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ALZHEIMER-LIKE CHANGES OF PROTEIN KINASE B AND GLYCOGEN SYNTHASE KINASE-3 IN RAT FRONTAL CORTEX AND HIPPOCAMPUS AFTER DAMAGE TO THE INSULIN SIGNALLING PATHWAY

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Abbreviations: GSK-3α/β, glycogen synthase kinase-3α/β); pGSK-3α/β, phosphorylated GSK-3α/β; Akt/PKB, protein kinase B; IR, insulin receptor; STZ, streptozotocin; TG, 5-thio-

D-glucose; GLUT2, glucose transporter-2; APP, amyloid precursor protein; icv,

intracerebroventricular.

Abstract

The insulin resistance brain state is related to the late-onset sporadic Alzheimer's disease, and alterations of insulin receptor (IR) and its downstream phosphatidylinositol-3 kinase signalling pathway have been found in human brain. Confirmation studies have not been performed in an experimental model probably related to the sporadic Alzheimer's disease, e.g., rats showing a neuronal IR deficit subsequent to intracerebroventricular (icv) treatment with streptozotocin (STZ). In this study, Western blot analysis performed 1 month after STZ-icv treatment showed an increase (+63%) in the level of phosphorylated glycogen synthase kinase - 3α/β (pGSK-3α/β) protein in the rat hippocampus, whereas the levels of GSK-3α/β and protein kinase B (Akt/PKB) remained unchanged. Three months after STZ-icv treatment, pGSK-3α/β and Akt/PKB levels tended to decrease (-8% and -9%, respectively). The changes were regionally specific, as a different pattern was found in frontal cortex. Structural alterations were also found, demonstrated as beta amyloid peptide like aggregates in brain capillaries of STZ-icv treated rats. Similar neurochemical changes and cognitive deficits were recorded in rats treated icv with 5-thio-D-glucose, a blocker of glucose transporter 2 (GLUT2) probably involved in brain glucose sensing. These results support the similarity of IR signalling cascade alteration and its consequences in STZ-icv treated rats, to those found in humans with sporadic Alzheimer's disease, and suggest a role of GLUT2 in its pathophysiology.

Running title:

Introduction

Research of the brain insulin system has been intensified in the last decade, particularly regarding its pathophysiology. There is a growing interest in discovering the role of neuronal insulin and its receptor in the brain, and particularly in Alzheimer's disease. Recent data indicate that brain insulin deficiency and insulin resistance brain state are related to the late-onset sporadic Alzheimer's disease (*de la Monte and Wands, 2005; Fulop et al., 2003; Hoyer, 2004; Hoyer and Frőlich, 2005*). The late-onset type of Alzheimer's disease is associated with glucose utilization abnormalities distributed all over the cerebral cortex, and particularly in structures with both high glucose demands and high insulin sensitivity (*Henneberg and Hoyer, 1995*). Neuronal glucose metabolism is under the regulation of neuronal insulin, and abnormalities in the brain glucose metabolism found in Alzheimer's disease have been suggested to be induced at the level of insulin signal transduction (*Hoyer, 2002; Hoyer and Frőlich, 2005*).

Two different types of insulin receptor (IR) have been found in adult mammalian brain, a peripheral type and a neuron-specific type of IR (*Baskin et al., 1983*). The major molecular structure and most of the biochemical properties of the neuronal IR are indistinguishable from those found in the periphery, although some structural and functional differences between the neuronal and the peripheral IR have been suggested (*Boyd and Raizada, 1983*). IR belongs to the receptor tyrosine kinase superfamily and neuronal IR signal transduction is similar to that at the periphery. Insulin signalling within the cell is mediated, in general, by two functional cascades, one acting through phosphatidylinositol-3 (PI3) kinase pathway (in the focus of our investigation), and the other acting through the mitogen activated protein kinase (MAPK) pathway (*Johnstone et al., 2003*). Briefly, activated IR recruits insulin receptor substrate (IRS) adapter protein to its phosphorylated docking site, which then becomes phosphorylated

on tyrosine residues and capable to recruit various SH2 domain-containing signalling molecules, among which also PI3 kinase (*Johnstone et al., 2003*). The activation of the PI3 kinase pathway transduces the signal to protein kinase B (Akt/PKB), which triggers glucose transporter GLUT4 translocation and consequently cellular glucose uptake in peripheral insulin-sensitive tissue as well as in some brain regions (*Johnstone et al., 2003; Vannucci et al., 1998*). In addition, Akt/PKB modulates the glycogen synthase kinase (GSK-3) pathway by phosphorylating GSK-3 at its serine 9 residue, thereby inactivating GSK-3 (*Cross et al., 1995*). GSK-3 may exist in two closely related isoforms, α involved in the regulation of amyloid-β peptides (Aβs) (*Phiel et al., 2003*), and β involved in tau-protein phosphorylation (*Ishiguro et al., 1993*). Major responses to insulin stimulated signalling through IRS recruited molecules include increased cell growth and survival, and inhibition of apoptosis *(Dudek et al., 1997; Puro and Agardh, 1984).* However, evidence accumulated from basic and clinical research have demonstrated that brain insulin and IR are involved in the brain cognitive functions, including learning and memory (*Zhao et al., 2004*).

The metabolism of amyloid precursor protein (APP), and the balanced phosphorylation of tau-protein (their alterations are the major neuropathological features of Alzheimer's disease) are therefore under the regulation of insulin/IR signal transduction. Factors that affect phosphorylation/dephosphorylation homeostasis of the elements in IR signal transduction are capable of modifying this cascade causing its dysfunction. In line with this, decreased brain insulin protein and its mRNA levels were found post mortem in the brain of people with Alzheimer's disease (*Craft et al., 1998; Steen et al., 2005*), while IR density was found to be increased *(Frolich et al., 1998; Preece et al., 2003; Steen et al., 2005*). Regarding the alterations of IR downstream signalling molecules, extensive abnormalities were observed to be associated with reduced levels of IRS mRNA and IRS-associated PI3 kinase (*Steen et al., 2005)*, whereas inconsistent results (i.e. of both increase and decrease) have been reported for

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alterations in Akt/PKB and GSK-3 enzymes recorded post mortem in the brain of patients with Alzheimer's disease (*Pei et al., 1997; Pei et al. 2003; Preece et al., 2003; Rickle et al., 2004; Steen et al., 2005*).

