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NINE-MONTH FOLLOW UP OF THE INSULIN RECEPTOR SIGNALLING CASCADE IN THE BRAIN OF STREPTOZOTOCIN RAT MODEL OF SPORADIC ALZHEIMER'S DISEASE

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Abstract

Sporadic Alzheimer disease (sAD) is associated with impairment of insulin receptor (IR) signalling in the brain. Rats used to model sAD develop insulin resistant brain state following intracerebroventricular treatment with a betacytotoxic drug streptozotocin (STZ-icv) but brain IR signalling has been mostly explored at one time-point and ≤3 months after the STZ-icv administration. We have investigated insulin signalling in the rat hippocampus at 5 time-points in a period ≤9 months after STZ-icv treatment. Male Wistar rats were given vehicle- (control) or STZ- (3mg/kg) icv injection and sacrificed 0.5, 1, 3, 6 and 9 months afterwards. Insulin-1 (Ins-1), IR, phospho- and total-glycogen synthase kinase 3-β (p/t-GSK-3β), phospho- and total-tau (p/t-tau) and insulin degrading enzyme (IDE) mRNA and/or protein were measured. Acute upregulation of tau and IR mRNA ($p<0.05$) was followed by a pronounced downregulation of Ins-1, IR and IDE mRNA ($p<0.05$) in the course of time. Acute decrement in p/t-tau and p/t-GSK-3β ratios ($p<0.05$) was followed by increment in both ratios (3-6 months, $p<0.05$) after which p/t-tau ratio demonstrated a steep rise and p/t-GSK-3β ratio a steep fall up to 9 months ($p<0.05$). Acute decline in IDE and IR expression ($p<0.05$) was followed by a slow progression of the former and a slow recovery of the latter in 3-9 months. Results indicate a biphasic pattern in time-dependency of onset and progression of changes in brain insulin signalling of STZ-icv model (partly reversible acute toxicity and progressive chronic AD-like changes) which makes this model an important tool in translational sAD research.

Keywords
sporadic Alzheimer's disease, streptozotocin, brain insulin signalling

Introduction

Late onset Alzheimer disease (sAD) is a chronic heterogeneous disease of aging characterized by a progressive cognitive decline (Nelson et al. 2009; Blennow et al. 2006). Neuropathologically, sAD is characterized by a loss of neurons and synapses, especially in hippocampus and cortex, the extracellular accumulation of amyloid-β (Aβ) peptide plaques, and the presence of intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein (Grundke-Iqbal et al. 1986, Goedert and Spillantini 2006)

A growing body of evidence has indicated that insulin homeostasis in the brain has an important role in the brain functioning. Insulin signalling plays a role in learning and memory, by modulating synaptic plasticity and activities of excitatory and inhibitory receptors such as glutamate and GABA receptors, and by triggering signal transduction cascades leading to alteration of gene expression that is required for long-term memory consolidation (Cardoso et al. 2009; Gasparini and Xu 2003; Zhao et al. 2004; Zhao et al. 2001). Insulin/insulin receptor (IR) signalling cascade in the brain is similar to the one at the periphery. Upon activation by insulin, IR phosphorylates different intracellular substrates, leading to the activation of two major signalling cascades; phosphoinositide 3-kinase (PI3-K) and the mitogen-activated protein kinase (MAPK) signalling pathway (Czech and Corvera 1999; Paz et al. 1996; Saltiel and Pessin 2002). GSK-3β isoform, which is downstream the PI3-K pathway, plays a role in tau-protein phosphorylation reducing its affinity for microtubules (Ishiguro et al. 1993; Hong et al. 1997). Insulin signalling cascade can affect IDE, highly conserved Zn^{2+} mettaloendopeptidase which also degrades Aβ and insulin whit a slight difference in Km (Zhao et al. 2004).

The idea that defective insulin signalling in the brain contributes to the Alzheimer disease (AD) pathogenesis was first proposed by Siegfried Hoyer 20 years ago (Hoyer 1994). Early abnormalities in brain glucose/energy metabolism have been found in structures with both high glucose demands and high insulin sensitivity, including those involved in cognitive function, which have suggested a role for impaired insulin signalling in the pathogenesis of sAD (Table 1) (Henneberg and Hoyer, 1995; Hoyer 2002, 2004).

Table 1 Literature overview of the IR-IP-3K signalling cascade dysfunction in the hippocampus of STZ-icv model and human sAD brain tissue postmortem.

	TIME AFTER STZ-ICV ADMINISTRATION			HUMAN sAD BRAIN POSTMORTEM
mRNA/protein in HPC	**≤ 1 month**	**> 1- ≤3 months**	**>3 months**	
insulin mRNA				↓(1)
IR mRNA	↓(2,3)		↓(4)	
IR protein	↓(2)			↓(1)
IR-TK activity		↑(3)		↓ (5)
p/t IRS 1 ratio	↓(2)	≈ (6)		
p/t PI-3K ratio		≈(6)		
Akt/PKB	↓(7)	↓(8)		
p/t Akt/PKB ratio	≈ (2)	≈(6)		↓ activity (9)
p/t GSK-3 ratio	↓(10)	↓(8)		↑ activity (equivalent to ↓ratio) (11)
mRNA tau			≈ (12)	↓(1)
p-tau (PHF-1)		↑(3) ≈ (6)		↑(13)
IDE	↓(7)			↓(14,15)

STZ streptozotocin; *icv* intracerebroventricular; *HPC* hippocampus; *IR* insulin receptor; *TK* tyrosine kinase; *IRS* insulin receptor substrate; *PI-3K* phosphatidylinositol-3 kinase; *Akt/PKB* protein kinase B; *p/t* phosphorylated/total; *p-tau* phosphorylated tau protein; *IDE* insulin degrading enzyme; *PHF*, paired helical filaments; ↓ decrease; ↑ increase; ≈ unchanged. Reference number in brackets: 1) Steen et al. 2005; 2) Agrawal et al. 2011; 3) Grünblatt et al. 2007; 4) Lee Y et al. 2014; 5) Frolich et al. 1998 ; 6) Chen Y et al. 2013; 7) Shingo et al. 2012; 8) Salkovic-Petrisic et al. 2006; 9) Rickle et al. 2004 ; 10) Shonsey et al. 2012; 11) Pei et al. 1997; 12) Park et al. 2013 ; 13) Castellani et al. 2008 ; 14) Qui and Folstein 2006; 15) Cook et al. 2003.

