



## Early and late neurodegeneration and memory disruption after intracerebroventricular streptozotocin

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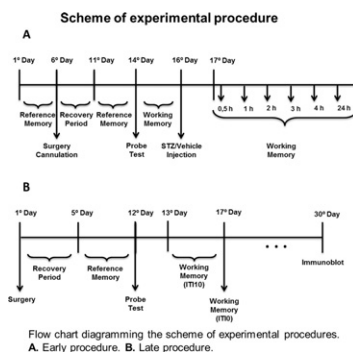
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### HIGHLIGHTS

- ▶ Intracerebroventricular streptozotocin impairs the reference and working memories.
- ▶ Working memory is disrupted as early as 3 h after STZ injection.
- ▶ Neuron degeneration was observed in hippocampus after 1 and 15 days of the injection.
- ▶  $\beta$ A peptide expression increases in the hippocampus, entorhinal cortex, and basal ganglia.
- ▶ Hyperphosphorylated Tau was seen in the entorhinal cortex, neocortex, and basal ganglia.

### GRAPHICAL ABSTRACT



### ARTICLE INFO

**Article history:**  
Received 31 May 2012  
Accepted 29 June 2012

**Keywords:**  
Alzheimer's disease  
Aging  
Neurodegeneration  
Glucose/energy metabolism  
Learning/memory  
Cognitive dysfunction  
Streptozotocin

### ABSTRACT

Glucose metabolism and insulin signaling disruptions in the brain have been proposed as a likely etiology of Alzheimer's disease. The aim of the present study was to investigate the time course of cognitive impairments induced by intracerebroventricular injection of streptozotocin (STZ) in rats and correlate them with the ensuing neurodegenerative process. Early and late effects of STZ were evaluated by using the reference and working memory versions of the Morris' water maze task and the evaluation of neurodegenerative markers by immunoblotting and the Fluoro-Jade C histochemistry. The results revealed different types of behavioral and neurodegenerative responses, with distinct time courses. We observed an early disruption on the working memory as early as 3 h after STZ injections, which was followed by degenerative processes in the hippocampus at 1 and 15 days after STZ injections. Memory disruption increases over time and culminates with significant changes in amyloid-beta peptide and hyperphosphorylated Tau protein levels in distinct brain structures. These findings add information on the Alzheimer's disease-like STZ animal model and on the mechanisms underlying neurodegenerative processes.

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## 1. Introduction

Alzheimer's disease (AD) is one of the most common causes of dementia in aged humans. It is clinically characterized by a general and progressive cognitive decline, especially memory deterioration. Most cases have a sporadic occurrence, and aging seems to be the main risk factor [1]. Typical neuropathological hallmarks of AD include both accumulations of senile plaques, corresponding to deposits of amyloid- $\beta$  (A $\beta$ ) peptide outside of neurons, and neurofibrillary tangles (NFT), corresponding to intracellular deposits of hyperphosphorylated Tau protein. *Post mortem* studies of AD patients reveal neuronal loss in the hippocampus, entorhinal cortex, amygdala, neocortex, and subcortical areas, including basal forebrain cholinergic neurons, dorsal raphe serotonergic neurons and locus coeruleus noradrenergic neurons [1]. Not surprisingly, the earliest symptoms of AD include explicit memory deficits, particularly when navigating in both familiar and unfamiliar environments [2], and disruption of executive functions, including working memory [1,3].

Molecular evidence raised the assumptions that trafficking of the amyloid precursor protein (APP) is under control of insulin signaling and insulin receptor tyrosine kinase [4,5] and that insulin regulates phosphorylation of tau protein via glycogen synthase kinase-3 activity [6,7]. In addition, insulin affects brain functions such as cognition and memory as shown by *in vivo* studies [8]. Functional studies have shown disruption in both cerebral glucose mobilization and energy metabolism either preceding or accompanying the initial stages of cognitive impairments in AD [9,10]. Accordingly, impairments of glucose metabolism and of the insulin signaling pathway have been proposed as a likely etiology of AD [5,9,11,12]. Indeed, behavioral changes reported in animal models of diabetes or Alzheimer's disease include disruption of performance in the reference memory version of the Morris' water maze spatial navigation task, the elevated plus maze, and the T-maze footshock avoidance paradigm [13–16]. Recently, Bird and colleagues [17] demonstrated that patients with mild AD exhibit impairments on topographical short-term memory even for very short periods of time, but not on non-spatial aspects of the landscapes. These data lead the authors to propose that “this memory function is particularly sensitive to the earliest stages of the disease”. Thus, behavioral results involving animal models of AD tested in the Morris' water maze task seem to be particularly relevant since this task evaluates spatial navigation, particularly when the animal is trained departing from different starting locations [18,19]. Therefore, on considering Bird and colleagues [17], it would be even more relevant to evaluate spatial navigation in animal models of AD by using a working memory version of the task, as that reported by Xavier and colleagues [19].

Intracerebroventricular (icv) injections of the diabetogenic drug streptozotocin (STZ), often employed to induce experimental diabetes mellitus in animals, have been used to investigate abnormalities of brain glucose metabolism [20–22] and thus for inducing an AD-type neurodegeneration. Biochemical, morphological and behavioral changes that follow icv injection of STZ in rodents seem to be equivalent to those seen in sporadic AD. For instance, STZ injection causes a reduction of glucose utilization and glycogen metabolism [23,24], impairments of insulin and insulin-like growth factor signaling [20,25], and impairments in the performance in memory tasks [21,26–29].

Even though performance in the reference memory version of Morris' water maze task is sensitive to STZ icv injection [26], that behavioral task does not seem to be the best alternative to evaluate both the early cognitive changes that follow administration of STZ in this animal model of AD and the progression of these symptoms, since it requires several days of training to be acquired and performance is maintained relatively stable for months after acquisition. In contrast, the working memory version of the Morris' water maze task, as described by Xavier et al. [19], appears to be a more suitable behavioral

test to investigate the progression of symptoms following icv injection of STZ.

Thus, the aim of present study was to investigate the time course of cognitive impairments induced by a single icv injection of STZ in rats and to correlate them with the ensuing neurodegenerative process. While the behavioral effects of STZ icv injection were evaluated by using reference and working memory versions of the Morris' water maze task, the expression of neurodegenerative markers was evaluated by immunoblotting and the Fluoro-Jade C histochemistry.

## 2. Methods

### 2.1. Subjects

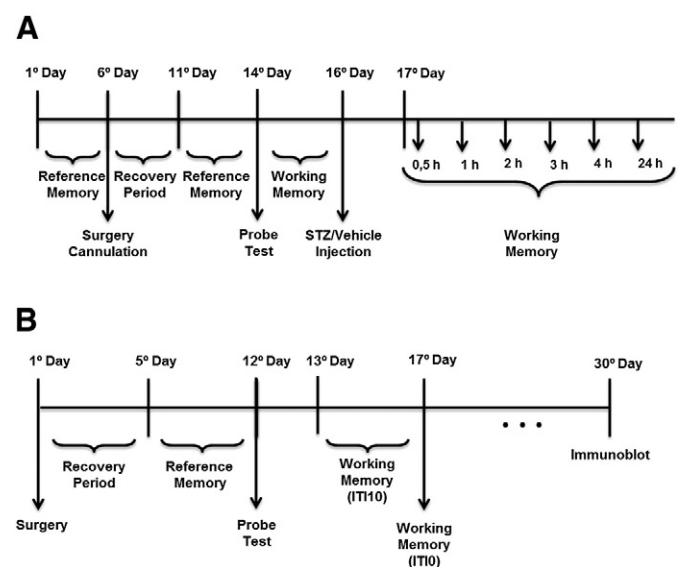
Adult male Wistar rats, weighing 250–350 g at the beginning of the experiments, obtained from the animal facilities of the Institute of Biomedical Sciences, University of São Paulo, were used in this study. The animals were housed in groups of 3–4 animals per conventional cage, maintained at 23 °C  $\pm$  2, in a 12 h light/12 h dark cycle (lights on at 06:00 h), with free access to food and water. These housing conditions lasted until the end of the experiments. The experiments were carried out in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by The Ethics Committee for Animal Research of the Institute of Biomedical Sciences, University of São Paulo (Protocol no 33/08/CEUA), which comply with national and international procedures of care of animals used in scientific research.

Independent groups of rats were used to investigate the early (Flow chart 1, part A) and late (Flow chart 1, part B) effects of STZ icv injection and the surgical procedures in each of these cases differed (see below). A third group of rats was used for histochemical analysis of neurodegeneration by the Fluoro-Jade C method.

### 2.2. Stereotaxic surgical procedure

Rats were anesthetized with an i.m. combination of ketamine (5 mg/100 g of body weight, b.w.) and xylazine (1 mg/100 g b.w.) and positioned in a stereotaxic frame.