Various experimental models of Alzheimer's disease have been introduced today, and actually no single model has been found to be truly representative of the sporadic type of Alzheimer's disease, unrelated to the genetic manipulations or inheritance. Therefore, considering the fact that Alzheimer's disease has now been recognised as an insulin resistant brain state, streptozotocin (STZ) intracerebroventricularly (icv) treated rat has become one of the proposed experimental models (*Hoyer, 2004; Lannert and Hoyer, 1998; Prickaerts et al., 1999; Sharma and Gupta 2001*). Namely, STZ is a drug selectively toxic for insulinproducing/secreting cells, which induces experimental diabetes mellitus in rats following peripheral administration of high doses (>65 mg/kg, intraperitoneally /ip/). However, central administration of very low STZ doses (1-3 mg/kg, icv) does not alter basal blood glucose (*Nitsch and Hoyer, 1991; Plaschke and Hoyer, 1993*), and does not produce diabetes mellitus, but brain glucose metabolism has been found to be markedly perturbed. Namely, icv treatment of STZ in a subdiabetogenic dose resulted in a decreased glucose utilization in 17 out of 35 brain areas (*Duelli et al., 1994*), an increase in lactate release from the brain (*Nitsch et al., 1989*), reduced activities of glycolytic enzymes (*Plaschke and Hoyer, 1993*), finnaly causing reduced formation of the energy rich compounds ATP and phosphocreatine (*Nitsch and Hoyer, 1991; Lannert and Hoyer, 1998*). Interestingly, the reduced glycolytic activity (capacity) was paralled by an increase in gluconeogenesis leading to unchanged concentrations of glucose and glycolytic compounds (*Plaschke and Hoyer, 1993*). Additionally, STZ icv treatment caused impairments of passive avoidance behaviour, and both, working and reference memory (*Blokland and Jolles, 1993; Lannert and Hoyer 1998; Mayer et al., 1990; Prickaerts et al., 1999*), likely to be due to a cholinergic

deafferentiation (*Hellweg et al., 1992*), and changes in monoaminergic neurotransmission (*Ding et al., 1992; Lackovic and Salkovic, 1990; Petrisic et al., 1997; Salkovic et al., 1995; Salkovic-Petrisic and Lackovic, 2003; Sharma and Gupta, 2001*). So far, different experimental and methodological approaches used by different investigators, clearly showed that icv STZ causes marked abnormalities in brain glucose/energy metabolism and behaviour. Alterations of IR and its downstream major signalling molecules have not been investigated in STZ-icv treated rats as an experimental model probably related to the sporadic Alzheimer's disease.

Diabetogenic action of STZ in the pancreas is preceded by its rapid selective uptake by B cells through the low affinity glucose transporter 2 (GLUT2) (*Hosokawa et al., 2001; Schnedl et al., 1994*). At the periphery, GLUT2 is a component of the signalling pathway involved in glucose sensing and regulation of insulin secretion from pancreatic beta cells (*Thorens et al., 1988*). However, GLUT2 has also been found in the rat and human central nervous system (*Brant et al., 1993; Leloup et al., 1994; Ngarmukos et al., 2001*), and its neuronal localisation and distribution relatively similar to glucokinase (the enzyme responsible for glucose metabolisam within the pancreatic beta cells) suggest GLUT2 involvement in glucose sensing in the brain as well (*Arluison et al., 2004; Arluison et al., 2004*). The effect of GLUT2 inhibition on IR signalling pathways in the brain, as well as on cognitive functions following icv administration of GLUT2 inhibitors has not yet been reported.

We report on alterations of the IR signalling cascade at the level of Akt/PKB and GSK-3 protein in the hippocampus and frontal cortex of STZ-icv and GLUT2 inhibitor-icv treated rats as well as on cognitive deficits in these animals, observed one and three months following the drug treatment.

Material and Methods

Material

Streptozotocin and 5-thio-D-glucose were purchased from Sigma-Aldrich Chemie (Munich, Germany). The anti-phospho-GSK-3α/β antibody (polyclonal, rabbit) and the anti-Akt/PKB antibody (polyclonal, rabbit) were purchased from Cell Signalling (Beverly, MA, USA); mouse (monoclonal) anti-GSK-3α/β antibody was purchased from BioSource International (Nievelles, Belgium); the polyclonal rabbit anti-human tau (K9JA) antibody was received as a gift from Dr. E-M Mandelkow (Max-Planck-Gesellschaft, Hamburg, Germany), although commerically originating from DAKOCytomation (Glostrup, Denmark). Anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibody were purchased from Cell Signalling (Beverly, MA, USA); chemiluminiscent Western blot detection kit was from Amersham Biosciences (Freiburg, Germany). Gels were from Novex (San Diego, CA, USA), and nitrocellulose membranes were from Invitrogen (Invitrogen GmbH, Karlsruhe, Germany).

Animals

Three-month-old male Wistar rats weighing 280-330 g (Department of Pharmacology, School of Medicine, University of Zagreb) were used throughout the study. Animals were kept on standardized food pellets and water *ad libitum*.

Drug treatments

For each experiment, rats were randomly divided in 3 groups (6 *per* group) and given general anaesthesia (chloralhydrate 300 mg/kg, ip), followed by injection of different drugs icv bilaterally into the lateral ventricle (2 µL/ventricle), according to the procedure described by Noble et al. (*Noble et al., 1967*). The following drug treatments were applied in a single dose: STZ (1 mg/kg, dissolved in 0.05M citrate buffer pH 4.5) in group I, 5-thio-D-glucose (TG)

(150 μg/kg, dissolved in the same vehicle as STZ) in group II, and an equal volume of vehicle icv in group III. Animals were sacrificed one and three months after the drug treatment. Brains were quickly removed, hippocampus and frontal cortex cut out, immediately frozen and stored at -80 °C. STZ-icv-treated animals had no symptoms of diabetes and steady-state blood glucose level did not differ in comparison with control animals.

Morris Water Maze Swimming Test

Cognitive functions were tested in Morris Water Maze Swimming Test (*Anger, 1991*). Adaptation of rats to the experimental environment and behavioural activity was done during two days before the experimental trials. On the first of these two days animals were subjected to 1 min of freely swimming in a pool (150x60 cm, 50 cm deep), with water temperature set at 25±1 °C, and on the second day rats were allowed to freely swim in the pool divided in four quadrants (I-IV). In the experimental trials, performed from day 1 to day 4, rats were thought to escape from water by finding an unseen rigid platform submerged about 2 cm below the water surface in quadrant IV. Stay on the platform was allowed for 15 s. One trial consisted of three starts, each from a different quadrant (I – III), separated by a 1-min rest period. Three consecutive trials were performed *per* day, separated by a 30-min rest period. After the third trial on day 4, the fourth trial was performed (starts from quadrants I-III) with a platform being removed from the pool, and the time spent in searching for the platform after entering quadrant IV was recorded. The cut off time was 1 min. Those rats who had no alterations in memory functions (control) were supposed to remember that the platform had previously been there, and, in line with that, to spend a long time swimming within quadrant IV, looking for the platform. In case of drug-induced deterioration of memory functions, rats were supposed to remember less intensively that the platform had been in quadrant IV, thus to spend less time in searching for the platform within this quadrant, in comparison with control rats.