In line with alternation of IR signalling pathway in the human sAD brain, intracerebroventricular (icv) administration of streptozotocin (STZ) to rats (STZ-icv model) with a purpose to induce brain insulin system dysfunction has emerged as a promising experimental approach to sAD research. Streptozotocin (STZ) is a drug toxic for insulin secreting/producing cells and IR (Szkudelski 2001, Kadowaki et al. 1984). Similar to what has been found in the human sAD brain, the glucose metabolism was found reduced in 17 of 35 brain areas in the STZ-icv treated rats (Duelli et al. 1994) and the activities of glycolytic key enzymes were found markedly decreased (Plaschke and Hoyer 1993) Recently, regionally specific glucose hypometabolism in FDG-PET imaging was found in monkeys 6 and 12 weeks after the STZ-icv treatment (Lee et al. 2014; Heo et al. 2011). Numerous changes have been found in the insulin/IR signalling cascade in the brain of STZ animal model as reviewed in Table 1. STZ-icv model has additionally been reported to develop also cerebral amyloid angiopathy in rats (appearing at 3 months and progressing up to 9 months after the STZ-icv) (Salkovic-Petrisic 2006; Salkovic-Petrisic 2011) and congophyllic amyloid deposition in mice (1 month after STZ-icv) (Sodhi et al. 2013, Wang et al.2014) as well as increased hippocampal APP mRNA after 4 weeks in rats (de la Monte et al. 2006) and normal APP mRNA expression 5 months after STZ-icv treatment in monkeys (Lee et al. 2014, Park et al. 2013). All were explored in STZ-icv model mostly at one time point only, usually up to 1 or 3 months after the STZ-icv treatment in rats and mice (Agrawal et al. 2010, Agrawal et al. 2011, Schido et al. 2013, Deng et al. 2009, Sodhi et al. 2013, Wang et al. 2014, Chen et al. 2013; Chen et al.2014) and up to 5 months in monkeys (Lee et al. 2014; Park et al., 2013; Heo

et al. 2011). Periods longer than 5 months post-STZ-icv injection have not been explored in regard to the brain insulin system in STZ-icv model of sAD.

We aimed to investigate the time-dependency in onset, development and progression of alternations in the brain insulin/IR signalling pathway in the STZ-icv rat model up to 9 months after the STZ-icv treatment.

Material and Methods

Material

The following chemicals were purchased from Sigma-Aldrich (Sigma, USA): STZ, Trizma® base (Cat. No. T1503.), Na_3VO_4 (Cat. No. S6508), EDTA 0.5 mol/L (Cat. No. E7889), protease inhibitor mix (Cat. No. P8340), sodium deoxycholate (Cat. No. D6750) and b-mercaptoethanol (Cat. No. M7154). Primary antibodies were purchased as follows: Mouse Anti-Insulin receptor (β-subunit), Monoclonal Mouse Anti-GSK-3α/β Unconjugated (Biosource, USA), Phospho-GSK-3β (Ser 9) (Cell Signalling Technology, Incorporated), Rabbit Polyclonal IDE (Chalbiochem, Germany), polyclonal rabbit anti-human tau (DAKO, Glostrup, Denmark) and monoclonal paired helical filaments (PHF)-1 anti-tau (recognizes tau phosphorylated at serine S-396 and S-404) (Sigma, USA). Monoclonal Anti- β-Actin Clone AC-15 (Sigma, USA) or Polyclonal Antibody to GAPDH (Imgenex, USA) were used as loading control. Anti-rabbit IgG, horseradish peroxidase-linked antibody and anti-mouse IgG, horseradish peroxidase linked antibody were purchased from Cell Signalling (Beverly, MA, USA). Chemiluminiscent Western blot detection kit was Lumi-Light^{PLUS} Western Blotting substrate (Roche Diagnostics. Germany). Nitrocellulose membranes were from Invitrogen (Invitrogen GmbH, Germany).

Animals

Three-to four-month-old male Wistar rats weighing 280–330 g (Department of Pharmacology, School of Medicine, University of Zagreb, Zagreb, Croatia) were used throughout the studies. Animals were kept on standardized food pellets and water ad libitum. Rats were randomly divided into two groups (five to six per group) and given general anaesthesia (ketamine 60 mg/kg /xylazine 5-10 mg/kg) followed by injection of streptozotocin (3 mg/kg) administered bilaterally into the left and right lateral ventricle according to the procedure described by Noble et al. (1967) and used in our experiments since 1990. Drug concentration and solution volume was adjusted according to the animal body weight, and a volume of 4 μL per 300 g body weight was administered (2 μL/ventricle). Control animals received bilateral icv injection of an equal volume of vehicle only. STZ-treated and respective control animals were killed 0.5, 1, 3, 6 and 9 months following the icv treatment.

For the protein and gene analyses, brains were quickly removed and cut into left and right half. The hippocampus (HPC) was cut out from the brain, immediately frozen and stored at -80°C. STZ icv-treated animals had no symptoms of systemic diabetes and steady-state blood glucose level did not differ in comparison with control animals.

Quantitative real-time RT-PCR

Total-RNA extraction

Isolation of total RNA was performed using RNeasy mini kit (Qiagen GmbH, Germany) for each animal and brain region separately. An additional step was added to the original protocol in order to receive a pure DNA free total-RNA. The total RNA was on column pre-treated

with DNase-I and the original protocol was then continued in order to have total RNA extraction. The RNA quality and quantity was assessed using the Experion electrophoresis (BioRad Laboratories, Hercules, CA, USA) which analyses the concentration of the total RNA and quality via the ratio of the 28S/ 18S ribosomal RNA. Only intact total RNA samples (with at least 28S/18S ration of 1.7) were used for the gene expression analysis.

Q-PCR

In order to measure the gene expression profile of insulin-1 (Ins 1), IR, IDE and tau quantitative real-time RT-PCR for mRNA samples isolated from rats' hippocampus were performed. Total RNA (1–0.4 mg) from each sample was reversetranscribed with random hexamer and oligo-dT primers using iScriptTM cDNA Synthesis Kit (BioRad Laboratories; Cat. No. 170–8890).. These were normalized to the house-keeping genes: beta-actin (ACTX), ribosomal 18S (Rnr1) and glyceraldehydes-3-phosphate dehydrogenase. The house-keeping genes were tested for their stability using the geNorm program (http://medgen.ugent.be/~jvdesomp/genorm/) (Vandesompele et al. 2002). After analysis for the most stable house-keeping genes, a normalization factor was calculated according to the program. Absence of DNA contamination was verified by amplifying the house-keeping gene, ribosomal 18S and run on gel to observe no product. Minus RT samples were tested simultaneously with experimental samples by quantitative RT-PCR.

Western blot

Tissue preparation. Hippocampal (one animal = one sample) tissue samples from the half of the rat brain were homogenized with three volumes of lysis buffer containing 10 mmol/L HEPES, 1 mmol/L EDTA, 100 mmol/L KCl, 1% Triton X-100, pH 7.5 and protease inhibitors 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 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°C.

