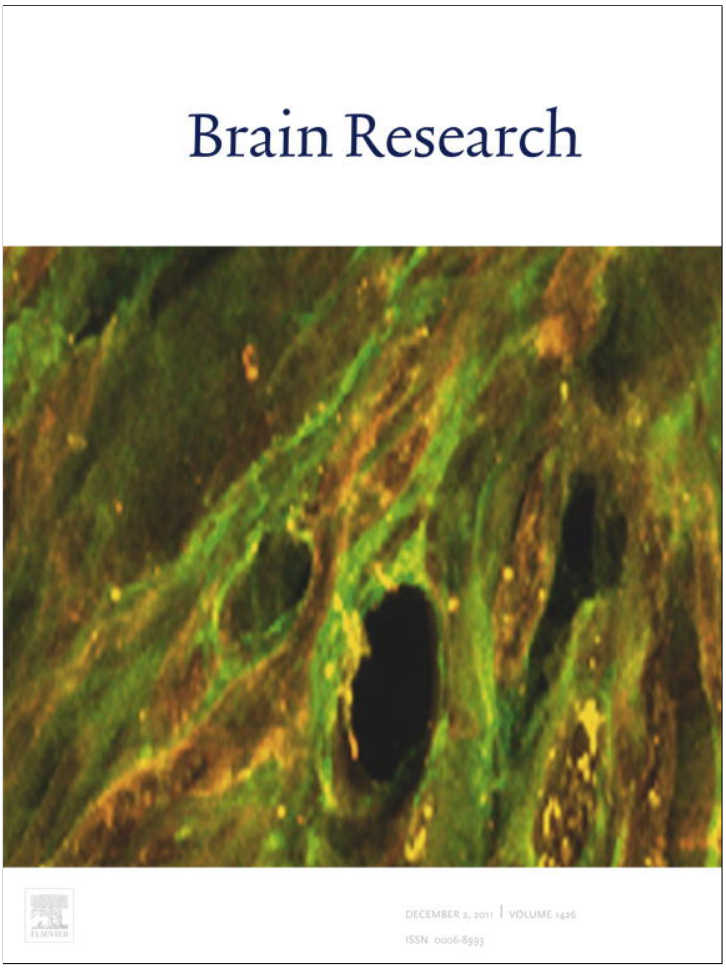

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Research Report

Lithium, phenserine, memantine and pioglitazone reverse memory deficit and restore phospho-GSK3β decreased in hippocampus in intracerebroventricular streptozotocin induced memory deficit model

Teresa Ponce-Lopez[a,*], *Gustavo Liy-Salmeron*[b], *Enrique Hong*[a], *Alfredo Meneses*[a]

[a]*Departamento de Farmacobiología, CINVESTAV. México City, Mexico*
[b]*Facultad de Ciencias de la Salud, Universidad Anáhuac México Norte, Huixquilucan Edo. de México, Mexico*

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ABSTRACT

Intracerebroventricular (ICV) streptozotocin (STZ) treated rat has been described as a suitable model for sporadic Alzheimer's disease (AD). Central application of STZ has demonstrated behavioral and neurochemical features that resembled those found in human AD. Chronic treatments with antioxidants, acetylcholinesterase (AChE) inhibitors, or improving glucose utilization drugs have reported a beneficial effect in ICV STZ-treated rats. In the present study the post-training administration of a glycogen synthase kinase (GSK3) inhibitor, lithium; antidementia drugs: phenserine and memantine, and insulin sensitizer, pioglitazone on memory function of ICV STZ-rats was assessed. In these same animals the phosphorylated GSK3β (p-GSK3β) and total GSK3β levels were determined, and importantly GSK3β regulates the tau phosphorylation responsible for neurofibrillary tangle formation in AD. Wistar rats received ICV STZ application (3 mg/kg twice) and 2 weeks later short- (STM) and long-term memories (LTM) were assessed in an autoshaping learning task. Animals were sacrificed immediately following the last autoshaping session, their brains removed and dissected. The enzymes were measured in the hippocampus and prefrontal cortex (PFC) by western blot. ICV STZ-treated rats showed a memory deficit and significantly decreased p-GSK3β levels, while total GSK3β did not change, in both the hippocampus and PFC. Memory impairment was reversed by lithium (100 mg/kg), phenserine (1 mg/kg), memantine (5 mg/kg) and pioglitazone (30 mg/kg). The p-GSK3β levels were restored by lithium, phenserine and pioglitazone in the hippocampus, and restored by lithium in the PFC. Memantine produced no changes in p-GSK3β levels in neither the hippocampus nor PFC. Total GSK3β levels did not change with either drug. Altogether these results show the beneficial effects of drugs with different mechanisms of actions on memory impairment induced by ICV STZ, and restored p-GSK3β levels, a kinase key of signaling cascade of insulin receptor.

* *Corresponding author at:* Tenorios 235, Granjas Coapa, México City 14330, Mexico. Fax: +52 55 54832863.
E-mail address: poncetere@hotmail.com (T. Ponce-Lopez).

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1. Introduction

The glycogen synthase kinase-3 (GSK-3) isoform has been proposed as the link between the two neuropathological hallmarks of AD (Grimes and Jope, 2001; Hernandez et al., 2002). GSK3 is a constitutively active, proline-directed serine/threonine kinase that plays a part in a number of physiological processes, such as glycogen metabolism (Welsh and Proud, 1993), gene transcription (Troussard et al., 1999) and apoptosis (Turenne and Price, 2001). GSK3 activity is modulated by insulin and WT signaling, both pathways act in a negative regulatory manner (Lizcano and Alessi, 2002; Saltiel and Kahn, 2001). *In-vitro* and *in-vivo* evidence points to a key role for GSK3 in promoting plaque amyloid and neurofibrillary tangle formation (Hooper et al., 2008). For instance, active GSK3 appears in neurons with pre-tangle changes (Pei et al., 1999) and increased GSK3 activity in the frontal cortex in AD (Leroy et al., 2007). Furthermore, GSK3 expression is upregulated in the hippocampus of AD patients (Blalock et al., 2004). Importantly, GSK3β phosphorylates tau in most sites that are hyperphosphorylated in neurofibrillary tangles (NFT) of transfected cells (Lovestone et al., 1994) and *in vivo* (Hong and Lee, 1997; Muñoz-Montaño et al., 1997). Thus, GSK3β may represent a target therapeutic mechanism for AD and other neurodegenerative diseases (Medina and Avila, 2010).

Central application of streptozotocin (STZ) in rodents has been associated with oxidative stress (Agrawal et al., 2009; Saxena et al., 2008, 2010; Sharma and Gupta, 2002), and histopathological changes (Saxena et al., 2010) decreased cholinergic transmission in the hippocampus (Agrawal et al., 2009; Ishrat et al., 2006; Prickaerts et al., 1999), decreased glucose/energy metabolism in cerebral cortical regions and hippocampus (Duelli et al., 1994; Plaschke and Hoyer, 1993), decreased insulin and insulin receptor (IR) gene/protein expression (Grünblatt et al., 2007) in the hippocampus and cortex along with hyperphosphorylated tau protein in the hippocampus (Grünblatt et al., 2007; Salkovic-Petrisic et al., 2006) and amyloid formation in leptomeningeal vessels (Salkovic-Petrisic et al., 2006) as long as 3 months after intracerebroventricular (ICV) STZ. All these changes have been associated with progressive deficits in memory, observed as early as 2 weeks after STZ-ICV administration (Lannert and Hoyer, 1998). Furthermore, several studies have reported that chronic treatment with antioxidants (e.g. resveratrol; Sharma and Gupta, 2002; melatonin; Saxena et al., 2010), natural antioxidants (e.g. cucurmin: Agrawal et al., 2010), drugs facilitating cholinergic neurotransmission (e.g. donepezil; Agrawal et al., 2009; Sonkusare et al., 2005) or improving glucose utilization and metabolism (e.g. pioglitazone; Pathan et al., 2006), lead to an enhancement of cognitive performance in ICV STZ-treated rats.