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Western blot

Tissue preparation.

Hippocampal (1 animal = 1 sample) and frontal cortical (3 animals = 1 sample, to get an adequate protein quantity) tissue samples from the left half of the rat brain were homogenized with 3 volumes of lysis buffer containing 10 mM HEPES, 1 mM EDTA, 100 mM KCl, and 1% Triton X-100, pH 7.5, and protease inhibitors coctail (1:100), and the homogenates were centrifuged at 600xg for 10 min. The supernatants were further centrifuged at 45 000 xg for 30 min at 4 ^{o}C, and the pellets were resuspended in 100 μL of the lysis buffer. Finally, the resuspended pellets were mixed with appropriate previous supernatants obtained after second centrifugation. Protein concentration was measured by Bradford Protein assay. Samples were frozen and stored at -80 ^{o}C.

Immunoblotting.

Equal amounts of total protein (150-200 μg *per* sample for enzyme, and 50 μg *per* sample for tau protein analyses) were separated by SDS-PAGE using 12% polyacrylamide gels and transferred to nitrocellulose membranes (*Schneppenheim et al., 1991*). The nitrocellulose membranes were blocked by incubation in 5% non-fat milk added to low salt washing buffer (LSWB) containing 10 mM Tris and 150 mM NaCl, pH 7.5, and 0.5% Tween 20, either overnight at 4 ^{o}C for GSK-3, or one hour at room temperature for pGSK-3, Akt/PKB, and total tau (recognizes total tau at C-terminal part, amino acids 243-441) analyses. Blocked blots were incubated either on the next day with primary anti-GSK-3α/β antibody (1:1000) for two hours at room temperature, or overnight at 4 ^{o}C with respective primary anti-pGSK-3 (1:1000), anti-Akt/PKB (1:1000), anti-total tau (1: 10 000), antibodies. Following the incubation, the membranes were washed three times with LSWB and incubated for 60 min at room temperature with secondary antibody solution (anti-mouse IgG, 1: 2000, for GSK-3 analysis, and anti-rabbit IgG, 1:5000, for all the rest). The specificity of the signal was checked on the control membranes that were not incubated with the primary antibody. After

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washing three times in LSWB, the membranes were immunostained using chemiluminiscence Western blotting detection reagents, signal captured and visualised with the Chemi Doc BioRad (UK) video camera system, or following exposition of membranes to the film (tau protein analysis).

Staining of beta-amyloid fibrils by modified alkaline Congo Red

Beta-amyloid fibrils were visualized by a modified alkaline Congo Red staining (Puchtler et al., 1962). In brief, frozen tissue sections (6 µm) are air dried for one hour. The sections are then incubated for 15 min in Mayer's Hematoxylin Solution and rinsed in tap water to display the nuclei. Subsequently, the tissue slices are incubated for 20 min in 80 % (v/v) ethanol, 3 % (m/v) NaCl, 1 % NaOH, to be then transferred to the Alkaline Congo Red Solution containing 80 % (v/v) ethanol, 3 % (m/v) NaCl, 1% NaOH, 0.5 % (m/v) Congo Red. For stain development, the slices are twice briefly rinsed in absolute ethanol and incubated for 5 min in xylene.

Statistics

In the Western blot analysis of hippocampal tissue 3 samples *per* treatment group were loaded on one gel, and two gels were analysed for one experiment. Therefore, densitometric values of samples from 2 treatments (STZ and TG) on each gel were expressed as percentage of the control group on the respective gel. In this way, values from two gels/immunoblots that belonged to the same experiment could be joined together, allowing for statistical analysis. Values were expressed as relative protein level (mean ± SE). Due to pooling the samples for the Western blot analysis of the frontal cortical tissue, there were only two samples (1 sample/3 animals) *per* treatment. Values were expressed as relative protein level (mean of two samples) and no statistical analysis was performed. In the Morris Water Maze Swimming Test, values were expressed as total time (s) spent in searching for the hidden platform in

quadrant IV during the three consecutive starts from quadrant I-III in the last experimental trial. Median values with a minimum-maximum range were presented. The significance of between-group differences was tested by Kruskal-Wallis ANOVA median test, followed by Mann-Whitney U-test, and p<0.05 considered statistically significant for all tests.

Ethics

Drug treatments and behavioural tests were carried out in Croatia under the guidelines of The Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised in 1985), according to the Croatian Act on Animal Welfare (NN 19/1999), and were approved by The Ethics Committee of the Zagreb University School of Medicine (No. 04-7672005-54).

Results

GSK-3α/β and pGSK-3α/β

In all experiments, Western blot analysis of GSK-3α/β and pGSK-3α/β protein showed a specific signal in the form of two bands at the positions of 47 kDa and 51 kDa, corresponding to the GSK-3β and GSK-3α form, respectively. The signal was densitometrically measured as joint α+β signal. However, it could be visually observed that on GSK-3α/β analysis, the β form signal was expressed with a lower intensity than α form, whereas on the analysis of phosphorylated GSK-3α/β (pGSK-3α/β), the α form signal was of a lower intensity than β form.

Hippocampus

Quantitative analysis of immunoblots indicated that GSK-3α/β levels in the hippocampal homogenates were not significantly changed 1 and 3 months following STZ and TG treatment, in comparison to the respective controls (Fig. 1). Contrary to that, pGSK-3α/β level

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in the hippocampus was significantly increased (+63%) 1 month following STZ treatment (Fig. 1), declining below the control value 3 months after STZ treatment (-8%), however, not reaching statistical significance (Fig. 1). Interestingly, TG treatment produced an increase (+35%) in pGSK-3α/β expression after 1 month, which, however, did not reach statistical significance (Fig. 1), also declining below the control value 3 months after the drug treatment (-8%) (Fig. 1). Relative pGSK-3α/β /GSK-3α/β ratio in hippocampal tissue was found increased (+50%) after the first month in STZ (+50%) and TG (+37%) icv treated rats in comparison to the controls (Fig. 3a), but fell down after the third post-treatment month, being -9% bellow the control levels in STZ icv treated, and +9% above the control levels in TG icv treated rats (Fig. 3a). The increase of pGSK-3/GSK-3 ratio after the first months was noticed for both, beta and alpha isoforms, when measured separately (Fig. 3b). However, 3 months after the treatment, the ratio of pGSK-3β/GSK-3β was decreased, while that of pGSK-3α/GSK-3α isoform was zero (Fig. 3b), as pGSK-3α band at that observational period was below the limit of detection.