Immunoblotting. Equal amounts of total protein (10 μg per sample) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 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 mmol/L Tris, 150 mmol/L NaCl, pH 7.5 and 0.5% Tween 20, 1 h at 25°C. Blocked blots were incubated overnight at 4°C with respective primary anti-total tau (1:10 000), anti-PHF-1-tau 1:300), anti-IR (1:1000), anti total GSK-3 α/ß (1:1000), anti pGSK-3 ß (1:300), anti IDE (1:1000) antibodies. Following the incubation, the membranes were washed three times with LSWB and incubated for 60 min at 25°C with secondary antibody solution (anti-rabbit IgG; 1:2000 and anti-mouse IgG; 1:500). The specificity of the signal was checked on the control membranes that were not incubated with the primary antibody. After washing three times in LSWB, the membranes were immunostained using chemiluminescence Western blotting detection reagents, signal captured and visualised with the Chemi Doc BioRad (Munich, Germany) video camera system or on the film.

Statistics

The differences of gene expression and densitometric values of proteins obtained in Western blot analysis were expressed as mean ± SEM, and the significance of between-group difference was tested by Mann–Whitney U-test. $p < 0.05$ was considered significant.

Ethics

The experiments carried out at the University of Zagreb, Croatia, were under the guidance of the Principles of Laboratory Animal care (NIH Publication No. 86-23, revised in 1985) according to the Croatian Act on Animal Welfare (NN135; 2006) and were approved by The Ethics Committee of the Zagreb University School of Medicine (No. 04-1343-2006).

Results

Gene expression profiles

IR mRNA

A significant increase in the expression of IR mRNA in HPC was found after 2 weeks (+40%, $p< 0.05$). It started to decline afterwards demonstrating a significant decrement at 3-month (-19%, $p< 0.05$), 6-month (-26%, $p<0.05$) and 9-month (-28%, $p<0.05$) time-point after the STZ-icv treatment in comparison to the respective control (Fig 1).

Ins1 mRNA

Expression of Ins1 mRNA was found unchanged 2 weeks after the STZ-icv treatment in comparison to the control but with a huge intra-group variability (Fig 1). Three months after the STZ-icv treatment Ins1 mRNA was significantly down-regulated (-84.5%, $p<0.05$) and stayed decreased at 6-month (-57%, $p<0.05$) and 9-month (-81%, $p<0.05$) time-points after the STZ-icv treatment (Fig 1). This was found to be the strongest effect of STZ-icv treatment on the insulin system in the HPC measured in our experiments during a 9-month follow up (Fig 1).

IDE mRNA

The effect of STZ-icv treatment on the gene expression profile of IDE mRNA in HPC seems to be partly similar to that of insulin mRNA (Fig 1). Expression of IDE mRNA was insignificantly increased by 14% compared to the control 2 weeks, after the STZ-icv treatment after which it started to decrease and became significantly decreased at 3-month (-25%, $p<0.05$) and 6-month (-38%, $p<0.05$) time-points after the STZ-icv treatment in comparison to the control (Fig 1). Decrement found at 9 months (-11%) was not significant compared to control (Fig 1).

Tau mRNA

Acute response seen 2 weeks after the STZ-icv treatment was demonstrated as tau mRNA upregulation (+66%, $p<0.05$), but then this reactive increase started to fall and became normalized 3 months after the treatment (Fig 1). It stayed unchanged 6 and 9 months after the STZ-icv treatment, respectively, indicating the high stability of tau gene expression (Fig 1).

Protein expression profiles

Insulin receptor (IR)

The protein expression of IR (recognising β-subunit of IR receptor) in HPC was found decreased 1 (-20%, $p<0.05$), 3 (-38%, $p<0.05$) and 6 (-28%, $p<0.05$) months after the STZ-icv treatment in comparison to the respective control group. No change was found 9 months after STZ-icv treatment (Fig 2).

Insulin degrading enzyme (IDE)

The expression of IDE in HPC was found decreased after 1 month (-58%, $p<0.05$) and stayed decreased 3 (-21% $p<0.05$), 6 (-26% $p<0.05$), and 9 (-38% $p<0.05$) months after the STZ-icv treatment in comparison to the control, respectively (Fig 3).

Total and phosphorylated glycogen synthase kinase -3 beta (t-GSK-3ß and p-GSK-3ß)

The expression of t-GSK-3ß remained unchanged until 6 months but then increased 9 months after the STZ-icv treatment for 47% ($p<0.05$) in comparison to the controls (Fig 4). The expression of p-GSK-3ß showed a biphasic response pattern in the course of time; acute decrease at 2 weeks (-40%, $p<0.05$), normalisation at 1 and 3 months, followed by an increase at 6 months (54%, $p<0.05$) and then decreased 9 months (-45%, $p<0.05$) after the STZ-icv treatment, respectively (Fig 4)

Total and phosphorylated tau (t-tau and p-tau) protein

The expression of t-tau in the HPC was found mildly increased at 6 months (+22%, $p<0.05$) and decreased 9 months (-32%, $p<0.05$) after the STZ-icv treatment in comparison to the control, respectively (Fig 5). The expression of p-tau in the HPC was found acutely decreased at 2 weeks (-26%, $p<0.05$) which was followed by a significant increase at 3 and 6 months (+90% and +25%, $p<0.05$) time points in comparison to the control (Fig 5). After normalization of the phosphorylated tau levels with the level of total tau (p/t tau ratio), significant increase in tau phosphorylation was seen 3 and 9 months after the STZ-icv treatment (Fig 5). This increase in hyperphosphorylated tau appears in the course of time after the changes in insulin/IR expression.

Summary of 9-month follow up of the protein expression of IR and IDE and of the ratio of p/t GSK-3β and p/t tau.

While IR expression shows a slow progressive decline up to 3 months following STZ-icv treatment, after which a slow tendency of normalization of this decline has been observed towards 9-month time-point (but being still significantly decreased after 6 months), IDE expression showed a biphasic pattern in the course of time (Fig 6). The acute decline in IDE expression seen after 1 month was followed by a tendency to reduce this decline which, however, remained significant at the 3-month time-point. From 3 months onward, IDE expression started to slowly and progressively decline again until 9 months after STZ-icv treatment (Fig 6). The activity of GSK-3β has been measured indirectly by a ratio of phospho- to total-GSK-3β expression. The results indicated a biphasic pattern in the time- course of GSK-3β activity (Fig 6). It was acutely increased after 2 weeks (decreased p/t GSK-3β ratio) and then was slowly returning back to control values (1 month), and moving towards the opposite direction (decreased activity seen as increased p/t GSK-3β ratio) until 6 months after the STZ-icv treatment (Fig 6). However from 6 to 9 months, the activity of GSK-3β demonstrated a steep increase (fall in p/t-GSK-3β ratio) (Fig 6). Changes of tau protein phosphorylation, demonstrated as p/t tau ratio, showed decreased tau phosphorylation up to 1 month, after which it started to rise and reached significantly high level at 3-month time-point which was transiently normalized at 6 months but was then followed by a steep rise of tau phosphorylation 9 months after the STZ-icv treatment (Fig 6).