In order to test early effects of STZ icv injection, a group of pre-trained (see Section 2.4) rats received permanent bilateral implants of 13-mm-long 23-gauge guide cannulae, positioned according



**Flow chart 1.** Flow chart diagramming the scheme of experimental procedures. A. Early procedure. B. Late procedure.

to the following stereotaxic coordinates: AP=1.0 mm, MD=1.5 mm, and DV=3.5 mm [30]. The implanted guide cannulae allowed later injection of either STZ (N=7) or citrate buffer (vehicle, N=5), by way of a 30-gauge infusion cannulae 13 mm long, when the subjects were awake and freely moving, thus allowing to follow behavioral changes after injection (see below).

In order to test late effects of STZ icv injection, rats without any pre-surgical behavioral training were subjected to bilateral injections of either STZ (3 mg/kg, b.w., Sigma, St. Louis, MO) freshly dissolved in citrate buffer (0.05 mol/L, pH 4.5; N=9) or the same volume of vehicle (control group, CTR; N=9) at the same stereotaxic coordinates. A volume of 4  $\mu$ L was injected in each hemisphere by way of glass micropipettes with tips ranging from 10 to 20  $\mu$ m, coupled to a pressure system. After injections, there was a 4-day recovery period before the beginning of behavioral testing (see Section 2.5), and after that, those subjects were used for immunoblotting analysis (see Section 2.6).

Histochemical analysis of neurodegeneration by the Fluoro-Jade C method was run in a group of rats subjected to this later surgical method (N=21).

### 2.3. Morris' water maze apparatus and general procedures of reference and working memory

The Morris' water maze apparatus and the procedures employed were similar to those described by Xavier and colleagues [19]. The pool was a round, black fiberglass tank, 200 cm diameter, 50 cm height, filled to a depth of 25 cm with water ( $26^{\circ}\text{C} \pm 1$ ), rendered opaque by the addition of milk. A movable circular plastic platform 9 cm in diameter, mounted on a plastic column, was placed in the pool about 2 cm below the water surface. The platform location depended on the behavioral testing procedure (see below). For descriptive data analysis, the pool was divided into four equal-area quadrants which bordered each other along imaginary lines which intersected the edge of the pool at the arbitrary cardinal start locations. The time spent within the training counter (an area three times the platform diameter, surrounding each platform position), as well as the number of entries within the counter, provided specific indexes of spatial location. The percentage of time spent within a 33-cm wide ring containing the platform (the critical ring), which external border was 33 cm distant from the pool border, helped to analyze the spatial bias of the animals in the pool. The swimming pool was located in a  $3.15 \times 4.00$  m room with several salient cues hanging on the walls. A video camera positioned approximately 290 cm over the center of the pool was connected to a video-tracking digitizing device (VP112, HVS Image Ltd., Hampton, UK) and sampled in units of 0.1 section by a computer system programmed to collect and analyze the animals' swim paths. Each trial in both the reference and the working memory versions of the task constituted placing the rat near the border of the pool (in one of the starting locations) and allowing it to swim until the platform was found. If the rat did not find the platform within 120 s, the animal was then manually guided to the platform, where it remained for a 10-s period.

In the water maze reference memory training task, the platform was located in a single, fixed position in the center of the critical quadrant. Each animal was exposed to four trials per session (one session a day), with an intertrial interval (ITI) of 10 min. Acquisition of the reference memory task was assessed by decreases of latency and path length, and both by increases of the percentage of time spent in the quadrant and by increases of the percentage of time spent within the ring where the platform was located. Training proceeded until the subjects reached an asymptotic level of performance.

A probe test, with the platform removed, was conducted 24 h after the end of the reference memory training phase. During this test, the animals were allowed to swim freely in the pool for 3 min. The

percentage of time the animal spent within the critical quadrant relative to the other quadrants, the amount of time the animal spent within the counter, and the number of times the animal swam across that counter (counter frequency), in three time bins of 60 s each, allowed assessment of long-term memory for platform location through the extent of spatial bias in the first time bin, and evaluation of the rate of extinction of this behavior by comparing the same parameter over the three consecutive time bins.

In the water maze working memory task, the animals were tested along four trials per session. The platform location was changed from session to session. Therefore, on the first trial of each session the subjects had to find the platform by scanning the pool. Then, on the second and remaining trials, the subjects could use the information about the platform location acquired in the previous trials in order to improve their performance along trials. The ITI could be either 10 min (ITI=10) or 0 min (ITI=0), thus stimulating maintenance of the critical information about the platform location for variable periods of time in each session. Maintenance of information about the platform location in working memory was assessed by decreases of latency and path length along trials within a session. When testing involved one session per day, the time spent within the critical previous session counter and the critical previous session counter frequency provided a 24-h delay test of memory for the precise former platform location [19].

### 2.4. Behavioral pre-injection training and post-injection testing soon after STZ administration (early effects)

Evaluation of early effects (see Flow chart 1, part A) of STZ on spatial working memory required training the subjects prior to the STZ injection. These pre-STZ injection procedures included (1) 5 sessions of pre-surgical training in the reference memory task, (2) execution of a surgery for implantation of the guide cannulae to be later used for administration of STZ, (3) a post-surgical 5-day recovery period, (4) 3 sessions of post-surgical training in the reference memory task, (5) one Probe Test session, and (6) 2 sessions of training in the working memory task, with an ITI of 10 min, in this sequence. These procedures led to complete acquisition of the spatial working memory task and, in addition, provided data of the subjects' pre-STZ injection level of performance in this task; this allowed allocation of the subjects for the groups (see below) according to their pre-injection performance, thus rendering homogeneity among the groups before the critical treatments. Twenty-four hours later, either STZ or vehicle (in the same dose and volume as described above) was injected bilaterally, along 5 min, via infusion cannulae inserted into the guide cannulae; the infusion cannulae was connected to a polyethylene tube connected to a Hamilton microsyringe. Then, the subjects were exposed to successive sessions of testing in the working memory version of the water maze task, with ITI=10 min. These sessions were run 0.5, 1, 2, 3, 4 and 24 h after the injection, thus allowing the follow up of the time course of cognitive disruption.

### 2.5. Post-injection behavioral testing of late effects of a single dose of STZ

After the 4-day period of surgery recovery (see Flow chart 1 part B), the subjects were exposed to (1) 7 sessions of training in the spatial reference memory version of the water maze task, (2) a Probe Test session, (3) 5 sessions of testing in the spatial working memory version of the water maze task, with ITI=10, and (4) one session of testing in the spatial working memory version of the task, with ITI=0, in order to evaluate performance of the subjects when the time required for maintenance of the spatial information in working memory was virtually zero.

## 2.6. Immunoblotting

Thirty days after the injections, the subjects involved in evaluation of the late behavioral effects of STZ and corresponding controls were decapitated under administration of an overdose of anesthesia and the brain rapidly collected and dissected. The entorhinal cortex, neocortex, basal ganglia, hippocampus, and hypothalamus, were individually collected, and homogenized in extraction buffer at 4 °C (Tris, pH 7.4, 100 mM; EDTA 10 mM; PMSF 2 mM; aprotinin 0.01 mg/ml). The protein concentration of the samples was determined by a protein assay (Bio-Rad, Hercules, CA, USA), and 50 µg of protein were subjected to 10% SDS-PAGE gel and electrotransferred to nitrocellulose membranes using a Trans-Blot cell system (Bio-Rad). The nitrocellulose membranes were then blocked and incubated overnight with a rabbit polyclonal antiserum against Aβ (H-43; Santa Cruz Technology, Santa Cruz, CA, USA), a mouse monoclonal antibody against Tau, a rabbit polyclonal antiserum against phosphorylated-Tau (pSer<sup>199/202</sup>; Sigma), a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP; Sigma), and a goat polyclonal antiserum against the synthesizing enzyme of acetylcholine, choline acetyltransferase (ChAT; Chemicon, Temecula, CA, USA). A loading control was conducted in all experiments by using an anti-GAPDH antibody (Santa Cruz). Specifically bound antibody was visualized using a chemiluminescence kit (ECL Kit; Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). Finally, the blots were densitometrically analyzed with Scion Image 4.0.2 (Scion Corporation, Frederick, MD).

## 2.7. Histochemical analysis by Fluoro-Jade C

After 1, 15, and 30 days of the STZ icv injections, subjects were deeply anesthetized with ketamine and xylazine and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PB). Brains were postfixed and then cryoprotected in a 30% sucrose solution in PB. Coronal sections (30 µm) of the frozen brains were cut on a sliding microtome and subjected to the fluorescent Fluoro-Jade C technique to evaluate degenerating neurons [31]. Briefly, gelatin- and chromoalumen-coated slides containing the brain sections were immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 min and in 70% alcohol, followed by distilled water for 2 min. Subsequently, after agitation in a solution of 0.06% potassium permanganate for 10 min, and washing in distilled water for 2 min, the material was incubated in a solution of 0.001% Fluoro-Jade C (Chemicon) in 0.1% acetic acid for 20 min, and washed three times in distilled water. The slides were then fully dried, cleared by immersion in xylene for 2–3 min, and coverslipped using DPX (Fluka, Milwaukee, WI). Finally, the material was analyzed on a fluorescence microscope equipped with a standard fluorescein filter, and digital images were collected.

