Alzheimer-like changes in protein kinase B and glycogen synthase kinase-3 in rat frontal cortex and hippocampus after damage to the insulin signalling pathway

Melita Salkovic-Petrisic,* Florian Tribl,† Manuela Schmidt,† Siegfried Hoyer‡ and Peter Riederer†

**Department of Pharmacology and Croatian Institute for Brain Research, School of Medicine, University of Zagreb, Zagreb, Croatia*

†Department of Clinical Neurochemistry, University Department of Psychiatry and Psychotherapy, University of Würzburg, Würzburg, Germany

‡Department of Pathology, University Clinic, University of Heidelberg, Heidelberg, Germany

Abstract

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

Keywords: Alzheimer's disease; glucose transporter 2; glycogen synthase kinase-3, hippocampus, protein kinase B, streptozotocin.

J. Neurochem. (2006) **96**, 1005–1015.

Research into the brain insulin system has intensified in the past decade, particularly regarding its pathophysiology. There is a growing interest in determining 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 the insulin-resistant brain state are related to late-onset sporadic Alzheimer's disease (Fulop *et al.* 2003; Hoyer 2004; De la Monte and Wands 2005; Hoyer and Frölich 2005). The late-onset type of Alzheimer's disease is associated with glucose utilization abnormalities 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 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 Frölich 2005).

Received July 8, 2005; revised manuscript received November 2, 2005; accepted November 2, 2005.

Address correspondence and reprint requests to Melita Salkovic-Petrisic, MD, PhD, Department of Pharmacology, School of Medicine and Croatian Institute for Brain Research, University of Zagreb, Salata 3, HR-10000 Zagreb, Croatia. E-mail: melitas@mef. hr

Abbreviations used: Akt/PKB, protein kinase B; CTL, control; GLUT, glucose transporter; GSK-3α/β, glycogen synthase kinase-3α/β; i.c.v., intracerebroventricular; IR, insulin receptor; IRS, insulin receptor substrate; LSWB, low-salt washing buffer; pAkt/PKB, phosphorylated Akt/PKB; pGSK-3α/β, phosphorylated GSK-3α/β; PI3, phosphatidylinositol-3; PP2A, protein phosphatase 2A; STZ, streptozotocin; TG, 5-thio-D-glucose.

© 2006 The Authors
Journal Compilation © 2006 International Society for Neurochemistry,

Two different types of insulin receptor (IR) have been found in adult mammalian brain, a peripheral type and a neurone-specific type (Baskin *et al.* 1983). The major molecular structure and most of the biochemical properties of the neuronal IR are indistinguishable from those of the peripheral form, although some structural and functional differences between the neuronal and the peripheral receptors have been suggested (Boyd and Raizada 1983). The 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 the phosphatidylinositol-3 (PI3) kinase pathway (the focus of our investigation), and the other acting through the mitogen-activated protein kinase 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 of recruiting various sic homology (SH)2 domain-containing signalling molecules, like PI3 kinase (Johnstone *et al.* 2003). Activation of the PI3 kinase pathway transduces the signal to protein kinase B (Akt/PKB), which triggers glucose transporter (GLUT)4 translocation and consequently cellular glucose uptake in peripheral insulin-sensitive tissue as well as in some brain regions (Vannucci *et al.* 1998; Johnstone *et al.* 2003). In addition, Akt/PKB modulates the glycogen synthase kinase (GSK)-3 pathway by phosphorylating GSK-3 at its serine 9 residue, thereby inactivating it (Cross *et al.* 1995). GSK-3 may exist in two closely related isoforms; the α isoform is involved in the regulation of β-amyloid peptides (Phiel *et al.* 2003) and the β isoform 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 (Puro and Agardh 1984; Dudek *et al.* 1997). However, evidence from basic and clinical research has demonstrated that brain insulin and the IR are involved in the brain cognitive functions, including learning and memory (Zhao *et al.* 2004).

The metabolism of amyloid precursor protein, and the balanced phosphorylation of tau protein, alterations of which are the major neuropathological features of Alzheimer's disease, are under the regulation of insulin–IR signal transduction. Factors that affect the 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 at autopsy in the brains of people with Alzheimer's disease (Craft *et al.* 1998; Steen *et al.* 2005), whereas IR density was increased (Frolich *et al.* 1998; Preece *et al.* 2003; Steen *et al.* 2005). As regards alterations in 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 (both an increase and decrease) have been reported for alterations in Akt/PKB and GSK-3 enzymes recorded post mortem in the brain of patients with Alzheimer's disease (Pei *et al.* 1997, 2003; Preece *et al.* 2003; Rickle *et al.* 2004; Steen *et al.* 2005).

Various experimental models of Alzheimer's disease have been developed, but none has been found to be truly representative of the sporadic type of Alzheimer's disease, which is unrelated to genetic manipulations or inheritance. Taken that Alzheimer's disease has now been recognized as an insulin-resistant brain state, the rat treated with intracerebroventricular (i.c.v.) streptozotocin (STZ) has been proposed as an experimental model for this disease (Lannert and Hoyer 1998; Prickaerts *et al.* 1999; Sharma and Gupta 2001; Hoyer 2004). STZ is selectively toxic to insulin producing/secreting cells, and induces experimental diabetes mellitus in rats following peripheral administration of high doses (> 65 mg/kg, i.p.) (Szkudelski 2001). However, central administration of very low STZ doses (1–3 mg/kg, i.c.v.) does not alter basal blood glucose levels (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. Specifically, treatment with an i.c.v. injection of STZ at a subdiabetogenic dose resulted in decreased glucose utilization in 17 of 35 brain areas (Duelli *et al.* 1994), an increase in lactate release from the brain (Nitsch *et al.* 1989) and reduced activity of glycolytic enzymes (Plaschke and Hoyer 1993), finally 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 parallelled by an increase in gluconeogenesis leading to unchanged concentrations of glucose and glycolytic compounds (Plaschke and Hoyer 1993). Additionally, i.c.v. STZ caused impairment of passive avoidance behaviour, and both working and reference memory (Mayer *et al.* 1990; Blokland and Jolles 1993; Lannert and Hoyer 1998; Prickaerts *et al.* 1999), most probably as a result of cholinergic deafferentiation (Hellweg *et al.* 1992) and changes in monoaminergic neurotransmission (Lackovic and Salkovic 1990; Ding *et al.* 1992; Salkovic *et al.* 1995; Petrisic *et al.* 1997; Sharma and Gupta 2001; Salkovic-Petrisic and Lackovic 2003). Various experimental and methodological approaches used by different investigators have clearly shown that i.c.v. STZ causes marked abnormalities in brain glucose and energy metabolism and behaviour. However, alterations in the IR and its major downstream signalling molecules have not yet been investigated in this potential model of sporadic Alzheimer's disease.

The diabetogenic action of STZ in the pancreas is preceded by its rapid selective uptake by B cells through the low-affinity GLUT2 (Hosokawa *et al.* 2001; Schnedl *et al.* 1994). Peripherally this transporter is a component of the signalling pathway involved in glucose sensing and

© 2006 The Authors
Journal Compilation © 2006 International Society for Neurochemistry,

regulation of insulin secretion from pancreatic β cells (Thorens *et al*. 1988). However, GLUT2 has also been found in rat and human CNS (Brant *et al*. 1993; Leloup *et al*. 1994; Ngarmukos *et al*. 2001). Its neuronal localization and distribution have been shown to be relatively similar to those of glucokinase (the enzyme responsible for glucose metabolisam within pancreatic β cells), suggesting the involvement of GLUT2 in glucose sensing in the brain as well (Arluison *et al*. 2004a; Arluison *et al*. 2004b). The effect of GLUT2 inhibition on IR signalling pathways in the brain, as well as on cognitive functions following i.c.v. administration of GLUT2 inhibitors, has not yet been reported.