Discussion

In the present study we have observed alterations in the expression and phosphorylation/activation of several components of the brain insulin/IR signalling cascade but we did not find a uniform downregulation of this pathway in the course of 9 months following the STZ-icv treatment. The changes we observed have demonstrated a kind of a biphasic time-dependent pattern in the 9-month time-course which could be divided in 3 periods; ≤1 month (acute response), >1 - <6 (subchronic response) and ≥6 months (chronic response). The results clearly show an acute response to STZ-icv application with upregulation of IR gene which is not accompanied by a change in IR protein expression as early as 2 weeks after STZ-icv administration but is rapidly followed by down-regulation in expression of both IR mRNA and protein at 1-month time-point, similar to what has been reported in literature for that post-icv-treatment period (Agrawal et al. 2011; Liu et al. 2013). These results suggest that IR gene is affected before IR protein as acute response to the STZ-icv treatment. Since we have not measured the brain insulin level it may be only speculated that it might be altered in response to a downregulation of Ins-1 mRNA observed 1 month after STZ-icv treatment but there are no literature data on the insulin content in the brain of this model to be compared with. On the other side, insulin is a substrate of IDE and the acute response in our experiments is also seen in IDE protein level which has demonstrated a marked tendency to decline during this period which became significant at 1-month time-point. These changes could be speculated to be caused by a decreased stability and/or increased degradation of IDE protein since they have not been associated with changes in IDE mRNA expression which remained unchanged in this acute phase, although we have not measured IDE activity which might be changed as well. Our data are in line whit Deng et al (2009) who has demonstrated decrement in the expression of IDE protein and its activity in HPC 3 weeks after the STZ-icv treatment.

Moving downstream the IR-PI-K3 signalling pathway to the GSK-3ß, an important enzyme which regulates the phosphorylation homeostasis of tau protein, our results demonstrate decreased ratio of p/t GSK-3ß in the acute phase, indirectly indicating the abundant activation of this enzyme as early as 2 weeks after the STZ-icv treatment. Decreased p/t GSK-3ß ratio has been reported in a period up to 1 month after the STZ-icv treatment by others as well (Deng et al. 2009; Shonsey et al. 2012; Young et al. 2014). However, in our experiments, this GSK-3β activation has not resulted in increased tau protein phosphorylation (PHF1) at this time-point when decreased p/t tau ration was found. Also, our experiments show that this early decline in p/t GSK-3ß ratio transiently vanishes in the course of time. It could not be excluded that decreased p/t tau ratio in the acute phase could be due to a possible activation of protein phosphates 2A (PP2A), which is the major tau dephosphatase (Plattner et al. 2006) but has not been measured in our experiments or reported in STZ-icv model at this time point by others. Some support for this hypothesis might be speculated from the finding of an acutely increased PP2A activity in the hippocampus of streptozotocin-type 2 but not type 1 diabetic animals (Liao et al, 2013), having in mind that sAD has been proposed to be a type 2 diabetes of the brain (Hoyer 1998). It should be also kept in mind that we have used the PHF-1 antibody which targets tau protein that might be phosphorylated not only by GSK-3ß, but also by Cdk5 and ERK1/2 kinases. Selection of antibodies which target different epitopes (e.g. only Ser396 or Ser 396/404 and many other epitopes) of phospho-tau protein might also account for some inconsistency in reports of increased p-tau in cerebrum, hypothalamus and HPC (Deng et al. 2009; de la Monte et al. 2006; Lester-Coll et al. 2006; Yang et al. 2014) and unchanged p/t tau ratio (Ser 199-200) in HPC (Santos et al. 2012) found up to 1 month after STZ-icv treatment Although the expression of tau mRNA has been increased as early as 2

weeks after STZ-icv administration, it seems to be just a transient acute effect since it has not affected the tau protein expression and tau gene expression has not been altered in any of the time-points afterwards. The work of others indicates that tau mRNA decreases 2 and 4 weeks after the treatment (de la Monte et al. 2006) which could be due to the fact that the research was done on the rat pups and because only one house keeping gene for normalisation of gene expression was used instead of 3 house-keeping genes used in our experiments.

In a period of 1 to 6 months post-icv treatment downregulation of IR and IDE and most markedly of Ins1 mRNA (3 months), respectively, has been observed. The progression of decrement in IR protein expression correlated with downregulation of the corresponding gene up to the 3-month time-point after which a magnitude of decrement in the protein level started slowly but persistently to decline in the course of time. Some reports indicate downregulation of IR mRNA in HPC observed as long as 5 months after the STZ-icv treatment (Yang et al. 2014; Lee et al. 2014) while others (de la Monte et al. 2006) did not found significant changes in the HPC 4 weeks after the STZ-icy treatment. The time-course of changes in IDE protein levels in our experiments did not correlate with changes in the corresponding gene as the former did not progressed but showed a tendency of diminishing the magnitude of decrement in a period from 1 to 3 months, after which a persistent decline was observed further on. Literature data indicate that IDE mRNA decreases 2 weeks after the STZ-icv treatment, the only time point at which IDE was investigated so far (Shingo et al. 2013)

A tendency to return from increased to normal values has been clearly and persistently present in the GSK-3β activity in the period of 1-3 months after STZ-icv administration, ending up in the opposite direction (decreased activity) at the 6-month time-point. Such a change in the GSK-3β activity seems not to correlate with alterations in the IR protein level in that period (decreased expression) but since we have not measured the IR tyrosine kinase activity, it could not be excluded that it might be increased at this time point to compensate for reduced IR protein level and consequently to lead to such changes in p/t GSK-3β ratio (activity). Additionally, it has to be kept in mind that GSK-3β phosphorylation homeostasis might be affected by different kinases besides IR-PI-3K-Akt/PKB pathway, like ERK which can phosphorylate GSK-3β on T48 site making GSK-3 β inactive, as regulated through IR-MAPK pathway (McCubrey et al. 2014). This speculation might be a possible explanation also of the tau hyperphoshorylation (PHF1) we observed in the period of 1-3 months after STZ-icv administration when GSK-3β activity was not increased. This could be because enzymes like ERK-1/2 and Cdk-5 have also been involved in the phosphorylation of tau protein on Ser396/404 (Wen et al. 2008; Takahaski et al. 2000; Ferrer et al. 2001). Literature data on ERK -1/2 level/activity in the brain of a STZ-icv model show insignificant increment of ERK1/2 activity in HPC (Agrawal et al. 2011) and significant decrement in phosphorylation of ERK (Liu et al. 2013) while there is no research on the Cdk-5 in the brain of STZ-icv model. Our preliminary experiments showed increased p-ERK expression measured at 1 and 9 months after STZ-icv treatment (unpublished data). Also, increased Cdk-5 expression and increase ERK-1/2 phosphorylation have been found in human sAD post-mortem (Takahaski et al. 2000; Ferrer et al. 2001).

Finally, the last part of the post-STZ-icv observational period in our research (6-9 months), clearly demonstrates that contrary to the no further progression in downregulation of genes explored (IR, Ins-1, IDE) from the previously observed level, alterations in protein expression or enzyme activity downstream the IR/PI-3K signalling pathway generally worsen; IDE level continue to decline, GSK-3β activity increases and tau hyperphosphorylation rises. The level of IR protein has been the only one which has returned to the control values after 9 months for

unknown reason but since we have not measured the tyrosine activity of IR, it could not be excluded that on a long-term, in spite of normalized IR level, its tyrosine activity might be decreased leading to downstream cascade dysfunction.