## 2.8. Peripheral blood glucose levels

Glycemia was measured for all subjects before surgery. Subjects involved in evaluation of late effects of STZ administration had glycemia also measured 15 and 30 days after STZ injection. Blood was collected by tail prick and glycemia was measured by a strip-operated blood glucose sensor (Accu-Check Active, Roche).

## 2.9. Data analysis

Reference memory water maze scores were analyzed using repeated measure analysis of variance (ANOVA) with Groups as the between-subject, and Sessions and Trials as the within-subject factors (SAS Institute, Inc., Cary, NC).

Probe-test scores were analyzed using repeated measure ANOVA having Group as the between-subject and Time Bin as the within-subject factors.

Early effects of STZ on working memory latency and path length for the four trials across the last pre-injection working memory session and the working memory sessions run 0.5, 1, 2, 3, 4 and 24 h after the injection were individually compared using repeated measures analysis of variance (ANOVA), with Group as the between-subject, and Session and Trials as the within-subject factors [19]. In all cases, an ANOVA was run for each individual parameter.

Late effects of STZ on working memory were evaluated by calculating the means of latency, path length, percentage of time spent within the critical training counter and counter frequency for the four trials across the last four days of testing with ITI of 10-min. These scores were compared to those obtained in the last session of training in the working memory task when the ITI was virtually zero. An individual ANOVA was run for each score having Groups as the between-subject factor and ITI and Trials as the within-subject factors.

Immunoblotting optical densities bands were first normalized in relation to the GAPDH in each experiment and then analyzed by Student's *t*-tests for independent measures in terms of changes in protein expression levels compared to controls.

## 3. Results

### 3.1. Behavioral tests

The swim speeds of the STZ and CTL subjects did not differ significantly along the behavioral testing. Therefore, it is unlikely that differences in other parameters reflect either motor or motivational impairments.

#### 3.1.1. Early behavioral effects of STZ

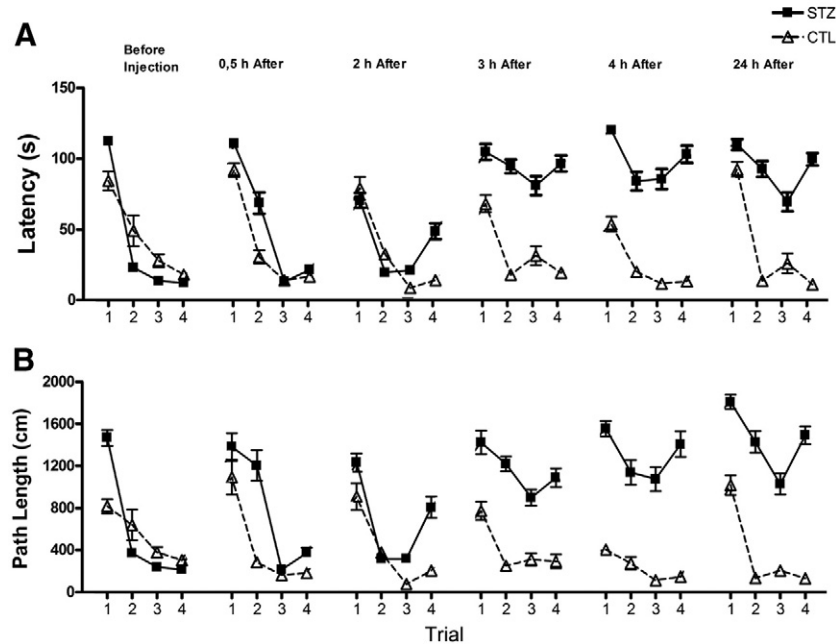
As expected, pre-STZ injection behavioral data analysis revealed lack of significant STZ and CTL group differences for (1) latency, path length and time percentual within the critical training quadrant along the 5 sessions of pre-surgical reference memory training, (2) time spent within the critical counter and counter frequency during the probe test, and (3) latency, path length and spatial bias towards the session-before critical location during the working memory test, indicating that the subjects allocation to the groups to be later injected with either STZ or vehicle was effective since no pre-treatment, significant behavioral differences were observed. In addition, data showed that the surgical procedure for implantation of the guide cannulae by itself (that occurred prior to the STZ injection) did not interfere with performance achieved at the end of the pre-surgical training in the reference memory task (data not shown).

Early effects of STZ on performance of the working memory task was assessed by requiring the rats to learn a new platform location in each of 6 sessions executed 0.5, 1, 2, 3, 4, and 24 h after STZ injection, in a matching to place procedure with four trials per session, and by comparing these results with those of a working memory session run just before the STZ injection. Fig. 1 shows performance in terms of latency (Fig. 1A) and path length (Fig. 1B). Note that for comparison purposes, data of the last working memory session prior to the injections are presented at the left panel of each figure.

Statistical analysis of the results and interaction effects are presented in Table 1. In relation to latency scores (Fig. 1A) repeated measures ANOVA revealed Group, Session and Trial main significant effects. ANOVA also revealed Group×Session, Group×Trial, Session×Trial, and Groups×Session×Trial interaction effects. In relation to path length scores (Fig. 1B) repeated measures ANOVA revealed Group, Session and Trial main significant effects. ANOVA also revealed a Group×Session interaction effect. Post hoc analyses revealed, as expected, lack of significant differences between performances of both



## Acute Effects of STZ Injection on Working Memory



**Fig. 1.** Early effects of intracerebroventricular STZ or vehicle injections on distinct working memory parameters in rats. Mean values  $\pm$  SEM are expressing the groups' performance in terms of latency (in seconds, A) and path length (in centimeters, B) in 4 trials, after 0.5, 2, 3, 4, and 24 h of injections. For comparison purposes, data of the last working memory session prior to injection were presented at the left panel of each figure. Data were analyzed using repeated measures analysis of variance (ANOVA) with Sessions (before injection, 0.5, 2, 3, 4, and 24 h after injection) as the within-subject factor, and Group (CTL and STZ) as the between-subject factor. Significance level adopted was  $P < 0.05$ .

groups in the session run before the injection, indicating that Groups performance prior to STZ injection was equivalent. In contrast, post hoc analyses revealed (1) significant latency and almost significant path length Groups differences in the second trial of the session run 0.5 h after STZ injection, but lack of significant latency and path length differences in the remaining trials within this session (see Fig. 1A and B, respectively), (2) no significant Groups differences in the session run 2 h after STZ injection, and (3) significant Groups differences in the sessions run 3, 4 and 24 h after the STZ injection. As Fig. 1 shows, while in the session run 0.5 h after STZ injection, STZ subjects exhibited disruption in acquisition (see trial 2), but not in maintenance (see trials 3 and 4) of the spatial information required for performance of the working memory task. In the sessions run 3, 4 and 24 h after STZ injection there was a marked disruption of acquisition and maintenance of the spatial information.

### 3.1.2. Late behavioral effects of STZ

STZ injection disrupted acquisition of the spatial navigation reference memory task in the water maze, as revealed by the significant Group effects and by the significant Session  $\times$  Group interaction effects for latency, path length, percentage of time within the critical quadrant and percentage of time within the critical annulus (Fig. 2A). ANOVA also revealed a significant Session effect for latency, percentage of time within the critical quadrant and percentage of time within the critical annulus. In addition, there were significant

