Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the author's institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/authorsrights

Author's Personal Copy

Life Sciences 188 (2017) 172–185

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Streptozotocin-induced hippocampal astrogliosis and insulin signaling malfunction as experimental scales for subclinical sporadic Alzheimer model

Farzaneh Rostami[a], Mohammad Javan[b], Ali Moghimi[d], Aliakbar Haddad-Mashadrizeh[a,c], Masoud Fereidoni[d,*]

[a] Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
[b] Department of Physiology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[c] Cell and Molecular Research group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran
[d] Rayan Center for Neuroscience and Behavior, Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran

ARTICLE INFO

Chemical compounds studied in this article:
Streptozotocin (PubChem CID: 29327)

Keywords:
Alzheimer's disease
Non-transgenic sAD model
Insulin resistance
Streptozotocin
Rodent
Pre-clinical stages

ABSTRACT

Aims: Insulin signaling malfunction has recently been suggested as a preliminary event involved in the etiology of Sporadic Alzheimer's disease (SAD). In order to develop insulin resistance-related SAD model, rats were treated with streptozotocin, intracerebroventricularly (icv-STZ). Nevertheless, given the lack of knowledge regarding sub-clinical stages of SAD, the current challenging issue is establishing a practical pre-clinical SAD model. Despite some proposed mechanisms, such as insulin malfunction, neuroinflammation, and gliosis, icv-STZ mechanism of action is not fully understood yet and Streptozotocin-induced rat model of Alzheimer has still major shortcomings.

Main methods: Using three STZ doses (0.5, 1, and 3 mg/kg) and three testing time (short-term, medium-term and long-term), we sought the best dose of STZ in order to mimic the characteristic feature of sAD in rats. So, we conducted a series of fifteen-week follow-up cognitive and non-cognitive studies. Besides, IR, tau and ChAT mRNA levels were measured, along with histological analysis of astrocyte, dark neuron numbers, and pyramidal layer thickness, in order to compare the effects of different doses of icv-STZ.

Key findings: STZ 3 mg/kg caused cognitive and insulin signaling disturbance from the very first testing-time. STZ1-injected animals, however, showed an augmented hippocampal astrocyte numbers in a short time; they, also, were diagnosed with disturbed insulin signaling in medium-term post icv-STZ-injection. Moreover, behavioral, molecular and histological impairments induced by 0.5 mg/kg icv-STZ were slowly progressing in comparison to high doses of STZ.

Significance: STZ1 and 0.5 mg/kg-treated animals are, respectively, suggested as a suitable experimental model of MCI, and sub-clinical stage.

1. Introduction

A growing body of evidence has indicated that sAD is multifactorial, with age being an important risk factor. Main neuropathological hallmarks identified in Alzheimer's brains are beta-amyloid deposits, hyperphosphorylated tau proteins, and astrogliosis [1]. Recently, dysregulated brain insulin signaling has been reported to play a pivotal role in AD pathology. Additionally, several studies have indicated that insulin malfunction induces beta amyloid accumulation and tau pathology in AD brain [2]. Thus, Alzheimer's disease has been argued to be a type III diabetes [3,4]. Given the specific nature of sAD, there is no clinical evidence on its early stage, and this makes making an appropriate model more difficult. Thus, a convenient model for the initiation phase of sAD could be the beginning step to explore the mechanisms involved in the pathology of Alzheimer disease. Several animal models have been established among which icv-STZ model (icv-STZ administration in rat) has been foregrounded as a hopefully suitable model to investigate the mechanism of sAD progression and explore the neuroprotective potential of some components up to now [5,6]. Importantly, this model could mimic some features of sporadic AD, such as disturbance in some aspects of learning and memory, beta-amyloid accumulation, hyperphosphorylation of tau protein (microtubule-associated protein tau, MAPT) and dysregulation of insulin-related gene expression (for instance, insulin, insulin receptor (IR), choline acetyltransferase (ChAT), and MAPT). In this regard, due to some data proposing insulin resistance as a possible mechanism of ip-STZ, and

* Corresponding author.
E-mail address: fereidoni@um.ac.ir (M. Fereidoni).

http://dx.doi.org/10.1016/j.lfs.2017.08.025
Received 13 March 2017; Received in revised form 23 August 2017; Accepted 26 August 2017
Available online 01 September 2017
0024-3205/ © 2017 Elsevier Inc. All rights reserved.

Author's Personal Copy

regarding the presence of insulin receptor in rat brain, it has been suggested that icv-STZ injection could induce central insulin resistance, but this mechanism has not been supported by the experimental data yet [6]. Also, Oxidative stress and subsequent neuroinflammation leading to the neuronal death is the second proposed icv-STZ mechanism [7,8]. With respect to the aforementioned evidence, sAD is a chronic and slowly progressing disease. However, STZ doses used in the present model did not turn out to, desirably, imitate the development and progression of AD pathologic features. Furthermore, almost all icv-STZ–based studies have ultimately presented first month post-icv-STZ injection as a suitable testing time. These limitations encouraged us to seek an appropriate dose and testing time of icv-STZ model in order to observe more similar progression in cognitive decline, along with the molecular and neuropathological characteristic features of sAD of slowly progressive nature. Up to now, researches have not comprehensively investigated up to 15-week post-injection behavioral, molecular and neuropathological effects of different doses of icv-STZ. In this regard, the effects of three distinct doses of icv-STZ (0.5, 1 and 3 mg/kg) were compared on these features in three post-injection time points, including short-term (3–5-weeks), medium-term (9–11 weeks) and long-term (13–15 weeks) effects.

2. Materials and methods

2.1. Animals and surgical procedures

Male Wistar rats (3 months old, weighing 280-300 g) were used throughout this study. Rats were housed in groups of 3–4 and kept under standard conditions of temperature (22 °C ± 2) and humidity (40–50%). A 12-h light/dark cycle was provided and animals had ad libitum access to food and water. For icv injection, the animals were anesthetized by i.p. injection of ketamine-xylazine mixture (100 mg/kg–10 mg/kg, respectively) and fixed in a stereotaxic apparatus. A single injection of STZ (0.5, 1 and 3 mg/kg) or saline was applied into the right lateral ventricle (0.8 mm posterior to bregma, 1.5 mm lateral to the sagittal suture, and 3.6 mm beneath the brain surface). Injection volume was 6 μl. The experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978), and were approved by the Ferdowsi University of Mashhad ethical review process.

2.1.1. Experimental setup

Group 1. Control: Rats undergone stereotaxic surgery and received icv-STZ vehicle (saline).

Group 2. STZ 0.5: Rats received STZ (0.5 mg/kg) on the 1st day.

Group 3. STZ1: Rats received icv-STZ (1 mg/kg) on the 1st day.

Group 4. STZ 3: Rats received icv-STZ (3 mg/kg) on the 1st day.

The animals were subjected to a battery of behavioral tests during 3–5, 9–11, and 13–15 weeks following icv injection. The behavioral studies included Accelerating Rotarod, Y Maze, and novel object recognition task (NORT) accompanying with Morris water maze. All observations were performed between 10.00 am and 16.00 pm. Equipment was cleaned with 70% ethanol between each test to eliminate olfactory cues. After every test battery, ten animals from each group were sacrificed by decapitation (Fig. 1), and their brains were removed quickly. Five brains were kept in 4% paraformaldehyde for 24 h and were then paraffin-embedded for histological studies on the hippocampus (hematoxylin and eosin and Congo-red staining). The hippocampi of the other brains were dissected and stored in − 70 °C for Real-time RT-PCR quantification of insulin receptor (IR), tau and choline acetyltransferase (ChAT).

2.2. Behavioral tests

2.2.1. Accelerating rotarod

In the first part of this study, short-term, medium-term and the long-term effects of icv-STZ injection on the rotarod performance were measured using an accelerating rotarod device (4–40 rpm Rota-Rod 7650; Ugo Basile, Comerio, Italy) in order to prove that the animal motor coordination was not affected by intraventricular drug injection and icv-STZ, per se, had no toxic effect on the animal's motor balance over the time; this was performed once and the mean delay time of the first falling off a rotating rod, known as fall latency, was calculated in each group. Animals were individually placed on a rod facing opposite to the direction of rotation. Each animal was placed on the rod rotating at a constant speed of 4 rpm and trained to remain balanced during training. One session of five trials (180 s each) was given, as described elsewhere, during the retention phase [9]. Inter-trial interval was 2 min. The speed of rotation was increased from 4 to 40 rpm during a 120-s period. Latency to fall was recorded automatically. The mean riding time was measured for each animal. In case of longer than 180 s riding duration, the rat was removed from the rod and the fall latency was assumed to be 180 s.

2.2.2. Open-field

This maze was used to measure the rat's exploratory and anxiety-like behavior. Animals with less anxiety, spend more time in the central area of the arena. The open-field arena was constructed using white Plexiglas with 75 cm length, 75 cm width and 36 cm high. The rats were individually placed in the center of the arena and the number of rearing, as well as the percentages of time spent in the central area of the maze was recorded during 5 min, as previously described with the time duration modification [10].

2.2.3. Y-maze

Y-maze alternation task was performed, as described above, to investigate icv-STZ effect on animals' working memory [11]. Briefly, animal was placed in the center of the maze to explore the arena freely, and spontaneous alternation behaviors were counted over an 8-min session. The function of working memory of animals was quantified as the percentage of alternation: (the number of alternations) / (total arm entries − 2) ∗ 100. Also, the number of arm entries was recorded as a locomotor activity of rat during an 8-min session of Y-maze alternation task. The apparatus consisted of three equal arms, measuring 40 × 15 × 30 cm oriented at 120° angles from each other. The number of different arm visits was considered as an index of exploratory activity of the animal in the Y-maze.

2.2.4. Novel object recognition test

This test has been designed to assess animal's recognition memory, as well as working memory, based on the rodent's preference to explore new objects. Nevertheless, animal's reference memory is not involved in this test. These advantages make this test useful to evaluate mild cognitive impairment in pre-clinical studies [12]. Here, based on rat's unconditioned preference for novelty, short-term, medium-term and the long-term effect of different doses of icv-STZ were evaluated, as reported previously with some modifications [13]. Briefly, the novel object recognition test was performed in three phases of habituation, familiarization (acquisition phase) and test (retention phase). During habituation phase, the animals were individually placed in the apparatus (an empty open-field arena measuring 75 × 75 × 36 cm) to explore the arena for five consecutive days, 15 min per day. On the test day, two identical objects (colored striped plastic bottles filled with NaCl) were placed in the opposite corners of the arena during the acquisition phase; the animal was released in the arena back to the objects and allowed to explore the objects freely for 3 min. Exploration was defined as pointing the head within 2 cm of the objects, sniffing, licking or biting the objects. Duration of time each rat explored each object was

