STREPTOZOTOCIN

DIABETES AND/OR COGNITIVE DECLINE INDUCTION

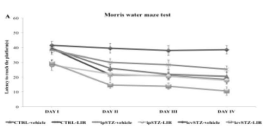
DAILY LIRAGLUTIDE TREATMENT

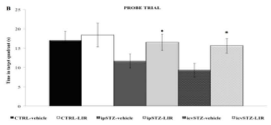
AFTER SIX WEEK OF TREATMENT

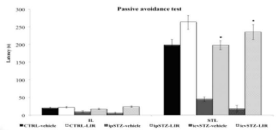
BEHAVIORAL TESTING

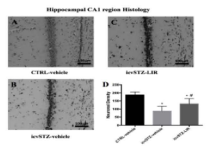
LIRAGLUTIDE IMPROVED LEARNING AND MEMORY

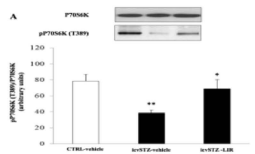
LIRAGLUTIDE HAD PRO-DEPRESSANT AND ANXIOLYTIC EFFECTS

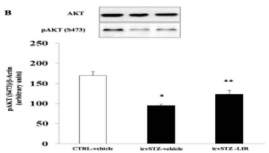












HISTOLOGICAL AND BIOCHEMICAL ANALISYS

LIRAGLUTIDE REDUCED HIPPOCAMPAL NEURONAL DEATH AND PREVENTED THE DECREASED PHOSPHORYLATION OF AKT AND P70S6K.

Liraglutide prevents cognitive decline in a rat model of streptozotocin-induced diabetes independently from its peripheral metabolic effects

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Abstract

Diabetes has been identified as a risk factor for cognitive dysfunctions. Glucagone like peptide 1 (GLP-1) receptor agonists have neuroprotective effects in preclinical animal models. We evaluated the effects of GLP-1 receptor agonist, liraglutide (LIR), on cognitive decline associated with diabetes. Furthermore, we studied LIR effects against hippocampal neurodegeneration induced by streptozotocin (STZ), a well-validated animal model of diabetes and neurodegeneration associated with cognitive decline. Diabetes and/or cognitive decline were induced in Wistar rats by intraperitoneal or intracerebroventricular injection of STZ and then rats were treated with LIR (300µg/kg daily subcutaneously) for 6 weeks. Rats underwent behavioral tests: Morris water maze, passive avoidance, forced swimming (FST), open field, elevated plus maze, rotarod tests. Furthermore, LIR effects on hippocampal neurodegeneration and mTOR pathway (AKT, AMPK, ERK and p70S6K) were assessed. LIR improved learning and memory only in STZ-treated animals. Anxiolytic effects were observed in all LIR-treated groups but *pro*-depressant effects in CTRL rats were observed. At a cellular/molecular level, intracerebroventricular STZ induced hippocampal neurodegeneration accompanied by decreased phosphorylation of AMPK, AKT, ERK and p70S6K. LIR reduced hippocampal neuronal death and prevented the decreased phosphorylation of AKT and p70S6K; AMPK was *hyper*-phosphorylated in comparison to CTRL group, while LIR had no effects on ERK. LIR reduced animal endurance in the rotarod test and this effect might be also linked to a reduction in locomotor activity during only the last two minutes of the FST. LIR had protective effects on cognitive functions in addition to its effects on blood glucose levels. LIR effects in the brain also comprised anxiolytic and *pro*-depressant actions (although influenced by reduced endurance). Finally, LIR protected from diabetes-dependent hippocampal neurodegeneration likely through an effect on mTOR pathway.

Abbreviations

AD, Alzheimer's disease;

APP/PS1, amyloid precursor protein/presenilin 1;

Aβ, β-amyloid;

CNS, central nervous system;

CTRL, control;

EPM, elevated plus maze;

FST, forced swimming test;

GLP-1R, glucagon-like peptide-1 receptor;

i.c.v., intracerebroventricular;

i.p., intraperitoneally;

IL, initial latency;

IT, immobility time.