We have studied alterations of the IR signalling cascade at the level of Akt/PKB and GSK-3 protein in the hippocampus and frontal cortex, along with cognitive deficits, 1 and 3 months after i.c.v. injection of STZ and GLUT2 inhibitor in rats.

Material and methods

Materials

STZ and 5-thio-D-glucose (TG) were purchased from Sigma-Aldrich Chemie (Munich, Germany). The anti-phosphorylated GSK-3α/β (pGSK-3α/β) antibody (polyclonal, rabbit) and the anti-Akt/PKB antibody (polyclonal, rabbit) were purchased from Cell Signaling (Beverly, MA, USA). Mouse (monoclonal) anti-GSK-3α/β antibody was purchased from BioSource International (Nievelles, Belgium). Polyclonal rabbit anti-human tau (K9JA) antibody was a gift from Dr E.-M. Mandelkow (Max-Planck-Gesellschaft, Hamburg, Germany), although commerically originating from DAKOCytomation (Glostrup, Denmark). Anti-rabbit IgG and anti-mouse IgG horseradish peroxidase-linked antibodies were purchased from Cell Signaling. The 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 GmbH (Karlsruhe, Germany).

Animals

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

Drug treatments

For each experiment, rats were divided randomly into three groups (six per group) and given general anaesthesia (300 mg/kg i.p. chloralhydrate), followed by i.c.v. injection of different drugs bilaterally into the lateral ventricle (2 μL/ventricle), as described previously (Noble *et al*. 1967). The first group of animals received a single dose of STZ 1 mg/kg, dissolved in 0.05 M citrate buffer pH 4.5, the second group had TG 150 μg/kg, dissolved in the same vehicle as STZ, and the third group received an equal volume of vehicle. Animals were killed 1 and 3 months after the drug treatment. Brains were quickly removed, and hippocampus and frontal cortex were dissected out, immediately frozen and stored at −80°C. Animals that received i.c.v. STZ had no symptoms of diabetes and the steady-state blood glucose level did not differ from that in control animals.

Morris water maze swimming test

Cognitive functions were tested using a Morris water maze swimming test (Anger 1991). Adaptation of rats to the experimental environment and behavioural activity was done for 2 days before the experimental trials. On the first day animals were subjected to 1 min of freely swimming in a pool (150 × 60 cm, 50 cm deep), with the water temperature set at 25 ± 1°C, and on the second day they were allowed to swim freely in the pool divided in four quadrants (I–IV). In the experimental trials, performed from day 1 to day 4, rats could escape from the water by finding an unseen rigid platform submerged about 2 cm below the water surface in quadrant IV. A 15-s stay on the platform was allowed. 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) after removal of the platform from the pool. The time spent searching for the platform after entering quadrant IV was recorded. The cut-off time was 1 min. Rats that had no alterations in memory functions (control) were expected to remember that the platform had previously been there and to spend a long time swimming within quadrant IV, looking for the platform. Rats with drug-induced deterioration of memory were expected to be less likely to remember that the platform had been in quadrant IV, and therefore to spend less time searching for the platform within this quadrant, in comparison with control rats.

Western blot

Hippocampal (one sample per animal) and frontal cortical (one sample from three animals, to get an enough protein) tissue samples from the left half of the rat brain were homogenized with three volumes of lysis buffer containing 10 mM HEPES, 1 mM EDTA, 100 mM KCl, 1% Triton X-100, pH 7.5, and a protease inhibitor cocktail (1 : 100), and the homogenates were centrifuged at 600 *g* for 10 min. The supernatants were further centrifuged at 45 000 *g* for 30 min at 4°C, and the pellets were resuspended in 100 μL lysis buffer. Finally, the resuspended pellets were mixed with appropriate previous supernatants obtained from the second centrifugation. Protein concentration was measured by Bradford protein assay. Samples were frozen and stored at −80°C.

Equal amounts of total protein (150–200 μg per sample for enzyme, and 50 μg per sample for tau protein analyses) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis 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, 150 mM NaCl, pH 7.5, and 0.5% Tween 20, either overnight at 4°C for GSK-3, or 1 h at 22°C for pGSK-3, Akt/PKB and total tau. Blocked blots were incubated either on the next day with primary anti-GSK-3α/β antibody (1 : 1000) for 2 h at room temperature, or overnight at 4°C with primary antibody against pGSK-3 (1 : 1000), Akt/PKB (1 : 1000) or total tau (1: 10 000); the latter antibody recognizes total tau at C-terminal amino acids 243–441. After incubation, the membranes were washed three times with LSWB

© 2006 The Authors

and incubated for 60 min at room temperature with secondary antibody solution (anti-mouse IgG, 1 : 2000, for GSK-3 analysis; anti-rabbit IgG, 1 : 5000, for the remainder). The specificity of the signal was checked on control membranes that were not incubated with primary antibody. After washing three times in LSWB, the membranes were immunostained using chemiluminiscence western blotting detection reagents. Signals were captured and visualized with the Chemi Doc video camera system (Bio-Rad, Hertfordshire, UK), or following exposure of membranes to film (tau protein analysis).

Staining of β-amyloid fibrils by modified alkaline Congo Red

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

Statistical analysis

In the western blot analysis of hippocampal tissue three samples per treatment group were loaded on one gel, and two gels were analysed for one experiment. Densitometric values of samples from two treatments (STZ and TG) on each gel were expressed as a percentage value for the control group on the respective gel. In this way, values from two gels/immunoblots that belonged to the same experiment could be combined, allowing for statistical analysis. Values were expressed as mean ± SEM relative protein level. Owing to pooling of the samples of frontal cortical tissue for western blot analysis, there were only two samples (one sample from three 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 (in seconds) spent searching for the hidden platform in quadrant IV during the three consecutive starts from quadrant I–III in the last experimental trial. Median (range) values are presented. The significance of between-group differences was tested by Kruskal–Wallis ANOVA median test, followed by Mann–Whitney *U*-test; $p < 0.05$ was 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 (National Institutes of Health 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 forms respectively. The signal was measured densitometrically as a joint α + β signal. However, it was evident visually that the β form was expressed with a lower intensity than the α form on GSK-3α/β analysis, whereas the α form signal was of lower intensity than the β form in the analysis of pGSK-3α/β.