Author's Personal Copy

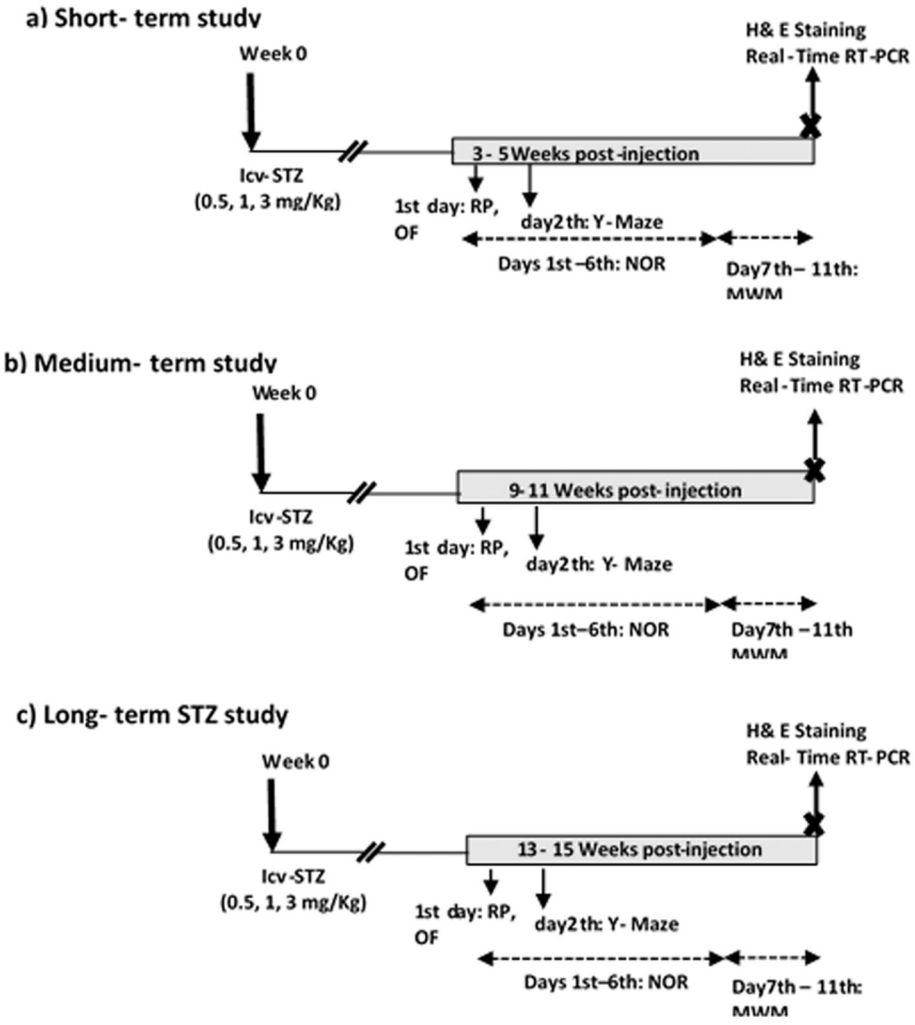

Fig. 1. Schematic representation of the experimental time scale. An Alzheimer-like behavior was induced by icv injection of STZ in 3-month-old rats. Animals were randomly assigned into four groups: (1) control group: rats underwent stereotaxic surgery and received STZ vehicle (saline); (2) icv-STZ 0.5 mg/kg: rats received STZ (0.5 mg/kg) into the right ventricle; (3) icv-STZ 1 mg/kg: injected intracerebroventricularly with 1 mg/kg dose of STZ; (4) icv-STZ 3 mg/kg: 3-month-old rats receiving 3 mg/kg icv-STZ. Thirty animals were randomly categorized into each group and, then, each group was divided into three subdivisions, each including 10 rats. Three different subdivisions of every group performed behavioral tests in a different testing time, i.e. short-term, medium-term and long-term studies. Animals were sacrificed after each battery of the behavioral experiment. The hippocampi of five rats were dissected and stored in − 70 °C for Real-Time RT-PCR and the other dissected brains were fixed (4% paraformaldehyde for 24 h) and reserved for hematoxylin-eosin (H & E) staining. As shown in Schematic figure, Rotarod performance (RP), and Open field (OF) were carried out on the first day, and Y-maze (alternation) test was conducted on the second day. Novel object recognition task (NOR) consisted of a five-day habituation phase, followed by the object recognition task, separated into two phases of acquisition and retention by itself, performed on the sixth day. From the 7th to 11th days of each experimental block, Morris water Maze (MWM) performed. Training phase consisted of four consecutive days, each consisting of four trial sections, 90 s for each; this was followed by 60 s probe trials on the 5th day.

measured, as *exploration time*. Then, the animal was returned to its home cage. After 1-hour inter-trial interval, during the 3-min retention phase, rats were returned to the arena with two objects exactly placed at the same position of acquisition phase objects. One object was identical to the previously presented object (familiar), and a new (novel) object (different object shape with the same salience). Again, the exploration time was recorded in the retention trial. The animal's exploratory preference was calculated as the percentage of time spent exploring the novel object divided by the total time spent exploring both objects during the test phase.

2.2.5. MWM

Learning and memory of the animals were assessed in the Morris water maze, based on the previous reported protocol, with some modifications [14]. A black circular water maze (120 cm diameter and 50 cm height) was placed in a darkened room and filled with water at room temperature (25 ± 1 °C). During habituation, the animals were individually placed into the tank and allowed to swim freely for 30 s. One day after habituation a black colored platform with an 8 cm diameter was hidden 1–2 cm below the water surface. Tank water was colored with a black non-toxic dye to hide the platform. The platform was placed in one of the four imaginary quadrants. The location of the platform was constant during the experiment. In the training phase, each animal received one session of 4 trials per day for 4 consecutive days. There was a 2-minute interval between trails. The rat was released from one of the four start points and had a maximum time of 90 s to locate the hidden platform during each trail. If the animal couldn't find the platform, it was manually guided to the platform and remained there for 30 s. The latency time to find the hidden platform was recorded as *escape latency*. During the probe trial on day 5, the platform was removed and the animal was allowed to swim for 60 s and the percentage of time spent in the goal quadrant was recorded.

2.3. Quantitative real-time RT-PCR

After each behavioral test block, using quantitative real-time polymerase chain reaction, insulin receptor (IR), tau and choline acetyltransferase (ChAT) mRNA levels were assessed in the hippocampi extracts. Insulin-1 gene expression data was omitted from the analysis due to low concentration and relative high threshold cycle (Ct.30).

2.3.1. Total RNA isolation and cDNA synthesis

Briefly, the left rat hippocampus was homogenized, and RNA was extracted using an RNX-Plus reagent (Cinnagen, Iran) according to the manufacturer's instructions. In the next step, total RNAs went through DNase-I digestion to get rid of DNA residues. The RNA quality was assessed via the 28S/18S ratios around 2 in 1% agarose gel electrophoresis. Furthermore, using Nanodrop ND-2000 Spectrophotometer (Thermo scientific), only samples whose ratios of the A260/A230 were > 1.8 and A260/280 ratios > 1.9 were used for the gene expression analysis. Total RNA (11 μl) from each sample was reverse transcribed using random hexamer, oligo-dT primers, and by RevertAid RT Reverse Transcription Kit (Fermentas, Canada).

Author's Personal Copy

Table 1
Primers used for quantification of gene expression and parameters.

Abbreviation	Gene name	Primer direction	Sequence (5′–3′)	Amplicon size(bp)
Ins1	Insulin 1	F	ACCATCAGCAAGCAGGTCATTGT	212
		R	CGGGTCCTCCACTTCACGAC	
IR	Insulin receptor 1	F	AGAGGTGGGCAATGTGACAG	165
		R	ATGCGGTACCCAGTGAAGTG	
MAPT	Microtubule associated protein	F	CGGAGGCAGTGTGCAAATAGTC	94
		R	TCCTGGCTTGTGATGGATGTTC	
ChAT	Choline acetyl transferase	F	CCATGACTGACCACAAGGCT	167
		R	TGGCTCTTTGCACAGGTCTC	
18srRNA	Ribosomal 18S	F	GCCTCACTAAACCATCCAATCG	160
		R	AACCCGTTGAACCCCATTCG	

2.3.2. Primer design, standard curve analysis and real-time RT-PCR

The information related to primers is shown in Table 1. Using the NCBI site (https://www.ncbi.nlm.nih.gov/tools/primer-blast), specific pairs of primers were designed, and to confirm the gene specificity of the primer sequences, BLAST searches were performed, and the results were checked to ensure the absence of multi-locus matching at the particular primer sites. The cDNA was diluted 1:10 (for tau, 18s rRNA) or 1:1 (for IR and ChAT), and 2 µl were amplified by real-time PCR in 20 µl reaction mixture (2 µl primers (5 ρmol each of forward and reverse primers), 6 µl DEPC water and 10 µl SYBR Green master mix) using 2× Real-time PCR master mix (Amplicon) for 40 cycles in a Rotor-Gene Q-Qiagen equipment under the following thermal conditions: 10 min preheating and 40 cycles of 95 °C; denaturation for 10 s at 95 °C; annealing for 1 min at 64a°C; extension for 1 min at 72 °C. Primer sequences were as follows: for IR, forward, 5′-AGAGGTGGGCAATGTGACAG-3′ and reverse, 5′-ATGCGGTACCCAGTGAAGTG-3′; for tau (microtubule-associated protein tau (Mapt)), forward, 5′-CGGAGGCAGTGTGCAAATAGTC-3' and reverse, 5′-TCCTGGCTTGTGATGGATGTTC-3′; for ChAT, forward, 5′-CCATGACTGACCACAAGGCT-3′ and reverse, 5′-TGGCTCTTTGCACAGGTCTC-3′; for 18srRNA, forward, 5′-GCCTCACTAAACCATCCAATCG-3′ and reverse, 5′-AACCCGTTGAACCCCATTCG-3′; and for Ins1, forward, 5′-AGAGGTGGGCAATGTGACAG-3′ and reverse, 5′-ATGCGGTACCCAGTGAAGTG-3.′ Ribosomal 18S (18srRNA) was used, as the reference gene, for normalization during quantification. A denaturing curve was performed after amplification to ensure the presence of specific amplification products. All reactions were run in triplicate. Standard curve for each amplification product was obtained from 10-fold dilutions of cDNA. The efficiency of amplification was also checked, and relative quantitation of gene expression was carried out using the procedure proposed by Schmittgen and Livak (ΔΔCt). Data is shown as the percentage of changes in comparison to the control group. The efficiency range of amplifications of tested genes was 96–110% (Table 1).

2.4. Brain sectioning, hematoxylin-eosin (H & E) and Congo-red staining

After each battery of behavioral tests, five rats were randomly selected out of each group for light anesthesia with CO2 and decapitation at 5, 11 and 15 weeks after icv-STZ injection. Rat hippocampi were removed quickly and stored in 4% paraformaldehyde solution for 24 h. Then, samples were subjected to graded ethanol and xylene transparency and finally embedded in paraffin. Continuous coronal slices (5 µm thickness) were prepared according to the Paxinos atlas of the rat brain for hippocampus (from-3.2 to − 4.2 mm bregma). Four sections were placed on each slide. The first two slides out of every six slides were selected for hematoxylin-eosin staining (seven pairs slides overall). Physical dissector method was used for quantitative analysis of dark neurons, astrocytes and pyramidal layer thickness after photography under the optical microscope (200 ×; Olympus, Tokyo, Japan). Briefly, in each pair of sections, the first section of the first slide, was assumed as the reference to which the first section of second one was compared. Counting was carried out using an unbiased frame and physical dissector rules. The cells observed in both sections were not considered in the total number [15]. Dark cells are degenerating darkly-stained neurons which are characterized as shrunken neurons with dark cytoplasm and pyknotic nuclei. Due to the invisible cytoplasm and the processes of astrocytes in this staining method, their identification was based on their specific unclear nucleoli and rounded or oval-shaped nuclei, which were larger than other glial nuclei [16].

To test whether icv-STZ model induces beta-amyloid deposition in the rat brain long-term after icv-STZ3 treatment, some rats were sacrificed at 24 and 36-week post-injection and their paraffin embedded brain sections were stained by Congo-red alone and with hematoxylin (Fig. 2) as described before [17,18]. Briefly, after deparaffinization and rehydration, brain sections were incubated 20 min in alkaline saturated NaCl solution and, then, in 0.5% alkaline Congo red solution for 30 min, followed by dehydration with 95% and 100% ethanol. For Congo-red/hematoxylin staining, after Congo-red step, a stain development step was performed involving dipping in absolute ethanol twice, followed by rinsing in water. Then, sections were incubated in Mayer's hematoxylin for 1 min and, then, thoroughly rinsed with water.