LIR, liraglutide;

mTOR, mammalian target of rapamycin;

MWM, Morris water maze;

NOS, nitric oxide synthase;

OF, open field arena;

p70S6K, p70 ribosomal protein S6 kinase;

PD, Parkinson's disease;

s.c., subcutaneously;

STL, step-through latency;

STZ, streptozotocin;

T2DM, type 2 diabetes mellitus;

TNF-α, tumor necrosis factor-alpha.

Keywords: Streptozotocin, diabetes mellitus, rats, cognitive functions, neurodegeneration, GLP-1 receptors, liraglutide, mTOR signaling.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a public health problem currently increasing in prevalence [1]. T2DM is a complex endocrine and metabolic disorder also accompanied by a negative impact on the central nervous system (CNS), particularly in the elderly [2], leading to diabetic encephalopathy and concomitant increased incidence of cognitive problems; the latter are particularly associated with atrophy of the hippocampal formation that is known to be also involved in learning and memory processing [2]. Patients with T2DM show volumetric abnormalities in the hippocampus and amygdala, similar to those found in patients with major depression [3]. Furthermore, neurocognitive deficits in working memory, attention and executive function have been reported in both individuals with diabetes and mood disorders [2]. The precise pathophysiology of cognitive dysfunction and neuronal damage in T2DM is not completely understood, but it is likely that impaired glucose control (hyperglycemia, hypoglycemia), vascular damage, and insulin resistance play significant roles [3]. None of the known antidiabetic drugs currently used for T2DM has a proven effect on cognitive decline in patients, even if, there are some data on the efficacy of metformin and other antidiabetic drugs in animal models of diabetes and Alzheimer's disease (AD) [4-6], however, the glucagon-like peptide-1 receptor (GLP-1R) agonists, a new class of antidiabetic drugs now used therapeutically in diabetic patients, also exert powerful neuroprotective properties in animal models of neurodegenerative diseases, cerebral ischemia and traumatic brain injury [7, 8], therefore might be valuable therapeutic tools for prevention of neurological comorbidity in T2DM. GLP-1 is an endogenous incretin (insulinotropic) peptide hormone secreted from the gastrointestinal tract, that plays a key physiological role in glucose homeostasis by enhancing pancreatic insulin secretion and by suppressing glucagon release and hepatic glucose output [9]. GLP-1 receptor (GLP-1R) agonists such as exenatide, liraglutide (LIR) and lixisenatide have been approved for treatment of T2DM [10]; in

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December 2014, liraglutide 3.0 mg was approved by the Food and Drug Administration and in March 2015 by the European Medicines Agency for obesity treatment. In addition to its peripheral metabolic effects, GLP-1 acts as a growth factor in the brain, inducing neurite growth and protecting against oxidative injury [11]. It is known that GLP-1Rs are expressed by pyramidal neurons in the hippocampus, hypothalamus, neocortex and Purkinje cells in the cerebellum and are involved in cell differentiation and neuroprotection as demonstrated both *in vitro* and *in vivo* [12]. GLP-1 and GLP-1 analogs also increase neuronal progenitor proliferation in the brain, as well as long-term potentiation and paired-pulse facilitation in the hippocampus [13, 14]; accordingly, GLP-1R knock-out mice have an impairment of memory formation [14].

