEM (n=8-10). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, comparison between sham and other groups.
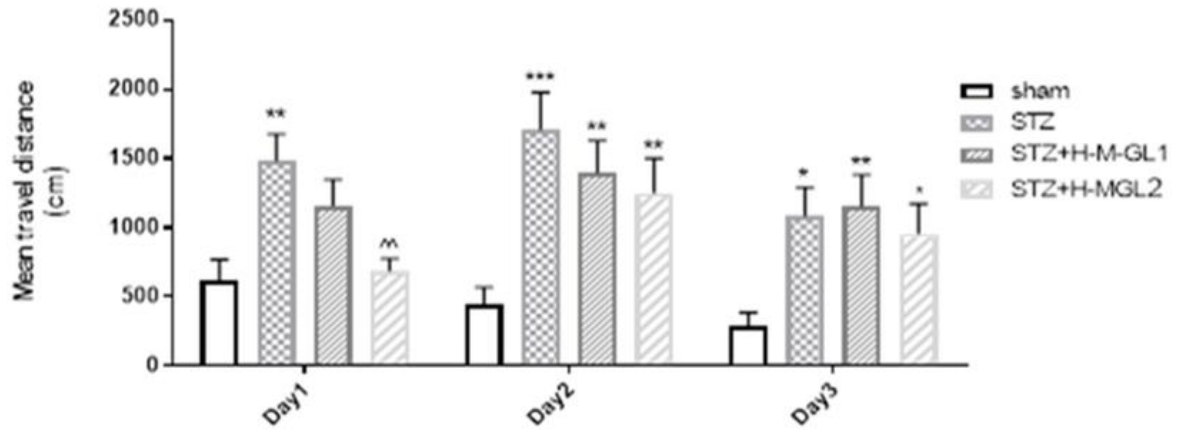
Figure 3. H-MGL decreased increased mean travel distance in MWM during training days following STZ injection. All values are represented as mean ± SEM (n=8-10). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, comparison between sham and other groups. ^^ $p < 0.01$ comparison between STZ and other groups.
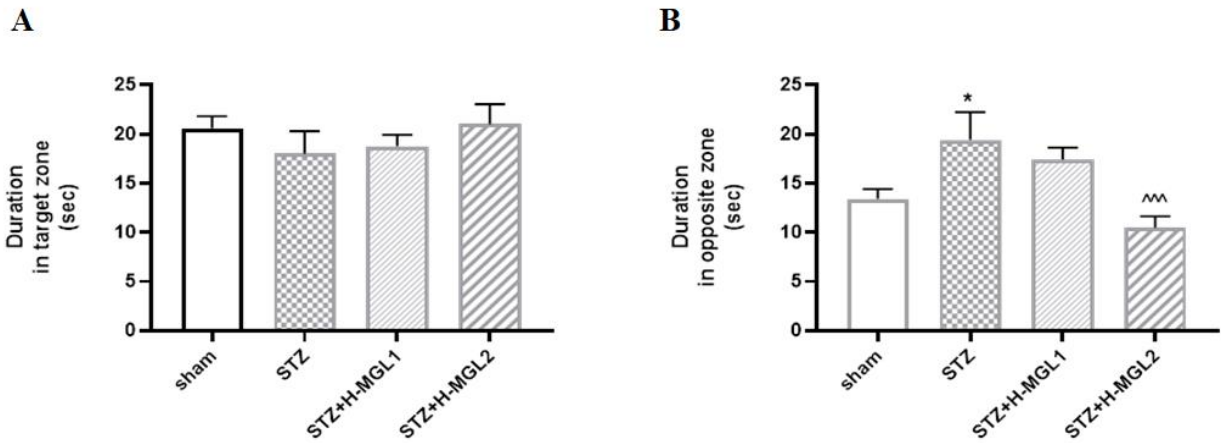
A