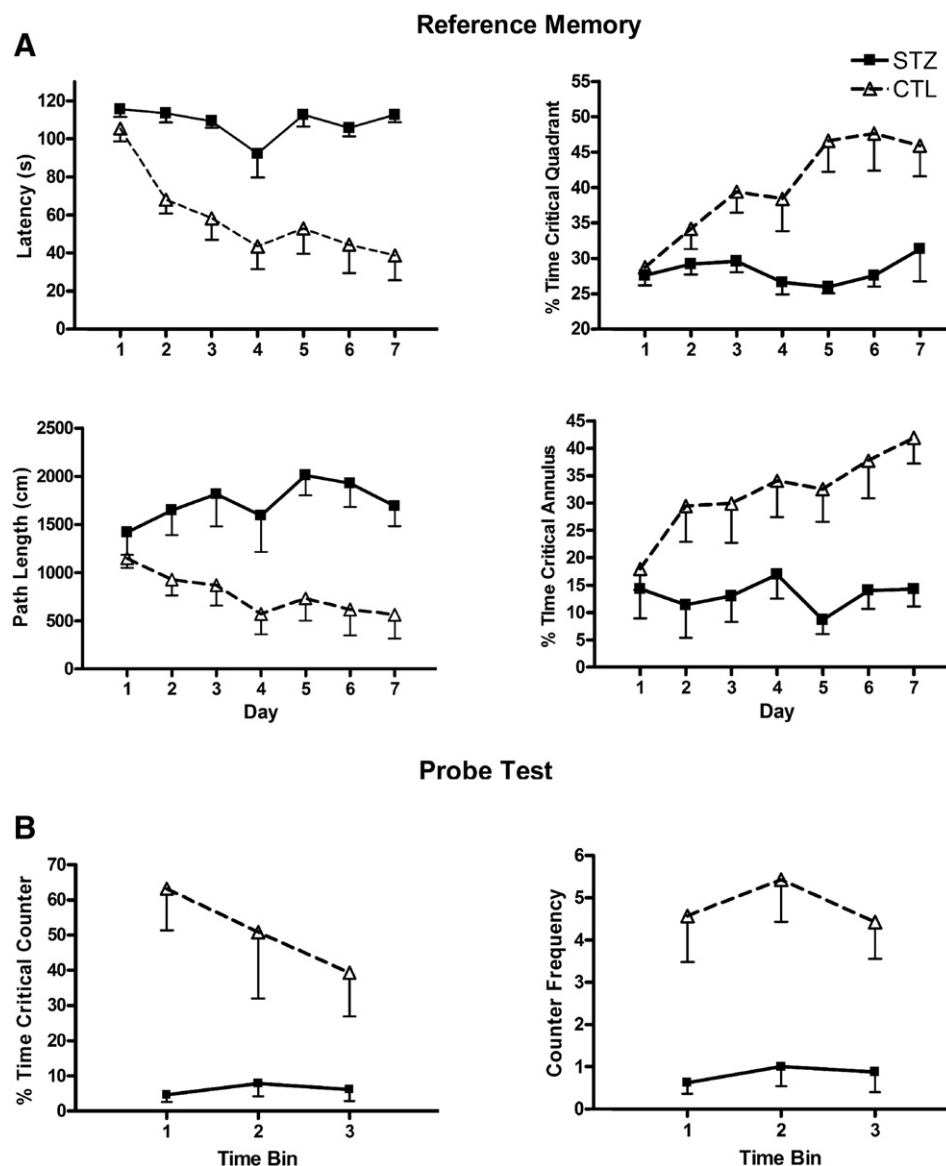
Trial effects for latency and path length, and a significant Trial  $\times$  Group interaction effect for latency. In fact, Fig. 2A shows that CTL subjects exhibited a typical reduction of latency and path length, associated with an increase in the percentage of time spent within the critical quadrant and within critical annulus, along sessions. In contrast, STZ group exhibited a much smaller (if any) reduction in both latency and path length, and a smaller increase in the percentage of time spent within the critical quadrant and critical annulus, thus indicating marked disruption of performance (Table 2).

In the probe test, when the platform was removed, the CTL subjects, as expected, spent substantial amount of time searching for the platform in its former location indicating that the animals remembered that spatial information (Fig. 2B). This behavior tends to extinguish along time bins (Fig. 2B), thus reflecting the flexibility of the animals to deal with the novel contingency involving the lack of the platform. In contrast, STZ subjects exhibited a marked disruption of performance, as reflected by significant reductions of the time spent within the critical counter, the counter frequency, the percentage of time spent within the critical counter, and by a close to significant reduction in the percentage of time spent within the critical quadrant.

In the working memory task the animals learned a new platform location every day, in a matching-to-place procedure involving 4 trials per day. At the beginning of the first trial of each day the platform location was unknown. However, the animals received this

**Table 1**  
Early behavioral effects of STZ.

| Before the test |       |        |         |        |                         |       |               |         |               |        |             |      |        | Post hoc 0.5 h |        | Post hoc 3, 4 and 24 h |        |      |
|-----------------|-------|--------|---------|--------|-------------------------|-------|---------------|---------|---------------|--------|-------------|------|--------|----------------|--------|------------------------|--------|------|
| Group           |       |        | Session |        | Session/trial/<br>group |       | Session/group |         | Session/trial |        | Trial/group |      | Trial  |                | Group  |                        | Group  |      |
| F 1,10          |       | P      | F 5,50  |        | F 15,150                |       | F 5,50        |         | F 5,150       |        | F 3,30      |      | F 3,30 |                | F 1,10 |                        | F 1,10 |      |
| LAT             | 14.78 | 0.003  | 7.02    | 0.0001 | 2.46                    | 0.006 | 12.60         | 0.0001  | 3.03          | 0.0009 | 4.17        | 0.03 | 112.97 | <0.0001        | 3.03   | 0.05                   | 6.66   | 0.03 |
| PL              | 21.19 | 0.0001 | 3.01    | 0.02   | nd                      | nd    | 6.33          | <0.0001 | 1.92          | 0.01   | 2.99        | 0.05 | 39.32  | 0.0001         | 4.21   | 0.07                   | nd     | nd   |



**Fig. 2.** Late effects of intracerebroventricular STZ or vehicle injections on distinct reference memory and probe test parameters in rats, evaluated by Morris' water maze task. Mean values  $\pm$  SEM of trials in the same group are expressing the performance in terms of latency (in seconds), path length (in centimeters), percent time in critical quadrant, and percent time in critical annulus over 7 days of sessions (4 trials per session) on reference memory task (A), percent time and frequency within the critical counter in each of three 1-min consecutive time bins in probe test (B). Data were analyzed using repeated measures analysis of variance (ANOVA) with the groups (CTL and STZ) as the between-subject factor, and day (1 to 7 or 1 to 4), time bin (1 to 3 trials) or trial (1 to 4) as the within-subject factor. Significance level adopted was  $P < 0.05$ .

information at the end of the first trial. Therefore, improvements of performance along the remaining trials within a day reflect the animals' ability to maintain the critical information about the platform location in working memory. The ITI was either 10 min (along 5 days) or virtually zero min (in the last day). Repeated measures ANOVA revealed significant Group and Trial main effects for latency and path length, and a significant Trial  $\times$  Group interaction effect for latency (Fig. 3A). These figures indicate that STZ subjects exhibit difficulties to deal with the spatial working memory task. ANOVA also revealed a close to significant Group main effect for percentage of time spent within the critical training counter and significant Trial and Trial  $\times$  Group interaction effects for counter frequency and percentage of time spent within the critical counter relative to other symmetrically located counters (Fig. 3A). These results show that the CTL subjects search for the platform in the area of the pool where the platform was located the day before, particularly in the first trial of each day then extinguishing this behavior along the remaining trials, thus indicating that they remember the prior

platform location. In contrast, STZ group does not exhibit this pattern of behavior, indicating that they do not remember the former platform location (Fig. 3A).

Together these results indicate that subjects submitted to STZ injection exhibited poorer performance in the working memory version of the water maze task as compared to CTL subjects (Fig. 3A).

As performance of the STZ group was disrupted, the ITI was reduced to 0 min in order to evaluate to which extent this could lead to improvement of performance as compared to that seen when the ITI was 10 min. However, ANOVA revealed lack of significant effects for ITI and its interaction with other factors, indicating that the disruption seen in STZ subjects was independent of the ITI (Fig. 3B).

### 3.2. Immunoblotting

Subjects injected with STZ exhibited significant changes of the A $\beta$  peptide, the ratio phosphorylated Tau/total Tau, and the GFAP levels in specific brain areas (Fig. 4 – data presented as the

**Table 2**  
Late behavioral effects of STZ.

| Reference memory |          |          |         |          |             |          |               |          |             |          |
|------------------|----------|----------|---------|----------|-------------|----------|---------------|----------|-------------|----------|
|                  | Group    |          | Session |          | Trial       |          | Session/group |          | Trial/group |          |
|                  | F 1,16   | <i>P</i> | F 6,96  | <i>P</i> | F 3,48      | <i>P</i> | F 6,96        | <i>P</i> | F 3,48      | <i>P</i> |
| LAT              | 9.64     | 0.05     | 2.93    | 0.02     | 8.43        | 0.001    | 2.63          | 0.05     | 5.08        | 0.01     |
| PL               | 10.60    | 0.005    | nd      |          | 13.37       | <0.0001  | 3.42          | 0.004    | nd          |          |
| QD4              | 16.60    | 0.0009   | 3.91    | 0.001    | nd          |          | 3.84          | 0.001    | 2.77        | 0.05     |
| ANB              | 9.64     | 0.006    | 2.93    | 0.01     | nd          |          | 2.93          | 0.02     | 2.78        | 0.05     |
| Probe test       |          |          |         |          |             |          |               |          |             |          |
|                  | Group    |          |         |          |             |          |               |          |             |          |
|                  | F 1,13   |          |         |          |             |          |               |          |             |          |
|                  | <i>P</i> |          |         |          |             |          |               |          |             |          |
| TC4              | 13.67    |          |         |          |             |          |               |          |             |          |
| F4               | 33.57    |          |         |          |             |          |               |          |             |          |
| QD4              | 17.17    |          |         |          |             |          |               |          |             |          |
|                  |          |          |         |          |             |          |               |          |             |          |
| Working memory   |          |          |         |          |             |          |               |          |             |          |
|                  | Group    |          | Trial   |          | Trial/group |          |               |          |             |          |
|                  | F 1,14   | <i>P</i> | F 3,42  | <i>P</i> | F 3,42      | <i>P</i> |               |          |             |          |
| LAT              | 13.86    | 0.002    | 14.63   | 0.0001   | 3.52        | 0.02     |               |          |             |          |
| PL               | 16.34    | 0.001    | 14.63   | <0.0001  | nd          |          |               |          |             |          |
| QDant            | 14.12    | 0.002    | 7.07    | 0.0006   | 3.27        | 0.04     |               |          |             |          |
| TCANT            | nd       |          | 10.85   | <0.0001  | 9.67        | <0.0001  |               |          |             |          |
| FANT             | nd       |          | 9.81    | <0.0001  | 5.65        | 0.003    |               |          |             |          |

percentage of variation relative to controls), as revealed by optical densitometry (Table 3). In contrast, there were no changes of ChAT expression in any of these brain regions.