Therefore, it seems that the phosphatidylinositol 3′ kinase–Akt–glycogen synthase kinase3 (PI3K–Akt–GSK3) signaling pathway that is deregulated via inhibition of the neuronal IR may function in ICV STZ model. Thus, the hypothesized activation of the PI3K/Akt pathway by therapeutic agents makes the assumption that they might have an inhibitory effect on GSK-3β. Nonetheless, no reports are available regarding the effect of drugs in phospho-GSK3β (p-GSK3β) in ICV STZ induced memory deficit model. Hence, in order to determine if the GSK3β was associated to memory deficits in the ICV STZ model lithium (a GSK3 inhibitor) was tested. Likewise phenserine, an AChE inhibitor, and memantine, a NMDA receptor antagonist, were used, which are two therapeutic strategies currently used for AD, and have improved memory deficit in behavioral tasks (Ikari et al., 1995; Zoladz et al., 2006) and neuroprotective activity (Nakamura et al., 2006; Shaw et al., 2001). Phenserine has already been tested in our laboratory (Meneses, 2002), demonstrating its effectiveness in attenuating scopolamine-induced and age-related memory impairments in rats in T-maze (Iijima et al., 1993; Ikari et al., 1995) neuroprotective activity and reducing Aβ levels by regulating βAPP translation (Shaw et al., 2001). Finally, pioglitazone, a PPARγ agonist, was also tested. Interestingly, it has been suggested that PPAR agonists could prevent memory deficit and neurodegeneration features in intracerebral (IC) STZ model (de la Monte et al., 2006). Hence, the aim of the present study was to assess the effect of mechanistically diverse therapeutic agents on the performance of an autoshaping task and p-GSK3 and total GSK3β levels in the hippocampus and prefrontal cortex (PCF) in ICV STZ-treated rats. Autoshaping has been useful to detect effects induced by aging, promnesic, amnesic and anti-amnesic effects, and molecular mechanisms underlying memory formation (see Meneses et al., 2011a, 2011b, 2011c). To our knowledge there is no previous evidence about the effects of administration of lithium, phenserine, memantine and pioglitazone on the associative autoshaping task and p-GSK3β and total GSK3β changes (Fig. 1).

2. Results

2.1. *Memory formation in ICV STZ-treated rats*

There were no significant differences in the conditioned responses (CR) between sham and citrate buffer (CB) groups neither in short- (STM) or long-term memory (LTM) (Fig. 2A). The CR were significantly [$F(2, 16) = 4.94$, $p < 0.0239$] lower in streptozotocin (STZ) group relative to sham and CB groups during STM (Fig. 2A). Likewise, during LTM (24 and 48 h), CR were significantly [$F(2,16) = 7.95$, $p < 0.0049$; $F(2,16) = 15.58$, $p < 0.003$] diminished in the STZ-treated group compared to sham and CB groups (Fig. 2A). On the other hand, in order to verify that the memory deficit induced by ICV STZ-treatment was not due to a decrease in locomotion or motivation for food, the head-pokes in the presence of conditioned stimulus (CS) (head-pokes/CS) into the food magazine were recorded (Meneses, 2007; Meneses & Hong, 1994; Meneses & Perez-Garcia, 2006). No differences were observed in head-pokes/CS in sham and CB groups during either STM or LTM (Fig. 2B). In the STZ group, we observed a significant [$F(2,16) = 8.61$, $p < 0.0036$; $F(2,16) = 5.27$, $p < 0.0197$; $F(2,16) = 8.09$, $p < 0.0046$] increase of head-pokes/CS over time in relation to sham and CB groups during both STM (1.5 h) and LTM (24 and 48 h; Fig. 2B).

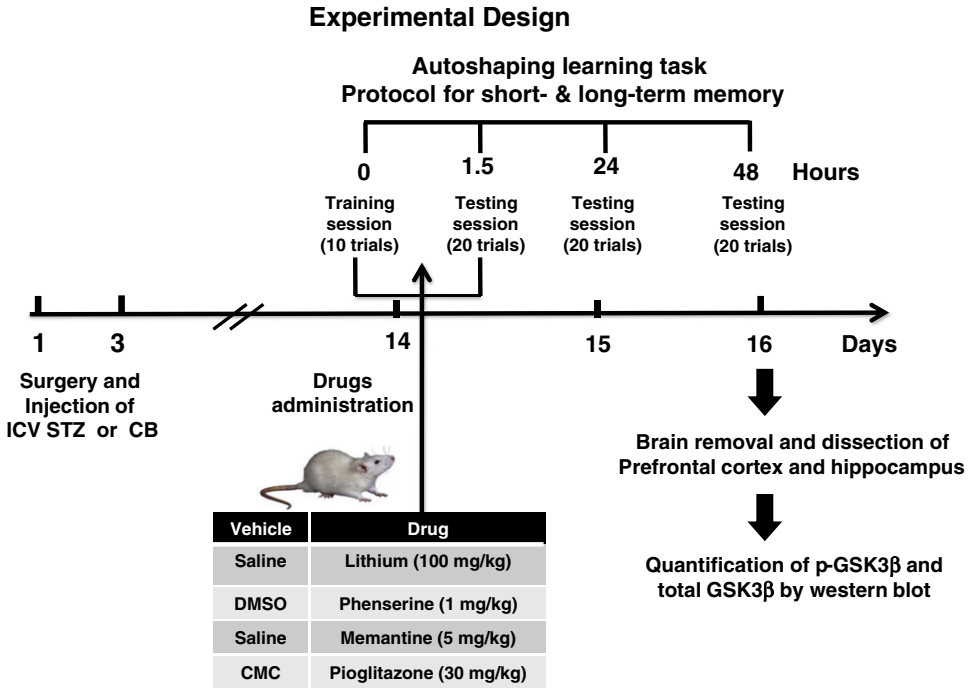

Fig. 1 – Under anesthesia Wistar rats were bilaterally injected in lateral ventricles with streptozotocin (STZ) (3 mg/kg) or citrate buffer (CB; vehicle) twice on day 1 and 3, after 2 weeks short- (STM) and long-term memory (LTM) were assessed in autoshaping, an associative learning task. The drugs or vehicles; saline, dimetil sulfoxide (DMSO) and carboximethylcelluose (CMC) were administrated post-training schedules. The animals were sacrificed on day 16, and the brains were removed and the hippocampus and prefrontal cortex were dissected in order to measure phosphorylation of GSK3β (p-GSK3β) and total GSK3β by western blot. ICV: intracerebroventricular.