Hippocampus

Quantitative analysis of immunoblots indicated that GSK-3α/β levels in hippocampal homogenates were similar to control values at 1 and 3 months after STZ and TG treatment (Fig. 1). In contrast, the pGSK-3α/β level in the hippocampus was significantly increased (+ 63%) 1 month

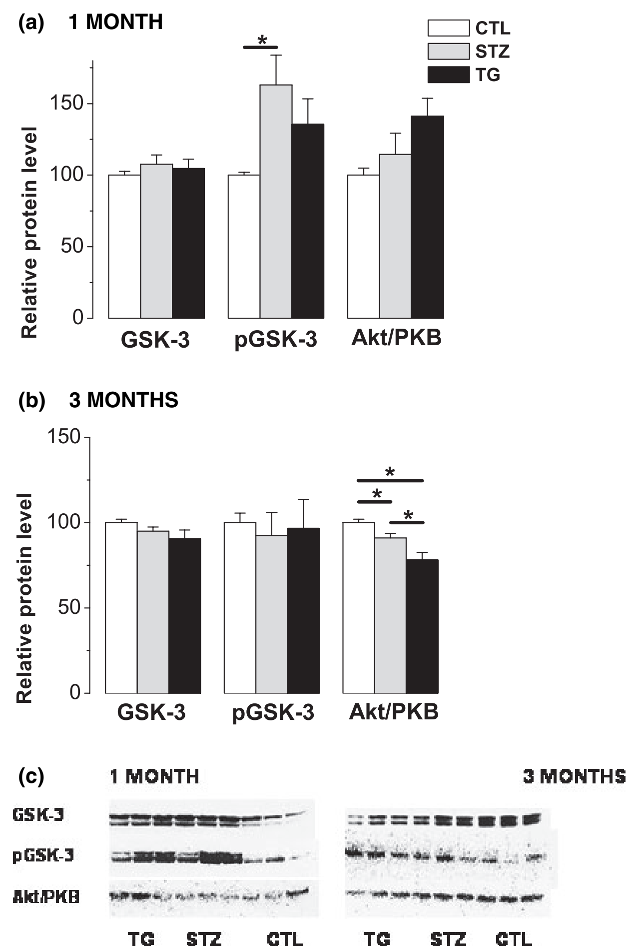

Fig. 1 Western blot analysis of GSK-3α/β, pGSK-3α/β and Akt/PKB protein in the hippocampus of rats treated i.c.v with streptozotocin or GLUT2 blocker TG (a) 1 month and (b) 3 months after drug treatment and in control animals (CTL). Values are mean ± SEM ($n = 5$–6). *$p < 0.05$ (Kruskal–Wallis ANOVA median test followed by Mann–Whitney *U*-test). (c) Blots from a typical experiment are presented (one sample from one animal). Lanes 1–3, TG treatment; lanes 4–6, STZ treatment; lanes 7–9, control group (CTL).

© 2006 The Authors

following STZ treatment (Fig. 1), but declined to below the control value at 3 months after STZ treatment (− 8%); the latter decrease was not statistically significant (Fig. 1). Interestingly, TG treatment produced a non-significant increase (+ 35%) in pGSK-3α/β expression after 1 month, (Fig. 1), which had also declined to below the control value at 3 months after the drug treatment (− 8%) (Fig. 1). The relative pGSK-3α/β to GSK-3α/β ratio in hippocampal tissue was increased after the first month in rats treated with i.c.v. STZ (+ 50%) or TG (+ 37%) in comparison to controls (Fig. 2a), but decreased after the third month, being 9% below control levels in the STZ group and 9% above control levels in TG-treated rats (Fig. 2a). The increase in pGSK-3 to GSK-3 ratio after the first month was noted for both β and α isoforms individually (Fig. 2c). However, by 3 months after the treatment, the ratio of pGSK-3β to GSK-3β had decreased, whereas that of pGSK-3α to GSK-3α was zero (Fig. 2c) because the pGSK-3α band was below the limit of detection.

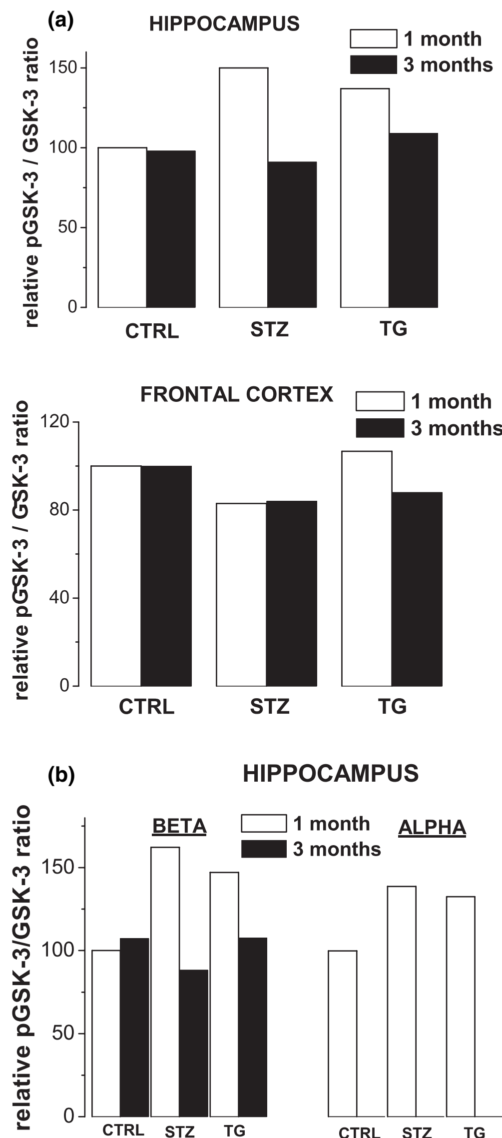

Fig. 2 Relative pGSK-3 to GSK-3 ratio in (a) hippocampus and (b) frontal cortex of rats after i.c.v. treatment with STZ or TG, and in control rats (CTL). In (a) and (b) the ratio was calculated from combined pGSK-3/GSK-3 α + β values. In (c) the ratio was calculated for β and α isoforms separately. Note that the pGSK-3α band was practically invisible 3 months after the treatment.

Frontal cortex

Quantitative analysis of GSK-3α/β immunoblots from pooled homogenates of frontal cortical tissue demonstrated that i.c.v. STZ and TG treatment induced mild changes that were ≤ 10% below or above the respective control value at both observation times (Fig. 3). STZ-induced alterations in

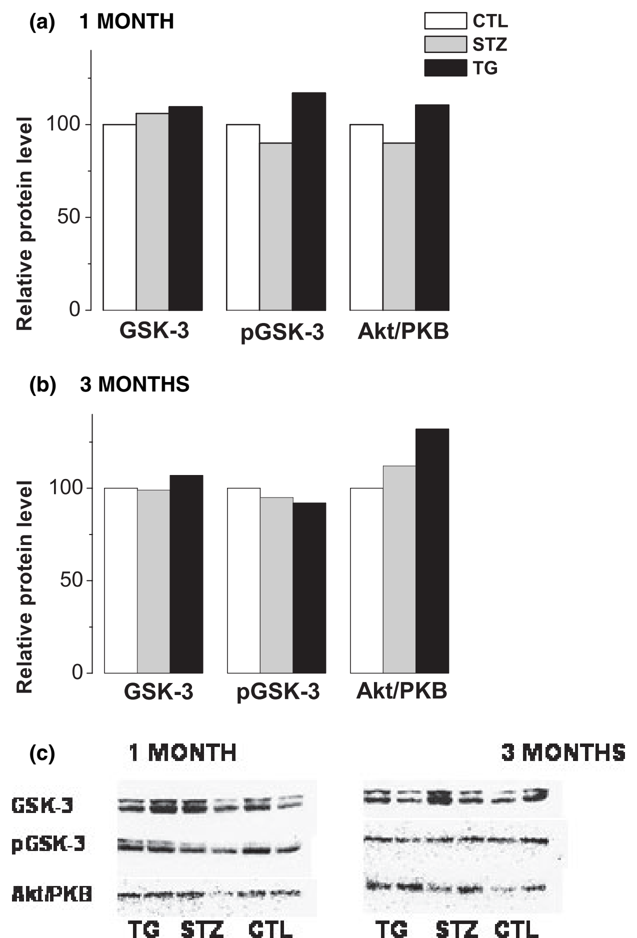

Fig. 3 Western blot analysis of GSK-3α/β, pGSK-3α/β and Akt/PKB protein in the frontal cortex of rats treated i.c.v with streptozotocin or GLUT2 blocker TG (a) 1 and (b) 3 months after drug treatment and in control animals (CTL). Values are mean of two samples (each sample represents tissue pooled from three animals). (c) Blots from a typical experiment are presented. Lanes 1–2, TG treatment; lanes 3–4, STZ treatment; lanes 5–6, control group (CRTL).