To conclude, our results provide evidence that the changes in the IR signalling cascade downstream the PI-3K pathway depend on the time past after the STZ-icv administration. The acute changes seem to be partly reversible up to 3-6 months while chronic and progressive changes are particularly manifested in a period longer than 6 months after STZ-icv administration. Interestingly, such a biphasic time pattern in brain insulin/IR signalling cascade correlates well with the cognitive deficits we have observed in a 9-month follow up of STZ-icv treated rats (manuscript in preparation). Therefore, it could be hypothesized that the acute changes might reflect the non-specific toxicity of STZ (known as cytotoxic agent) while the chronic changes seem to correlate more with corresponding ones observed in human sAD brain, which particularly might be true for the tau and amyloid pathology development and progression. Awareness of such a difference in a time-course of changes in the brain insulin system in STZ-icv model of sAD might be of utmost importance in translational sAD research. Both acute and chronic STZ-icv-induced changes might represent a valuable tool, the former to explore the possible ethiopathogenesis of sAD and mechanisms of its prevention, and the latter to assess novel approaches in the disease modifying therapy of sAD, and should, therefore, be exploited accordingly.

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Figures

Fig 1 Normalized gene expression of insulin receptor, insulin-1, tau protein and insulin degrading enzyme in the hippocampus of streptozotocin-intracerebroventricularly-treated rats. Gene expression measured via RT-PCR 0.5, 3, 6 and 9 months after the streptozotocin-intracerebroventricular (STZ-icv) treatment. Control is presented as zero line and changes of gene expression in the streptozotocin group are expressed (mean±SEM) as percentage of control. **IR,** insulin receptor; **Ins 1,** insulin-1; **IDE,** insulin degrading enzyme; **tau,** tau protein. *p<0.05 vs respective control at each time point by Mann-Whitney U-test.

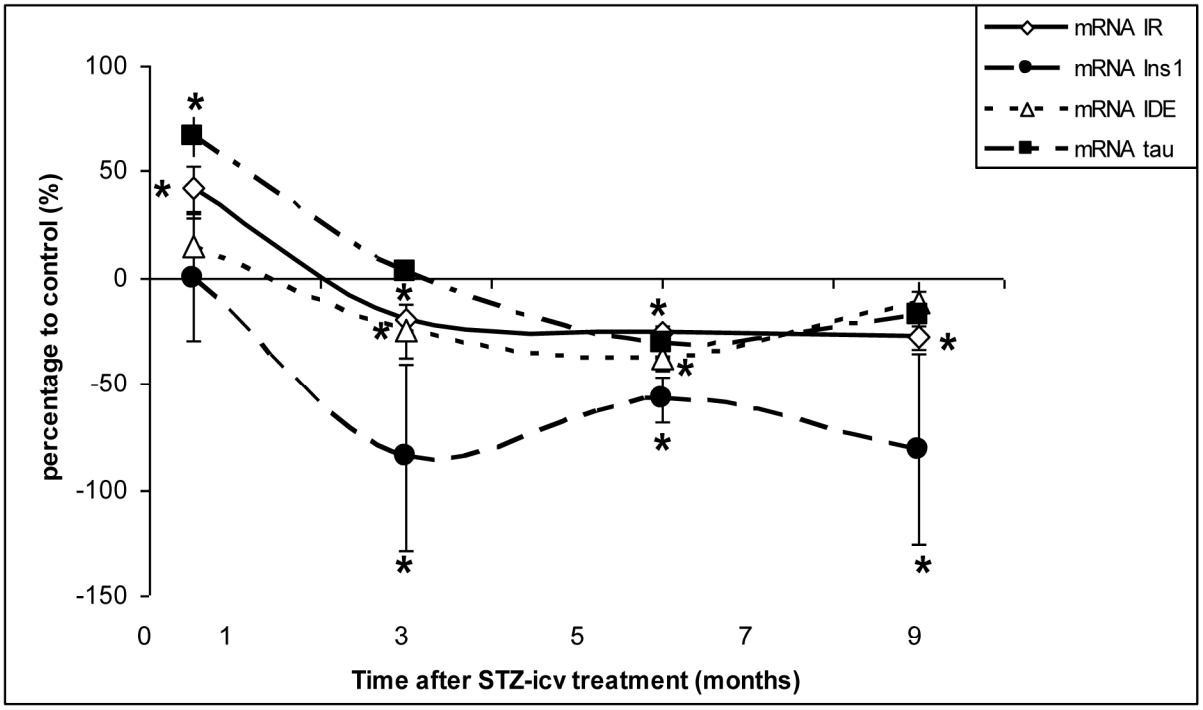
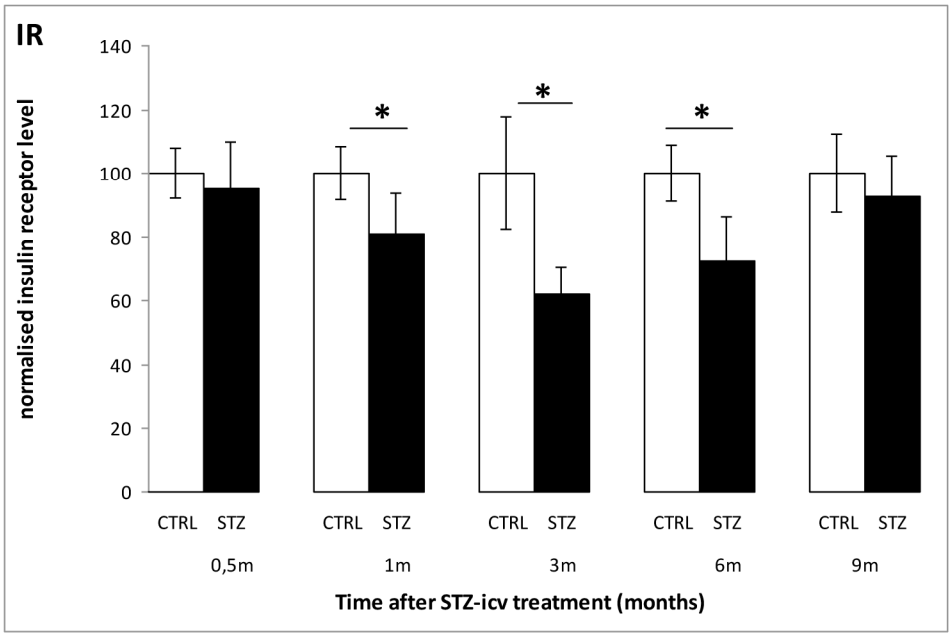