2.2. *Effects of lithium, phenserine, memantine and pioglitazone on STZ induced memory deficit*

In the STZ-, CB+lithium and STZ+lithium-treated groups a significant [$F(3, 22)=5.21$, $p<0.0085$] reduction on CR was observed in comparison to CB control group at STM (Fig. 3A). However, in CB+lithium- and STZ+lithium-treated groups there were no significant changes in the number of CR relative to CB group (at 24 and 48 h) (Fig. 3A); while in STZ+lithium-treated group there was a significantly [$F(3, 22)=6.18$, $p<0.0041$; $F(3, 22)=4$, $p<0.0003$] increased CR, during LTM as compared to STZ (at 24 and 48 h; Fig. 3A). On the other hand, CB+phenserine-treated animals did not show significant differences on CR in comparison to CB control group, and STZ+phenserine did not modify the CR at STM (Fig. 3B). CB+phenserine- and STZ+phenserine-treated groups did not change the CR relative to CB control whereas in STZ+phenserine group there was a significantly [$F(3,22)=4$, $p<0.0203$; $F(3, 22)=7.10$ $p<0.0021$] increased CR, during LTM as compared to STZ (at 24 and 48 h; Fig. 3B). In contrast, CB+memantine- (5 mg/kg, i.p.) and STZ+memantine-treated groups did not significantly change CR as compared to the CB control during STM and LTM (Fig. 3C). While, in STZ+memantine group CR was significantly [$F(3, 22)=4$, $p<0.0058$; $F(3, 22)=4$, $p<0.0011$; $F(3, 22)=4$, $p<0.0081$] higher than STZ-treated rats during STM and LTM (Fig. 3C). Lastly, CB+pioglitazone (30 mg/kg p.o.) and STZ+pioglitazone did not significantly modify CR as compared to CB control during STM and LTM; whereas STZ+pioglitazone significantly [$F(3, 22)=4$, $p<0.0035$; $F(3, 22)=4$, $p<0.0012$; $F(3, 22)=4$, $p<0.0006$] increased CR relative to STZ rats in STM and LTM (Fig. 3D).

2.3. *Basal levels of p-GSK3β and total GSK3β in untrained vs. trained ICV CB animals*

The values of p-GSK3β and total GSK3β in the hippocampus and PFC of untrained ICV CB rats were considered as basal, and were compared with those of trained ICV CB rats. The p-GSK3β levels of untrained and trained animals were slightly or significantly [$F(3, 11)=37.60$, $p<0.0001$] increased in hippocampus and PFC (Fig. 4B), respectively.

2.4. *Effects of lithium, phenserine, memantine and pioglitazone on phosphorylation and total GSK3β in hippocampus and PFC of trained STZ-treated rats*

2.4.1. *Hippocampus*

Quantitative analysis of immunoblots indicated that p-GSK3β levels in hippocampal were significantly [$F(5, 17)=21.48$, $p<0.0001$] decremented in STZ group in comparison with CB control group (Fig. 5B). Lithium, phenserine, memantine and pioglitazone treatment did not produce significant differences in p-GSK3β levels between STZ-treated and CB control

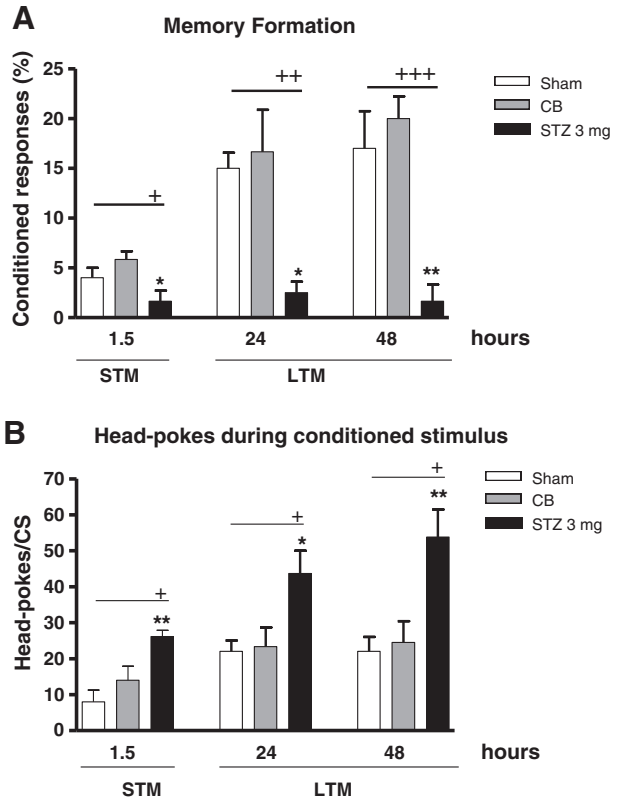

Fig. 2 – (A) Performance in short (STM) and long term memory (LTM), (B) number of head-pokes into the food-magazine during the presence of conditions stimulus (CS) after 2 weeks of intracerebroventricular (ICV) streptozotocin (STZ; 3 mg/kg) twice on day 1 and 3, after in an autoshaping task. Values are expressed as mean ± SEM of % conditioned responses or number of head-pokes of each group (n = 5–6). Significant differences (*p < 0.05, **p < 0.01, +p < 0.05, ++p < 0.01, +++p < 0.001) in citrate buffer (CB; control) and STZ groups *vs.* sham group; ANOVA (one way) followed by Tukey test.

animals. However, except memantine, all other drugs significantly [F (5, 17) = 21.48, p < 0.0001] increased p-GSK3β levels relative to STZ-treatment in the hippocampus (Fig. 5B). Likewise, treatments did not significantly [F (5, 17) = 3, p > 0.05] modify the total GSK3β levels in STZ-treated animals with respect to CB or STZ groups (Fig. 5C). In the hippocampus the p-GSK3β/GSK3β was significantly [F (5, 17) = 11.73, p < 0.0003], decreased of STZ and STZ + memantine-treated rats relative to CB values; nevertheless, lithium, phenserine and pioglitazone showed no differences with CB control group (Fig. 5D). The relative p-GSK3β/GSK3β ratio was significantly [F (5, 17) = 11.73, p < 0.0003] augmented in ICV STZ + all other drugs in comparison with ICV STZ-treatment (Fig. 5D).

2.4.2. *Prefrontal cortex*

Western blots showed that in PFC p-GSK3β values were significantly [F (3,11) = 1672, p < 0.0001] decremented in STZ rats in comparison with CB control values (Fig. 6B). In contrast, in the hippocampus lithium was the only drug that produced a significant [F (5,17) = 1109, p < 0.0001] increment of p-GSK3β in STZ-treated rats in comparison with STZ alone.

Phenserine, memantine and pioglitazone treatment had no effects in p-GSK3β levels of STZ-treated group with respect to STZ alone. Similarly, the drug treatments did not modify the total GSK3β levels in the STZ-treated group regarding CB or STZ groups (Fig. 6B). But the relative p-GSK3β/GSK3β ratio in PFC was significantly [F (5,17) = 7.39, p = 0.0022] decreased of STZ-treated rats compared with CB group (Fig. 6C).

3. Discussion

Given that GSK3β plays a key role in the pathogenesis of AD, the present study investigated the effects of four mechanistically different agents on memory and *ex-vivo* p-GSK3β and total GSK3β production in ICV STZ-treated rat. ICV STZ rats submitted to an autoshaping task showed STM and LTM deficits as indicated by a significant decrease of conditioned responses. That such as memory impairment was not due to a decrease in locomotion or motivation for food, since STZ-treated group increased their number of head-pokes/CS during memory deficit, suggests that the locomotor activity and motivation for eating remained and was even augmented; however, these animals were unable to form the association between behaviors (i.e., CR), CS and foods. STZ-treatment seems to be able to elicit increased food- and water-intake (see e.g., Vishwakarma et al., 2010). In contrast, CB control animals exhibit an inverse relationship between the increased CR (i.e., memory formation) and head-pokes/CS over time and progressively improved memory (see also Meneses, et al., 2011c). Therefore, control rats were able to associate the CS (press-lever response) and the food-pellet delivery (Meneses, 2007; Meneses & Hong, 1994; Meneses & Perez-Garcia, 2006). Notably, while total GSK3β remained unchanged, in trained (*vs.* untrained) control animals p-GSK3β levels increased (slightly) in the hippocampus and (significantly) in the PFC (Fig. 4B), which is consistent with evidence that PFC and hippocampus mediate STM and LTM (see Meneses et al., 2011a; 2011b; 2011c). The p-GSK3β levels decreased, the total GSK3β levels did not change and the relative p-GSK3β/total GSK3β ratio decreased in hippocampus and PFC in STZ-treated rats, this indirectly suggests an increase in the active GSK3β form (Salkovic-Petrisic et al., 2006). GSK3β is highly regulated by phosphorylation, for instance, phosphorylation of tyr216 is required for basal activity and high levels of phosphorylation of this residue result in GSK3β being active in resting cells (Hughes et al., 1993). On the other hand, phosphorylation of ser9, by kinases, leads to inactivation of GSK3β, overriding the activation induced by phosphorylation of tyr216 (Bhat et al., 2000; Frame and Cohen, 2001). Conversely, decreased Ser9-phosphorylation increases the activity of GSK3. As a result, measurements of changes in the state of p-GSK3 reflect modulation of its activity (Frame and Cohen, 2001). Hence, the STZ induced memory impairment was associated to decreased p-GSK3β levels (or increased GSK3β activity) in the hippocampus and PFC, brain structures involved in memory aspects. Interestingly cognitive impairments in the Morris water maze and new object recognition test that persisted in aged (18 months) rats were associated to,