Frontal cortex

Quantitative analysis of GSK-3α/β immunoblots in the pooled homogenates of the frontal cortical tissue demonstrated that STZ-icv and TG-icv treatment induced mild changes that were by ≤10% below or over the respective control values at both observation times (Fig. 2). STZ-induced alterations in pGSK-3α/β expression were also mild, less than -10% below the control values (Fig. 2). TG-icv treatment induced an increase (+20%) in pGSK-3α/β expression after the first month, which declined below the control values after three months (-10%) (Fig. 2). As previously stated, no statistical analysis was performed. Relative pGSK-3α/β /GSK-3α/β ratio in frontal cortical tissue was equally decreased after the first (-17%) and the third (-16%) months post STZ icv treatment, in comparison to the controls (Fig. 3a), while

in TG icv treated rats, this ratio decrement was found only after the third month (-12%) (Fig. 3a).

Akt/PKB

Western blot analysis of Akt/PKB protein (antibody detecting total levels of endogenous Akt1, Akt2 and Akt3 proteins) showed a specific signal in the form of one band at the position of 60 kDa.

Hippocampus

Quantitative analysis of immunoblots indicated that Akt/PKB level in the hippocampal homogenates of STZ treatment was mildly increased (+14%) but not statistically significantly changed after one month, and mildly, although statistically significantly decreased (-9%) three months after STZ treatment (Fig. 1). In TG treated animals Act/PKB level tended to increase (+41%) after the first month, yet not reaching statistical significance due to intra-group variation, but was significantly lowered (-22%) after three months (Fig. 1).

Frontal cortex

Western blot analysis of Akt/PKB expression in the pooled tissue of frontal cortex demonstrated a mild acute decrease (-10%) in STZ treated rats after one month, which turned to a mild increase three months after the treatment (+14%) (Fig. 2). However, Akt/PKB values seemed to gradually increase following TG treatment, from +11% to +32% after one and three months, respectively (Fig. 2). As previously stated, no statistical analysis was performed.

Tau protein

A preliminary experiment was done to see if any change could be observed at the tau protein level. The immunoreaction of total tau (recognizing total tau protein at C-terminal part, amino acids 243-441) increased significantly in the hippocampus of STZ icv treated rats, in comparison to the control animals (Fig. 4), demonstrating changes at the tau protein level, as well.

Congophilic deposits in the rat brain

Representative sections of human and rat brain are shown in Fig. 5. Fig. 5(a, b) shows beta-amyloid aggregates in the human AD brain intensively stained by Congo Red. In the final state of AD beta-amyloid aggregates can be seen in blood vessels and as cerebellar plaques. The examination of the rat brain after the three month treatment with STZ icv reveals diffuse congophilic deposits in the blood vessels, but not yet within the brain (c). Congophilic deposits were additionally monitored with cross-polarized light, where they show characteristic green autofluorescence (d). Untreated control animals, however, are devoid of such autofluorescencent deposits (e,f).

Morris Water Maze Swimming Test

In Morris Water Maze Swimming Test all STZ treated rats demonstrated significant cognitive deficits in learning and memory function (Fig. 6). In each experiment, STZ-icv treated animals spent significantly less time in search for the hidden platform, in comparison to the control group (Fig. 6). Relative changes in comparison to the control group were more pronounced after three months (-46%) than after one month (-33%) (Fig. 6). Interestingly, TG-treated rats also demonstrated similar cognitive deficits, spending less time searching for the hidden platform than control rats in all observation periods (-51% and -37% below the controls) (Fig. 6). However, in respect to STZ-icv treated rats, TG-icv treated rats

demonstrated more severe cognitive deficits and spent significantly less time in search after one month, but significantly less severe cognitive deficits after three months (Fig. 6).

Discussion

Insulin and IR signalling participate in a variety of region-specific functions in the central nervous system, through mechanisms not necessarily associated with glucose regulation (*Schulingkamp et al., 2000*). Among them, growing evidence suggest that IR signalling modulates neuronal excitability and synaptic plasticity (*Skeberdies et al., 2001; Wan et al., 1997*), consequently affecting cognitive functions like learning and memory (*Zhao et al., 2004*). In line with this, deterioration of IR signalling has been found to be associated with sporadic Alzheimer's disease (*Hoyer, 2002; Hoyer and Frőlich, 2005*). However, IR and its signalling cascade have not been investigated either in STZ-icv treated rats as an experimental model probably related to the sporadic Alzheimer's disease, or in a model of the disturbed brain glucose sensing system.

In the hippocampus of STZ-icv treated rats we found alterations at the level of Act/PKB - GSK-3 enzymes that are downstream the PI-3 kinase pathway of the IR signalling cascade. The level of phosphorylated GSK-3α/β enzyme was significantly increased at one month following the treatment, and fell down below the control values at three months following the treatment. However, these changes were not followed by alterations of non-phosphorylated GSK-3α/β, which remained unchanged. Considering brain GSK-3 research in Alzheimer's disease in humans, inconsistent results have been reported; increased brain (*Pei et al., 1997*) and unchanged hippocampal and hypothalamic (*Steen et al., 2005*) levels of total GSK-3α/β protein, unchanged cortical GSK-3α mRNA levels (*Preece et al., 2003*), unchanged hippocampal and hypothalamic GSK-3β levels (*Steen et al., 2005*), and also unchanged (*Pei*

et al., 1997), reduced (*Swatton et al., 2004*) and increased (*Steen et al., 2005*) GSK-3α/β activity. In respect to animal models, a significant increase in GSK-3 activity has been reported in the hippocampus of Tet/GSK-3beta transgenic mice that have also been proposed as an experimental model of Alzheimer's disease (*Hernandez et al., 2002*). However, our results are the first report of GSK-3 investigation in the brain of the STZ-icv rat experimental model, probably related to the sporadic type of Alzheimer's disease. Changes found in our experiments could suggest some effects at the level of GSK-3 phosphorylation or pGSK-3 dephosphorylation, since total GSK-3α/β levels were unchanged, both in the hippocampus and in the frontal cortex, corresponding to the findings in human Alzheimer's disease (*Steen et al., 2005)*, whereas the level of pGSK-3 was increased. Decrement of the relative pGSK-3/GSK-3 ratio in hippocampal tissue of STZ icv treated rats from the first month-measured over-control level (+50%) to the third month-measured bellow-control level (-9%) could indirectly suggests increment of non-phosphorylated, therefore, active GSK-3 form (Fig. 3) with the duration of observational period. This tendency of decrement of phospho/total enzyme ratio was demonstrated in both, alpha and beta GSK-3 isoform, and seemed to be particularly pronounced after the third month in the former isoform (Fig. 3). However, the activity of GSK-3, whose alterations could not be excluded, has not been measured in our experiments. Increased ratio after first month in hippocampus could represent an acute change which, in line with decreased ratio after the third month, could not be compensated with the time in STZ icv rat model, as observed in both isoforms, particularly in GSK-3 alpha one. Indirectly suggested increment in non-phosphorylated, active GSK-3 in the brain of STZ icv rat model could lead to tau hyperphosphorylation. Our preliminary experiments have indeed shown some alterations at the level of tau protein, the meaning of which is not clear yet, but more extensive analyses of tau protein in STZ icv rat model, needed to clarify this issue, are in preparation.