2.5. Statistical analysis

Data was expressed as mean ± SEM. The results of RP, OF, Y-maze, the percentage of the time in the correct quadrant in MWM, along with molecular and histological results were subjected to Repeated measure Two-Way ANOVA followed by Bonferroni post-test. Also, One-Way ANOVA followed by Tukey post-test was performed to compare the effect of icv-STZ injection on Latency(s) to platform in MWM and NORT exploratory preferences. Student's paired *t*-test was used to compare the effect of icv-STZ treatment on the time spent exploring the familiar versus the novel object using GraphPad Prism version 5.0, with significance determined at the $p < 0.05$.

3. Results

3.1. Although Icv-STZ did not alter balance and locomotor activity in rats, it induced the expression of anxiety-related behavior

3.1.1. Rotarod performance

Animal's performance in rotarod was not affected by icv-STZ injection in all groups (see Fig. 3a); there was also no significant difference in terms of *time* and *time* × *dose effect* ($F < 1$ *for all comparisons*).

3.1.2. Y-maze arm entries

During an 8-min session of Y-maze alternation task, the number of arm entries was recorded as a locomotor activity of rats. The effects of dose, time and time × dose were not statistically significant ($F < 1$ *for all cases*) (see Fig. 3b). All these results confirmed that icv-STZ injection had no destructive effect on the balance and exploratory behavior of animals at different testing times.

Author's Personal Copy

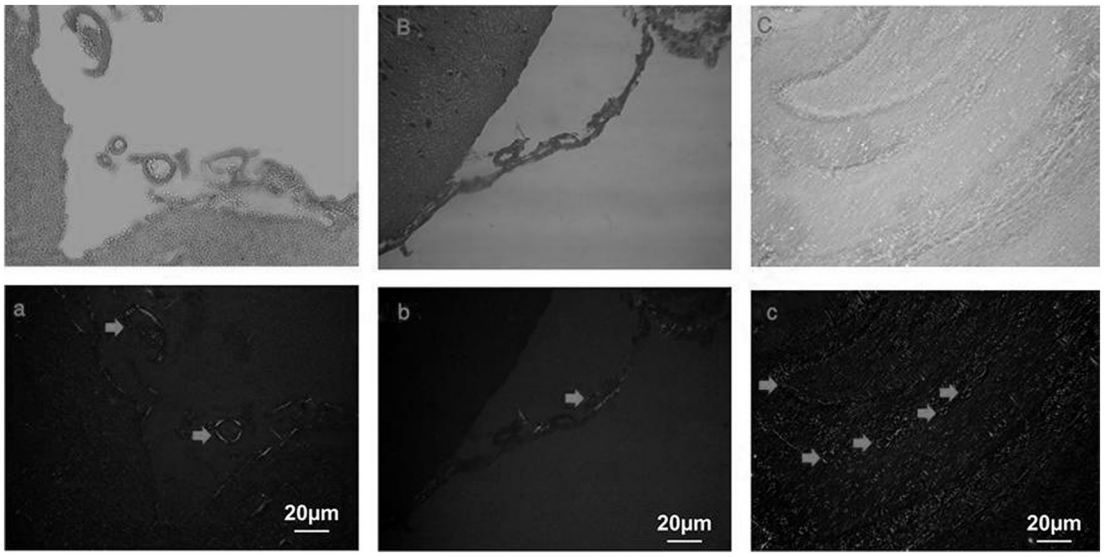

Fig. 2. Amyloid deposits in the meninges' blood vessels (A, B), and hippocampus (C) of rats 24 weeks (A, B) or 36 weeks (C) after 3 mg/kg icv-STZ injection. Figures with lowercase letter's labels represent auto-fluorescent signals of upper figures under cross-polarized light. STZ injected animals sacrificed 24 weeks or 36 weeks after icv-STZ injection, and after removal and fixation of their brain, paraffin-embedded brains were cut in 6 μm sections and beta-amyloids deposits stained with the colour of the Congo alone (A and C) and with hematoxylin (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.1.3. Open-field test

Exploratory behaviors of animals were assessed by measuring animals' rearing frequencies within a 5-min open-field test and no significant alteration in exploratory behaviors of animals was detected over time. Also, neither time nor time × STZ dose interaction were significant (*F < 1 for both effects of dose and time, in all groups*) (see Fig. 3c). The percent of the time each rat spent in the central area of open-field apparatus was also measured to compare the general anxiety-related behavior of animals. Unlike exploratory-related behaviors, although a strong dose effect was detected in terms of anxiety-related behavior of animals ($F_{dose\ (3,\ 36)} = 32.99$; $p_{dose} < 0.0001$), similar trend was achieved neither in time effect nor in interaction effect (*F < 1 for all comparisons*). Furthermore, both 1 and 3 mg/kg doses of icv-STZ could induce a constant reduction in the total duration of time

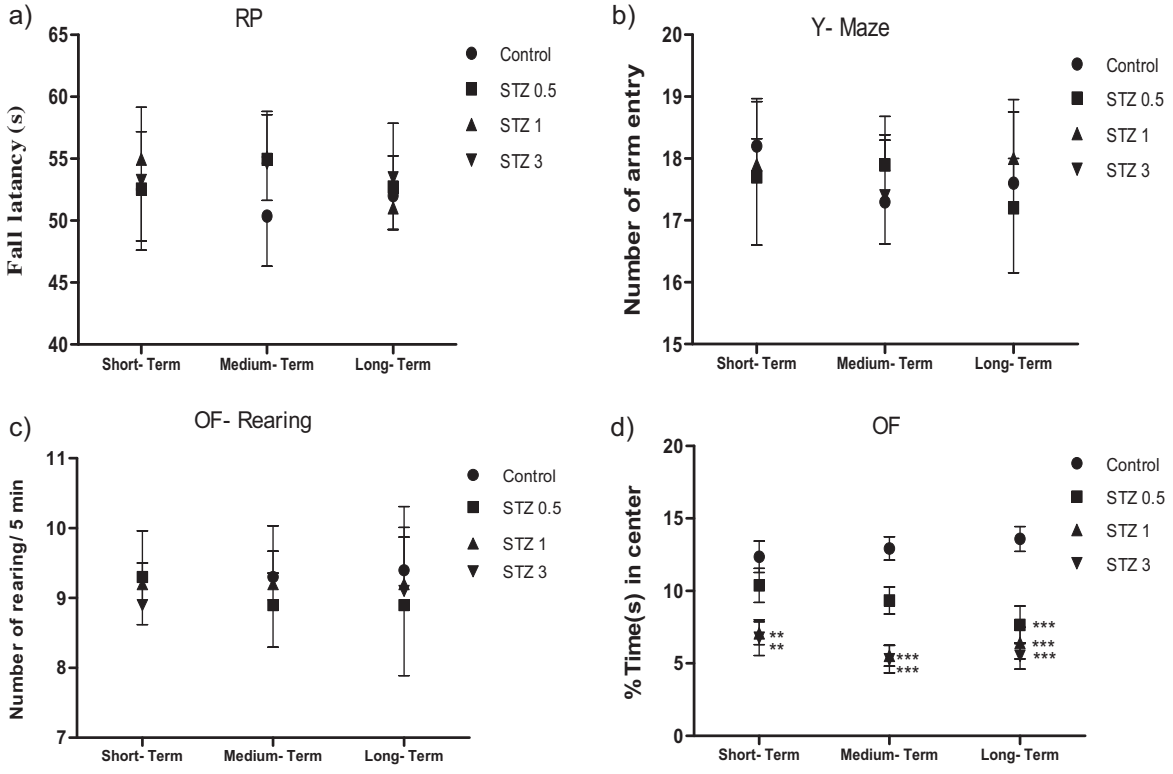

Fig. 3. Three-month follow-up of motor activity, coordination, exploratory and general anxiety behaviors of icv-STZ-injected animals. Motor balance and coordination of the rats were assessed in accelerating Rotarod (a). The overall number of Y-maze arm entries was recorded as an indicator of loco-motor activity (b). Rearing frequencies (c) and percent of times each animal spent in the central area of open field apparatus (d) were measured as indices of exploratory behavior and general anxiety, respectively. Icv-STZ injection did not affect motor balance skill (a) and could not impair animal tendency to explore environment at all testing times (b and c). However, a strong dose effect was detected on anxiety behavior of animals (d). Data are reported as mean ± SEM. **, $p < 0.01$ and ***, $p < 0.001$ vs. Control.

Author's Personal Copy

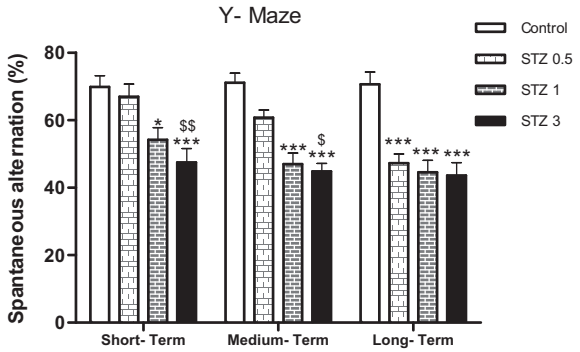

Fig. 4. Evaluation of animals' working memory at three different testing times using the Y-maze test. Streptozotocin induced progressive dose- and time-dependent reduction in the working memory ability of the animals in Y-maze, as shown by spontaneous alternation percentage. Data are reported as mean ± SEM. *, $p < 0.05$ and ***, $p < 0.001$ vs. Control rat. $, $p < 0.05$ and $$, $p < 0.01$ vs. STZ 0.5 mg/kg rat (n = 10).

each animal spent in the central area of the apparatus from the very beginning of the study (from 4 weeks onward); however, animals which took 0.5 mg/kg icv-STZ showed increased anxiety-related behaviors only from the third testing time. In long-term study, all icv-STZ groups spent less time, in comparison to the control group, in the central region of open-field apparatus and there was no statistical difference between three groups receiving icv-STZ.

3.2. Icv-STZ caused significant impairment in rat's working memory

Y-maze alternation task was performed to investigate the effect of icv-STZ injection on the working memory of animals. In this regards, spontaneous alternation behavior of animals was counted over an 8-min session. There was a significant dose and time-related decline in the spontaneous alternation behavior of rats ($F_{dose\ (3,\ 36)} = 33.58$; $p_{dose} < 0.0001$ and $F_{time\ (2,\ 36)} = 6.144$; $p_{time} = 0.0034$). However, no interaction was observed between dose and age ($F_{interaction\ (6,\ 36)} = 2.03$; $p_{interaction} = 0.07$) (Fig. 4). Moreover, alternation behavior of 1 or 3 mg/kg icv-STZ-injected animals was drastically reduced from four weeks after injection in comparison to their age-matched control groups. However, as Fig. 4 illustrates, icv-STZ 0.5 mg/kg was unable to reduce animal's working memory over the time period of 4 and 10 weeks later. Nonetheless, all three STZ doses could induce a severe impairment in working memory in long-term study. There were, also, significant differences between STZ 3- and STZ 1-injected rats' performance in alternation Y-Maze until 14 weeks after injection.