The neuroprotective effects of GLP-1 have been widely demonstrated in cultured neurons [15]. Human neuroblastoma cell lines over-expressing GLP-1Rs are protected from oxidative stress-induced cell death [16]. Excitotoxic L-glutamate-induced death of cultured rat hippocampal neurons is also decreased by GLP-1 [17]. The dual actions of GLP-1 in pancreatic β-cells and in neurons have recently generated therapeutic interest, considering that T2DM is a risk factor also for AD. Thus, GLP-1 has beneficial effects not only for the treatment of diabetes but also produces significant neuroprotection in animal models of cerebral ischemia and AD [18, 19]. Indeed, GLP-1 reduces endogenous levels of β-amyloid (Aβ) in the rodent brain [20] and intracerebroventricular (i.c.v.) administration of GLP-1 enhances associative and spatial learning [13]. Furthermore, most GLP-1 agonists show remarkable neuroprotective effects in different neurodegenerative disorders such as AD, Parkinson's disease (PD) and stroke by reducing β-amyloid plaques, preventing loss of synapses and memory impairments, and reducing oxidative stress and chronic inflammatory responses in the brain. Recent studies have established that exenatide, a stable GLP-1R agonist, enhances neuronal progenitor proliferation in the brain of diabetic mice [21] and

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reduces endogenous levels of Aβ in transgenic AD mice [20]. Similarly, LIR has neuroprotective effects in rats [22]. It also has neuroprotective effects in an amyloid precursor protein/presenilin 1 (APP/PS1) mouse model of AD [23], increases neurogenesis, improves cognitive function and reduces amyloid plaque deposition in a mouse model of AD [19, 24] and is now being tested in clinical trials for the treatment of AD patients [25]. LIR additionally attenuates the neuronal damage following cerebral ischemia in rats by preventing apoptosis and decreasing oxidative stress [26]. Recently, lixisenatide was tested in the APPswe/PS1DE9 mouse model of AD in comparison with LIR, showing that lixisenatide was equally effective but more potent [27]. Therefore, GLP-1 agonists apparently have a potential role to facilitate neuronal network repair in cortical tissue and consequently could have beneficial effects in patients with neurodegenerative disorders. Based on this background, we tested the effects of LIR on cognitive decline associated with diabetes mellitus in the established streptozotocin (STZ) rat model; furthermore, to exclude any influence of LIR effects on blood glucose levels, we studied the effects of LIR against the hippocampal neurodegeneration induced by i.c.v. injection of STZ, a well-validated model of neurodegeneration associated with cognitive impairment, not accompanied by diabetes [28]. Finally, in order to clarify the neuroprotective mechanism of action of LIR, we studied its effects on the mammalian target of rapamycin (mTOR) signaling pathway, which is known to be involved in cognitive functions and several neurological diseases [29].

2. Materials and Methods

2.1. Animals

Male Wistar rats (6 weeks old; *n*=190) were obtained from Charles River Laboratories s.r.l. (Calco, Lecco, Italy). Animals were housed three/five per cage under controlled environmental conditions. Procedures involving animals and their care were conducted in

conformity with international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept). The experimental protocols and procedures described in this manuscript were approved by the local ethical committee of the University of Catanzaro, Italy. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Drugs

Tiletamine/zolazepam (1:1; Zoletil 100®; VIRBAC S.r.l., Milan, Italy) was administered 50mg/kg intraperitoneally (i.p.); STZ (N-(methylnitrosocarbamoyl)-α-D-glucosamine; Sigma Aldrich, Milan, Italy), was dissolved in 0.01M sodium citrate buffer (pH4.5) and administered i.p. at a dose of 40mg/kg or i.c.v. at a dose of 3mg/kg. LIR (Victoza®) was obtained from Novo Nordisk S.p.A. (Rome, Italy) as pre-filled pens containing 18mg LIR in 3mL solution; 250µL of this solution was diluted in 5mL of saline solution (0.9% NaCl) to obtain a final solution of 300µg/mL and administered subcutaneously (s.c.) at a dose of 300µg/kg per day [30]. All drug solutions were freshly prepared immediately prior to administration.