### 3.2.1. A $\beta$ peptide

Animals injected with STZ, relative to controls, showed a significant increase of A $\beta$  peptide expression in several brain regions (the specific statistical differences are presented in Fig. 4A). As Fig. 4A shows, there was a 40.7% increase in the expression of A $\beta$  in the enthorinal cortex and a 41.8% increase in the basal ganglia. In addition, the hypothalamus presented a more pronounced variation in the peptide expression, with an increase of 86.6% relative to controls. The hippocampus and neocortex of STZ subjects did not show significant changes in A $\beta$  levels relative to corresponding brain regions of CTL subjects.

### 3.2.2. Ratio phosphorylated Tau/total Tau

Relative phosphorylation values were calculated by the ratio between phosphorylated Tau and total Tau levels, and considered for statistical analysis (Fig. 4B). Animals injected with STZ, relative to CTL, showed a significant increase of the relative Tau phosphorylation in the majority of brain regions analyzed (the specific statistical differences are presented in Fig. 4B).

There was a significant increase of 63% in the relative Tau phosphorylation levels in the neocortex and a marked increase of 298.7% in basal ganglia. The only structure that exhibited a decrease in the relative Tau phosphorylation levels was the enthorinal cortex (43.88%). Other structures, such as the hippocampus and the hypothalamus presented no significant differences in the relative Tau phosphorylation between animals of the STZ and CTL groups.

### 3.2.3. GFAP

Animals injected with STZ, relative to CTL subjects, showed significant increments of GFAP expression in the neocortex, hippocampus and basal ganglia, and a significant decrement in GFAP expression in the enthorinal cortex (the specific statistical differences are presented in Fig. 4C). There was a 284.9% increase of GFAP in the neocortex, 205.71% increase in the hippocampus, and 162.8% increase in the basal ganglia. Similarly to the observed for the relative Tau phosphorylation,

the enthorinal cortex exhibited a decrease of GFAP levels (71.86%) after STZ injections. The hypothalamus exhibited no significant changes of GFAP after STZ injections.

### 3.3. Fluoro-Jade C

Fluoro-Jade C histochemistry was used to reveal neurodegeneration in STZ-injected animals. As expected, CTL animals did not show any labeling whatsoever. In contrast, STZ injected animals exhibited stained fibers and neurons in distinct brain areas, at different time points. For instance, one day after STZ injection, Fluoro-Jade C-positive neurons were seen in the hypothalamus, near the third ventricle, and in the septal area. In addition, labeled perikarya and neuropil in the hippocampal CA1 pyramidal layer were also seen after one day of the STZ injection (Fig. 5). Similar results were observed after 15 days, with labeled neurons in the striatum and labeled perikarya and neuropil in the hippocampal CA1 pyramidal layer (Fig. 6). On the other hand, we did not observe any staining in any brain region after 30 days of STZ injection.

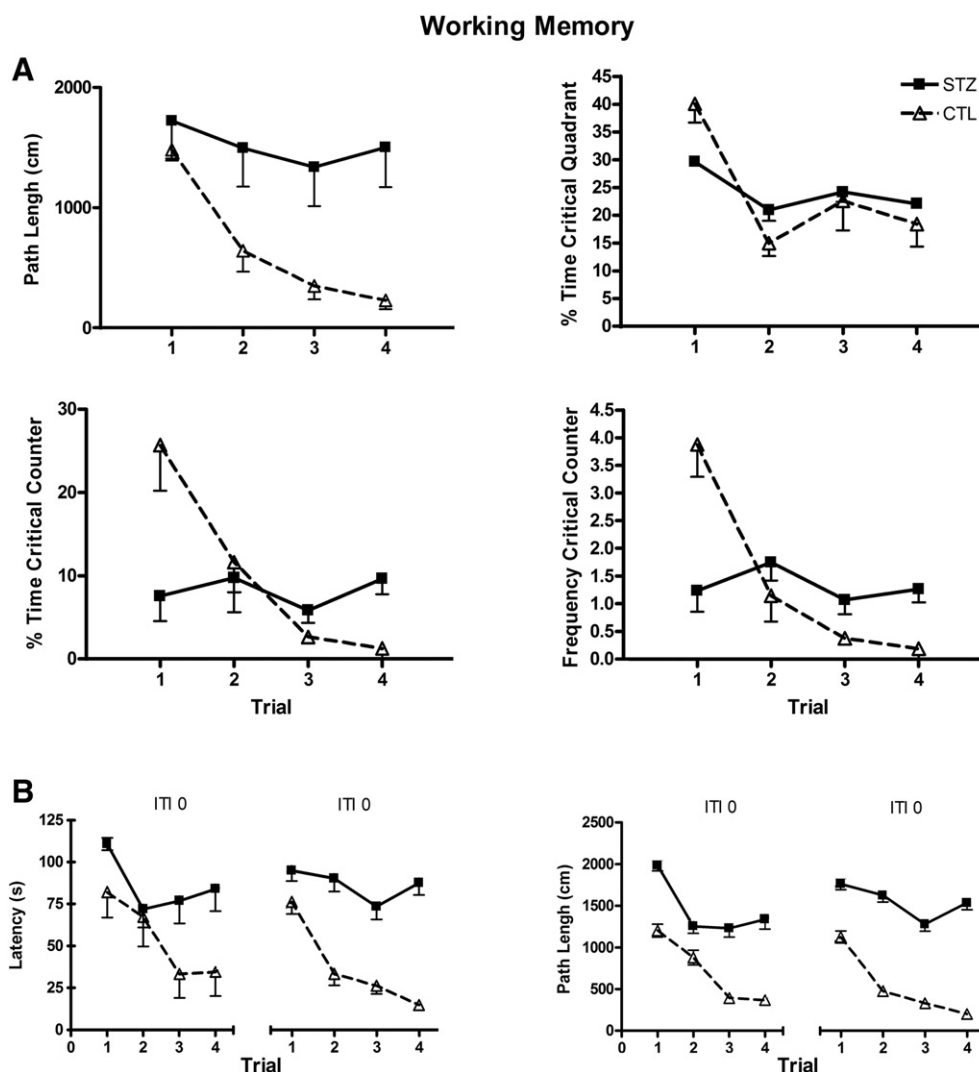
### 3.4. Peripheral blood glucose level

There were no significant group differences in the peripheral blood glucose levels at any time point after the injections (data not shown).

## 4. Discussion

Our results revealed behavioral and molecular changes, with distinct time courses, that followed icv STZ injection. A disruption on the working memory was observed as early as 3 h after injections, which was followed by degenerative processes in the hippocampus at 1 and 15 days after STZ injections. Memory disruption increases over time and culminates with significant changes of  $\beta$ A peptide and hyperphosphorylated Tau protein levels in distinct brain structures.

Alzheimer's disease, clinically characterized by a progressive loss of cognitive functions, especially a pronounced decline of memory [1], is the most common cause of dementia in aged humans and the proportion of elderly people in world populations is gradually



**Fig. 3.** Late effects of intracerebroventricular STZ or vehicle injections on distinct working memory parameters in rats, evaluated by Morris' water maze task. Mean values  $\pm$  SEM of trials in the same group are expressing the performance in terms of path length (in centimeters), percent time within critical counter, percent time within critical quadrant, and frequency within the critical counter over 4 days of sessions (4 trials per session) with intertrial intervals of 10 min (ITI10) on working memory task (A) latency (in seconds) and path length (in centimeters) in one session (4 trials) with intertrial intervals of 0 min (ITI0) and 10 min (ITI10) on working memory task (B). Data were analyzed using repeated measures analysis of variance (ANOVA) with the groups (CTL and STZ) as the between-subject factor, and day (1 to 7 or 1 to 4) or trial (1 to 4) as the within-subject factor. Significance level adopted was  $P < 0.05$ .

increasing. In addition to aging as the main risk factor, other factors may play important roles in pathogenesis of AD, including changes in energy metabolism, oxidative stress, pro-death gene activation, and the cholinergic system [11,32]. In an attempt to understand the pathogenesis of AD, different groups have employed both *in vivo* and *in vitro* approaches. However, none of these models seem to actually mimic the whole disease process [33].