© 2006 The Authors
Journal Compilation © 2006 International Society for Neurochemistry,

pGSK-3α/β expression were also mild, less than 10% below control values (Fig. 3). TG treatment induced an increase (+ 20%) in pGSK-3α/β expression after the first month, which declined to below the control value after 3 months (− 10%) (Fig. 3). No statistical analysis was performed. The relative pGSK-3α/β to GSK-3α/β ratio in frontal cortical tissue was equally decreased after the first (− 17%) and the third (− 16%) months after i.c.v. STZ treatment, in comparison to controls (Fig. 2a), whereas in TG-treated rats a decline in this ratio was observed only after the third month (− 12%) (Fig. 2a).

Akt/PKB

Western blot analysis of Akt/PKB protein (antibody detected 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 the Akt/PKB level in hippocampal homogenates treated with STZ was slightly but not significantly increased (+ 14%) by 1 month, and mildly but statistically significantly decreased (− 9%) at 3 months after STZ treatment (Fig. 1). In TG-treated animals the Akt/PKB level tended to increase (+ 41%) after the first month, although the increase was not statistically significant owing to intragroup variation, but was significantly lower (− 22%) after 3 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 1 month, which turned to a mild increase by 3 months after the treatment (+ 14%) (Fig. 2). However, Akt/PKB values seemed to gradually increase following TG treatment, by 11 and 32% after 1 and 3 months respectively (Fig. 3).

Tau protein

A preliminary experiment was performed to determine whether any change could be observed at the tau protein level. The immunoreaction of total tau (recognizing total tau protein at C-terminal amino acids 243–441) increased significantly in the hippocampus of STZ-treated rats in comparison to levels in control animals (Fig. 4).

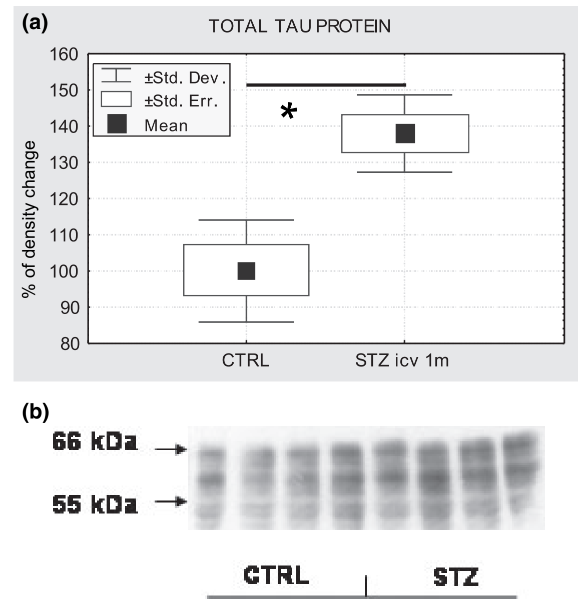

Fig. 4 Western blot analysis of total tau protein visualized by K9JA antibody (recognizes C-terminal part of tau protein) in the hippocampus of rats 1 month after i.c.v. STZ (a) Quantitative analysis. $^{*}p < 0.05$ (Mann–Whitney *U*-test). Representative blots are shown in (b). Lanes 1–4, control group (CRTL); lanes 5–8, STZ group.

Congophilic deposits in the rat brain

Representative sections of human and rat brain are shown in Fig. 5. Figures 5(a) and (b) show β-amyloid aggregates in human Alzheimer's disease brain intensively stained by Congo Red. In the final stages of the disease β-amyloid aggregates can be seen in blood vessels and as cerebellar plaques.

Examination of rat brain 3 months after treatment with i.c.v. STZ revealed diffuse congophilic deposits in the blood vessels, but not within the brain (Fig. 5c). Congophilic deposits were additionally monitored with cross-polarized light, where they showed characteristic green autofluorescence (Fig. 5d). Untreated control animals, however, were devoid of such autofluorescent deposits (Figs 5e and f).

Morris water maze swimming test

In the Morris water maze swimming test all STZ-treated rats demonstrated significant cognitive deficits in learning and memory function (Fig. 6). In each experiment, STZ-treated animals spent significantly less time searching for the hidden platform than control rats (Fig. 6). Changes in comparison to the control group were more pronounced after 3 months (− 46%) than after 1 month (− 33%) (Fig. 6). Interestingly, TG-treated rats demonstrated similar cognitive deficits, spending less time searching for the hidden platform than control rats in all observation periods (51 and 37% below control values) (Fig. 6). However, compared with STZ-treated rats, TG-treated rats demonstrated more severe cognitive deficits and spent significantly less time searching after 1 month, but showed significantly less severe cognitive deficits after 3 months (Fig. 6).

Discussion

Insulin and IR signalling participate in a variety of region-specific functions in the CNS, through mechanisms not necessarily associated with glucose regulation (Schulingkamp

© 2006 The Authors

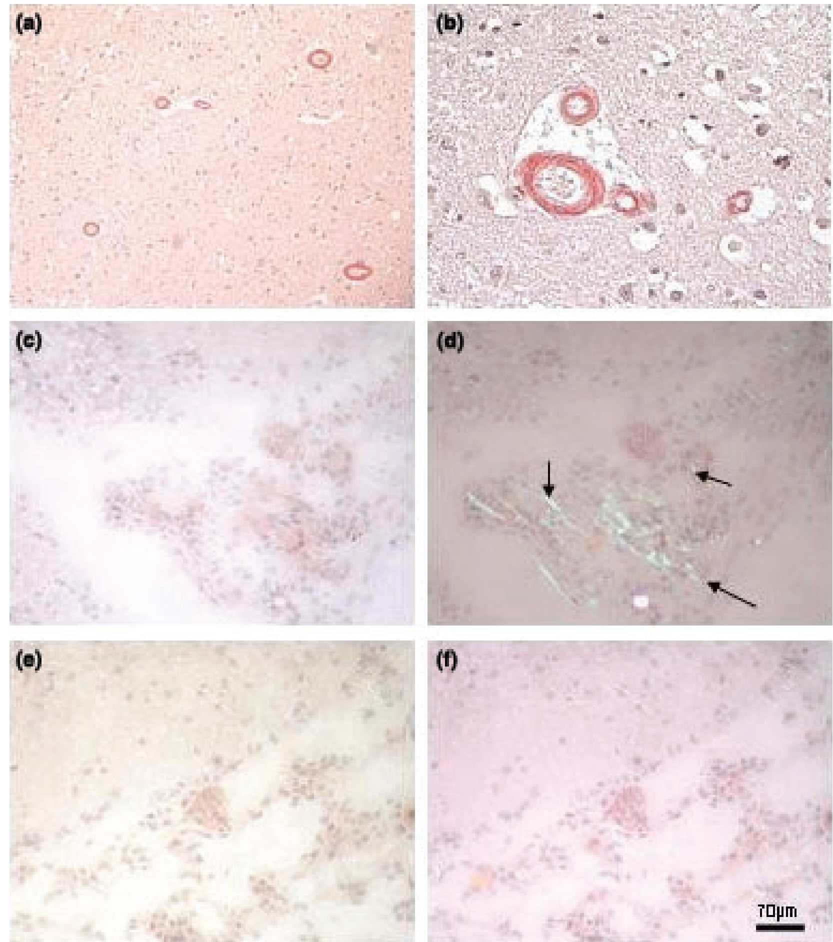

Fig. 5 β-Amyloid peptide deposits in meningeal capillaries of i.c.v. streptozotocin-treated rats 3 months after drug treatment, visualized by Congo Red staining. (a, b) Tissue sections of human Alzheimer's disease brain show β-amyloid, visualized as characteristic congophilic deposits. (c) Brain tissue section from rat 3 months after treatment with STZ exhibits diffuse congophilic deposits (arrows), which are autofluorescent by cross-polarized light (d). (e, f) Untreated control animals do not exhibit such autofluorescent congophilic material.