3.3. Animal's spatial reference learning and memory impaired following icv-STZ injection

Animal's spatial learning was assessed using Morris water Maze. Rats received 4 × 90 s acquisition trials per day over four consecutive days and the mean latency time spent to find the hidden platform was measured. All rats gradually learned to locate the escape platform, so that the escape latency decreased significantly during the training phase, at all testing times and in all different groups involved in the study (*Short-term study: control group (F (3, 27) = 27.79; $p < 0.0001$); STZ 0.5 (F (3, 27) = 21.99; $p < 0.0001$); STZ 1 (F (3, 27) = 17.62; $p < 0.0001$); STZ3 (F (3, 27) = 16.05; $p < 0.0001$). Medium-term study: Fcontrol (3, 27) = 23.6; $p < 0.0001$, FSTZ 0.5 (3, 27) = 13.88; $p < 0.0001$, FSTZ1 (3, 27) = 8.215; $p = 0.0005$ and FSTZ3 (3, 27) = 20.43; $p < 0.0001$. Long-term study: Fcontrol (3, 27) = 30.06; $p < 0.0001$, FSTZ 0.5 (3, 27) = 9.322; $p = 0.0002$, FSTZ1 (3, 27) = 12.75; $p < 0.0001$ and FSTZ3 (3, 27) = 19.82; $p < 0.0001$*). To compare the daily efforts of different groups of rats, One-Way ANOVA test with Tukey post-test was performed. Results showed that in the short-term study, only rats receiving 3 mg/kg icv-STZ had difficulty in finding the hidden platform from the second training day in comparison to the vehicle treated group. Also, significant differences were detected between groups (Fig. 5a).

In the medium-term study, a significant increase was observed in the latency time for STZ 1 and 3 to locate the submerge platform, compared to STZ 0.5 and control groups. Severe spatial learning impairment in STZ 3 group was associated with an irregular learning process graph (Fig. 5b). Finally, all STZ doses could severely decrease

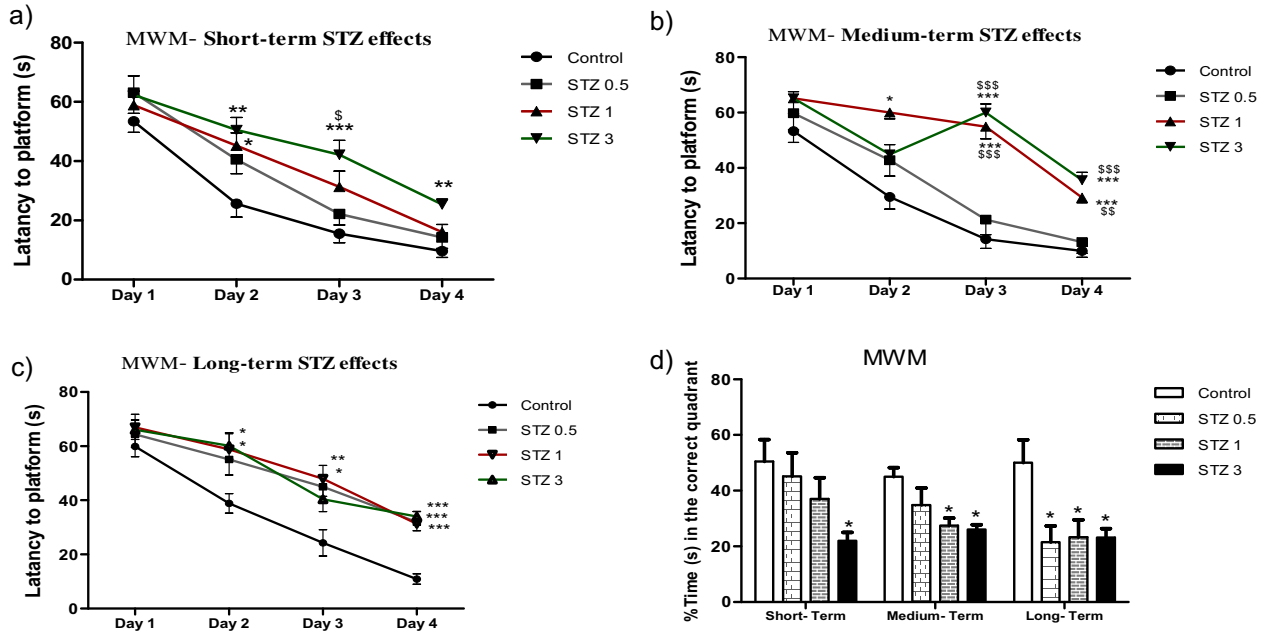

Fig. 5. The effect of different doses of streptozotocin on the spatial learning and memory of rats in Morris water maze test over time. During the training phase of the test, only rats receiving icv-STZ (3 mg/kg) had difficulty in finding the hidden platform in short-term studies (a). In medium-term studies (b), STZ 1 and 3 induced severe impairment in spatial learning ability as compared to STZ 0.5 and control groups. In long-term studies (c), all STZ doses reduced spatial learning ability of animals. A significant dose-dependent decline, assessed by percentage of time spent in the goal quadrant in the probe trial, was observed in the spatial memory of STZ-injected animals (d). Data are reported as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ vs. Control rat. $, $p < 0.05$; $$, $p < 0.01$ and $$$, $p < 0.001$ vs. STZ 0.5 mg/kg rat (n = 10).

Author's Personal Copy

animal spatial learning, as compared to control group, from the second day of training process in the long-term study (Fig. 5c). In the probe trial, percentage of time each animal spent in the goal quadrant was calculated to evaluate spatial reference memory of animals. Accordingly, a significant dose-dependent decline was observed in animal's spatial memory ($F_{dose\ (3,\ 36)} = 9.88$; $p_{dose} < 0.0001$). Time did not turn out to be an effective factor ($F_{time\ (2,\ 36)} = 2.93$; $p_{time} = 0.06$) and no relationship was found between dose and age ($F_{interaction\ (6,\ 36)} = 1.32$; $p_{interaction} = 0.26$). Our data revealed a permanent decrease in the percentage of time STZ 3 group animals spent exploring the correct quadrant, as compared to the age-matched control group, from the first testing time (Fig. 5d). Besides, an enduring spatial memory reduction was detected in STZ1-treated animals from the medium-term testing onward. An extended time needed to find the hidden platform was observed by STZ 0.5 treated animals only in the long-term study. There was not any significant difference between icv-STZ groups' spatial memory performance.

3.4. Icv-STZ decreased animal's performance in the one-trial novel object recognition task

To evaluate the effects of icv-STZ injection on rat's unconditioned preference for novelty, the novel object recognition task was conducted in short-, medium- and long-term after injection. First, across three-min retention phase, using a one-sample *t*-test, we calculated the *novel object percent preference* for vehicle-treated animals after the one-hour inter-trial interval. It was significantly above 50 in all three testing time, implying a well-worked familiar object retention (short-term effects, M = 67.17, SE = 4.09, t = 4. 19, p = 0.0023; medium-term effects, M = 69.28, SE = 4.34, t = 4. 44, p = 0.0016; long-term effects, M = 70.02, SE = 1.77, t = 11. 34, p = 0.0001). Then, each object exploration time was measured using Paired *t*-test. No statistically intrinsic preference was observed among all groups for the left or the right objects in the acquisition phase (Fig. 6(a, c, e)). In the retention phase, high doses of icv-STZ (1 and 3) could impair animals' preferences for novelty from the first testing time, which is determined by reducing the amount of time exploring the new object. In spite of that, 0.5 mg/kg STZ did not significantly impair the retention of the familiar object until the medium-term study (Fig. 6b, d, f). In addition, the percentage of animal's exploratory preference was calculated as the ratio of the time spent exploring the new object (Tn) over time spent exploring the new and the familiar objects (Tf), i.e. [Tn / (Tn + Tf)] × 100. The object exploratory performance of each group was compared to other groups using One Way-ANOVA and Tukey post-test. STZ 3 mg/kg-injected animals from the first testing time and STZ 1 mg/kg-treated rats from the medium-term study were permanently unable to distinguish between the novel and familiar objects, but STZ 0.5 mg/kg impaired animal's performance only in the long-term study. In the medium-term section of behavioral tests, an obvious but not significant reduction was seen in 0.5 mg/kg icv-STZ injected animals' preferences for novelty, but these animals had remarkable difficulty in recognizing the familiar object only in the long-term study (Fig. 6g).

So, according to the results listed here, STZ at the dose of 0.5 mg/kg, in comparison to other doses, could induce cognitive impairment deteriorating over time. In order to determine the best time for implementing behavioral tests after icv-STZ injection (to be able to mimic the early stages of sporadic Alzheimer's disease), time-dependent cognitive performance of STZ 0.5 mg/kg injected animals in NORT was measured using repeated measure One-Way ANOVA; as shown in Fig. 6, STZ-induced deficit had a time dependent manner.

3.5. Icv-STZ-induced gene expression alternations have a dose and time dependent manner

After each behavioral test block, gene expression changes were measured using hippocampal RNA extracts from the fresh brain samples of decapitated animals. Insulin-1 gene expression had very low concentration and relative high threshold cycle in rat hippocampus (Ct > 30); consequently, the data related to this item was excluded from the study.

3.5.1. IR mRNA

IR expression levels showed a dose-dependent manner. Although there was no significant time effect, the interaction of STZ dose × time turned out to be significant (Table 2). There was an early dramatic rise in IR expression level in the short-term study in STZ3 group which decreased over the time. Also, it took 11 weeks to observe an increase in IR expression in STZ 1-treated animals (Medium-term study). Nevertheless, it didn't last more than four weeks and IR expression decreased in the long-term study in this group. STZ 0.5 treated animals experienced an augmentation in IR mRNA levels in the long-term study (Fig. 7a).

3.5.2. Tau mRNA

Although dose had a remarkable effect on the expression level of Tau mRNA, time had no significant effect. Yet, STZ dose × time had a significant relationship with Tau expression (Table 2). Our result also revealed a fluctuation trend in Tau mRNA levels over time in STZ 3 mg/kg group in comparison to the age-matched control group, so that Tau mRNA expression levels were significantly above the control group in the first testing time (Short-term effects, p < 0.0001). Tau mRNA expression levels was almost at the same level of the control expression amount in the second testing time (Medium-term effects), and, finally, there was a measurable decline in Tau mRNA levels in the long-term study, as compared to control group (Long-term effects, p < 0. 01). STZ1 caused the gradual increase in Tau mRNA expression only after 11 weeks (medium-term study). Then, it decreased 4 weeks later. STZ 0.5 could not change Tau mRNA levels until the study reached long-term stage. Also, there was a significant difference between various STZ doses over the time (Fig. 7b).

3.5.3. ChAT mRNA

There was a dose, time and dose × time interaction effect on the ChAT expression values. Our data showed a gradual decrease in ChAT mRNA over time in all STZ treated animals, and this reduction was observed earlier in the higher dose, but the lower doses showed a more slowly change in ChAT levels over time (Table 2).