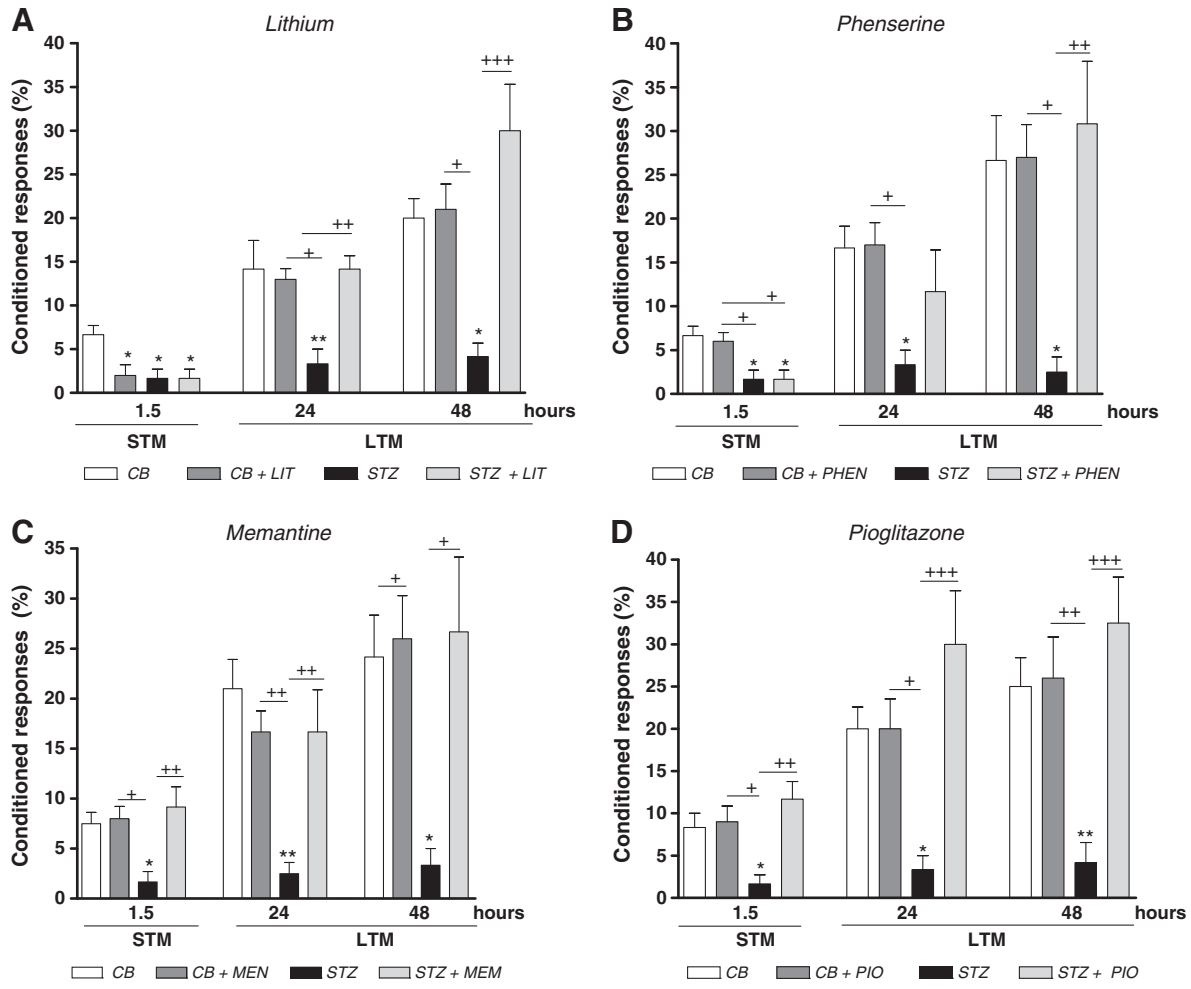

Fig. 3 – Effects of post-training systemic administration (i.p.) of (A) lithium (LIT; 100 mg/kg), (B) phenserine (PHEN; 1 mg/kg), (C) memantine (MEM; 5 mg/kg) and oral administration of (D) pioglitazone (PIO; 30 mg/kg) on short (STM) and long term memory (LTM) in an autoshaping task after 2 weeks of intracerebroventricular (ICV) streptozotocin (STZ; 3 mg/kg; twice). Values are expressed as mean ± SEM of % conditioned responses of each group (n = 5–6). Significant differences (*p < 0.05, **p < 0.01) in citrate buffer (CB) + drug or STZ or STZ + drug *vs.* CB control group, (+p < 0.05, ++p < 0.01, +++p < 0.001), CB + drug or STZ + drug *vs.* STZ group; ANOVA (one way) followed by Tukey test.

among other changes, decreased p-GSK3β levels (Solas et al., 2010). Next, we investigated the effect of lithium, a GSK3 inhibitor. Lithium reversed LTM deficit and restored p-GSK3β levels in the hippocampus and PFC in STZ-treated rats. It seems that deregulation of p-GSK3β was involved in memory deficit and neuroprotective effect in ICV STZ-treated rats. Consistent with these results, specific inhibition of GSK3β activity by lithium prevents increased tau phosphorylation and spatial memory impairment resulting from PI3K and PKC inhibition (Liu et al., 2003). Also, in transgenic mice (Tet/GSK-3β) conditionally overexpressing GSK3β in the hippocampal and cortical neurons showed a memory deficit (Hernandez et al., 2002).

Notwithstanding the different vehicles used herein had no effect on memory, including DMSO, which is consistent with diverse data. For instance, Sharifzadeh et al. (2005) reported that intrahippocampal infusion of DMSO produced no significant alterations on the memory retention test regarding naïve and animals infused with saline. Also, Kang et al. (2004) reported that DMSO *per se* had no effect on p-GSK3β. Notably, in this work phenserine (1 mg/kg; an AChE inhibitor) reversed LTM deficit (48 h) induced by ICV STZ-treatment and restored p-GSK3β levels in hippocampus, but not in PFC. Previously, a study showed that donepezil (another AChE inhibitor) improves memory and reduces AChE activity in ICV STZ rats model (Sonkusare et al., 2005). In agreement with our results, donepezil increased p-GSK-3β levels and phosphorylated Akt, which regulates the phosphorylation (Ser9) GSK3, and decreased phosphorylated tau in amyloid-β1-42 induced neurotoxicity in cortical neurons of rats; these neuroprotective effects were blocked by PI3K inhibitor (Noh et al. 2009).