et al. 2000). Growing evidence suggests that IR signalling modulates neuronal excitability and synaptic plasticity (Wan *et al*. 1997; Skeberdies *et al*. 2001), consequently affecting cognitive functions such as 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 Frölich 2005). However, IR and its signalling cascade have not been investigated either in rats treated with i.c.v. STZ, suggested as an experimental model of sporadic Alzheimer's disease, or in rats with a disturbed brain glucose-sensing system.

In the hippocampus of STZ-treated rats we found alterations at the level of Aκt/PKB–GSK-3 enzymes that are downstream in the PI3 kinase pathway of the IR signalling cascade. The level of pGSK-3α/β was significantly increased at 1 month following treatment, and then decreased to below control values by 3 months. However, these changes were not followed by alterations in non-phosphorylated GSK-3α/β, levels of which remained unchanged. Inconsistent results have been reported with respect to brain GSK-3 levels in Alzheimer's disease in humans; 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 unchanged (Pei *et al*. 1997), reduced (Swatton *et al*. 2004) and increased (Steen *et al*. 2005) GSK-3α/β activity, have been noted. A significant increase in GSK-3 activity has been reported in the hippocampus of Tet/GSK-3β transgenic mice, another proposed experimental model of Alzheimer's disease (Hernandez *et al*. 2002).

The present study represents the first investigation of GSK-3 in the brain of the i.c.v. STZ-treated rat. Changes found in our experiments suggest effects at the level of GSK-3 phosphorylation or pGSK-3 dephosphorylation, as total GSK-3α/β levels were unchanged in both hippocampus and frontal cortex, corresponding to findings in human Alzheimer's disease (Steen *et al*. 2005), whereas the level of pGSK-3 was increased. A decrease in the relative pGSK-3 to GSK-3 ratio in hippocampal tissue of STZ-treated rats between the first and third months, from 50% above the control level to 9% below the control level respectively, indirectly suggests an increase in the non-phosphorylated, active GSK-3 form with time (Fig. 2). This tendency towards

© 2006 The Authors

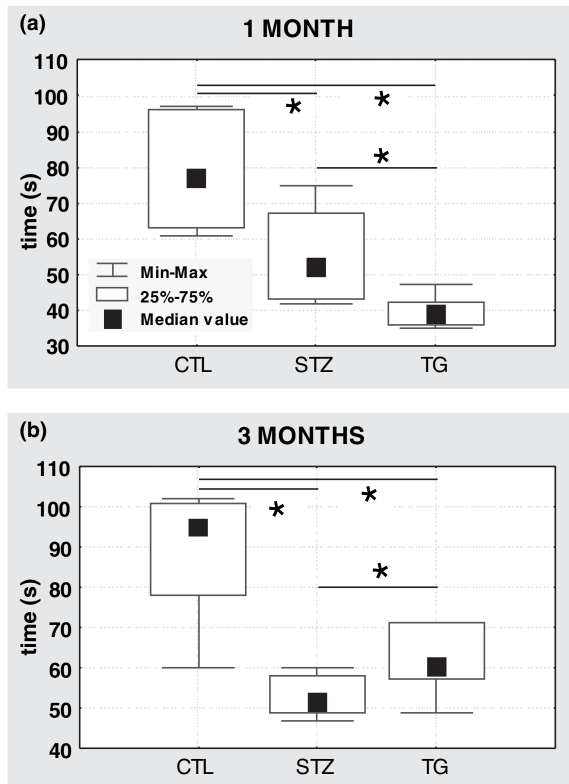

Fig. 6 Memory function in Morris water maze swimming test (a) 1 month and (b) 3 months after i.c.v. treatment with STZ or TG and in control rats (CTL). Deficits were measured as the time spent searching for a hidden platform in the quadrant in which the platform had been placed in training trials. The better the memory had been preserved, the longer the rats were searching for the platform, and *vice versa*. *$p < 0.05$ (Kruskal–Wallis ANOVA median test followed by Mann–Whitney *U*-test).

a decrease in the ratio of phosphorylated to total enzyme was demonstrated for both α and β isoforms, and seemed particularly pronounced after 3 months for the α isoform (Fig. 2). However, the activity of GSK-3, whose alterations cannot be excluded, was not measured in our experiments. The increased ratio observed in the hippocampus of STZ treated rats after the first month might represent an acute change, as afterwards the ratio declined. Possibly, induced changes of these enzymes could not be compensated and the pGSK-3/GSK-3 ratio decline continued below the control values after the third observation month. The suggested increase in non-phosphorylated, active GSK-3 in the brain of STZ-treated rats might lead to tau hyperphosphorylation. Preliminary experiments indeed showed some alterations at the level of tau protein, but the significance of these is not clear and more extensive analyses of tau protein in the i.c.v. STZ rat model are under way.

The pGSK-3a/β antibody used in our western blot analysis detects endogenous levels of GSK-3 when phosphorylated at Ser 21 of GSK-3α or Ser 9 of GSK-3β. Ser21/9 phosphorylation and thus inactivation of GSK-3 is mediated by Akt/PKB, which lies downstream in the IR-activated PI3 kinase pathway (Cross *et al*. 1995). The total level of Akt/PKB in hippocampal tissue samples was unchanged after 1 month and slightly decreased after 3 months, and small changes (± 10%) were noted in the frontal cortex of STZ-treated rats. These data suggest that this protein was quite resistant to damage over the 3-month period of observation in this experimental model. In line with this, levels of total GSK-3 protein were found to be unchanged. These results are in agreement with the finding that Akt/PKB levels were unchanged in the brain of patients with Alzheimer's disease at autopsy (Steen *et al*. 2005). The same authors reported decreased levels of phosphorylated Akt/PKB (pAkt/PKB) and pGSK-3, and the latter were also found to have decreased moderately over 3 months in our proposed experimental model of Alzheimer's disease. Our i.c.v. STZ rats could be thought of as a model which enables follow up of pathological changes in relation to disease stage and severity. Therefore, 1 and 3 month-long observation period in rats might represent early and subchronic stage of disease, respectively, in contrast to post-mortem studies on brains of humans with, in general, severe end-stage sporadic Alzheimer's disease. In line with this, we report structural changes in STZ-treated rats, localised in brain capillaries only, in comparison to disease in humans where both brain capillaries and tissue have been affected (Fig. 5). Namely, structural changes at the level of β-amyloid peptide accumulation, resembling those in human Alzheimer's disease, were observed in the i.c.v. STZ rat model. The conformational transition of β-amyloid peptides from α-helices to β-sheets strongly favours the formation of β-amyloid fibrils, which give rise to pathological protein aggregates called amyloid plaques. β-Amyloid fibrils stained with Congo Red showed green autofluorescence on cross-polarization. Congo Red, which is known to specifically bind to β-amyloid fibrils (Klunk *et al*. 1989; Balbirnie *et al*. 2001), has been used widely in histological staining procedures for the evaluation of β-amyloid aggregates in human (Ladewig 1945) and murine (Li *et al*. 2005) tissues. A possible relationship between structural changes and the decrease in pGSK-3α to GSK-3α ratio, which suggests an increase in non-phosphorylated active GSK-3α, known to be involved in Aβ regulation (Phiel *et al*. 2003), cannot be excluded. The severity of STZ-induced effects has been demonstrated to be dose dependent following its peripheral (persistent or transient diabetes, morphological alterations of islet insulin-immunoreactive cells; Junod *et al*. 1969; Ar'Rajab and Ahren 1993) and central (neurochemical alterations of brain monoamine level; Lackovic and Salkovic 1990) administration, supporting the stage- and severity-dependent hypothesis of manifestation of pathological changes. Furthermore, regarding human Alzheimer's disease, a statistically signifi-