The phospho-GSK-3α/β antibody used in our Western blot analysis detects endogenous levels of GSK-3 when phosphorylated at Ser 21 of GSK-3α or Ser9 of GSK-3β. Ser21/9 phosphorylation and thus inactivation of GSK-3 are mediated by Akt/PKB that is downstream the IR-activated PI3 kinase pathway (*Cross et al., 1995*). Unchanged level of total Akt/PKB in the hippocampal tissue samples after one month, and a small decrease after three months as well as mild (~+/-10%) changes of this protein in the frontal cortex of the STZ treated rats may suggest this protein itself to be quite resistant to damage in the course of the 3-month period of observation in this experimental model. Consequently, this may have a reflection on total GSK-3 protein, which, in line with this, we found to be unchanged. These results are in agreement with literature data on unchanged Akt/PKB levels in the brain of patients with Alzheimer's disease post mortem (*Steen et al., 2005*). The same authors report on decreased levels of pAkt/PKB and pGSK-3, which were also found to have moderately decreased during the 3-month course of the probable experimental Alzheimer's disease in our experiments. This could suggest the possible relation to differences in the disease staging and severity. Our STZ icv (1 mg) rat model refers to the early pathological changes, contrary to data obtained post-mortem from humans with, in general, severe, end-stage of sporadic AD disease. The issue of early pathological changes could involve the different velocity of structural changes development in STZ icv rat experimental model in comparison to humans. Some structural changes at the level of beta-amyloid peptide accumulation, resembling those in human AD, have been observed in STZ icv rat model. The conformational transition of beta-amyloid peptides from alpha-helices to beta-sheets strongly favours the formation of beta-amyloid fibrils, which give rise of pathological protein aggregates called amyloid plaques. Beta-amyloid fibrils stained by Congo red show green autofluorescence on cross-polarization. Congo red, which is known to specifically bind to beta-amyloid fibrils (*Klunk et al., 1989; Balbirnie et al., 2001*), has widely been used in histological staining procedures for the evaluation of beta-amyloid aggregates in human (*Ladewig, 1945*) and murine tissues (*Li et al.,*

2005). A possible relationship of these structural changes to decrement of pGSK-3 α/GSK-3 α ratio which suggests increment of non-phosphorylated, active GSK-3 α known to be involved in Aβ regulation (*Phiel et al., 2003*) can not be excluded. Dose-dependent severity of STZ-induced effects have been demonstrated following its peripheral (persistent or transient diabetes, morphological alterations of the islet insulin-immunoreactive cells /*Ar'Rajab et al, 1993; Junod et al., 1969/*), and central (neurochemical alterations of brain monoamine level /*Lackovic and Salkovic, 1990/*) administration, supporting this stage- and severity-dependent hypothesis of alteration manifestation. Furthermore, regarding human Alzheimer's disease, a statistically significant positive correlation seen in the human tissue between Akt/PKB activities or pAkt/PKB levels and Braak staging for the neurofibrillary changes supports this explanation (*Pei et al., 2003, Rickle et al.,2004*). Increased Akt/PKB protein levels found in frontal cortex (*Pei et al., 2003*) and increased Akt/PKB activity found in temporal cortex but not in frontal cortex (*Rickle et al., 2004*) of patients with Alzheimer's disease post mortem, suggest the possible regional pattern of changes. Our results of highly increased pGSK-3α/β level found after one month in the hippocampus but not in the frontal cortex as well as of decreased Akt/PKB level in the hippocampus but increased in the frontal cortex after three months in STZ-icv treated rats are consistent with these reports.

We did not measure the activity of Akt/PKB, and it can not be excluded that the increased level of hippocampal pGSK-3α/β found one month after STZ-icv treatment was the consequence of increased Akt/PKB activity, as reported elsewhere (*Rickle et al., 2004*). However, beside the Akt/PKB, numerous kinases can phosphorylate GSK-3β at Ser9, such as protein kinase C, involved in signalling of G-protein linked receptors (*Kaytor and Orr, 2002*). Furthermore, the increased pGSK-3 level could be related to inactivity and/or decreased levels of phosphatase that dephosphorylates GSK-3, among which serine/threonine protein phosphatase 1 (PP1) and 2A (PP2A) has been mentioned (*Bennecib et al., 2000; Milward et*

al., 1999; Hoyer and Frőlich, 2005). PP2A is a negative regulator of the insulin PI3/Akt/PKB signalling pathway that dephosphorylates and thereby inactivates Akt/PKB, and to a minor extent dephosphorylates, and thereby activates GSK-3 (*Milward et al., 1999*). PP2A mRNA expression was found to be significantly reduced in the hippocampus of sporadic Alzheimer's disease brain (*Vogelsberg-Ragaglia et al., 2001*). Immunoblotting analyses revealed a significant reduction in the total amount of PP2A in frontal and temporal cortex that matched the decrease in PP2A activity in the same region, and was further supported by the finding of lower PP2A expression in immunohistochemical studies of the brains from patients with Alzheimer's disease (*Gong et al., 1995; Sontag et al., 2004*). A recent finding of up-regulation of the endogenous PP2A inhibitors in the neocortex of patients with Alzheimer's disease further supports this hypothesis (*Tanimukai et al., 2005*). Therefore, it could not be excluded that in STZ-icv experimental model, some neurochemical changes are related to the possible lower activity/protein level of PP2A, not investigated in STZ-icv treated rats so far. Furthermore, GSK-3β is involved in phosphorylation of tau protein, which in the hyperphosphorylated form builds neurofibrillary tangles, important pathological features of Alzheimer's disease (*Kaytor and Orr, 2002*). Interestingly, recent data demonstrate that involvement of GSK-3 is not necessary to obtain hyperphosphorylated tau *in vivo*, indicating that inhibition of PP2A, an enzyme that can directly dephosphorylate tau, is likely the predominant factor in inducing tau hyperphosphorylation (*Planel et al., 2001*).

In line with literature reports of STZ-icv induced cognitive deficits (*Hoyer, 2004; Lannert and Hoyer, 1998; Prickaerts et al., 1999; Sharma and Gupta, 2001*), a decreased memory function was also found in STZ-icv treated rats in our experiments, demonstrated as a decline in time spent in search for the hidden platform within the appropriate quadrant where the platform had been placed in training trials.