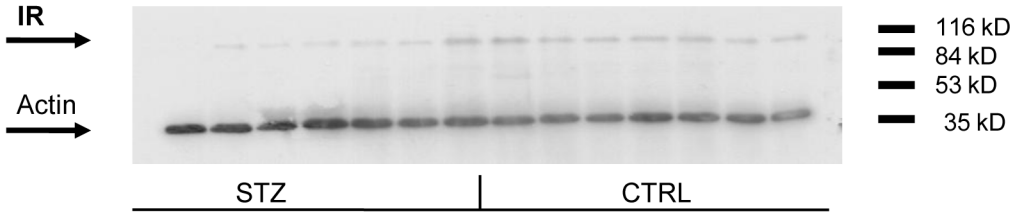
Fig 2 Protein expression of insulin receptor in the hippocampus of streptozotocin-intracerebroventricularly-treated rats. The expression of IR was measured by Western blot analysis using the antibody recognising β-subunit of IR receptor. **a**-bars show normalised expression of protein where control is presented as 100 % and changes of protein expression in the streptozotocin group are expressed (mean±SEM) as percentage of control; **b-**Western blot analysis of the protein expression at the time point with significant difference between the treatment groups. * $p<0.05$ vs control measured by Mann-Withny U-test. **IR,** insulin receptor; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **STZ,** streptozotocin**; CTRL,** control.

a

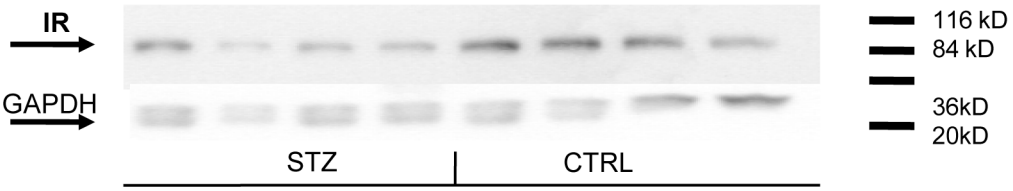


b

1 month



3 months



6 months

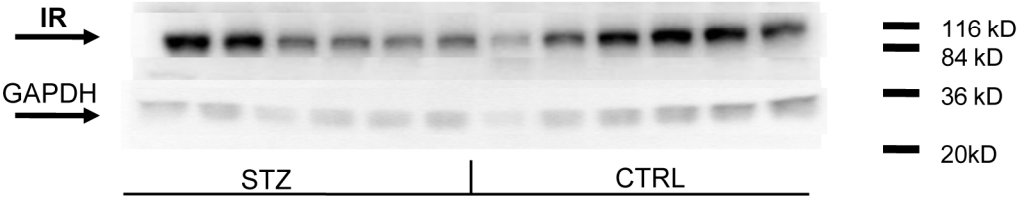
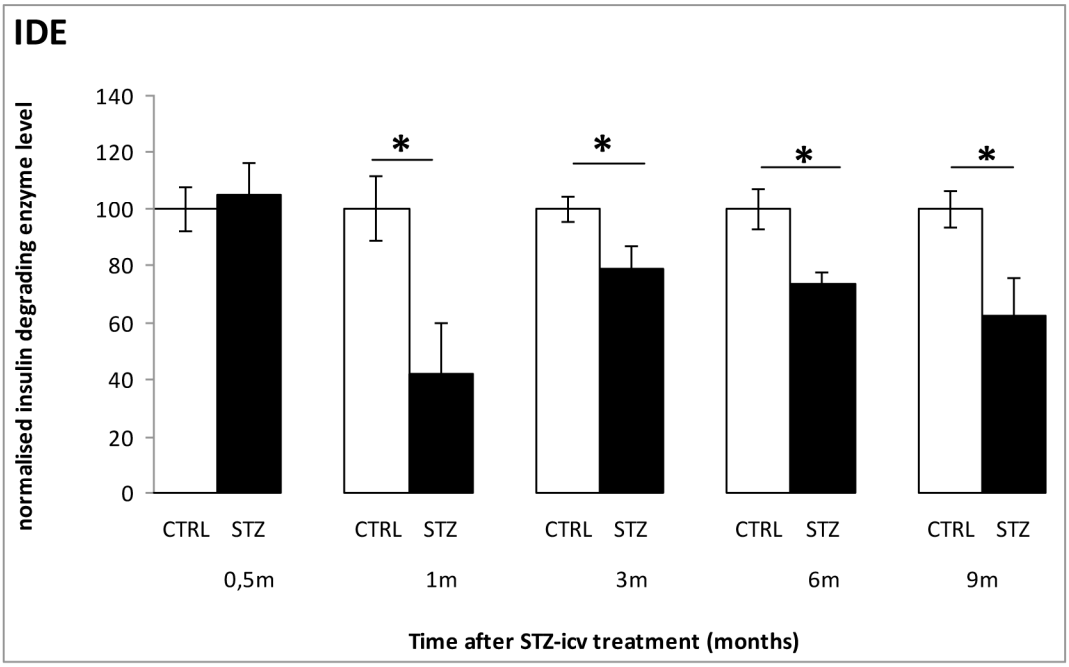


Fig 3 Protein expression of insulin degrading enzyme in the hippocampus of streptozotocin-intracerebroventricularly-treated rats. The expression of IDE was measured by Western blot analysis**. a**- bars show normalised expression of protein where control is presented as 100 % and changes of protein expression in the streptozotocin group are expressed (mean±SEM) as percentage of control; **b-**Western blot analysis of the protein expression at the time point with significant difference between the treatment groups. * $p<0.05$ vs control measured by Mann-Withny U-test. **IDE,** insulin degrading enzyme; **STZ,** streptozotocin**; CTRL,** control.

a


IDE
normalised insulin degrading enzyme level
140
120
100
80
60
40
20
0
*
*
*
*
CTRL STZ
CTRL STZ
CTRL STZ
CTRL STZ
CTRL STZ
0,5m
1m
3m
6m
9m
Time after STZ-icv treatment (months)