The hypothesis of glucose metabolism and insulin signaling disruption as a likely etiology for AD has been investigated by different groups [5,9,11,21,22]. Behavioral and molecular findings which follow glucose metabolism and insulin signaling disruption seem to mimic at least some aspects of the AD. For instance, icv injection of STZ leads to cognitive impairments in memory tasks including the Morris' water maze, the passive avoidance, and the elevated plus-maze [26–29]. However, these studies assessed memory long after disruption of glucose metabolism and insulin signaling, restricting tagging of the progressive symptoms of the disease. The underlying assumption, derived from Bird and colleagues [17] study with humans, was that the working memory version of the Morris' water maze task is more suitable to investigate early AD-like symptoms following icv STZ injection in animals. In the present study, early and late effects of icv STZ injection on cognitive

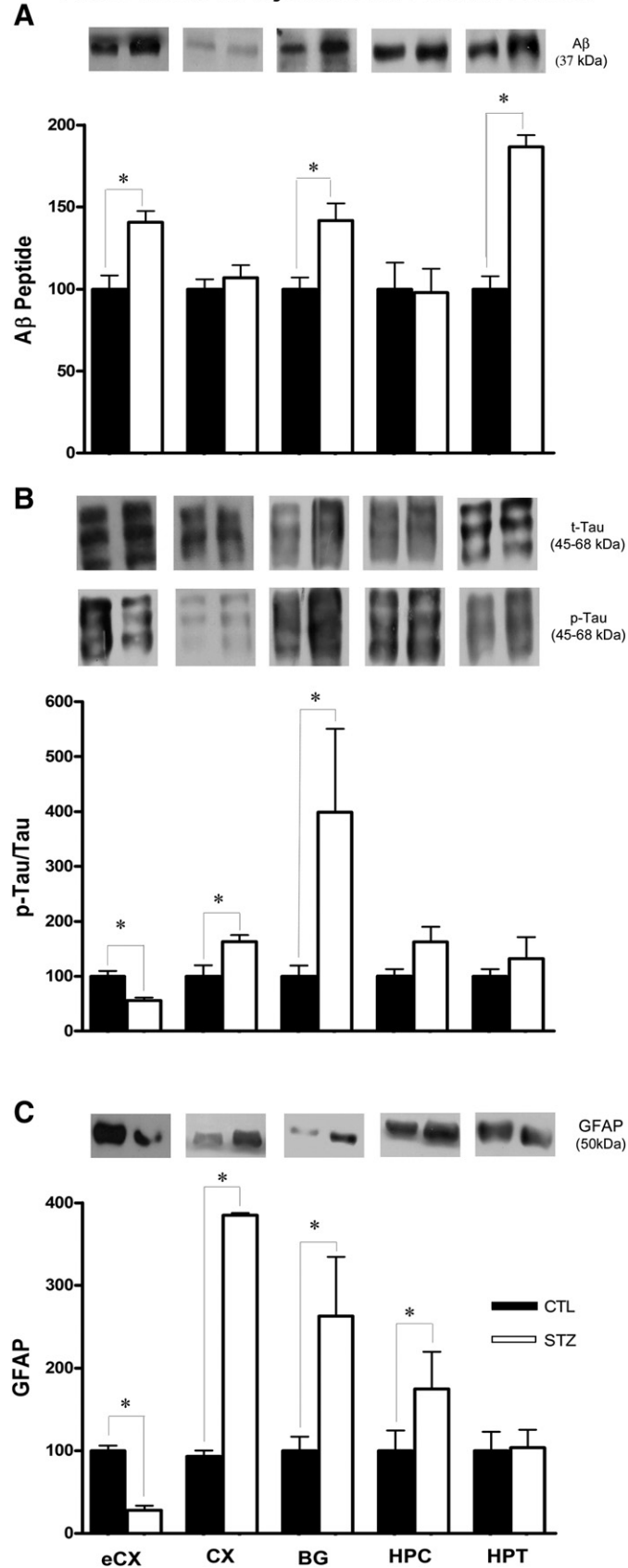
function were evaluated using the spatial working and the spatial reference memory versions of the Morris' water maze task, as a model for the disease progression.

The present results revealed different types of cognitive disruptions after icv STZ injection, with distinct time courses. However, data revealed a sustained disruption of acquisition and maintenance of the spatial information about the platform location starting 3 h after STZ injection. Cognitive impairments were marked and expressed both as increased latency and path length to find the platform in any of the four trials within each session, and as a smaller improvement of performance along trials within each session. These results indicate that STZ subjects exhibit substantial difficulties to acquire and/or maintain spatial information about the platform location along trials within a session, about 3 h or longer after the icv injection. These STZ-induced disruptions were also reflected on the searching pattern in the first trial of each session, when none of the groups know the platform location. As far as we know, this is the first report of early STZ-induced disruption of rats performance in a working memory task, which could be related to Bird and colleagues [17] observation that patients at the earliest stages of the AD exhibit mild short-term memory impairments. It is well known that AD is a



lengthy and progressive neurodegenerative disease. There have been reports that the onset dementia appears clinically observed at least a decade after the onset of the disease at the molecular level [34,35].

### Late Effects of injection on Protein Levels



Accordingly, atrophy in the enthorinal cortex and hippocampus may be detected before the occurrence of clinically relevant symptoms [36]. This lack of clinical symptoms associated with ongoing detectable neurodegenerative processes may be related to the use of clinical tests insensitive to the ongoing changes.

The behavioral disruption observed in the present study shortly after the STZ icv injection may be a result of direct alkylating and oxidizing effects of this drug [37,38], which could be sufficiently intense in brain regions reached by the drug to trigger neuronal death. In addition, certain brain regions might be more susceptible to the STZ effects because of ineffective defenses against DNA damage or against reactive oxygen species. For example, the high expression levels of transient receptor potential channels in hippocampal neurons [40] could render them more susceptible to reactive oxygen species, thus leading to neurodegenerative processes related to AD [41]. STZ-induced necrosis in pancreatic beta cells results from its DNA alkylating properties and generation of reactive oxygen species [37]. The STZ structure is similar to that of glucose, which facilitates its access to beta cells via low-affinity type 2 glucose transporter (GLUT2) [38,42,43]. It is not known if comparable mechanisms take place in neurons. Nevertheless, there have been reports that GLUT2 is expressed in the nervous system, particularly in the dentate gyrus and distinct nuclei of hypothalamus [39,44,45]. Therefore, it seems plausible to hypothesize that STZ gets into these neurons via GLUT2 [21]. On the other hand, Grünblatt and colleagues [22] observed impairments of memory in rats pre-treated with inhibitors of GLUT2 and then submitted to icv injections of STZ, suggesting that other transporters might also be involved. Even though the present study did not specifically address this question, it seems interesting to note that degenerating neurons, revealed by Fluoro-Jade C, were found in the hypothalamus one day after the STZ injection. Not surprisingly, hypothalamus has been described to contain high levels of GLUT2 [39,44]. In addition, Shoham and colleagues [46] observed an enlargement of the third ventricle after STZ injection, consistent with the hypothesis of neuronal loss in the hypothalamus. The results of the present study related to early evaluations favor the idea that the degenerative processes which follow icv STZ injection may be revealed both histologically in the hypothalamus, hippocampus and septal area from 24 h after the drug injection, and behaviorally as early as 0.5 h after the injection, being more prominent after 3 h, when the adequate behavioral task is employed. Even though there are analogous cognitive changes in this animal model of AD and the mild cognitive disruption in patients at early stages of AD, it seems important to emphasize that this model should be seen cautiously when compared to the human pathology.