The results of our experiments with GLUT2 blocker, i.e. (TG) icv treatment came as a surprise. A single TG-icv treatment induced longlasting neurochemical effects in the hippocampus, which mostly resembled those induced by STZ-icv treatment, e.g., a tendency to increase in pGSK-3α/β level after one month, unchanged GSK-3α/β levels during the 3-month observation period, and a decrease in Akt/PKB level after three months, which was more pronounced than the one induced by STZ at the same time. Thus, it could be speculated that by blocking the intracellular glucose uptake and consequently its intracellular metabolism and possible glucose sensing, TG-icv treatment induced local conditions in the brain that could be similar to the impaired brain glucose uptake and metabolism found in human sporadic Alzheimer's disease and in STZ-icv treated rats proposed as a probable experimental model of this disease, as reviewed elsewhere (*Hoyer, 2004; Hoyer and Frőlich, 2005*). The finding of cognitive deficits on Morris Water Maze Swimming Test in TG-icv treated rats, which were similar or even more severe than in STZ-icv treated rats after one month, supported this hypothesis. These results are in agreement with the finding that 3 weeks after STZ-icv injection the ultrastructure of rat frontoparietal cortical neurons was similar to that observed after iv application of non-metabolizable glucose analogue 2-deoxyglucose (*Grieb et al., 2004*). A small improvement in cognitive deficits in comparison to STZ-icv treatment, but still persistent deficits in comparison with control treatment, seen at three months of TG-icv treatment, could suggest involvement of some compensatory mechanisms and factors yet unexplored in such an experimental model.

In conclusion, STZ-icv probably induces experimental, sporadic Alzheimer's disease in rats that is associated with acute increase in pGSK-3α/β level and subsequent decreasing tendency in pGSK-3α/β and Akt/PKB levels in the hippocampus. No changes or less intensive changes found in the same periods of observation in frontal cortex suggest regional specificity of changes in this probable experimental model of Alzheimer's disease. The icv treatment with

the blocker of GLUT2, a glucose transporter suggested to be related with brain glucose sensing, induces neurochemical changes and cognitive deficits that are, in general, similar to those induced by STZ-icv treatment. This is the first report of altered IR-PI3 kinase downstream signalling pathway in STZ-icv rats that supports the hypothesis of the STZ-icv rats being a probable experimental model of sporadic Alzheimer's disease. Also, a possible role of GLUT2 in the pathophysiology of sporadic Alzheimer's disease, at least in its probable experimental animal models, has been suggested.