© 2006 The Authors

cant positive correlation noted in human tissue between Akt/PKB activities or pAkt/PKB levels and Braak staging for the neurofibrillary changes supports this hypothesis (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 at autopsy in temporal cortex but not in frontal cortex (Rickle *et al*. 2004), of patients with Alzheimer's disease suggest a possible regional pattern of changes. Our findings of a highly increased pGSK-3α/β level after 1 month in the hippocampus but not in the frontal cortex, and a decreased Akt/PKB level in the hippocampus but an increase in the frontal cortex after 3 months, in i.c.v. STZ-treated rats are consistent with these reports.

We did not measure the activity of Akt/PKB, and it cannot be excluded that the increased level of hippocampal pGSK-3α/β found 1 month after STZ treatment was the consequence of increased Akt/PKB activity, as reported elsewhere (Rickle *et al*. 2004). However, besides Akt/PKB, numerous kinases can phosphorylate GSK-3β at Ser 9, such as protein kinase C, involved in signalling of G protein-linked receptors (Kaytor and Orr 2002). Furthermore, the increased pGSK-3 level might be related to inactivity and/or decreased levels of the phosphatase that dephosphorylates GSK-3; serine–threonine protein phosphatase 1 and 2A (PP2A) have been mentioned in this context (Chen *et al.* 1992; Millward *et al*. 1999; Bennecib *et al*. 2000; Hoyer and Frölich 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 (Millward *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 brains from patients with Alzheimer's disease (Gong *et al*. 1995; Sontag *et al*. 2004). A recent finding of up-regulation of endogenous PP2A inhibitors in the neocortex of patients with Alzheimer's disease further supports this hypothesis (Tanimukai *et al*. 2005). Therefore, it cannot be excluded that in the i.c.v. STZ experimental model some neurochemical changes are related to a lower activity/protein level of PP2A. This requires further investigation. 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 for hyperphosphorylation of tau *in vivo*, indicating that inhibition of PP2A, an enzyme that can directly dephosphorylate tau, is probably the predominant factor in inducing tau hyperphosphorylation (Planel *et al*. 2001).

In line with published reports of i.c.v. STZ-induced cognitive deficits (Lannert and Hoyer 1998; Prickaerts *et al*. 1999; Sharma and Gupta 2001; Hoyer 2004), decreased memory function was found in STZ-treated rats in our experiments, demonstrated as a decline in time spent searching for the hidden platform within the appropriate quadrant.

The results of our experiments with TG, a GLUT2 blocker, were surprising. A single i.c.v. TG treatment induced long-lasting neurochemical effects in the hippocampus, which mostly resembled those induced by i.c.v. STZ treatment, for example a tendency to an increase in pGSK-3α/β level after 1 month, unchanged GSK-3α/β levels over the 3-month observation period, and a decrease in Akt/PKB level at 3 months; the latter was more pronounced than that induced by STZ at the same time point. Thus, it might be speculated that, by blocking intracellular glucose uptake and consequently its intracellular metabolism and possible glucose sensing, TG induced local conditions in the brain that might be similar to the impaired brain glucose uptake and metabolism found in human sporadic Alzheimer's disease and in i.c.v. STZ-treated rats, as reviewed elsewhere (Hoyer 2004; Hoyer and Frõlich 2005). The finding of cognitive deficits in the Morris water maze swimming test in TG-treated rats, which were more severe than those in STZ-treated rats after 1 month, supported this hypothesis. These results are in agreement with the finding that 3 weeks after i.c.v. STZ injection the ultrastructure of rat frontoparietal cortical neurones was similar to that observed after i.v. application of non-metabolizable glucose analogue 2-deoxy-glucose (Grieb *et al*. 2004). The finding of a small improvement in cognitive deficits at 3 months in TG-treated mice compared with STZ-treated animals, but a persisting deficit in comparison with controls, suggests the involvement of some as yet unknown compensatory mechanisms and factors in this experimental model.

In conclusion, i.c.v. STZ probably induces experimental, sporadic Alzheimer's disease in rats, characterized by an acute increase in pGSK-3α/β level and subsequent tendency to a decline in pGSK-3α/β and Akt/PKB levels in the hippocampus. The finding of smaller changes in frontal cortex over the same observation period suggests that changes in this model are region specific. Intracerebroventricular treatment with a blocker of GLUT2, a glucose transporter suggested to be involved in brain glucose sensing, induced neurochemical changes and cognitive deficits that were generally similar to those induced by i.c.v. STZ treatment. This is the first report of an altered IR-PI3 kinase downstream signalling pathway in i.c.v. STZ-treated rats and the data support the hypothesis that these rats represent an experimental model of sporadic Alzheimer's disease. Furthermore, a possible role of GLUT2 in the pathophysiology of sporadic Alzheimer's disease, at least in this experimental animal model, is suggested.

© 2006 The Authors
Journal Compilation © 2006 International Society for Neurochemistry,

Acknowledgements

The research was supported by the Ministry of Science, Education and Sports, Republic of Croatia (project no. 0108253) and by Deutscher Akademischer Austausch Dienst through a collaborative (Germany, Bosnia and Herzegovina, and Croatia) project (no. A/04/20017), within the frame of the Stability Pact for South-eastern Europe programme. We thank Dr E.-M. Mandelkow for the tau antibody, Professor Dr W. Roggendorf for suggestions regarding histological analysis and Mrs B. Hrzan for technical assistance.

References

Anger W. K. (1991) Animal test systems to study behavioral dysfunctions of neurodegenerative disorders. *Neurotoxicology* **12**, 403–413.

Ar'Rajab A. and Ahren B. (1993) Long-term diabetogenic effect of streptozotocin in rats. *Pancreas* **8**, 50–57.

Arluison M., Quignon M., Thorens B., Leloup C. and Penicaud L. (2004a) Immunocytochemical localization of the glucose transporter 2 (GLUT2) in the adult rat brain. II. Electron microscopic study. *J. Chem. Neuroanat.* **28**, 137–146.

Arluison M., Quignon M., Nguyen P., Thorens B., Leloup C. and Penicaud L. (2004b) Distribution and anatomical localization of the glucose transporter 2 (GLUT2) in the adult rat brain – an immunohistochemical study. *J. Chem. Neuroanat.* **28**, 117–136.