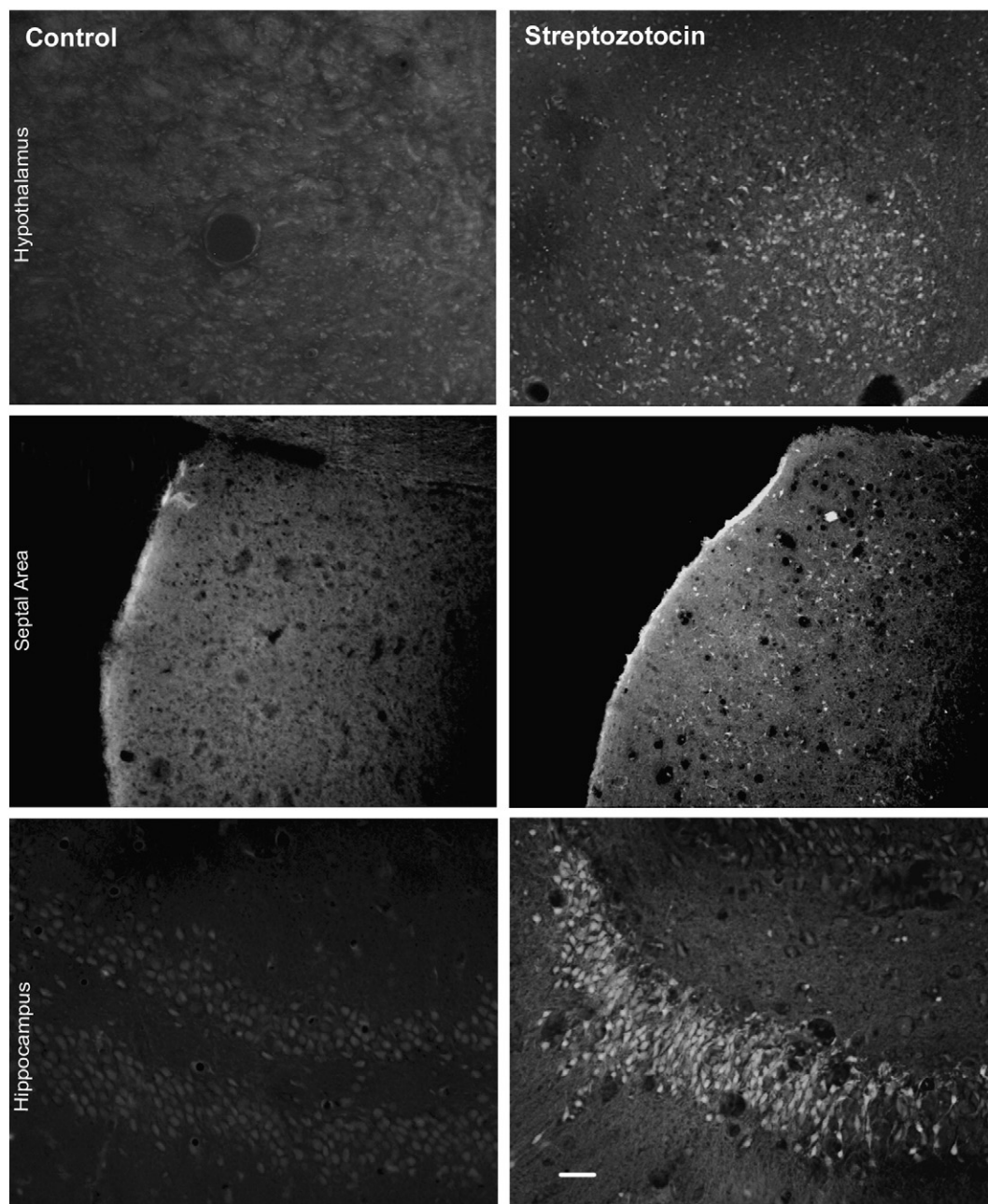
Prior studies described the development of long-lasting learning and memory disturbances which follow icv injection of STZ to abnormalities in neuronal glucose metabolism, decreased insulin receptor and insulin resistance [5,6,21,48,49]. The results of the present study do not conflict with this possibility. The late effects of STZ injection reported in the present study generally confirm and extend data of other groups using the same animal model [7,9,12,14,15]. In short, STZ-injected animals showed impairments of performance in the reference memory version, in the probe test, and in the working memory version of the Morris' water maze task. In the reference memory

**Fig. 4.** Late effects of intracerebroventricular STZ or vehicle injections on protein levels in distinct brain regions measured by immunoblotting analysis (data presented as the percentage of variation relative to controls). Samples from the homogenate containing 50 µg of protein were subjected to SDS-PAGE gel and electrotransferred to nitrocellulose membranes. Graphs express changes in protein levels (percentage) of STZ group in relation to CTL group of Aβ (A), ratio phosphorylated Tau/Tau total (B), and GFAP (C). As a loading control GAPDH was performed in all experiments (Supplementary Figure). eCX = enthorinal cortex, CX = cortex, BG = basal ganglia, HPC = hippocampus, and HPT = hypothalamus. Data (mean values ± SEM) were analyzed using absolute values obtained from optical densitometry and submitted to *t*-test statistical analysis. Significance level adopted was *P* < 0.05.

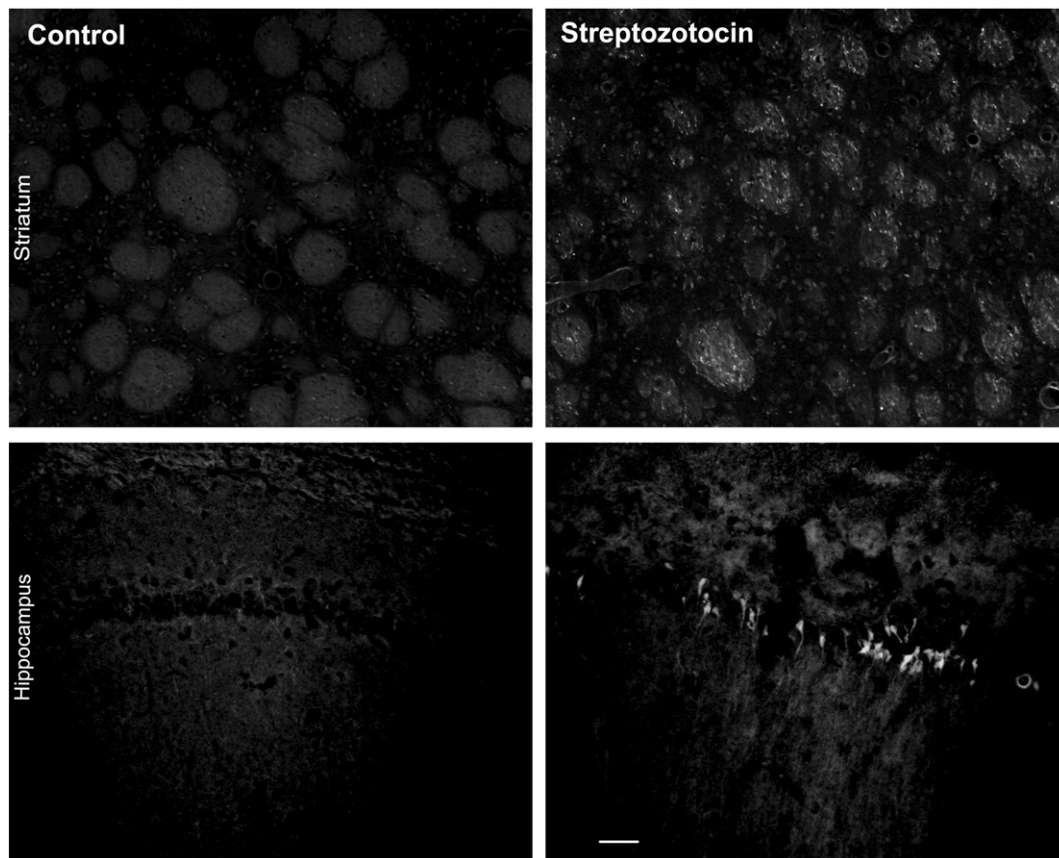
**Table 3**  
Immunoblotting analysis of protein expression with densitometry.<sup>a</sup>