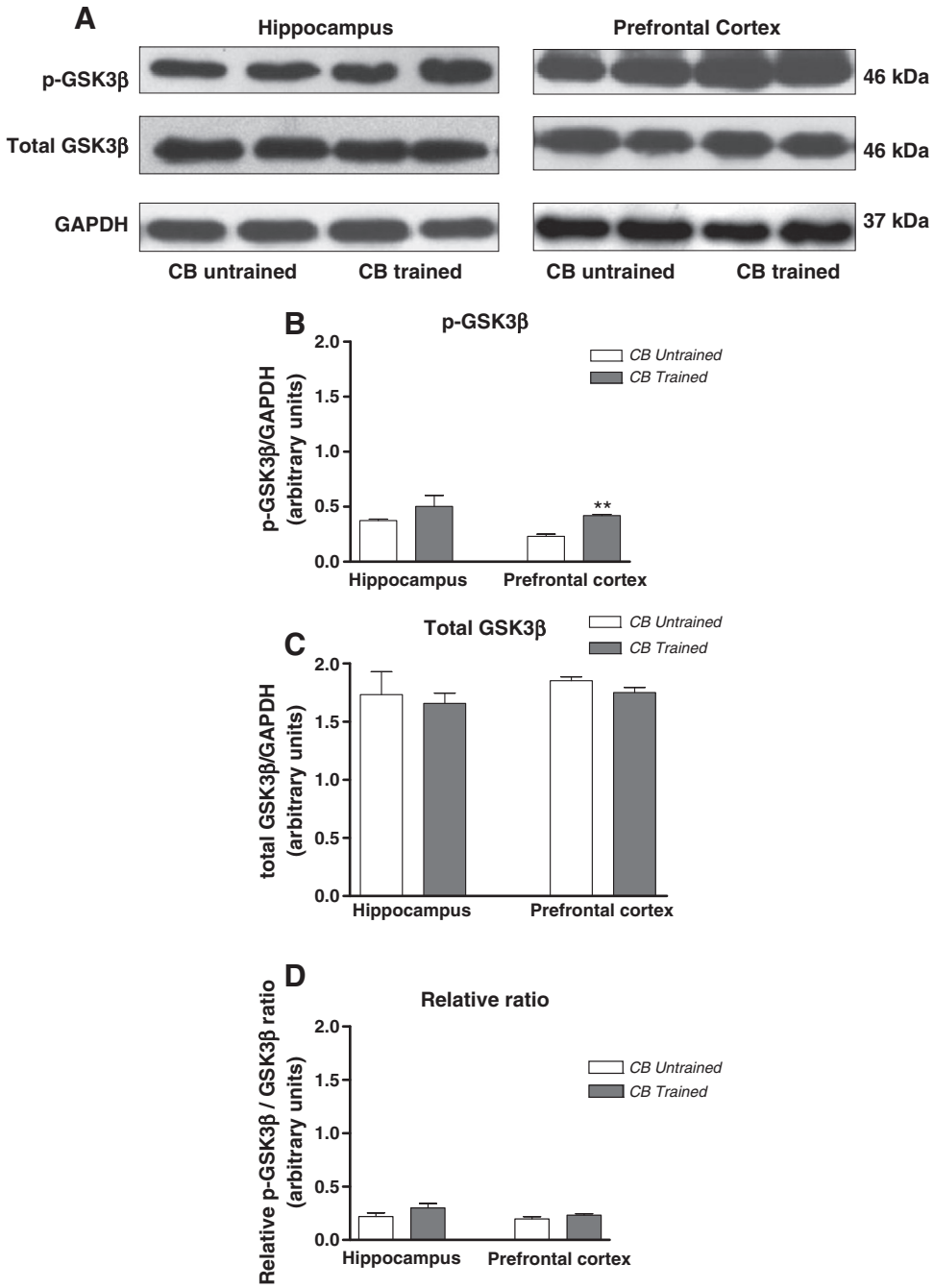

Fig. 4 – Comparison of phosphorylated (p) GSK3β levels and total GSK3β in the hippocampus and prefrontal cortex of untrained and trained rats treated with intracerebroventricular (ICV) citrate buffer (CB). (A) Representatives immunoblots of p-GSK3β and total GSK3β, (B) p-GSK3β levels, (C) total GSK3β levels and (D) relative p-GSK3β/GSK3β ratio. Hippocampal and prefrontal cortex protein extracts were immunoblotted for phospho-GSK3β (Ser9) and total GSK3β. Quantification of both enzymes was normalized against GAPDH. Data expressed as mean ± SEM, n = 3 animal per group. Significant differences (*p < 0.05), t-test.

Although, in this study the Akt was not measured, recently Agrawal et al. (2011) reported a significant decrease in IR expression, phosphorylation of IRS-1 and Akt in the CA3 region in ICV STZ-treated rats. In other study Hoshi et al. (1996) showed that GSK3 phosphorylated and inactive piruvate deshydrogenase (PDH), which converts piruvate to acetyl CoA in mitochondria. Herein, inactivation of PDH by βA exposure resulted in dysfunction of mitochondria contributing to neuronal death through failure of energy metabolism and leading to a reduced level of acetylcholine (Ach) in cholinergic neurons due to decreased acetyl CoA production. The phenserine neuroprotective activity had been already reported, by reducing βA levels via regulating βAPP translation (Shaw et al., 2001). Therefore, phenserine, in addition to

increasing the availability of ACh (via AChE inhibition), it augmented p-GSK3β. This mechanism of action may occur by means of: 1) Akt phosphorylation, that in turns phosphorylates GSK3β, and 2) PDH activation, which would increase acetyl CoA production and consequently ACh synthesis.

Unlike of phenserine, pioglitazone (30 mg/kg) and memantine (5 mg/kg) reversed both STM and LTM impairment in ICV