3.6. Histopathological changes in the rat hippocampus as hallmarks of the advanced stages after icv-STZ injection

Brain sections of the STZ3-treated animals were initially stained with Congo-red or Congo-red/hematoxylin. The collected data proved the occurrence of amyloid deposits in the meninges' blood vessels 24 weeks of STZ3 (Fig. 2A and B) and hippocampus (Fig. 2C) 36 weeks after injection. Then, rats' Hippocampal sections were stained with hematoxylin-eosin (Figs. 8 and 9) and the number of Dark neurons, astrocytes, and the pyramidal layer thickness was compared between all groups (Fig. 10). The number of dark degenerating neurons in the different hippocampus regions seemed statistically dose and time dependent, and there was a considerable interaction effect for time and treatment in CA1 and CA3 but not DG (Table 3). Also, there was an important dose effect in CA1 astrocyte numbers. Furthermore, a considerable dose × time interaction was observed in this region. Though, time was not effective factor. From a statistical point of view, although astrocyte numbers in CA3 turned out to have the greatest dose-dependent manner, there was not any significant time and interaction effect. DG astrocytes numbers did not show any consequential dose, time, and interaction effect. Lastly, CA1 pyramidal layer thickness looked statistically dose and time dependent, and dose × time interaction was significant, too. Although CA3 thickness was significantly affected by STZ dose, time did not have great influence on this layer width, and the

Author's Personal Copy

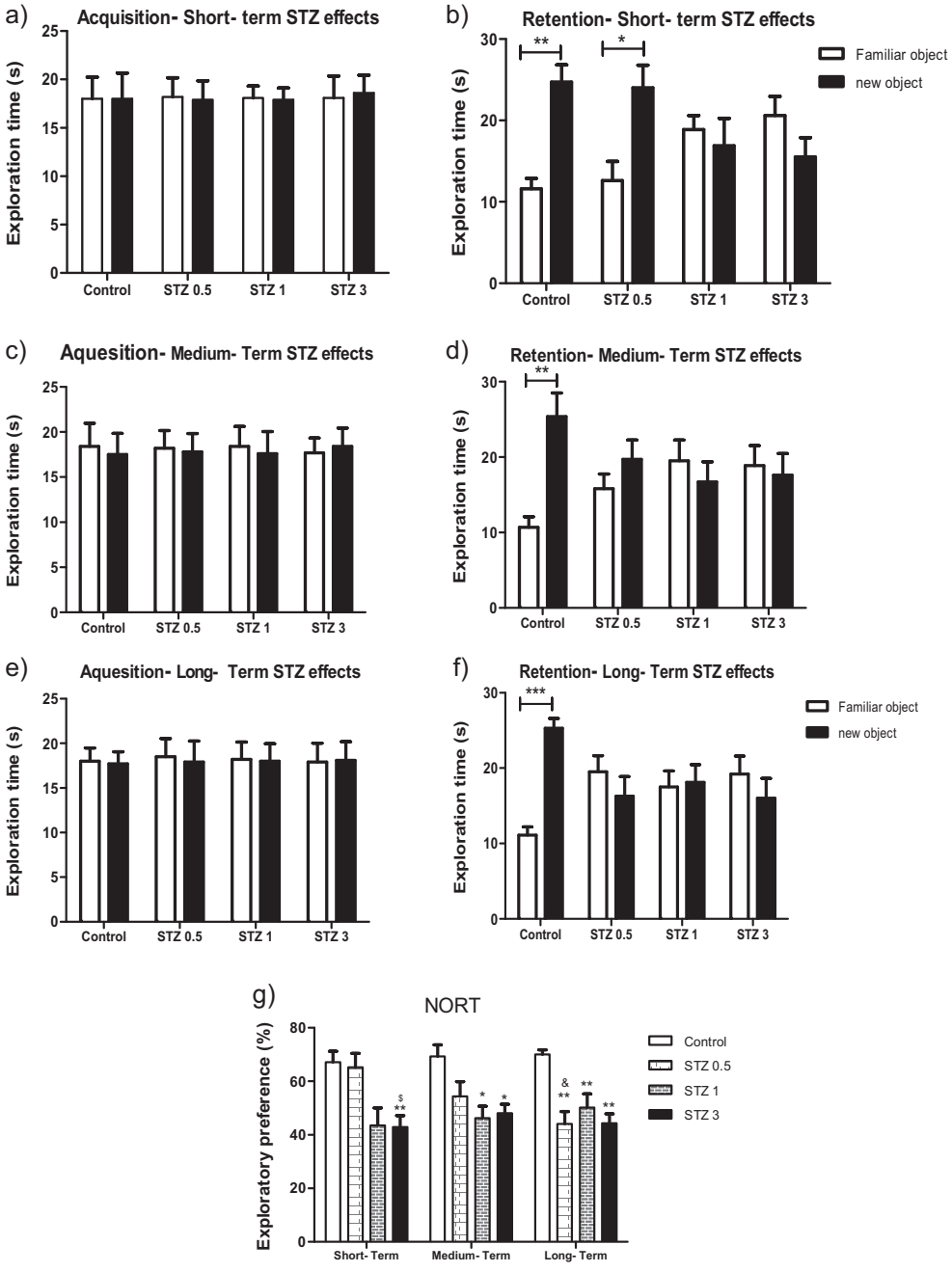

Fig. 6. Three-month follow-up of animal cognitive performance after icv-STZ injection in one trial novel object recognition task. During the acquisition phase (a, c, and e) no preference for either objects was observed in short-term (a), medium-term (c) and long-term studies (e). In the retention phase (b, d, and f), although STZ 1 and 3 could impair animals' preferences for novelty from the very first testing time, STZ 0.5-performance did not cause any impairment until the medium-term study. Exploratory preference was calculated for each time ((Tn – Tf / total exploring time) ∗ 100) as an index of distinguishing efficiency (g). STZ. Data are presented as mean ± SEM. Paired *t*-test was used to measure icv-STZ injection effects on each object exploration time in the acquisition and retention phase. One-way ANOVA was applied to compare Exploratory preference results, *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. Control rat. $, $p < 0.05$ vs. STZ 0.5 mg/kg rat. Repeated measure ANOVA statistic results for STZ 0.5 mg/kg effect over time: &, $p < 0.05$ vs. the first testing time (short-term STZ effects) (n = 10).

Table 2
Statistical information of Real-Time RT-PCR analysis related to insulin-related gene expression in rats treated with three different doses of icv-STZ (0.5, 1, and 3 mg/kg).

Genes	Factors and interaction	Degree of freedom	Mean square	*F*	p-Value
IR	Dose	3	3594	4.64	0.016
	Time	2	78.44	0.15	0.86
	Interaction	6	11,460	22.52	< 0.0001
Tau	Dose	3	3040	8.61	0.0012
	Time	2	1391	2.26	0.12
	Interaction	6	21,074	34.2	< 0.0001
ChAT	Dose	3	20,618	3.16	< 0.0001
	Time	2	4073	7.32	0.0024
	Interaction	6	1674	3	0.02

Different doses of icv-STZ, potentiate disturbed insulin-related gene transcripts in rat hippocampal, which were sometimes dose-dependent or time-dependent, or both.

interaction of time × dose was not significant (Table 3). Actuarial valuation, using Bonferroni multiple comparison Post-test, revealed a decrease in the pyramidal layer thickness along with an increased dark-cells count in STZ 3 mg/kg-injected animals over time. In the short-term studies, STZ 1-treated animals showed an increase, compared to the control and STZ 3-injected groups, in astrocyte numbers, which, of course, gradually decreased over the time. In medium-term study, STZ1 induced an increased astrocyte number detected in DG; moreover, it showed a significant increase in the number of dark neurons in CA1 and CA3 and a decrease in CA1 thickness in the long-term study. The histological evaluation revealed that, although with more time delay, STZ 0.5 mg/kg could mimic STZ 1 effects on hippocampal cells and astrocytes. In the medium-term study, STZ 0.5-injected rats showed a significant increase in CA1 and CA3 astrocyte numbers and in DG in the long-term study. However, no significant change was detected in the number of dark neurons and the pyramidal layer thickness, even, 15 weeks after the injection in STZ 0.5 group (Fig. 10).

Author's Personal Copy

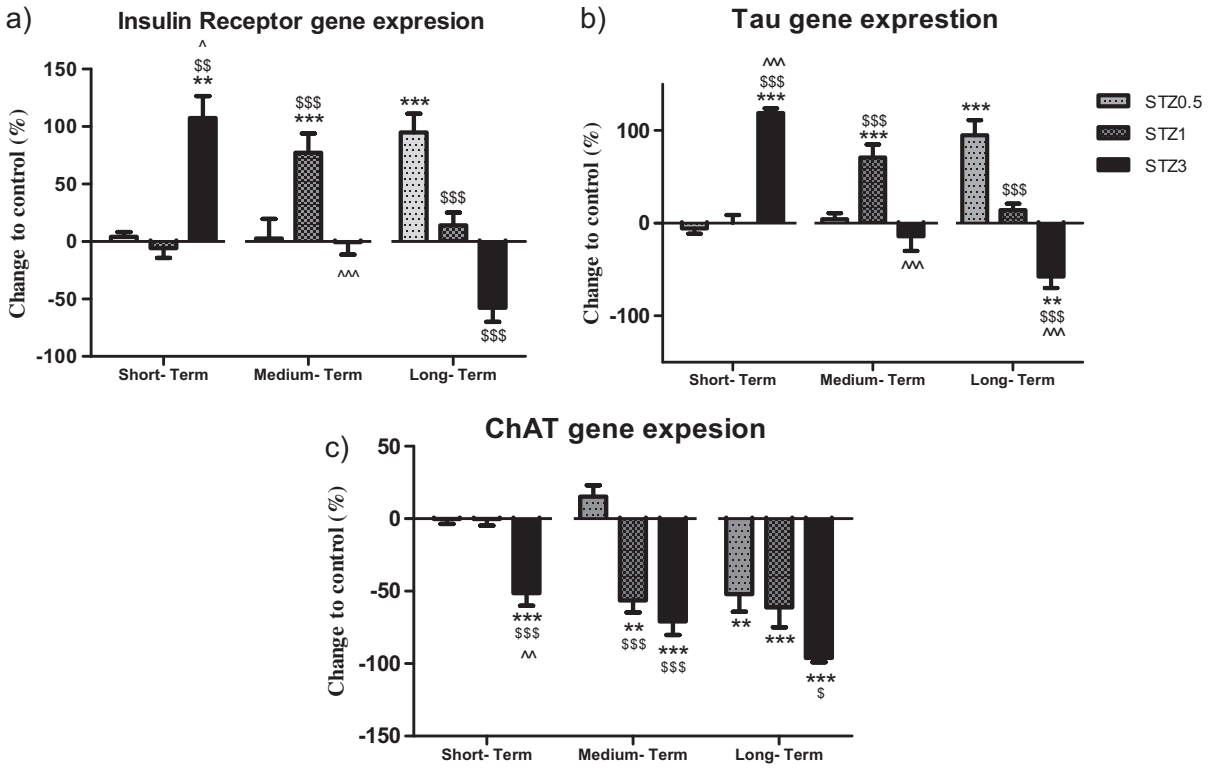

Fig. 7. Icv-STZ administration can affect the expression of insulin-related genes with a dose and time-dependent manner. The effects of icv-STZ on insulin receptor gene expression (a) Tau gene expression (b) and choline acetyltransferase (ChAT) (c) were measured by real-time RT-PCR. Data are reported as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ vs. Control rat. $, $p < 0.05$; $$, $p < 0.01$ and $$$, $p < 0.001$ vs. STZ 0.5 mg/kg rat. ^, $p < 0.05$; ^^, $p < 0.01$ and ^^^, $p < 0.001$ vs. STZ 1 mg/kg rat (n = 5).

4. Discussion

Recent evidence implies the existence of some noncognitive neuropsychiatric symptoms in sAD patients [19]. Despite studies which have claimed that icv-STZ increases the locomotor activity of the animal [14,20], there are numerous reports, like the present study, suggesting that animals treated with icv-STZ have normal motor activity [8,21,22]. Additionally, like others [14,23], we report increased anxiety-related behaviors in icv-STZ-treated animals. Low dose of STZ (0.5 mg/kg) could increase anxiety-related behaviors in rats in long-term study.

Cognitive dysfunction is a critical marker of icv-STZ action [24]. A large body of evidence demonstrates impaired learning and memory in icv-STZ-treated rats at various time points after injection (from 24 h to 3 months after the injection of STZ [22,24,25]. STZ3 was chosen, in the present study, as the best-known criterion against which other STZ doses can be measured. STZ3-injected animals exhibited severe deterioration of cognitive decline [14]. The collected data revealed that cognitive impairments of icv-STZ are dose-dependent. Icv-STZ 1 mg/kg causes a deteriorative spatial memory impairment in the Morris test only from 2.5 months after injection. Only in one study the effects of dose 1 are unstable and reversed 2.5 months after injection [26]. In other studies, as in ours, damaging effects of 1 mg/kg dose of STZ are mainly progressive [24,27,28].