Balbirnie M., Grothe R. and Eisenberg D. S. (2001) An amyloid-forming peptide from the yeast prion Sup35 reveals a dehydrated beta-sheet structure for amyloid. *Proc. Natl Acad. Sci. USA* **98**, 2375–2380.

Baskin D. G., Porte D. Jr, Guest K. and Dorsa D. M. (1983) Regional concentrations of insulin in the rat brain. *Endocrinology* **112**, 898–903.

Bennecib M., Gong C. X., Grundke-Iqbal I. and Iqbal K. (2000) Role of protein phosphatase-2A and -1 in the regulation of GSK-3, cdk5 and cdc2 and the phosphorylation of tau in rat forebrain. *FEBS Lett.* **485**, 87–93.

Blokland A. and Jolles J. (1993) Spatial learning deficit and reduced hippocampal ChAT activity in rats after an icv injection of streptozotocin. *Pharmacol. Biochem. Behav.* **44**, 491–494.

Boyd F. T. Jr and Raizada M. K. (1983) Effects of insulin and tunicamycin on neuronal insulin receptors in culture. *Am. J. Physiol.* **245**, C283–C287.

Brant A. M., Jess T. J., Milligan G., Brown C. M. and Gould G. W. (1993) Immunological analysis of glucose transporters expressed in different regions of the rat brain and central nervous system. *Biochem. Biophys. Res. Commun.* **192**, 1297–1302.

Chen J., Martin B. L. and Brautigan D. L. (1992) Regulation of protein serine–threonine phosphatase type-2A by tyrosine phosphorylation. *Science* **257**, 1261–1264.

Craft S., Peskind E., Schwartz M. W., Schellenberg G. D., Raskind M. and Porte D. Jr (1998) Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype. *Neurology* **50**, 164–168.

Cross D. A. E., Alessi D. R., Cohen P., Andjelkovich M. and Hemmings B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785–789.

De la Monte S. M. and Wands J. R. (2005) Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. *J. Alzheimer Dis.* **7**, 45–61.

Ding A., Nitsch R. and Hoyer S. (1992) Changes in brain monoaminergic neurotransmitter concentrations in rat fater intracerebroventricular injection of streptozotocin. *J. Cereb. Blood Flow Metab.* **12**, 103–109.

Dudek H., Datta S. R., Franke T. F., Birnbaum M. J., Yao R., Cooper G. M., Segal R. A., Kaplan D. R. and Greenberg M. E. (1997) Regulation of neuronal survival by the serine–threonine protein kinase Akt. *Science* **275**, 661–665.

Duelli R., Schröck H., Kuschinsky W. and Hoyer S. (1994) Intracerebroventricular injection of streptozotocin induces discrete local changes in cerebral glucose utilization in rats. *Int. J. Dev. Neurosci.* **12**, 737–743.

Frolich L., Blum-Degen D., Bernstein H. G. *et al.* (1998) Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. *J. Neural Transm.* **105**, 423–438.

Fulop T., Larbi A. and Douziech N. (2003) Insulin receptor and ageing. *Pathol. Biol.* **51**, 574–580.

Gong C. X., Shaikh S., Wang J. Z., Zaidi T., Grundke-Iqbal I. and Iqbal K. (1995) Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. *J. Neurochem.* **65**, 732–738.

Grieb P., Kryczka T., FiedorowiczZ. M., Frontczak-BaniewiczZ. M. and Walski M. (2004) Expansion of the Golgi apparatus in rat cerebral cortex following intracerebroventricular injections of streptozotocin. *Acta Neurobiol. Exp. (Warsz.)* **64**, 481–489.

Hellweg R., Nitsch R., Hock C., Jaksch M. and Hoyer S. (1992) Nerve growth factor and choline acetyltransferase activity level in the rat brain following experimental impairment of cerebral glucose and energy metabolism. *J. Neurosci. Res.* **31**, 479–486.

Henneberg N. and Hoyer S. (1995) Desensitization of the neuronal insulin receptor: a new approach in the etiopathogenesis of late-onset sporadic dementia of the Alzheimer type (SDAT)? *Arch. Gerontol. Geriatr.* **21**, 63–74.

Hernandez F., Borrell J., Guaza C., Avila J. and Lucas J. J. (2002) Spatial learning deficit in transgenic mice that conditionally over-express GSK-3beta in the brain but do not form tau filaments. *J. Neurochem.* **83**, 1529–1533.

Hosokawa M., Dolci W. and Thorens B. (2001) Differential sensitivity of GLUT1- and GLUT2-expressing beta cells to streptozotocin. *Biochem. Biophys. Res. Commun.* **289**, 1114–1147.

Hoyer S. (2002) The brain insulin signal transduction system and sporadic (type II) Alzheimer's disease: an update. *J. Neural Transm.* **109**, 341–360.

Hoyer S. (2004) Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. *Eur. J. Pharmacol.* **490**, 115–125.

Hoyer S. and Frölich L. (2005) Brain insulin function and insulin signal transduction in sporadic Alzheimer disease, in *Research Progress in Alzheimer's Disease and Dementia* (Sun M. K., ed.). Nova Science, New York (in press).

Ishiguro K., Shiratsuchi A., Sato S., Omori A., Arioka M., Kobayashi S., Uchida T. and Imahori K. (1993) Glycogen synthase kinase 3 beta is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS Lett.* **325**, 167–172.

Johnstone A. M., Pirola L. and Van Obberghen E. (2003) Molecular mechanisms of insulin receptor substrate protein-mediated modulation of insulin signalling. *FEBS Lett.* **546**, 32–36.

Junod A., Lambert A. E., Stauffacher W. and Renold A. E. (1969) Diabetogenic action of streptozotocin: relationship of dose to metabolic response. *J. Clin. Invest.* **48**, 2129–2139.

Kaytor M. D. and Orr H. T. (2002) The GSK3 beta signaling cascade and neurodegenerative disease. *Curr. Opin. Neurobiol.* **12**, 275–278.

Klunk W. E., Pettegrew J. W. and Abraham D. J. (1989) Quantitative evaluation of congo red binding to amyloid-like proteins with a beta-pleated sheet conformation. *J. Histochem. Cytochem.* **37**, 1273–1281.

Lackovic Z. and Salkovic M. (1990) Streptozotocin and alloxan produce alterations in rat brain monoamines independently of pancreatic beta cells destruction. *Life Sci.* **46**, 49–54.

© 2006 The Authors

Ladewig P. (1945) Double refringence of amyloid Congo red complex in histological section. *Nature* **156**, 81–82.

Lannert H. and Hoyer S. (1998) Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. *Behav. Neurosci.* **112**, 1199–1208.

Leloup C., Arluison M., Lepetit N., Cartier N., Marfaing-Jallat P., Ferre P. and Penicaud L. (1994) Glucose transporter 2 (GLUT2): expression in specific brain nuclei. *Brain Res.* **638**, 221–226.

Li J. P., Galvis M. L., Gong F., Zhang X., Zcharia E., Metzger S., Vlodavsky I., Kisilevsky R. and Lindahl U. (2005) *In vivo* fragmentation of heparan sulfate by heparanase overexpression renders mice resistant to amyloid protein A amyloidosis. *Proc. Natl Acad. Sci. USA* **102**, 6473–6477.

Mayer G., Nitsch R. and Hoyer S. (1990) Effects of changes in peripheral and cerebral glucose metabolism on locomotor activity, learning and memory in adult male rats. *Brain Res.* **532**, 95–100.