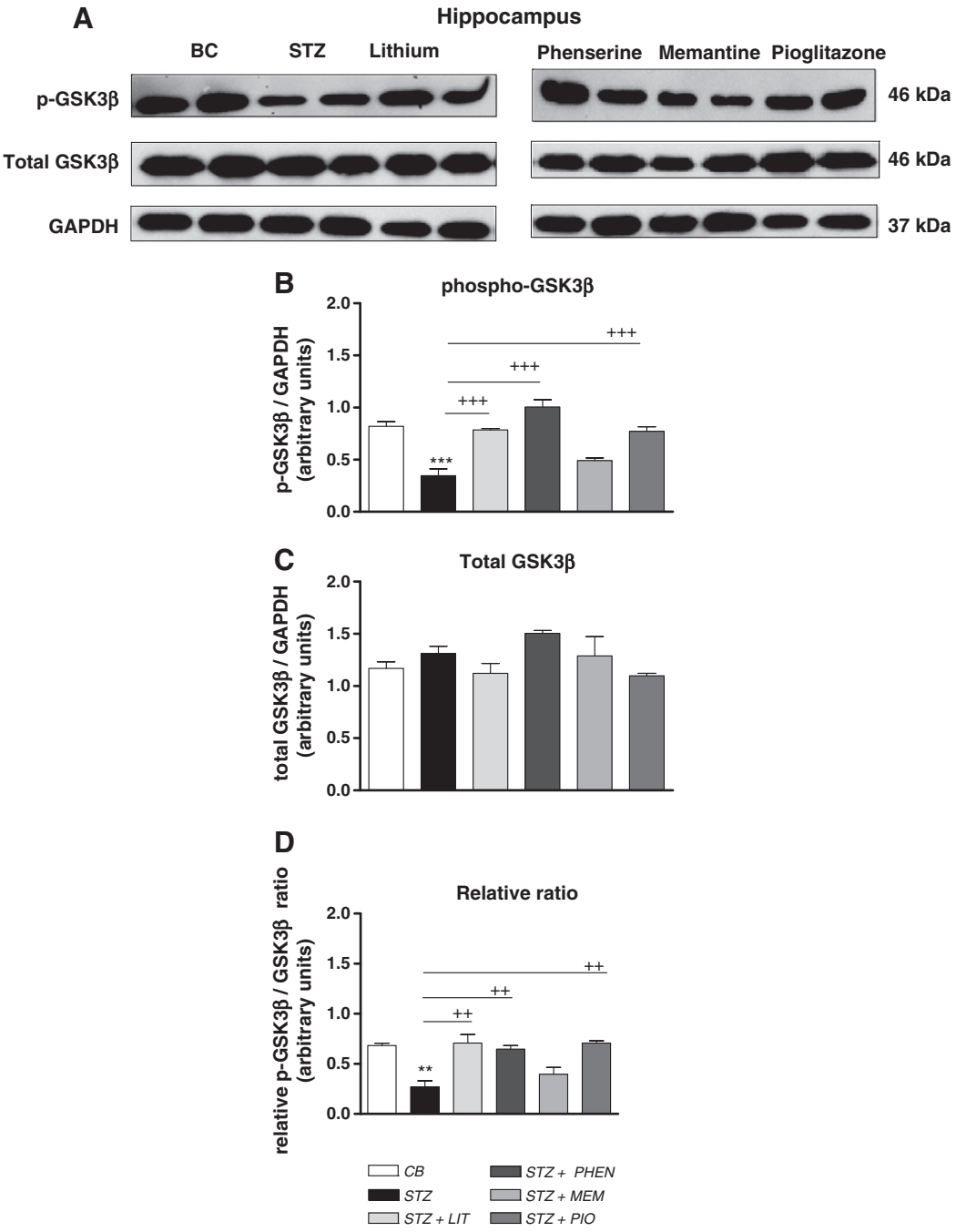

Fig. 5 – Effect of lithium (LIT; 100 mg/kg, i.p.), phenserine (PHEN; 1 mg/kg, i.p.), memantine (MEM; 5 mg/kg, i.p.) and pioglitazone (PIO; 30 mg/kg, o.p.) on phospho-GSK3β (p-GSK3β) and total GSK3β levels in the hippocampus 2 weeks following intracerebroventricular (ICV) streptozotocin (STZ) administration of trained rats. (A) Representatives immunoblots of p-GSK3β and total GSK3β, (B) p-GSK3β levels, (C) total GSK3β levels and (D) relative p-GSK3β/GSK3β ratio. Hippocampal protein extracts were immunoblotted for p-GSK3β (Ser9) and total GSK3β. Quantification of both enzymes was normalized against GAPDH. Data expressed as mean ± SEM, n = 3 animal per group. Significant differences in (*p < 0.05, **p < 0.01, ***p < 0.001) citrate buffer (CB) + drug or STZ or STZ + drug *vs.* CB control group, (++p < 0.01), STZ + drug *vs.* STZ group; one way ANOVA followed by Tukey test.

STZ-treated rats; nevertheless only pioglitazone significantly reestablished p-GSK3β levels to control values in hippocampus, but not in PFC. It is known that pioglitazone increases insulin-sensitive GLUT4 (Young et al., 1995) facilitating glucose utilization and metabolism in peripheral tissues (Yonemitsu et al., 2001). Formerly, Pathan et al. (2006) showed

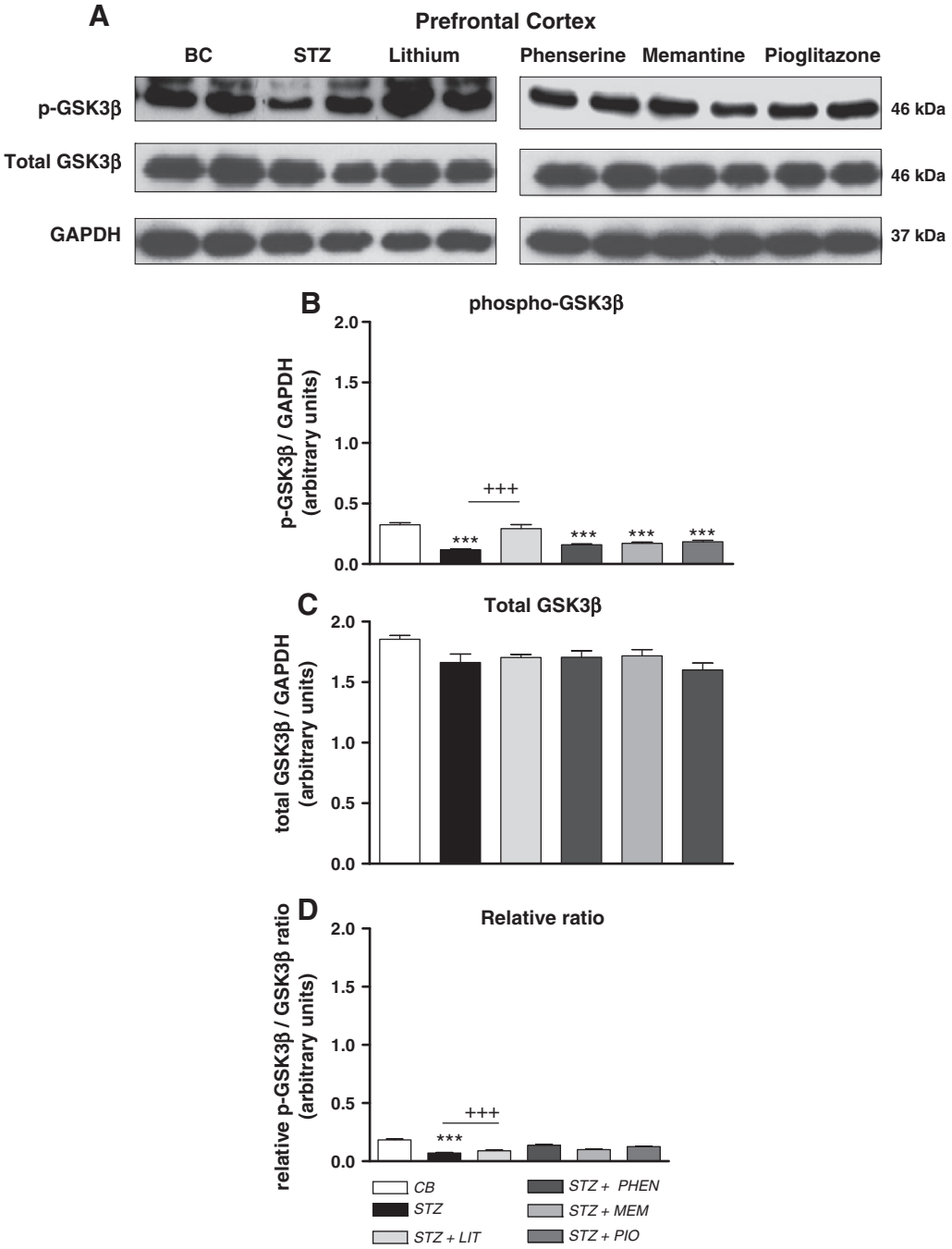

Fig. 6 – Effect of lithium (LIT; 100 mg/kg, i.p.), phenserine (PHEN; 1 mg/kg, i.p.), memantine (MEM; 5 mg/kg, i.p.) and pioglitazone (PIO; 30 mg/kg, o.p.) on phospho-GSK3β (p-GSK3β) and total GSK3β levels in the prefrontal cortex 2 weeks following intracerebroventricular (ICV) streptozotocin (STZ) in trained rats. (A) Representative immunoblots of p-GSK3β and total GSK3β, (B) p-GSK3β levels, (C) total GSK3β levels and (D) relative p-GSK3β/GSK3β ratio. Prefrontal cortex protein extracts were immunoblotted for p-GSK3β (Ser9) and total GSK3β. Quantification of both enzymes was normalized against GAPDH. Data expressed as mean ± SEM, n = 3 animal per group. Significant differences in (*p < 0.001) citrate buffer (CB) + drug or STZ or STZ + drug *vs.* CB control group, (+p < 0.05, +++p < 0.001), STZ + drug *vs.* STZ group; one way ANOVA followed by Tukey test.**