In a recent study which used three doses of icv-STZ (3, 1 and 0.3 mg/kg), all doses showed biphasic cognitive effects with acute and chronic phase of action during passive avoidance test [27]. The acute effect of 0.3 mg/kg dose of STZ approached normal mode three months post-injection, but, then, caused chronic cognitive impairment. Given that amygdala and hippocampus are involved in this test, while our study used MWM, which is hippocampus-dependent test, it is not possible to generalize the results of the mentioned study to the present one. However, progressive cognitive effect of all dose of icv-STZ was proved in both studies.

A simple comparison of the results of behavioral and histological tests of the present study with similarly conducted ones would indicate that increasing the injection frequency of one dose causes the same effect of the higher dose injection. For example, although rats' spatial memory remained untouched ten days after unilateral and single injection of 1.5 mg/kg icv-STZ [29], three-time icv injection of 1 mg/kg STZ caused significant spatial memory impairment two weeks later [24]. A previous study, also, reported that mouse spatial memory remained intact 21 days after two injection of 0.5 mg/kg icv-STZ [30].Assuming that two injections of 0.5 mg/kg has the same effect as one injection of 1 mg/kg icv-STZ, the results of this study are consistent with ours.

Weinstock et al. showed that unilateral icv administration of single-dose of STZ 1.5 mg/kg does not result in spatial memory impairment 40 days after injection. Meanwhile, three bilateral injection of 0.5 mg/kg dose resulted in severe spatial memory disruption [31]. Considering that multiple icv injections, itself, can induce neuroinflammation [5] and given that a part of the icv-STZ effects is acute toxic effects which is non-specific and sometimes remains up to three months after injection [6]; it seems that this multiple icv injection induces increased incidence of non-specific cognitive and neuropathological abnormalities in such studies.

STZ effect on insulin-related gene expressions showed a dose- and/or time-dependent manner. Previous studies have evaluated IR expression levels of icv-STZ-injected animals (1 or 2 mg/kg) up to 3 or 5 months after icv-STZ application [24,32]. In another longitudinal research, hippocampal mRNA levels of IR, and Tau were evaluated up to nine months after icv-STZ [33]. One study also showed icv-STZ-induced reduction in ChAT mRNA level up to six weeks after injection [34,35]. These results support our finding in icv-STZ (3 mg/kg)-treated rats.

Recent studies have reported neuroinflammation and hippocampal gliosis induced by icv-STZ before cognitive impairments in rodents [36]. Our data proved that STZ3 injection could induce hippocampal

Author's Personal Copy

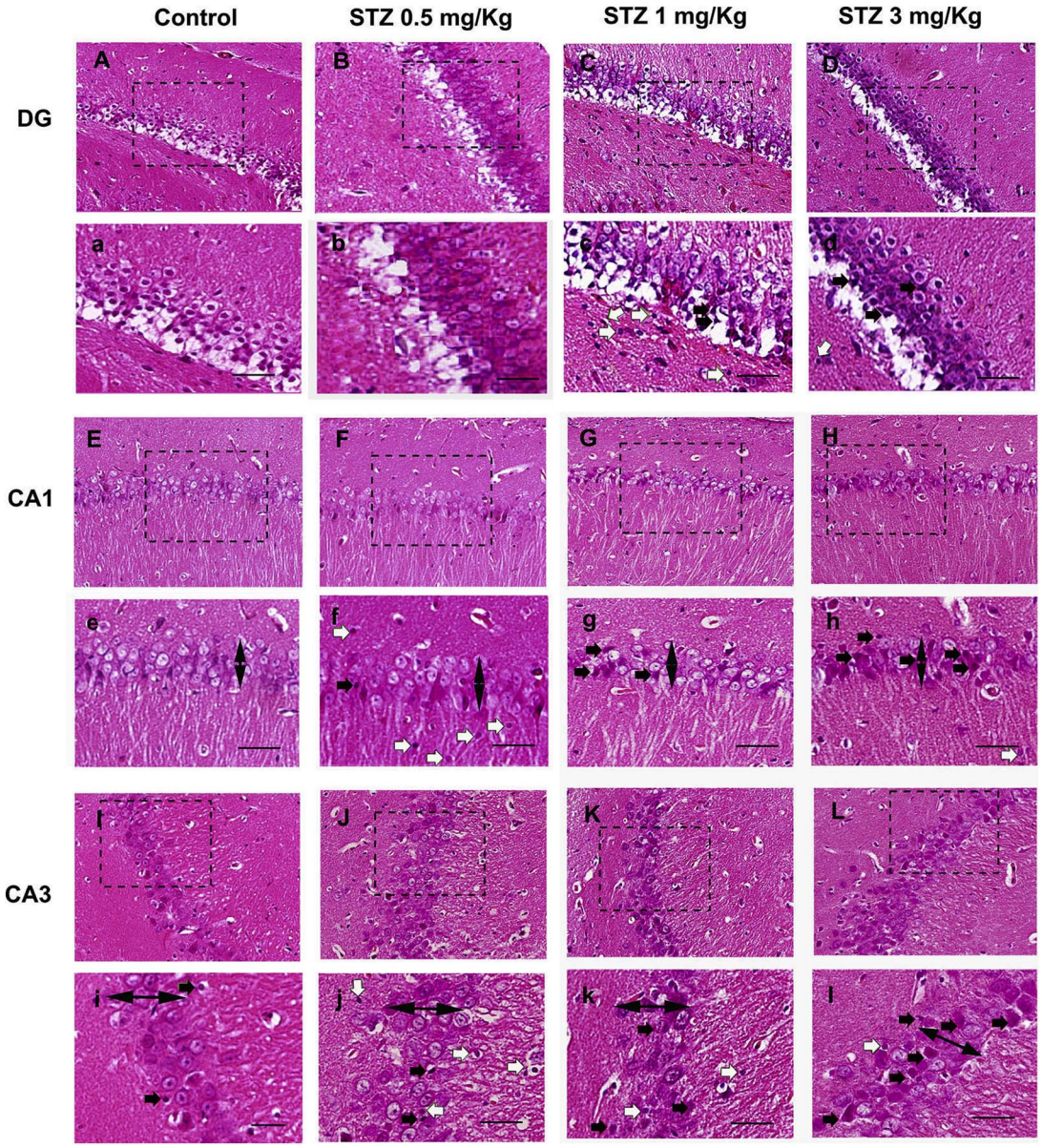

Fig. 8. Histological analysis of hippocampal dark neurons, astrocytes and pyramidal layer thickness 11 weeks after icv-STZ injection in DG (A–D), CA1 (E–H) and CA3 (I–L). Figures with lowercase letters represent more magnification of upper enclosed areas with dotted rectangles. Only 3 mg/kg dose of STZ could induce an increase in the number of CA1 and CA3 dark cells (black arrows). On the other hand, in comparison to control group, STZ 0.5 caused an increase in the number of CA1 and CA3 astrocytes (white arrows). Also, STZ1 induced astrocyte augment in DG. Counting was carried out using unbiased frame and physical dissector rules. Two-headed arrows represent the specific area from which pyramidal layer thickness was calculated. Scale bar represents 60 μm.

beta-amyloid deposition 9 months after injection (Fig. 2). Our histological observations also implied a significant dose and time-dependent increase in the number of degenerating darkly-stained neurons in the different hippocampus regions. Like our study, in a nine-month follow-up study performed in 2015 [27], STZ 3 mg/kg receiving animals showed progressive tauopathy and amyloid accumulation in hippocampus from 3 and 6 months, respectively. These results are consistent with our observation. Also, in this study, STZ 1-induced morphological deficits progressed slower.

Based on aforementioned results, neuropathological effects of icv-STZ emerged with more delay after insulin-related gene expression alternations. SO, it is expected that STZ 0.5 ultimately induce CA1 dark neuron augmentation along with pyramidal layer narrowing over time (manuscript in preparation), and this seems to be similar to the neuropathological characteristic feature of sAD with slowly progressive nature. This finding is inconsistence with a previous study, where STZ 0.3 mg/kg morphological changes were reported to be reversible [27].

Although single injection of 1.25 mg/kg STZ to transgenic mouse caused spatial memory impairment 6 months later, no apparent necrotic and apoptosis change was observed in animals' brain [37]. We observed pyknotic cells 3.5 months after 1 mg/kg icv-STZ injection. Then, it is likely for brain recovery process to a decrease in the number of pyknotic cells 6 months after injection. In another study conducted on monkeys, the amount of hippocampal Tau gene transcripts was similar to control group five months after three icv-injection of 2 mg/kg doses of STZ [38], which is not consistent with the other study on Rats [33], signifying the potency of the monkey's brain recovery system.

Recent evidence has suggested insulin signaling disturbance and/or nitroso-oxidative stress induction, as two proposed icv-STZ mechanisms, to induce cognitive deficits [14,27,35]; however, the significance of each one at the distinct STZ dose is not known yet. STZ 0.5 induced slowly progressive alternations in the amounts of insulin responsive gene transcripts (approximately in the first month post-astrocyte accumulation in CA1 i.e. 15 weeks post-injection) along with progressive

Author's Personal Copy

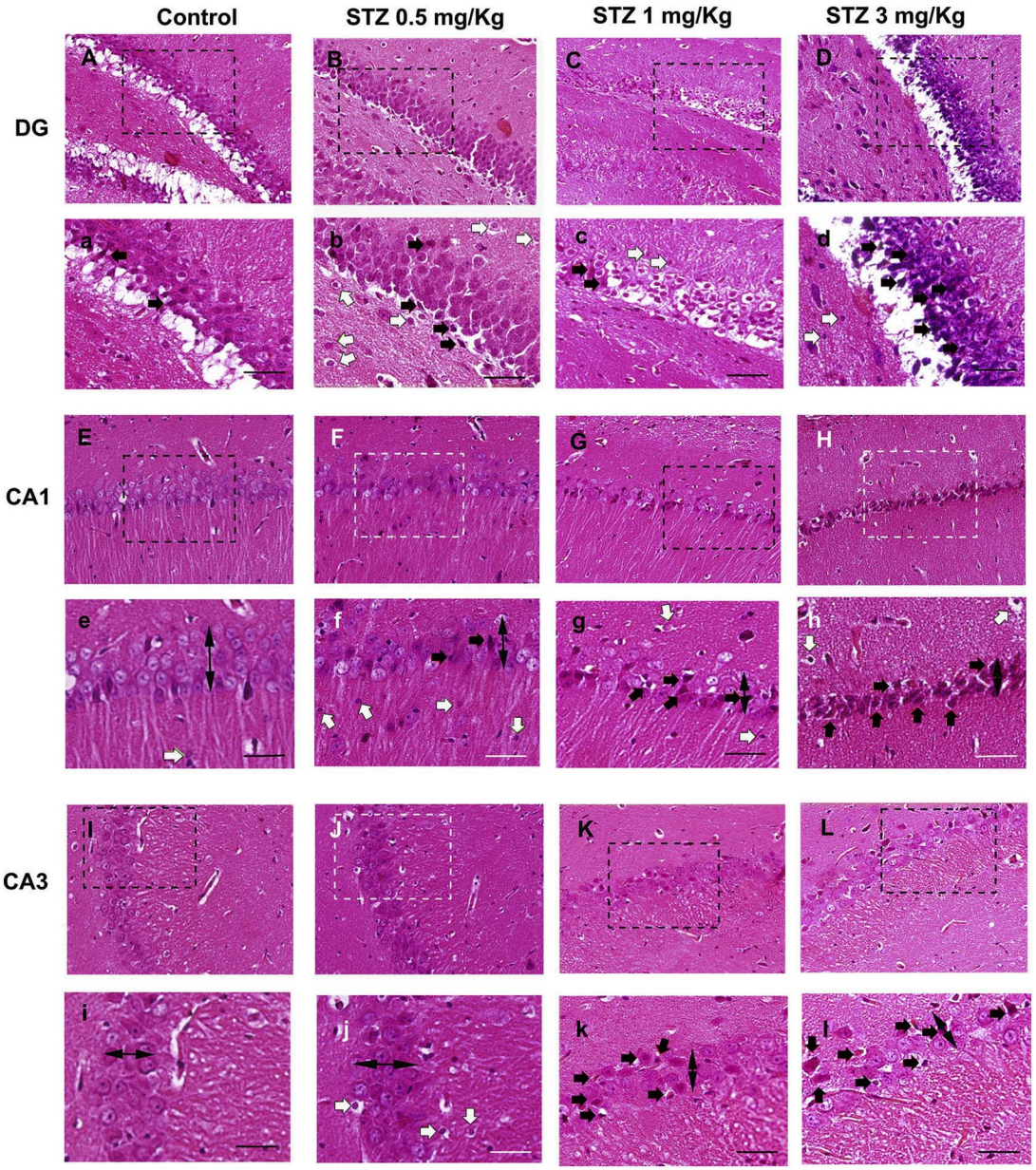

Fig. 9. Histological analysis of hippocampal dark neurons, astrocytes and pyramidal layer thickness 15 weeks after icv-STZ injection in DG (A–D), CA1 (E–H) and CA3 (I–L). Figures with lowercase letters represent more magnification of upper enclosed areas with dotted rectangles. 3 mg/kg Streptozotocin induced an increase in the number of dark cells (black arrows) in all hippocampal regions and a significant decrease of the pyramidal layer thickness (two-headed arrows). Hippocampal sections of STZ1-injected rat showed an increase in the number of dark neurons in CA1 and CA3 and a decrease in pyramidal layer thickness, in comparison to the control group. However, STZ 0.5 could only increase the number of DG astrocytes (white arrows) in the long-term study. Counting was carried out using unbiased frame and physical dissector rules. Two-headed arrows represent the specific area from which pyramidal layer thickness was calculated. Scale bar represents 60 μm.