b

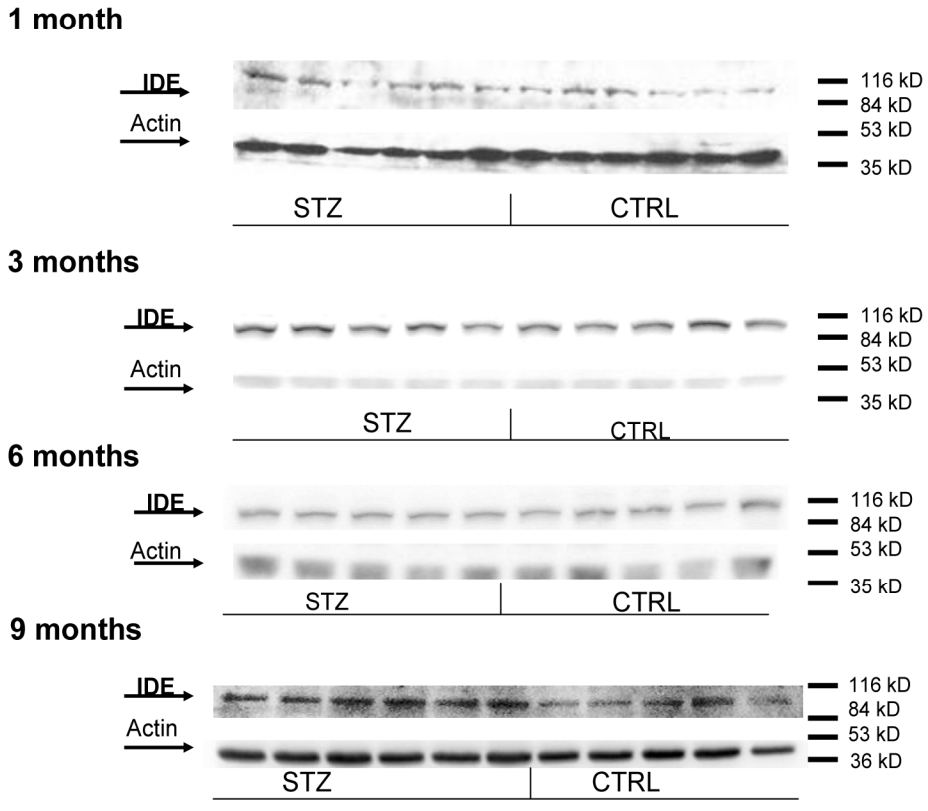
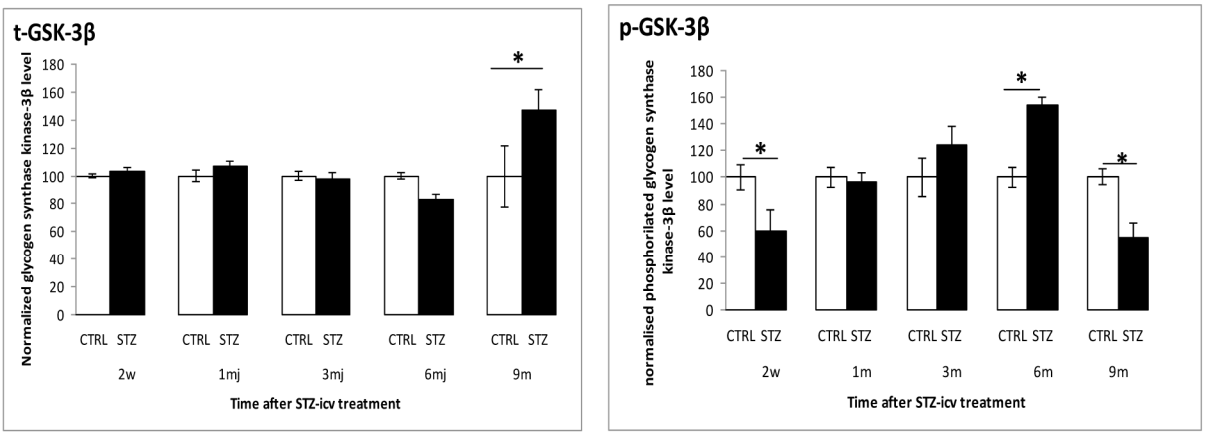

1 month
IDE
Actin
116 kD
84 kD
53 kD
35 kD
STZ
CTRL
3 months
IDE
Actin
116 kD
84 kD
53 kD
35 kD
STZ
CTRL
6 months
IDE
Actin
116 kD
84 kD
53 kD
35 kD
STZ
CTRL
9 months
IDE
Actin
116 kD
84 kD
53 kD
36 kD
STZ
CTRL

Fig 4 Protein expression of glycogen synthase kinase-3β in the hippocampus of streptozotocin-intracerebroventricularly-treated rats. The expression of glycogen syhthase kinase-3β was measured by Western blot analysis**. a**- bars show normalised expression of protein where control is presented as 100 % and changes of protein expression in the streptozotocin group are expressed (mean±SEM) as percentage of control; **b-**Western blot analysis of the protein expression at the time point with significant difference between the treatment groups. * $p<0.05$ vs control measured by Mann-Withny U-test. **t-GSK-3ß,** total glycogen synthase kinase-3β; **p-GSK-3ß,** phospho-GSK-3ß; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **STZ,** streptozotocin**; CTRL,** control.

a


t-GSK-3β
Normalized glycogen synthase kinase-3β level
180
160
140
120
100
80
60
40
20
0
CTRL STZ
2w
1mj
3mj
6mj
9m
Time after STZ-icv treatment
p-GSK-3β
normalised phosphorilated glycogen synthase kinase-3β level
1m
3m
6m

b

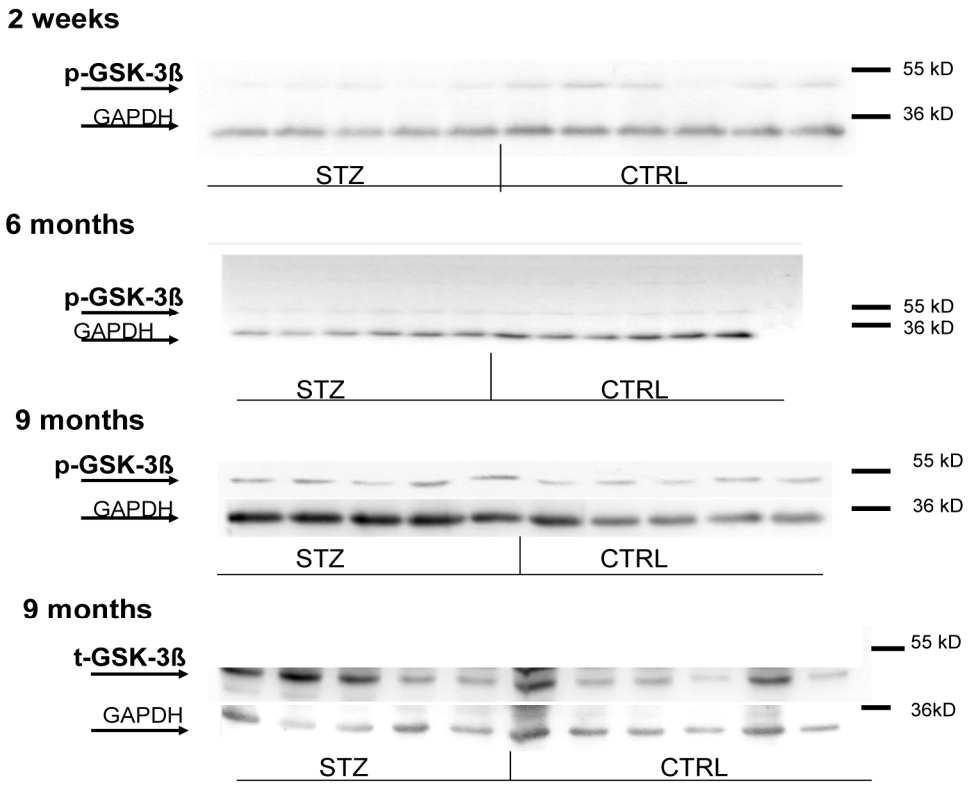
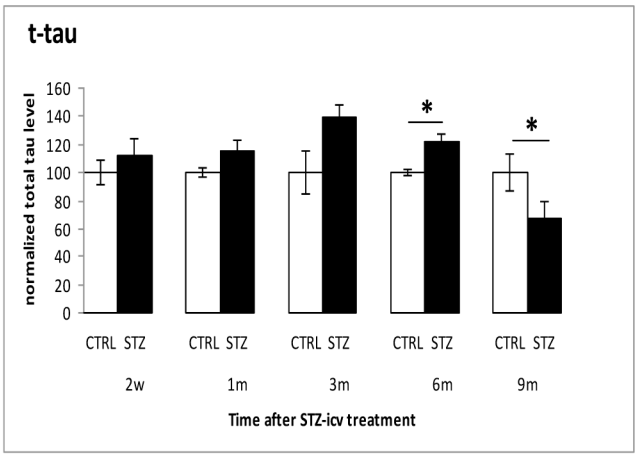
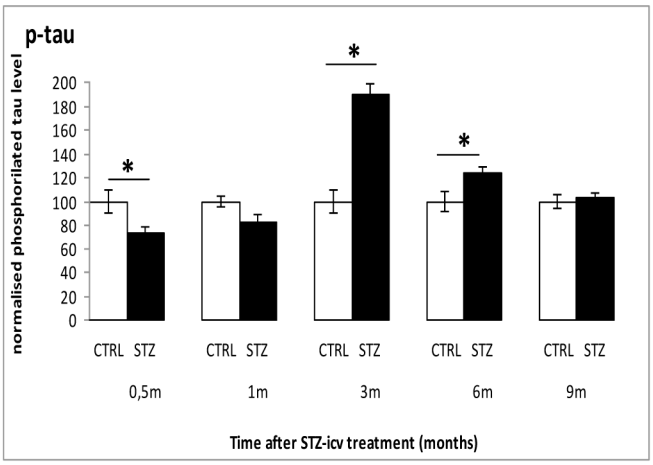