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Fig. 1. Western blot analysis of GSK-3α/β, pGSK-3α/β and Akt/PKB protein in the hippocampus of rats intracerebroventricularly (icv) treated with streptozotocin and glucose transporter GLUT2 blocker, 5-thio-D-glucose 1 and 3 months after drug treatment. Values of bars are expressed as mean ± SE (N=5-6). Blots from a typical experiment are presented (1 sample = 1 animal). TG, 5-thio-D-glucose icv-treated group (from left, lanes 1-3); STZ, streptozotocin icv-treated group (lanes 4-6); CTL, control group (lanes 7-9). *p<0.05 by Kruskal-Wallis ANOVA median test followed by Mann Whitney U test.
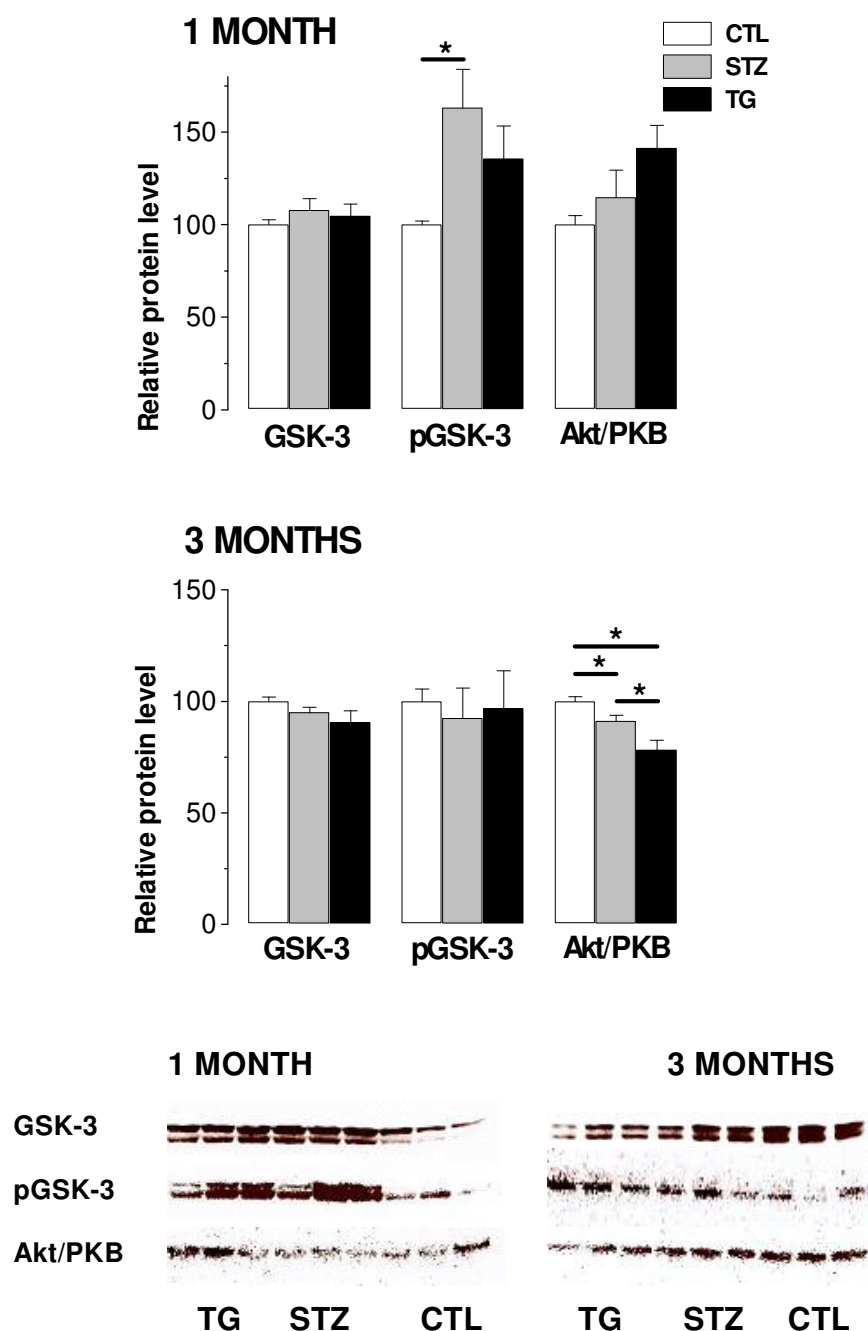
Fig. 2. Western blot analysis of GSK-3α/β, pGSK-3α/β and Akt/PKB protein in the frontal cortex of rats intracerebroventricularly (icv) treated with streptozotocin and glucose transporter GLUT2 blocker, 5-thio-D-glucose 1 and 3 months after drug treatment. Values of bars are expressed as a mean of two samples (1 sample represents tissue pooled from three animals). Blots from a typical experiment are presented (2 samples *per* group). TG, 5-thio-D-glucose icv-treated group (from left, lanes 1-2); STZ, streptozotocin icv-treated group (lanes 3-4); CTL, control group (lanes 5-6).
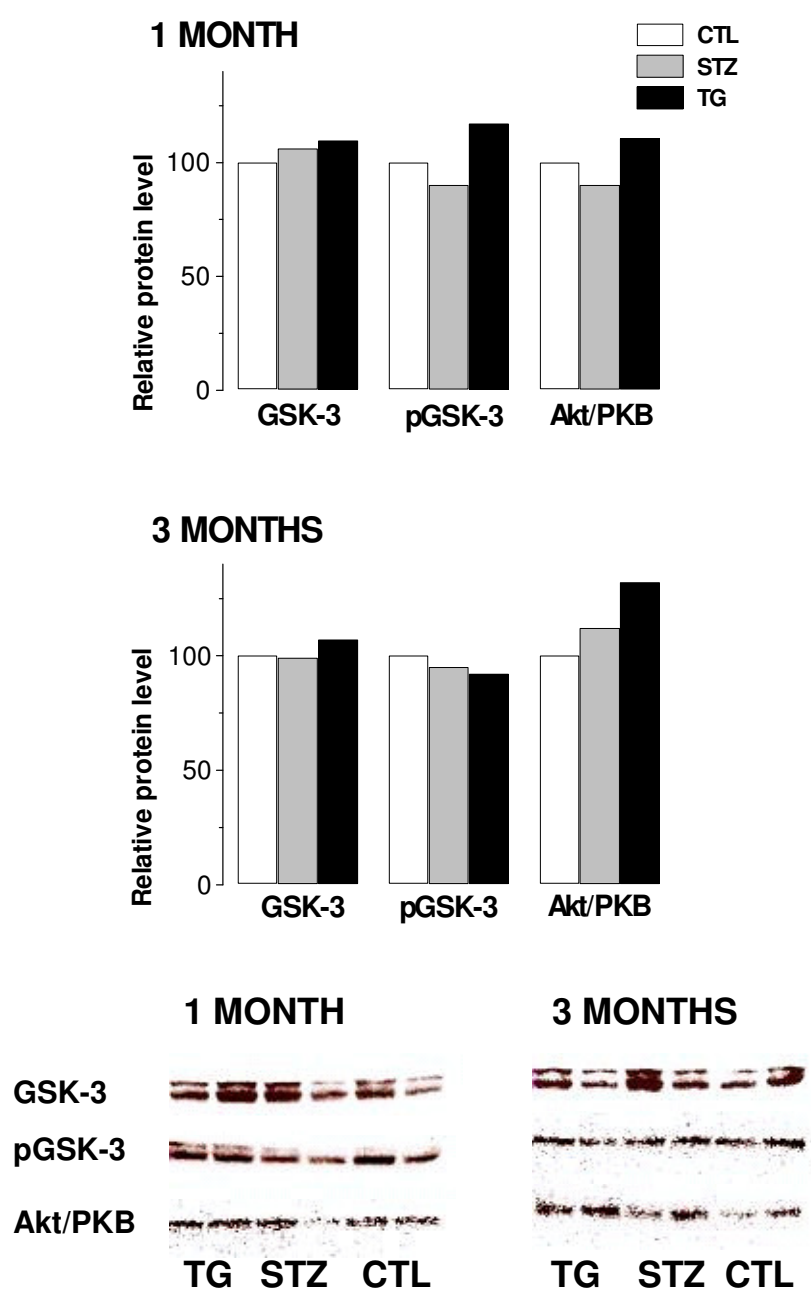
Fig. 3. Relative pGSK-3/GSK-3 ratio in hippocampus and frontal cortex of streptozotocin and glucose transporter GLUT2 blocker, 5-thio-D-glucose, intracerebroventricularly (icv) treated rats.Ratio is calculated either from combined pGSK-3/GSK-3 α+β values (**a**), or from separated pGSK-3α/GSK-3α and pGSK-3β/GSK-3β values (**b**) where pGSK-3 alpha isoform band is practically invisible 3 months after the treatment. CTRL, control group; STZ, streptozotocin icv-treated group; TG, 5-thio-D-glucose icv-treated group.