B

Figure 4. H-MGL restored some factors level in probe day following STZ injection. (A) the time spent in the target zone and (B) the time spent in the opposite zone. All values are represented as mean ± SEM (n=8-10). * $p < 0.05$, comparison between sham and STZ group. ^^^ $p < 0.001$ comparison between STZ and other groups.
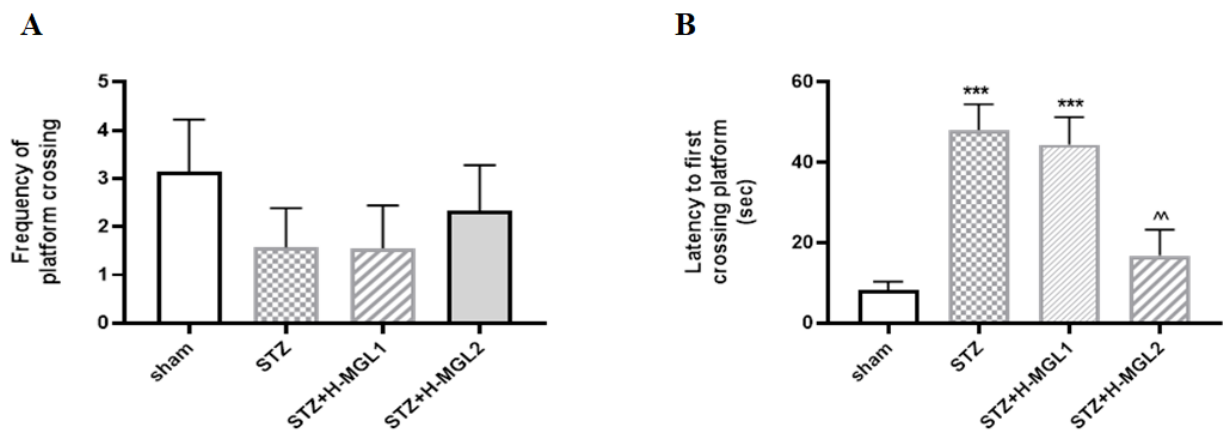
19

A

B

Figure 5. H-MGL restored some factors level in probe day following STZ injection., (A) the platform crossing frequency and (B) the mean latency to the first cross of the platform. All values are represented as mean ± SEM (n=8-10). *** $p < 0.001$, comparison between sham and STZ group. ^^ $p < 0.01$ comparison between STZ and other groups.
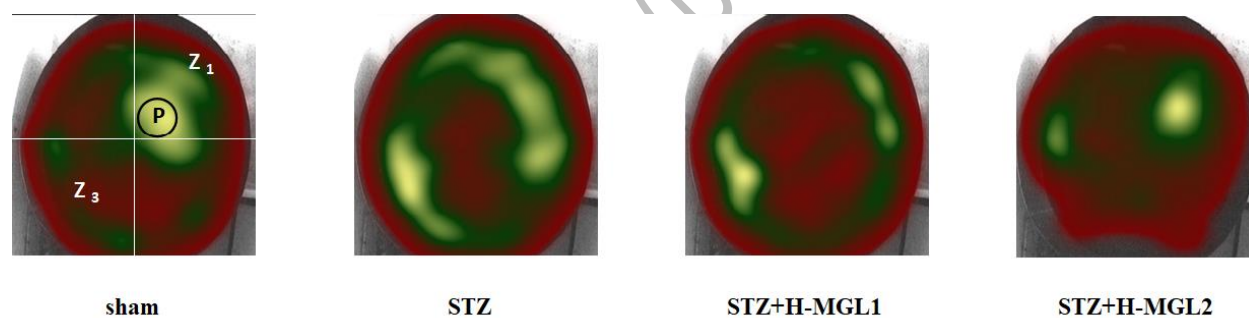

sham   STZ   STZ+H-MGL1   STZ+H-MGL2

Figure 6. The performance of animals treated with STZ and H-MGL on the probe day as heatmap visualization. Z_1: target zone. Z_3: opposite of target zone. P: platform
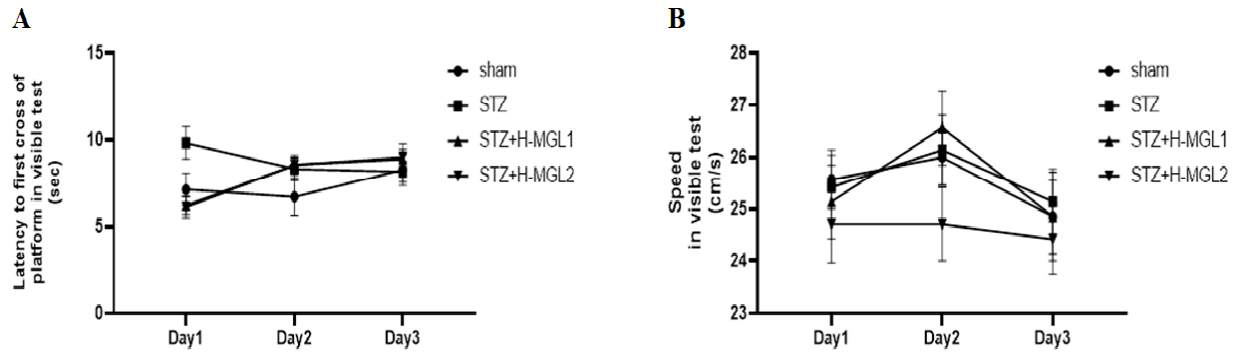
Figure 7. STZ and H-MGL couldn't effect on mean escape latency (A) and mean swimming speed (B) in visible test. All values are represented as mean ± SEM (n=8-10).