that chronic treatment with pioglitazone (30 mg/Kg, o.p.) improved cognitive performance, lowered oxidative stress and improved cerebral glucose utilization in ICV STZ rats. In the present study, we confirm the beneficial effect of pioglitazone on memory and increased p-GSK3β levels, which might involve improved glucose utilization (Pathan et al., 2006) and increased sensitivity to IR and subsequently activation of the PI3K/Akt pathway along with increased p-GSK3β levels. The latter suggests a possible neuroprotective role of pioglitazone, supporting the evidence that a PPAR agonist increased p-GSK3β and reduced tau phosphorylation in the hypothalamus in the intracerebral (IC) STZ model (de la Monte et al., 2006). Undoubtedly, further experiments are required to demonstrate the effect of pioglitazone on IR expression, PI3K/Akt pathway and tau phosphorylation in ICV STZ treated rat model. Concerning memantine, although it restored memory deficit, the dose (5 mg/kg) used was not able to modify the p-GSK3 levels neither in hippocampal or PFC tissues. We select these doses because it is reported that acute 5 mg/kg i.p. in rats can probably be considered of therapeutic relevance for its use in AD (Zoladz et al., 2006). Nonetheless, a study reported that memantine (50 mg/kg) *in vivo* increased p-GSK3β after 2 h in mouse brain (de Sarno et al., 2006). It should be noted that in our hands memantine at 20 mg/kg produced seizures and behavioral alterations (data not shown). Notwithstanding, the present results might be indicating that central STZ application involved NMDA receptor function, since memantine reversed memory deficit. According to our best knowledge this is the first study that determined the effect of an NMDA receptor antagonist on memory performance in the model of ICV STZ treated rats.

Finally, changes in the total levels of GSK3β were not observed with any of the drugs used, and the relative p-GSK3β/total GSK3β ratio increased with drugs (except memantine) in ICV STZ-treated group. This means that lithium, phenserine and pioglitazone modulated GSK3β activity just in the hippocampus, even though, lithium was able to regulate GSK3β activity in the PFC.

Taken together, these data suggest that insufficient inhibitory GSK3β control is associated to ICV STZ memory deficit model, demonstrated by a decrease in p-GSK3β levels in the hippocampus and associated to STM and LTM; hence GSK3β might be an important mediator of memory formation and therapeutic target. Lithium, phenserine and pioglitazone treatment reversed memory deficit and restored the inhibitory activity of GSK3β. Hence, the ICV STZ-treated rat model appears to be an excellent *in-vivo* tool to study the memory impairment and the down- and up-regulation of the cascade of neurodegeneration in sporadic AD and for investigating effective treatments to prevent or reverse memory deficits. Definitely this pharmacological model is for modeling sporadic AD (Hoyer, 2000; Salkovic-Petrisic, 2008) and in this work we did not test any of the hallmarks of AD. It should be noted that in the ICV STZ-treated rat model phosphorylation tau changes and beta amyloid would not be seen earlier than one (Grünblatt et al., 2007; Salkovic-Petrisic et al., 2006) and three (Salkovic-Petrisic et al., 2006) months, respectively. Currently, we are measuring total tau and phospho-tau in brain ICV STZ treated rats after 3 months by western blot (unpublished data). Undoubtedly, future works should explore diverse questions, e.g., amyloid plaque formation, the effects of phenserine, memantine, etc., on p-GSK3β during memory formation. In order to determine if the present results are reliable, subsequent works should use other memory models.

4. Experimental procedures

4.1. *Animals*

Male Wistar 3-month-old rats were used. Animals were placed together in a cage (10 per cage) prior to STZ injection and on an individual cage after STZ administration during recovery time after surgery (1 week), then they were placed together during the experimental phase. They were maintained at 22±2 °C and 12 h light/dark cycle. Rats had free access to food and water 1 week previous to experimental manipulation. The experimental protocol was revised and approved by the Institutional Review Committee (CICUAL; project no. 047/02) for the use of animal subjects in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85–23, revised 1985).

4.2. *Animal groups*

Animals were randomly divided into three groups: untrained, training sham-operated and trained animals. Untrained group was food-deprived, did not receive autoshaping sessions, were left in their home-cage and handled daily in order to reproduce the handling conditions of trained rats. It was used to estimate the basal level of p-GSK3β and total GSK3β in ICV CB rats. From trained groups, some rats were sham-operated; others were ICV injected with either citrate buffer (CB) or STZ and were treated with vehicle or drug (see Table 1). They were trained on the autoshaping task, and were also food-deprived, received an autoshaping training immediately followed by post-training vehicle or drug administration. After that, they were given training/testing sessions 1.5 h following autoshaping training and drug administration (STM) and 24 and 48 h (LTM) later untrained and trained groups were sacrificed at this moment.

4.3. *Surgical procedure and ICV administration of STZ*

Adult male Wistar rats weighing 320–340 g were anesthetized with ketamine (75 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). The animal body was placed in position in the stereotaxic apparatus and in its head a midline saggital incision was made in the scalp. Then, holes were drilled in the skull on both sides over the lateral ventricles. The following coordinates were used for ICV injection: 0.8 mm posterior to Bregma, 1.5 mm lateral to saggital suture, 3.6 mm ventral from the surface of the brain (Sharma and Gupta, 2002). Coordinates for placement of cannulae were determined by using the atlas of Paxinos and Watson (2005). As either artificial CSF (Saxena et al., 2008, 2010, Sharma and Gupta, 2002) or the citrate buffer (Grünblatt et al., 2007; Pathan et al., 2006; Salkovic-Petrisic et al., 2006; Sonkusare et al., 2005) is often used as vehicle for STZ, hence herein the latter was used. So the STZ was dissolved in CB (pH 4.5) just prior to injection. The STZ group

Table 1 – Experimental trained groups.

Subgroup treatment	n	Subgroup treatment	n
Saline	6	Saline	6
DMSO	6	DMSO	6
CMC	6	CMC	6
Saline + lithium (100 mg/kg; i.p.)	5	Saline + lithium (100 mg/kg; i.p.)	5
Saline + phenserine (1 mg/kg; i.p.)	5	Saline + phenserine (1 mg/kg; i.p.)	5
DMSO + memantine (5 mg/kg; i.p.)	5	DMSO + memantine (5 mg/kg; i.p.)	5
CMC + pioglitazone (30 mg/kg; o.p.)	5	CMC + pioglitazone (30 mg/kg; o.p.)	5

ICV: intracerebroventricular, CB: citrate buffer, STZ: streptozotocin, DMSO: dimethyl sulfoxide, CMC: carboxymethylcellulose

was injected bilaterally with STZ (3 mg/kg) in two divided doses, on days 1 and 3. The concentration of STZ was adjusted so as to deliver 4 μL of the solution. In the control group were given an ICV injection of the same volume of CB on 1 and 3 day as in STZ injected rats. Post-operatively, the rats were maintained in an individual cage and received free access to food and water during 1 week, after that they were placed together until the last experimental day. After that period, body weights were reduced to 85% by gradually reducing food intake. In the following week, animals were submitted to behavioral tasks (2 weeks after ICV STZ injection).