cognitive decline over time. Based on previous studies, icv-STZ can induce oxidative stress, microglia activation and astrocyte aggregations in the brain, which consequently lead to cognitive decline [36]. Recent studies have shown that increased GFAP expression in activated astrocytes reduces synaptic function [39], and this cognitive impairment has been shown to occur prior to ChAT expression deficiency [36]. Supposing that all STZ doses have the same mechanism, differing from one to another in severity and duration of delay before the onset of symptom, we can, based on previous studies, emphasize that STZ3 induces astrocyte augmentation earlier than first time-point in our study and maybe due to severe oxidative stress induced by icv-STZ [28,36]. Also, further disturbances in insulin-related genes expression lead to neuronal degeneration in the medium-term post-STZ3 injection, causing the reduction of pyramidal layer thickness in long-term. STZ1 did not increase hippocampal mRNA levels of insulin-related gene expression in the short-term, probably due to the induction of less severe oxidative stress and needing more time to progress, which, ultimately, results in an insulin signaling disturbance in the medium-term; this might be why STZ1 could not potentate spatial memory impairment in MWM test in our short-term study. Based on these finding, we suggest stimulated astrogliosis and disturbed insulin responsive gene expression as a critical step which increases the overall probability of cognitive impairment. Another limitation of the present study might be the use of non-specific method for staining and counting astrocyte in rat hippocampus. On the other hand, we only measured mRNA level of these genes. Further investigation will be necessary to complete and confirm our data.

We propose IR, Tau and ChAT gene alternations accompanied with prior astrogliosis to participate in icv-STZ-induced cognitive decline long before neuronal cell death. Like STZ3, STZ1 caused moderate

Author's Personal Copy

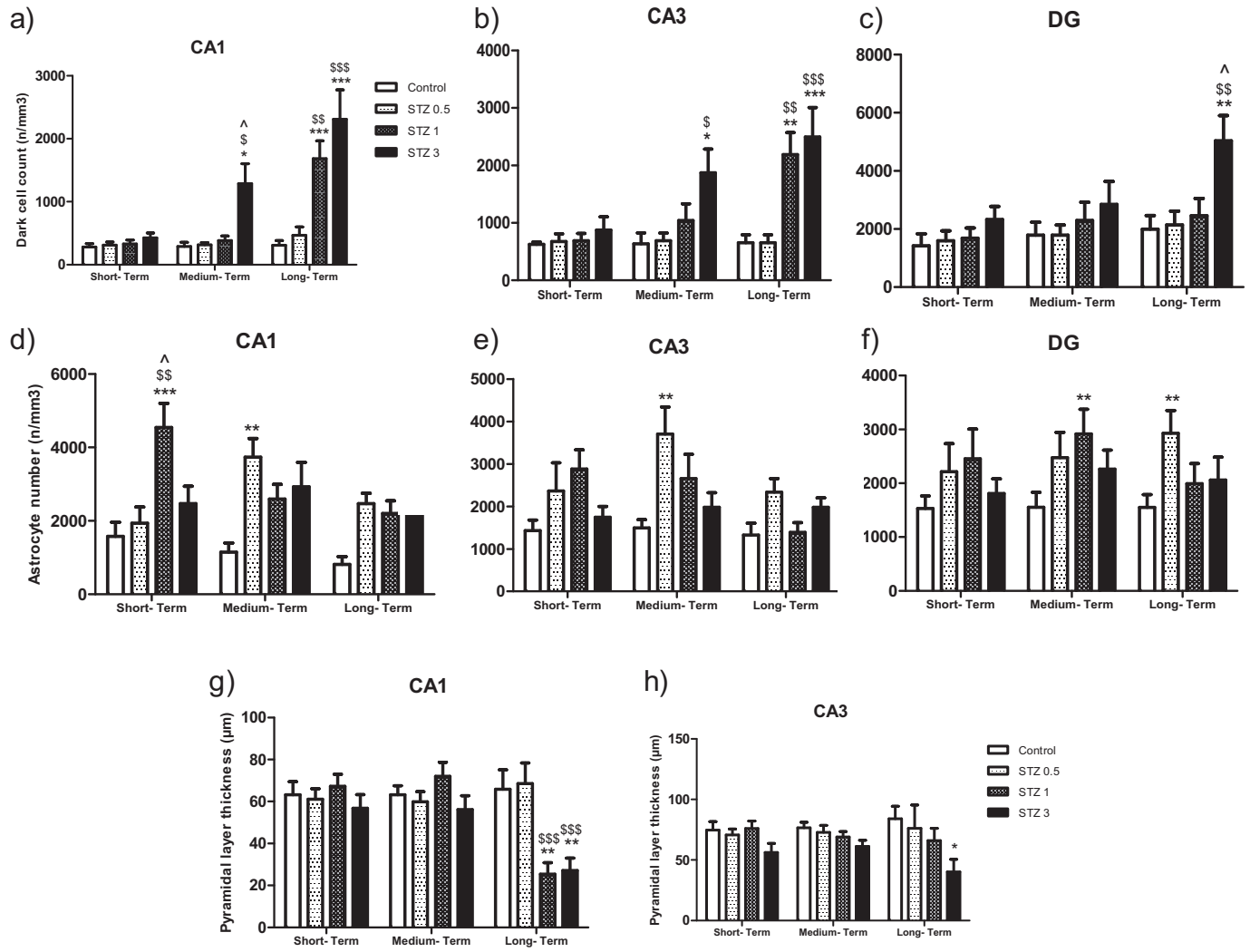

Fig. 10. Quantification of STZ-induced alterations in hippocampal dark neurons (a, b, c), astrocytes (d, e, and f) and pyramidal layer thickness (g and h) over time. Statistical analysis of the number of dark cells in different regions of the hippocampus (a–c) showed that destructive effects of STZ 3 were more serious, and caused significant cell death in all hippocampal areas. Also, compared to the control group, STZ 1 significantly increased the number of dark neurons in the CA1 and CA3 in the long-term study. Statistical analysis of astrocytes density in different areas of the hippocampus (d–f) showed that while STZ 1 increased astrocyte numbers of CA1 and DG in the short-term and long-term study, the effect of other doses was not significant in the short-term period after injection. STZ 0.5 could increase the number of CA1 and CA3 astrocyte in the medium-term and DG in long-term study. However, STZ 3 could not induce significant change in the number of astrocyte in the present study. STZ induced CA1 pyramidal layer narrowing with a time and dose dependent manner (g and h). High dose of STZ (3) decreased pyramidal layers (CA1 and CA3) thickness significantly only in long-term study. Destructive effects of STZ were primarily observed in CA1 and CA3. Apart from a rise in the number of DG astrocytes, no significant impairment was observed in the hippocampus of STZ 0.5-injected animals, even in the long-term study. Data is presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ compared with control group. $, $p < 0.05$; $$, $p < 0.01$ and $$$, $p < 0.001$ vs. STZ 0.5 mg/kg rat. ^, $p < 0.05$ vs. STZ 1 mg/kg rat ($n = 5$).

cognitive deficit and increased anxiety-related behavior from the 4th week onwards. In contrast to STZ3, however, STZ1-induced alterations in insulin-related gene transcripts were detected from 11 weeks after injection. Importantly, mild cognitive impairment (MCI due to Alzheimer), characterized by progressive decline in episodic memory of patients, has been known as one of the earliest clinically detectable stages of sAD with amyloid, and tau pathology [40]. According to the findings of the present study, we propose 13–15 weeks post-STZ1 injection as an appropriate time point to investigate mild cognitive impairment (MCI) stage of sAD. However, MCI is known as a progressive stage of sAD with neuroinflammation as its serious risk factor, and new medical therapies have only reduced its rate of progression [41,42]. So, it is very important to explore a suitable animal model of sub-clinical stages in order to prevent sAD pathology before MCI. According to our STZ 0.5-related data, which revealed a moderate deficit in the cognition ability and also increased anxiety behavior in animals 9–11 weeks after injection, we propose here, for the first time, STZ 0.5 as an appropriate STZ dose and 9–11 weeks after STZ 0.5 injection as a suitable time point to investigate the sub-clinical stage of sAD in rats.

5. Conclusion

Despite more than two decades since the introduction of icv-STZ model, disease progression has not been properly imitated by recently proposed STZ doses and research timescales. Here, we, for the first time, provide evidence that STZ 0.5 can be an appropriate STZ dose and 9–11 weeks after STZ 0.5 injection could be a suitable time-point to investigate cognitive abilities of the sub-clinical rat model of sAD. So, this experimental model may be useful to evaluate the potential effects of various intrinsic or extrinsic factors on the development, progress, as well as the prevention of sAD cognitive symptoms. Even so, it is apparent that further research is needed to clarify and confirm these findings.

Author's Personal Copy

Table 3
Statistical information related to Two-way ANOVA analysis of histological studies in rats treated with icv-STZ 0.5, 1 and 3 mg/kg.

	Region	Factors and interaction	Degree of freedom	Mean square	*F*	p-Value
Astrocyte count	DG	Dose	3	3,103,000	1.86	0.18
		Time	2	448,579	1.27	0.3
		Interaction	6	510,534	1.4	0.23
	CA1	Dose	3	10,910,000	9.4	0.0008
		Time	2	2,234,000	2.36	0.11
		Interaction	6	3,635,000	3.84	0.0054
	CA3	Dose	3	5,214,000	6	0.0061
		Time	2	2,452,000	3.14	0.057
		Interaction	6	1,318,000	1.7	0.16
Dark neurons	DG	Dose	3	8,844,000	3.92	0.028
		Time	2	6,775,000	6.47	0.0044
		Interaction	6	1,729,000	1.65	0.17
	CA1	Dose	3	1,170,000	13.76	0.0001
		Time	2	3,491,000	25.54	< 0.0001
		Interaction	6	3,919,000	7.63	< 0.0001
	CA3	Dose	3	4,285,000	6.69	0.0039
		Time	2	3,084,000	15.1	< 0.0001
		Interaction	6	1,115,000	5.44	0.0006
Pyramidal layer thickness	CA1	Dose	3	997.4	4.34	0.02
		Time	2	1649	7.97	0.0016
		Interaction	6	1068	5.16	0.0003
	CA3	Dose	3	1896	5.18	0.01
		Time	2	62.56	0.15	0.86
		Interaction	6	276.7	0.67	0.67

Hippocampal histopathogenesis potentiated by various doses of STZ, exhibits different behaviors which were dose-, time-, or dose/time-dependent, in different areas of the hippocampus.