| Brain areas | Group | $\beta$ A        |         |                   | Tau               |         |                    | GFAP             |         |                    | ChAT            |         |                    |
|-------------|-------|------------------|---------|-------------------|-------------------|---------|--------------------|------------------|---------|--------------------|-----------------|---------|--------------------|
|             |       | Optic density    | P-value | % of change       | Optic density     | P-value | % of change        | Optic density    | P-value | % of change        | Optic density   | P-value | % of change        |
| eCx         | CTL   | 1 $\pm$ 0.082    |         |                   | 1 $\pm$ 0.98      |         |                    | 1 $\pm$ 0.64     |         |                    | 1 $\pm$ 0.21    |         |                    |
|             | STZ   | 1.41 $\pm$ 0.68  | 0.0089  | $\uparrow$ 40.7   | 0.56 $\pm$ 0.054  | 0.0052  | $\downarrow$ 43.88 | 0.28 $\pm$ 0.53  | 0.0010  | $\downarrow$ 71.86 | 0.88 $\pm$ 0.12 | 0.6656  | $\downarrow$ 11.71 |
| BG          | CTL   | 1 $\pm$ 0.071    |         |                   | 1 $\pm$ 0.193     |         |                    | 1 $\pm$ 0.168    |         |                    | 1 $\pm$ 0.036   |         |                    |
|             | STZ   | 1.42 $\pm$ 0.104 | 0.0063  | $\uparrow$ 41.8   | 3.99 $\pm$ 1.519  | 0.0407  | $\uparrow$ 298.7   | 2.63 $\pm$ 0.71  | 0.0271  | $\uparrow$ 162.80  | 0.94 $\pm$ 0.08 | 0.4876  | $\downarrow$ 6.45  |
| HPT         | CTL   | 1 $\pm$ 0.077    |         |                   | 1 $\pm$ 0.131     |         |                    | 1 $\pm$ 0.229    |         |                    | 1 $\pm$ 0.08    |         |                    |
|             | STZ   | 1.87 $\pm$ 0.071 | 0.0001  | $\uparrow$ 86.6   | 1.32 $\pm$ 0.392  | 0.5107  | $\uparrow$ 31.82   | 1.04 $\pm$ 0.21  | 0.9126  | $\uparrow$ 3.82    | 1.07 $\pm$ 0.09 | 0.5670  | $\uparrow$ 7.30    |
| HPC         | CTL   | 1 $\pm$ 0.016    |         |                   | 1 $\pm$ 0.131     |         |                    | 1 $\pm$ 0.157    |         |                    | 1 $\pm$ 0.13    |         |                    |
|             | STZ   | 0.98 $\pm$ 0.014 | 0.9271  | $\downarrow$ 2.06 | 1.62 $\pm$ 0.276  | 0.0897  | $\uparrow$ 61.65   | 3.06 $\pm$ 0.31  | 0.0040  | $\uparrow$ 205.71  | 1.05 $\pm$ 0.06 | 0.7756  | $\uparrow$ 4.80    |
| Cx          | CTL   | 1 $\pm$ 0.058    |         |                   | 1 $\pm$ 0.201     |         |                    | 1 $\pm$ 0.038    |         |                    | 1 $\pm$ 0.048   |         |                    |
|             | STZ   | 1.07 $\pm$ 0.076 | 0.4984  | $\uparrow$ 7.00   | 1.63 $\pm$ 0.1221 | 0.0212  | $\uparrow$ 63.00   | 3.85 $\pm$ 0.030 | 0.0003  | $\uparrow$ 284.90  | 0.83 $\pm$ 0.11 | 0.1653  | $\downarrow$ 17.37 |

<sup>a</sup> Values correspond to arbitrary densitometry units (mean  $\pm$  SEM). Absolute values were analyzed using Student *t*-test. Changes are given as percentage of STZ group in relation to CTL group.



**Fig. 5.** Fluoro-Jade C histochemistry staining in distinct brain regions after 1 day of the intracerebroventricular STZ or vehicle injections. Digital images of rat brain sections showing Fluoro-Jade C-positive neurons in the hypothalamus, septal area, and perikarya and neuropil hippocampal CA1 pyramidal layer of STZ groups, indicating the degenerating process. Scale bar = 50  $\mu$ m.



**Fig. 6.** Fluoro-Jade C histochemistry staining in distinct brain regions after 15 days of the intracerebroventricular STZ or vehicle injections. Digital images of rat brain sections showing Fluoro-Jade C-positive neurons in the striatum and perikarya and neuropil hippocampal CA1 pyramidal layer of STZ groups, indicating the degenerating process. Scale bar = 50  $\mu$ m.

version, despite the marked disruption of STZ animals relative to CTL subjects, there was some improvement of performance along repeated training, indicating that STZ subjects acquired relevant information about the presence of the platform in the pool, and had a general idea of where to find it. This pattern of results is typical of animals with hippocampal damage [19] and seems to be related to the kind of behavioral strategy used by the animals to perform this task. The use of multiple starting locations during training, as employed in the present study, stimulates the use of cognitive mapping. Rats with hippocampal damage lose their ability to use cognitive maps but not the other strategies [47]. Thus, they usually rely on the remaining, less precise, strategies to perform the task. These results render plausible to hypothesize that icv STZ injection interferes with hippocampal functions. STZ-injected rats seem to remember some clues out of the maze and that guides them to the side of the pool where the platform was located. In addition, STZ-injected rats showed, over the time bin intervals, a slight increase in entry frequency and time spent at the critical counter, which may suggest that after frequent exposure to environmental stimuli they have improved their ability to rescue critical information from memory.

As Olton [50] defended, tasks requiring reference memory emphasize relevant information applicable to all trials. Thus, the Morris' water maze task corresponds to a spatial reference memory task if the platform position is kept constant throughout all training sessions. In a different way, working memory refers to maintenance of information about specific personal and temporal contexts. Therefore, it maintains relevant information applicable to a specific trial that does not apply to others. Xavier and colleagues [19], however, defended that if a different position for the hidden platform is used on each session, per day of training in the Morris' water maze, the critical spatial information about the platform location would be

acquired on the first trial of every session, being applicable to the remaining trials of that specific session but not to other sessions. In this way, one should to consider it as a spatial working memory task. Thus, this behavioral task would correspond to the rodents' analogous to topographical short-term memory-sensitive task reported by Bird and colleagues [17] that was sensitive to early stages of humans' AD.

The late effects of STZ injection on performance of the working memory task were marked. That is, relative to CTL subjects, STZ-injected subjects did not exhibit any improvement of performance along trials independently on being tested with an ITI of 10 min or even when the ITI was virtually 0 min. In other words, STZ-injected rats did not benefit from information about the platform location obtained on the previous trials within a session. In fact, when comparing the results of working memory tests with ITI10 and ITI0, we can clearly see that working memory was impaired in STZ rats only when the intervals were 10 min. When the interval was virtually zero, STZ rats showed a significant improvement. This fact suggests that the lesioned rats have some kind of information acquisition, whereas their retention mechanisms are highly compromised.

The histochemical analysis after periods longer than 15 days, are less conclusive. Degenerative process is visible after 15 days of the injection but not after 30 days when the cognitive impairments are still noticeable. This apparent disparity may suggest that in periods longer than 15 days, the neurons are going through a recovery process, a period in which there is no longer neuronal death. Other possibility is that the time points analyzed are critical to the efficiency of histochemical technique of Fluoro-Jade. The Fluoro-Jade is an acidic dye which seems to have affinity by a presumably basic molecule expressed during the degeneration process of neurons [31]. However, the results obtained with immunoblotting technique seem to validate this animal model as a neurodegenerative AD-like model, as significant changes of  $\beta$ A peptide



and phosphorylated Tau protein levels, both major hallmarks of AD, were observed after 30 days of drug injection. In most studies using *in vivo* models for induction of AD, such as transgenic animal models or the model of STZ icv injection, is seldom found the description of increased  $\beta$ A or its aggregation [51]. Our work has shown, however, a significant increase of the expression of  $\beta$ A in the hypothalamus, entorhinal cortex and the basal ganglia. We also observed hyperphosphorylation of Tau, especially in the entorhinal cortex, neocortex, and basal ganglia. In fact, Grünblatt and colleagues [22] showed that icv injection of STZ at low doses causes the hyperphosphorylation of Tau in the hippocampus. It is curious, however, that we did not find changes in these molecule levels in the hippocampus, a structure mainly related to memory function and that seems to be the target of a degenerative process, as seen in the behavioral and histochemical analyses. On the other hand, significant changes in GFAP levels were observed in the hippocampus, as well as in other structures, indicating that at this time point after the STZ injection, an inflammatory process is installed. The increase of GFAP is associated with oxidative and inflammatory reactions and, indirectly, with neurodegeneration. Exacerbated glial response and especially an increase in the activation of astrocytes and microglia in the vicinity of plaques have also been reported in AD [52,53].

The cholinergic system is implicated in consolidation and recovering of memory, and is severely disrupted in AD [1,54,55]. Previous studies have shown, for example, a reduction in ChAT activity of [56,57] and an increase in the function of acetylcholinesterase [58,59] in this animal model. However, although our behavioral data on late STZ effects showed clearly a degenerative process of structures related to memory function, we did not find any change of ChAT levels in our immunoblotting analysis in any of the evaluated structures.

As the molecular data point sometimes to distinct directions in relation to behavioral data, we can suggest that STZ acted during this long term period in a non-lethal manner in some group of neurons that did not undergo cell death in the initial periods after the injections. This could mean that the drug in subtoxic doses could somehow trigger biochemical changes required for the development of a pathological process similar to that found in AD. Occasionally, such changes would be incompatible with cell viability and culminate in neuronal collapse.

In conclusion, we show that icv injection of STZ triggers negative effects on rat behavioral performance and in neurodegenerative processes. These early processes seem to persist at the behavioral level and might culminate with changes in molecular levels. These findings could add information on the AD-like STZ animal model, as well as on the mechanisms underlying neurodegenerative diseases.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.physbeh.2012.06.019>.

## Disclosure statement

The authors disclose no conflicts of interest.

## Acknowledgments

This study has been supported by FAPESP, CAPES-PROEX, and CNPq, Brazil (A.S.T., G.F.X.). T.O.S. and C.H.Y.M. were recipients of fellowships from FAPESP.

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