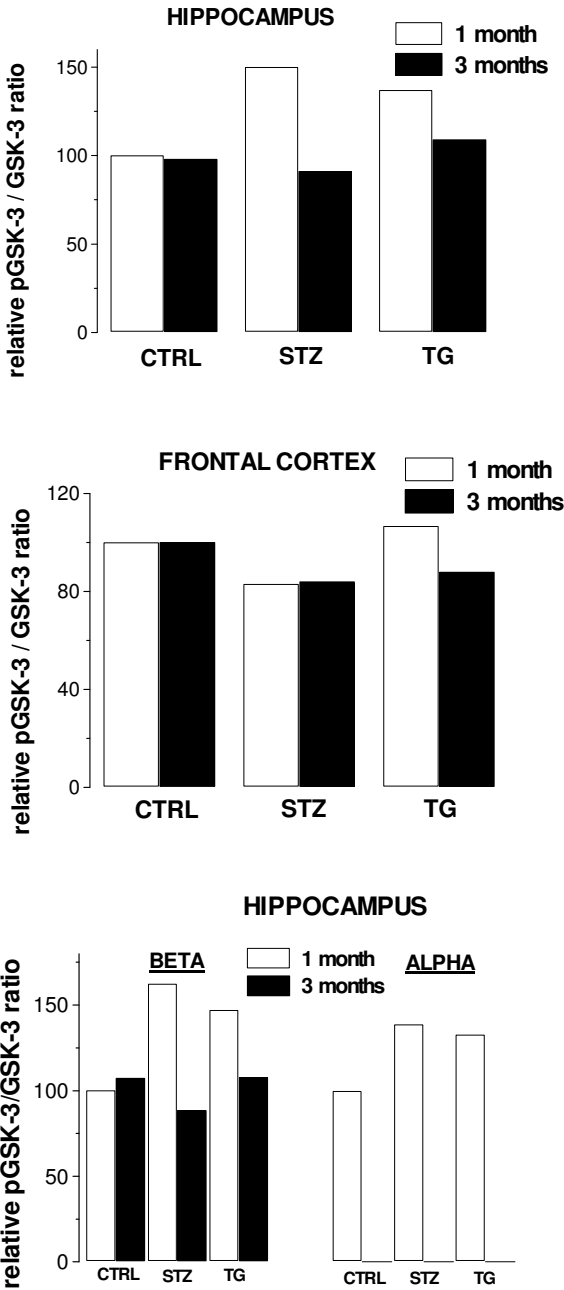
Fig. 4. Western blot analysis of total tau protein visualized by K9JA antibody (recognizing C-terminal part of tau protein) in the hippocampus of rats intracerebroventricularly (icv) treated with streptozotocin 1 month after drug treatment. STZ, streptozotocin icv-treated group (lanes 5-8); CTRL, control group (lanes 1-4). *p<0.05 by Mann Whitney U test.
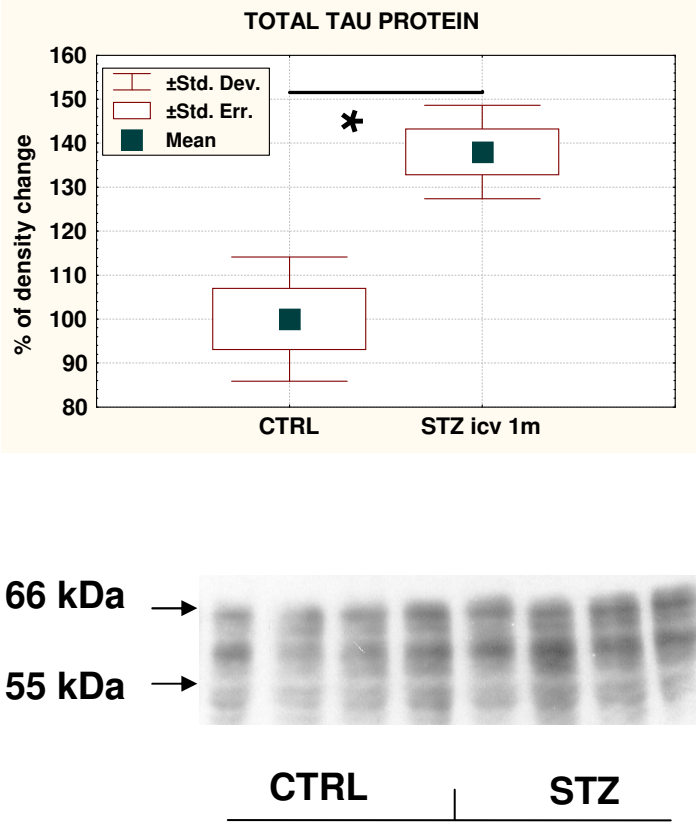
Fig. 5. Beta amyloid peptide depositions in meningeal capillaries of streptozotocin intracerebroventricularly treated rats 3 months following the drug treatment, visualized by Congo red staining. (a,b) Tissue sections of a human AD brain show beta-amyloid as visualized by characteristical congophilic deposits. (c) The brain tissue section of a rat after treatment of 3 months with STZ exhibits diffuse congophilic deposits, which are autofluorescent by cross-polarized light (d). Untreated control animals do not exhibit such autofluorescent congophilic material (e,f).
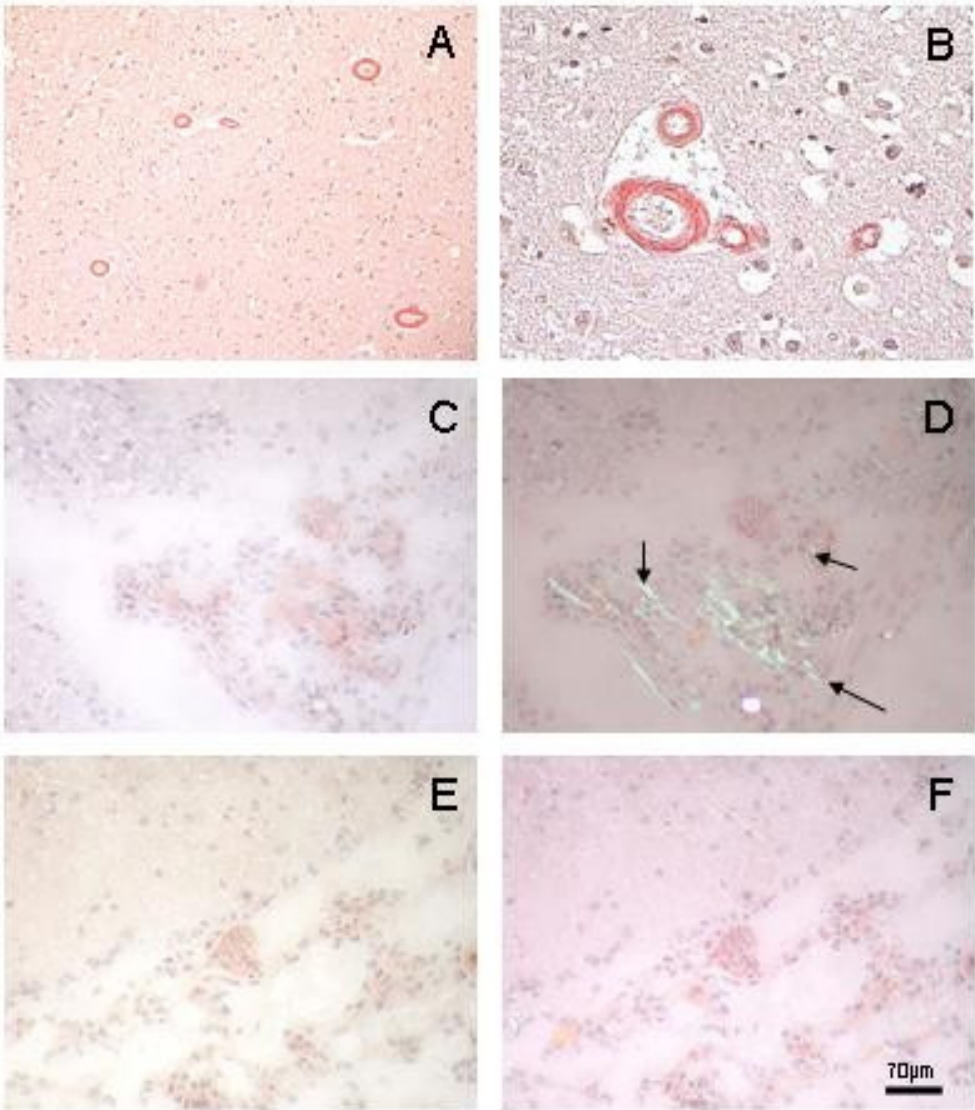
Fig. 6. Memory function in Morris Water Maze Swimming Test of rats intracerebroventricularly (icv) treated with streptozotocin and glucose transporter GLUT2 blocker, 5-thio-D-glucose. Deficits were measured as the time spent in search for the hidden platform after entering the quadrant where the platform had been placed in training trials. The better the memory had been preserved, the longer the rats were searching for the platform, and *vice versa*. Values are expressed as a median and minimum-maximum value range. CTL, control group; STZ, streptozotocin icv-treated group; TG, 5-thio-D-glucose icv-treated group. *p<0.05 by Kruskal-Wallis ANOVA median test followed by Mann Whitney U test.