2 weeks
p-GSK-3ß
GAPDH
55 kD
36 kD
STZ
CTRL
6 months
9 months
t-GSK-3ß
36kD

Fig 5 Protein expresion of tau protein in the hippocampus of streptozotocin-intracerebroventricularly-treated rats. The expression of tau protein was measured by Western blot analysis**. a**- bars show normalised expression of protein where control is presented as 100 % and changes of protein expression in the streptozotocin group are expressed (mean±SEM) as percentage of control;; **b-**Western blot analysis of the protein expression at the time point with significant difference between the treatment groups. * $p<0.05$ vs control measured by Mann-Withny U-test. **t-tau,** total tau protein; **p-tau,** phospho-tau protein; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **STZ,** streptozotocin**; CTRL,** control.

a


t-tau
normalized total tau level
160
140
120
100
80
60
40
20
0
CTRL STZ
2w
1m
3m
6m
9m
Time after STZ-icv treatment


p-tau
normalised phosphorilated tau level
200
180
160
140
120
100
80
60
40
20
0
CTRL STZ
0,5m
1m
3m
6m
9m
Time after STZ-icv treatment (months)

b

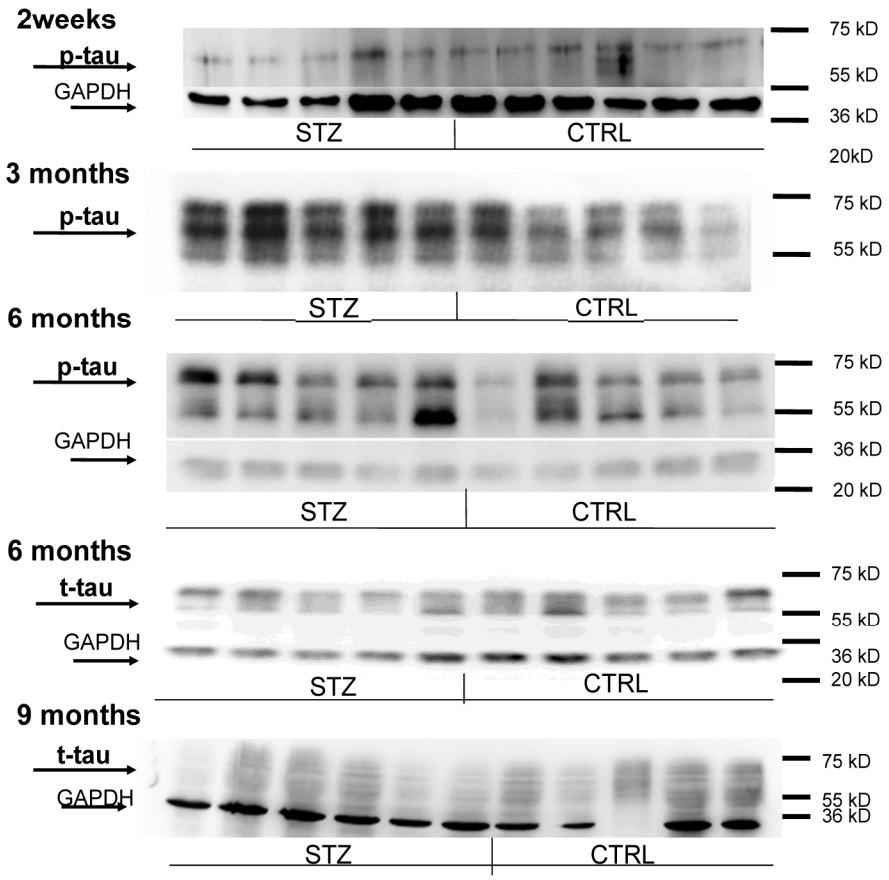

2weeks
p-tau
GAPDH
STZ
CTRL
75 kD
55 kD
36 kD
20kD
3 months
p-tau
75 kD
55 kD
6 months
p-tau
GAPDH
75 kD
55 kD
36 kD
20 kD
6 months
t-tau
GAPDH
75 kD
55 kD
36 kD
20 kD
9 months
t-tau
GAPDH
75 kD
55 kD
36 kD

Fig 6 The time-course of changes in the protein expression of insulin receptor and insulin degrading enzyme, and in the ratio of phosphorylated to total tau protein and glycogen synthase kinase-3β, respectively, measured in the hippocampus of streptozotocin-intracerebroventricularly-treated rats. Protein expression was measured by Western blot analysis. Control is presented as zero line and changes of protein/ratio expression in the streptozotocin group are expressed (mean±SEM) as percentage of control. **IR,** insulin receptor; **IDE,** insulin degrading enzyme; **p/t-tau,** phospho/total tau protein; p/t-GSK-3ß, phospho/total glycogen synthase kinase-3β. **STZ-icv,** streptozotocin-intracerebroventricularly. *p<0,05 vs control measured by Mann-Withny U-test.