4.4. *Drugs administration*

Lithium chloride (LiCl; Sigma-Aldrich) and memantine (Sigma-Aldrich) was dissolved in saline (0.9%), phenserine (Sigma-Aldrich) was dissolved in dimethyl sulfoxide 10% (DMSO: Sigma-Aldrich) and they were systemic injected (i.p.). The suspension of pioglitazone (Actos®; E. Lilly Company) was prepared in 1% carboxymethyl cellulose (CMC; Sigma-Aldrich) and administered orally. After 2 weeks of ICV STZ-treatment animals received autoshaping training and immediately after the (acute) post-training drugs and respective vehicle administration. Doses used were taken from the literature: lithium 100 mg/kg (Selenica et al., 2007; Tomasiewicz et al., 2006), phenserine 1 mg/kg (Meneses 2002), memantine 5 mg/kg (Zoladz et al., 2006) and pioglitazone 30 mg/kg (Pathan et al., 2006). These doses were selected based on works where behavioral and neurochemical changes were recorded.

4.5. *Behavioral protocol*

4.5.1. *Autoshaping learning task*

In an autoshaping or sign-tracking setting, a hungry animal is placed in a conditioning chamber to find food pellets (unconditioned stimulus [US]) in the food-magazine and is then given a Pavlovian sequential pairing (stimulus–stimulus [S-S]) of a lighted key or a retractable-illuminated lever (conditioned stimulus [CS]) and food (US). After a number of such presentations, the animal approaches the CS and presents instrumental responses (conditioned response [CR]), such as peck, nose-poke, and contactor lever-press. Then, CR or autoshaped responses result from the S-S association and are sustained by response–stimulus (R-S) association (Meneses, 2003).

Importantly, within the continued progress of behavioral task development, a P/I-A task combines both Pavlovian and instrumental conditioning. These offer the opportunity to study hippocampus-mediated declarative memory and striatum-mediated R-S "habit formation" (Meneses 2002). Furthermore, P/I-A, except for magazine training, is almost completely automatized, considerably reducing human intervention. It is sensitive to small increases or decreases in various behavioral parameters (i.e., not measuring the same event twice), including sign tracking (i.e., conditioned behavior directed toward the localized retractable and illuminated lever; CS), and goal tracking (i.e., the place where the US is delivered). The latter is quite important, as it allows the study of bidirectional expression of an enhanced or impaired memory formation. P/I-A clearly separates training for testing sessions, and it has been useful to detect changes in memory formation elicited by drugs or aging (Meneses, 2003).

4.5.2. *Food magazine and autoshaping training*

Individually, each rat was placed in an experimental chamber for a habituation period (≈15 min) with access to 50 food-pellets (45 mg each) previously placed into the food magazine. The criterion was that once the animal ate all 50 food-pellets and presented 150 nose-pokes (as measured by a photocell) into the food-magazine, the autoshaping training program was initiated (Meneses, 2003; 2007).

The autoshaping program had been reported previously (Meneses, 2007), and this consisted of discrete trials. A trial began with the presentation of a retractable and illuminated lever for 8 s (conditioned stimulus; CS) followed by a food-pellet (unconditioned stimulus; US) delivery. There was an inter-trial interval time (ITI) of 60 s. When the animal pressed the CS, it was considered a conditioned response (CR), which shortened the trial, retracted the lever, turned off the light, and a US was delivered. The CR increment or decrement was considered an index for enhancement or impairment in memory consolidation, respectively. There was an autoshaping training session (10 trials) lasting nearly 12 min, and three training/testing (20 trails each) sessions, lasting nearly 24 min. All sessions were conducted over three consecutive days. The autoshaping training session was followed by consecutive training/testing sessions at 1.5 h for short-term memory (STM), and 24 and 48 h for long-term memory (LTM). In addition, increases or decreases in the head-pokes/CS were used as a motivation measure for food-pellets and explorative behavior.

4.6. *Determination of phospho-GSK3β and total GSK3β*

4.6.1. *Tissue preparation*

Subsequent to the last autoshaping training/testing session (48 h) CB, STZ and STZ + drugs groups, and untrained CB rats were sacrificed by decapitation and used for determinate p-GSK3β and total GSK3β levels. Brains were quickly removed, placed on ice and the prefrontal cortex and hippocampus dissected (from CA1 area to dentate gyrus) for each group according to Paxinos and Watson (2005). Coordinates for the prefrontal cortex from Bregma to interaural were 3.72 to

12.72 mm and for the hippocampus −2.92 to 6.08 mm. Hippocampal and prefrontal cortex (one sample per animal to get an enough protein) tissue samples from the rat brain were homogenized with three volumes of lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, protease inhibitors (PMSF, aprotinin, leupeptin and pepstatin). The lysates were centrifuged at 12,000 *g* per 20 min at 4 °C. The supernatants were removed and kept in new Eppendorf tubes. Samples were frozen and stored at −70 °C until further analysis. Total protein concentration was determined. The amount of protein was assayed according to the method of Bradford (1976) (Sigma; Cat. No. B6916). For calibration curve BSA standard was used (Sigma). The concentration was determined by measurement of the absorbance at 595 nm.

4.6.2. *Western blot*

Equal amounts of total protein (20 μg per sample for enzyme for analyses) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% polyacrylamide gels and transferred to PVDF membranes (Kurien and Scofield, 2003; 2006). The PVDF membranes were blocked by incubation in 5% non-fat milk added to phosphate buffered saline (PBS-T) containing 137 mM de NaCl, 2.7 mM de KCl, 10 mM de Na_2HPO_4, 2 mM de KH_2PO_4, pH 7.4, 0.5% Tween 20, 1 h at 22 °C for p-GSK3β and total GSK3β. Blocked blots were incubated on the next day with primary antibody anti-phospho-GSK3β (Ser9) rabbit (1:1000, Cell Signaling, Inc), total GSK3β (27 C10) rabbit (1:1000, Cell Signaling, Inc.) and anti-GAPDH clone 6C5 (1:5000, Milipore, Inc.) overnight at 4 °C. After incubation, the membranes were washed three times with PBS-T 1% and incubated for 1 h at room temperature with secondary antibody solution anti-rabbit IgG (1:2000) for p-GSK3β analysis and for total GSK3β (1:5000, Milipore, Inc.), and anti-mouse IgG (1:10,000, Milipore, Inc.) for GAPDH. The specificity of the signal was checked on control membranes that were not incubated with primary antibody. After washing three times in PBS, the membranes were immunostained using chemiluminescence western blotting detection reagents (Bio Rad) and exposure to an X-ray film. Relative optical density of bands was analyzed using MCID gel analysis software, Imaging Research Inc.

4.7. *Statistical analysis*

The values of the conditioned responses (CR) were expressed as a percentage of the total trials (10 or 20) per session (mean ± SEM) in the autoshaping test, meaning that, e.g., 2–3 CR corresponded to 20–30%. It should be noted that the basal level of CR is almost zero (see Meneses, 2003; Meneses et al., 2011a; 2011c). The head pokes/CS was determined during the training/testing sessions (mean ± SEM). Both, CR and head pokes were analyzed by means of one-way ANOVA followed by Tukey test post-hoc. In all comparisons, $p<0.05$ was used as a criterion for significance. The *n* per group was 5–6 animals. The p-GSK3β and total GSK3β values were expressed as means (±SEM) and they were analyzed by Student's *t*-test for the CB untrained *vs.* CB trained groups (two groups) and by one-way ANOVA (three or more groups) followed by Tukey test post-hoc for all the rest of the groups. In all comparisons $p<0.05$ was considered as significant. The *n* per group were 3 animals. The statistical software used was GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

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