Millward T. A., Zolnierowicz S. and Hemmings B. A. (1999) Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem. Sci.* **24**, 186–191.

Ngarmukos C., Baur E. L. and Kumagai A. K. (2001) Co-localization of GLUT1 and GLUT4 in the blood–brain barrier of the rat ventromedial hypothalamus. *Brain Res.* **900**, 1–8.

Nitsch R. and Hoyer S. (1991) Local action of the diabetogenic drug streptozotocin on glucose and energy metabolism in rat brain cortex. *Neurosci. Lett.* **128**, 199–202.

Nitsch R., Mayer G. and Hoyer S. (1989) The intracerebroventricularly streptozotocin-treated rat: impairment of cerebral glucose metabolism resembles the alterations of carbohydrate metabolism of the brain in Alzheimer's disease. *J. Neural Transm. Park. Dis. Dement. Sect.* **1**, 109–110.

Noble E. P., Wurtman R. J. and Axelrod J. (1967) A simple and rapid method for injecting H3-norepinephrine into the lateral ventricle of the rat brain. *Life Sci.* **6**, 281–291.

Pei J. J., Tanaka T., Tung Y. C., Braak E., Iqbal K. and Grundke-Iqbal I. (1997) Distribution, levels, and activity of glycogen synthase kinase-3 in the Alzheimer disease brain. *J. Neuropathol. Exp. Neurol.* **56**, 70–78.

Pei J. J., Khatoon S., An W. L. *et al.* (2003) Role of protein kinase B in Alzheimer's neurofibrillary pathology. *Acta Neuropathol. (Berl.)* **105**, 381–392.

Petrisic S. M., Augood S. J. and Bicknell R. J. (1997) Monoamine transporter gene expression in the central nervous system in diabetes mellitus. *J. Neurochem.* **68**, 2435–2441.

Phiel C. J., Wilson C. A., Lee V. M.-Y. and Klein P. S. (2003) GSK-3α regulates production of Alzheimer's disease amyloid-β peptides. *Nature* **423**, 435–439.

Planel E., Yasutake K., Fujitas S. C. and Ishiguro K. (2001) Inhibition of protein phosphatase 2A overrides tau protein kinase I/glycogen synthase kinase 3β and cyclin-dependent kinase 5 inhibition and results in tau hyperphosphorylation in the hippocampus of starved mouse. *J. Biol. Chem.* **276**, 34 298–34 306.

Plaschke K. and Hoyer S. (1993) Action of the diabetogenic drug streptozotocin on glycolytic and glycogenolytic metabolism in adult rat brain cortex and hippocampus. *Int. J. Dev. Neurosci.* **11**, 477–483.

Preece P., Virley D. J., Costandi M., Coombes R., Moss S. J., Mudge A. W., Jazin E. and Cairns N. J. (2003) Beta-secretase (BACE) and GSK-3 mRNA levels in Alzheimer's disease. *Brain Res. Mol. Brain Res.* **116**, 155–158.

Prickaerts J., Fahring T. and Blokland A. (1999) Cognitive performance and biochemical markers in septum, hippocampus and striatum of rats after an i.c.v. injection of streptozotocin: a correlation analysis. *Behav. Brain Res.* **102**, 73–88.

Puchtler H., Sweat F. and Levine M. (1962) On the binding of Congo red by amyloid. *J. Histochem. Cytochem.* **10**, 355–364.

Puro D. G. and Agardh E. (1984) Insulin-mediated regulation of neuronal maturation. *Science* **225**, 1170–1172.

Rickle A., Bogdanovic N., Volkman I., Winblad B., Ravid R. and Cowburn R. F. (2004) Akt activity in Alzheimer's disease and other neurodegenerative disorders. *Neuroreport* **15**, 955–959.

Salkovic M., Sabolic L. and Lackovic Z. (1995) Striatal dopaminergic D1 and D2 receptors after intracerebroventricular application of alloxan and streptozotocin in rat. *J. Neural Transm.* **100**, 137–145.

Salkovic-Petrisic M. and Lackovic Z. (2003) Intracerebroventricular administration of betacytotoxics alters expression of brain monoamine transporter genes. *J. Neural Transm.* **110**, 15–29.

Schnedl W. J., Ferber S., Johnson J. H. and Newgard C. B. (1994) STZ transport and cytotoxicity: specific enhancement in GLUT2-expressing cells. *Diabetes* **43**, 1326–1333.

Schneppenheim R., Budde U., Dahlmann N. and Rautenberg P. (1991) Luminography – a new, highly sensitive visualization method for electrophoresis. *Electrophoresis* **12**, 367–372.

Schulingkamp R. J., Pagano T. C., Hung D. and Raffa R. B. (2000) Insulin receptors and insulin action in the brain: review and clinical implications. *Neurosci. Biobehav. Rev.* **24**, 855–872.

Sharma M. and Gupta Y. K. (2001) Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment. *Life Sci.* **68**, 1021–1029.

Skeberdies V. A., Lan J., Zheng X., Zukin R. S. and Bennett M. V. (2001) Insulin promotes rapid delivery of *N*-methyl-D-aspartate receptors to the cell surface by exocytosis. *Proc. Natl Acad. Sci. USA* **98**, 3561–3566.

Sontag E., Luangpirom A., Hladik C., Mudrak I., Ogris E., Speciale S. and White C. L. III (2004) Altered expression levels of the protein phosphatase 2A ABalphaC enzyme are associated with Alzheimer disease pathology. *J. Neuropathol. Exp. Neurol.* **63**, 287–301.

Steen E., Terry B. M., Rivera E. J., Cannon J. L., Neely T. R., Tavares R., Xu X. J., Wands J. R. and de la Monte S. M. (2005) Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease – is this type 3 diabetes?. *J. Alzheimer Dis.* **7**, 63–80.

Swatton J. E., Sellers L. A., Faull R. L., Holland A., Iritani. S. and Bahn S. (2004) Increased MAP kinase activity in Alzheimer's and Down syndrome but not in schizophrenia human brain. *Eur. J. Neurosci.* **19**, 2711–2719.

Szkudelski T. (2001) The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiol. Res.* **50**, 537–546.

Tanimukai H., Grundke-Iqbal I. and Iqbal K. (2005) Up-regulation of inhibitors of protein phosphatase-2A in Alzheimer's disease. *Am. J. Pathol.* **166**, 1761–1771.

Thorens B., Sarkar H. K., Kaback H. R. and Lodish H. F. (1988) Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney and beta-pancreatic islet cells. *Cell* **55**, 281–290.

Vannucci S. J., Koehler-Stec E. M., Li K., Reynolds T. H., Clark R. and Simpson I. A. (1998) GLUT4 glucose transporter expression in rodent brain: effects of diabetes. *Brain Res.* **797**, 1–11.

Vogelsberg-Ragaglia V., Schuck T., Trojanowski J. Q. and Lee V. M. (2001) PP2A mRNA expression is quantitatively decreased in Alzheimer's disease hippocampus. *Exp. Neurol.* **168**, 402–412.

Wan Q., Xiong Z. G., Man H. Y., Ackerley C. A., Braunton J., Lu W. Y., Becker L. E., MacDonald J. F. and Wang Y. T. (1997) Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin. *Nature* **388**, 686–690.

Zhao W.-Q., Chen H., Quon M. J. and Alkon D. L. (2004) Insulin and the insulin receptor in experimental models of learning and memory. *Eur. J. Pharmacol.* **490**, 71–81.

© 2006 The Authors
Journal Compilation © 2006 International Society for Neurochemistry,