Conflict of interest

The authors declare no conflict of interest related to this study.

Acknowledgment

Authors wish to thank Zohre Abbasi for her kind assistance in behavioral studies. We also thank Dr. Fatemeh Behnam and Leila Zare for technical assistance in real-time RT-PCR. This work was funded by Ferdowsi University of Mashhad, Mashhad, Iran (Research project No. 3/37057).

References

[1] C. Li, R. Zhao, K. Gao, Z. Wei, M. Yaoyao Yin, L. Ting Lau, et al., Astrocytes: implications for neuroinflammatory pathogenesis of Alzheimer's disease, Curr. Alzheimer Res. 8 (1) (2011) 67–80.
[2] M. Schubert, D. Gautam, D. Surjo, K. Ueki, S. Baudler, D. Schubert, et al., Role for neuronal insulin resistance in neurodegenerative diseases, Proc. Natl. Acad. Sci. U. S. A. 101 (9) (2004) 3100–3105.
[3] R. Kandimalla, V. Thirumala, P.H. Reddy, Is Alzheimer's disease a type 3 diabetes? A critical appraisal, Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. 1863 (5) (2017) 1078–1089.
[4] J. Leszek, E. Trypka, V.V. Tarasov, G. Md Ashraf, G. Aliev, Type 3 diabetes mellitus: a novel implication of Alzheimers disease, Curr. Top. Med. Chem. 17 (12) (2017) 1331–1335.
[5] L. Shi, Z. Zhang, L. Li, C.A. Hölscher, Novel dual GLP-1/GIP receptor agonist alleviates cognitive decline by re-sensitizing insulin signaling in the Alzheimer icv. STZ rat model, Behav. Brain Res. 327 (2017) 65–74.
[6] P. Grieb, Intracerebroventricular streptozotocin injections as a model of Alzheimer's disease: in search of a relevant mechanism, Mol. Neurobiol. 53 (3) (2016) 1741–1752.
[7] T.B. Bassani, J.M. Bonato, M.M. Machado, V. Cóppola-Segovia, E.L. Moura, S.M. Zanata, et al., Decrease in adult neurogenesis and neuroinflammation are involved in spatial memory impairment in the streptozotocin-induced model of sporadic Alzheimer's disease in rats, Mol. Neurobiol. (2017) 1–17.
[8] M. Sharma, Y. Gupta, Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment, Life Sci. 68 (9) (2001) 1021–1029.
[9] K. Mizoguchi, M. Yuzurihara, A. Ishige, H. Sasaki, T. Tabira, Chronic stress impairs rotarod performance in rats: implications for depressive state, Pharmacol. Biochem. Behav. 71 (1) (2002) 79–84.
[10] E. Schiørring, Amphetamine induced selective stimulation of certain behaviour items with concurrent inhibition of others in an open-field test with rats, Behaviour 39 (1) (1971) 1–17.
[11] P. Galeano, P.V. Martino Adami, S. Do Carmo, E. Blanco, C. Rotondaro, F. Capani, et al., Longitudinal analysis of the behavioral phenotype in a novel transgenic rat model of early stages of Alzheimer's disease, Front. Behav. Neurosci. 8 (2014) 321.
[12] E. Moscardo, B. Salvetti, S. Becchi, G. Bertini, P. Fabene (Eds.), The Novel Object Recognition Test in Rodents: Which Are the Essential Methodological Aspects. Measuring Behavior, Citeseer, 2012.
[13] B. Grayson, N. Idris, J. Neill, Atypical antipsychotics attenuate a sub-chronic PCP-induced cognitive deficit in the novel object recognition task in the rat, Behav. Brain Res. 184 (1) (2007) 31–38.
[14] Y. Chen, Z. Liang, J. Blanchard, C.-L. Dai, S. Sun, M.H. Lee, et al., A non-transgenic mouse model (icv-STZ mouse) of Alzheimer's disease: similarities to and differences from the transgenic model (3xTg-AD mouse), Mol. Neurobiol. 47 (2) (2013) 711–725.
[15] M. Hosseini-sharifabad, E. Esfandiari, A. Hosseini-sharifabad, The effect of prenatal exposure to restraint stress on hippocampal granule neurons of adult rat offspring, Iran. J. Basic Med. Sci. 15 (5) (2012) 1060–1067.
[16] J. Pittella, G. Brasileiro-Filho, The significance of paired astrocyte nuclei in normal human nervous tissue, J. Anat. 151 (1987) 101.
[17] D.M. Wilcock, M.N. Gordon, D. Morgan, Quantification of cerebral amyloid angiopathy and parenchymal amyloid plaques with Congo red histochemical stain, Nat. Protoc. 1 (3) (2006) 1591–1595.
[18] M. Soheili, M.R. Tavirani, M. Salami, Clearance of Amyloid Beta Plaques From Brain of Alzheimeric Rats by *lavandula angustifolia*, (2012).
[19] J. Cerejeira, L. Lagarto, E. Mukaetova-Ladinska, Behavioral and psychological symptoms of dementia, Front. Neurol. 3 (2012).
[20] G. Mayer, R. Nitsch, S. Hoyer, Effects of changes in peripheral and cerebral glucose metabolism on locomotor activity, learning and memory in adult male rats, Brain Res. 532 (1–2) (1990) 95–100.
[21] J.M. Gutierres, F.B. Carvalho, M.R.C. Schetinger, P. Marisco, P. Agostinho, M. Rodrigues, et al., Anthocyanins restore behavioral and biochemical changes caused by streptozotocin-induced sporadic dementia of Alzheimer's type, Life Sci. 96 (1) (2014) 7–17.
[22] N. Rajasekar, C. Nath, K. Hanif, R. Shukla, Intranasal insulin improves cerebral blood flow, Nrf-2 expression and BDNF in STZ (ICV)-induced memory impaired rats, Life Sci. 173 (2017) 1–10.
[23] Y. Chen, Z. Liang, Z. Tian, J. Blanchard, C.-L. Dai, S. Chalbot, et al., Intracerebroventricular streptozotocin exacerbates Alzheimer-like changes of 3xTg-AD mice, Mol. Neurobiol. 49 (1) (2014) 547–562.
[24] E. Grünblatt, M. Salkovic-Petrisic, J. Osmanovic, P. Riederer, S. Hoyer, Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein, J. Neurochem. 101 (3) (2007) 757–770.
[25] M. Salkovic-Petrisic, A. Knezovic, J. Osmanovic-Barilar, U. Smailovic, V. Trkulja, P. Riederer, et al., Multi-target iron-chelators improve memory loss in a rat model of sporadic Alzheimer's disease, Life Sci. 136 (2015) 108–119.
[26] J. Mehla, M. Pahuja, Y.K. Gupta, Streptozotocin-induced sporadic Alzheimer's disease: selection of appropriate dose, J. Alzheimers Dis. 33 (1) (2013) 17–21.
[27] A. Knezovic, J. Osmanovic-Barilar, M. Curlin, P.R. Hof, G. Simic, P. Riederer, et al., Staging of cognitive deficits and neuropathological and ultrastructural changes in streptozotocin-induced rat model of Alzheimer's disease, J. Neural Transm. 122 (4) (2015) 577–592.
[28] A. Kraska, M.D. Santin, O. Dorieux, N. Joseph-Mathurin, E. Bourrin, F. Petit, et al., In vivo cross-sectional characterization of cerebral alterations induced by intracerebroventricular administration of streptozotocin, PLoS One 7 (9) (2012) e46196.
[29] A. Blokland, J. Jolles, Brief communication, Pharmacol. Biochem. Behav. 44 (2) (1993) 491–494.
[30] S. Tota, H. Awasthi, P.K. Kamat, C. Nath, K. Hanif, Protective effect of quercetin against intracerebral streptozotocin induced reduction in cerebral blood flow and impairment of memory in mice, Behav. Brain Res. 209 (1) (2010) 73–79.
[31] M. Weinstock, N. KIRSCHBAUM-SLAGER, P. Lazarovici, C. Bejar, M.B. Youdim, S. Shoham, Neuroprotective effects of novel cholinesterase inhibitors derived from rasagiline as potential anti-Alzheimer drugs, Ann. N. Y. Acad. Sci. 939 (1) (2001) 148–161.
[32] Y. Lee, Y.-H. Kim, S.-J. Park, J.-W. Huh, S.-H. Kim, S.-U. Kim, et al., Insulin/IGF signaling-related gene expression in the brain of a sporadic Alzheimer's disease monkey model induced by intracerebroventricular injection of streptozotocin, J. Alzheimers Dis. 38 (2) (2014) 251–267.
[33] J.O. Barilar, A. Knezovic, E. Grünblatt, P. Riederer, M. Salkovic-Petrisic, Nine-month follow-up of the insulin receptor signalling cascade in the brain of streptozotocin rat model of sporadic Alzheimer's disease, J. Neural Transm. 122 (4) (2015) 565–576.
[34] Y. Chen, Z. Tian, Z. Liang, S. Sun, C.-L. Dai, M.H. Lee, et al., Brain gene expression of a sporadic (icv-STZ mouse) and a familial mouse model (3xTg-AD mouse) of Alzheimer's disease, PLoS One 7 (12) (2012) e51432.
[35] N. Lester-Coll, E.J. Rivera, S.J. Soscia, K. Doiron, J.R. Wands, S.M. de la Monte, Intracerebral streptozotocin model of type 3 diabetes: relevance to sporadic Alzheimer's disease, J. Alzheimers Dis. 9 (1) (2006) 13–33.
[36] S. Shoham, C. Bejar, E. Kovalev, D. Schorer-Apelbaum, M. Weinstock, Ladostigil prevents gliosis, oxidative–nitrative stress and memory deficits induced by intracerebroventricular injection of streptozotocin in rats, Neuropharmacology 52 (3) (2007) 836–843.
[37] K. Plaschke, J. Kopitz, M. Siegelin, R. Schliebs, M. Šalković-Petrišić, P. Riederer,

Author's Personal Copy

et al., Insulin-resistant brain state after intracerebroventricular streptozotocin injection exacerbates Alzheimer-like changes in Tg2576 AβPP-overexpressing mice, J. Alzheimers Dis. 19 (2) (2010) 691–704.
[38] S.-J. Park, Y.-H. Kim, Y. Lee, K.-M. Kim, H.-S. Kim, S.-R. Lee, et al., Selection of appropriate reference genes for RT-qPCR analysis in a streptozotocin-induced Alzheimer's disease model of cynomolgus monkeys (*Macaca fascicularis*), PLoS One 8 (2) (2013) e56034.
[39] C.E. Finch, Neurons, glia, and plasticity in normal brain aging, Neurobiol. Aging 24 (2003) S123–S127.
[40] M.S. Albert, S.T. DeKosky, D. Dickson, B. Dubois, H.H. Feldman, N.C. Fox, et al., The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease, Alzheimers Dement. 7 (3) (2011) 270–279.
[41] P. Bermejo, S. Martín-Aragón, J. Benedí, C. Susín, E. Felici, P. Gil, et al., Differences of peripheral inflammatory markers between mild cognitive impairment and Alzheimer's disease, Immunol. Lett. 117 (2) (2008) 198–202.
[42] J.C. Morris, M. Storandt, J.P. Miller, D.W. McKeel, J.L. Price, E.H. Rubin, et al., Mild cognitive impairment represents early-stage Alzheimer disease, Arch. Neurol. 58 (3) (2001) 397–405.