2.3. Induction of diabetes

Diabetes was induced, after 12-hour overnight fasting, in Wistar rats by i.p. injection of two low-doses of STZ (40mg/kg with an interval of 10 days) [31], while control (CTRL) rats were given vehicle only. Diabetes was verified 48h after the last STZ administration by quantifying blood glucose levels by means of an automated glucometer (Freestyle optium; Abbot, Italy) through a blood sample obtained from the tail vein. Rats with blood glucose levels higher than ≥250mgd/L were considered to be diabetic and used in the present study.

In order to test LIR effects in diabetic rats, one week after diabetes development (week 1), animals were divided into 4 groups as follows: 1) CTRL-vehicle, *n*=38; 2) CTRL-LIR, *n*=28;

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3) ipSTZ-vehicle, *n*=24; 4) ipSTZ-LIR, *n*=24. LIR was administered s.c. at a dose of 300µg/kg once a day for 6 consecutive weeks between 9 and 10am [32]. Control rats (not receiving LIR) were injected daily with saline solution s.c. (Figure 1).

2.4. Induction of cognitive impairment

I.c.v. administration of a sub-diabetogenic dose of STZ produces long-term and progressive learning and memory deficits in rats [33]. Central STZ administration in low doses (1-3mg/kg, i.c.v.) does not produce diabetes as it does after peripheral administration, but induces a dysfunction of brain energy metabolism, oxidative stress, cognitive impairment, and cholinergic deficiency in the hippocampus, leading to cognitive deficits [33]. This protocol was used in order to study the effect of LIR on cognitive impairment, excluding any possible indirect effects of the drug mediated by control of peripheral blood glucose levels. All rats were implanted, under a mixture of tiletamine/zolazepam (see section 2.2), using a Kopf stereotaxic instrument, with a guide cannula for i.c.v. administration into the lateral ventricle (0.8mm posterior to bregma, 1.6 mm lateral to sagittal suture, and 3.6mm beneath the surface of brain) [34]. The injection cannula was lowered 2 mm beyond the edge of the guide cannula into the lateral ventricle. Either vehicle or STZ (3mg/kg) was infused, in two divided doses, the injection was repeated 2 days after the first STZ injection (1.5 mg/kg per day of injection) or vehicle in a volume of 5µL via a Hamilton syringe connected to a CMA/100 infusion pump. The injection cannula was withdrawn 1 min following infusion. On day 3, the cannula was removed and the wound was sutured. After the second i.c.v. infusion of STZ, rats were divided into two groups as follows: 1) icvSTZ-vehicle *n*=34; 2) icvSTZ-LIR *n*=34, LIR (300µg/kg s.c. daily) was administered for 4 consecutive weeks, every day between 9 and 10am. Control rats were injected daily with saline solution s.c. (Figure 1).

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2.5. Body weight and blood glucose levels

Body weight was measured weekly in every group for the entire duration of the experiments. Fasting (12 hours food deprivation) glucose level was measured in blood taken from the tail vein by a glucometer (Freestyle optium; Abbot Laboratories, Italy) in every experimental group weekly.

2.6. Behavioral tests

For behavioral tests, animals of every group were divided in subgroups (n=8) in order to avoid exposure of the same animal to too many tests (for details see Supporting information). Subgroup I: Morris water maze (MWM); subgroup II: passive avoidance; subgroup III in the following order: forced swimming test (FST); open field arena (OF); elevated plus maze (EPM); subgroup IV: rotarod test (to simulate treadmill exercise). In subgroup II, the inter-test interval was at least 1 day (range 1-2 days). All behavioral tests were carried out under controlled conditions of temperature and humidity; light intensity was dependent on the respective experimental setup. All tests, with the exception of the rotarod test and passive avoidance, were carried out with the support of EthoVision XT8 software (Noldus; The Netherlands). LIR last injection was always administered 1h before the test session in every group. Every experimental animal group was evaluated in the tests starting at 9:00am and finishing before 11:00am in order to avoid possible circadian alteration of test results [35].

2.6.1. Morris water maze test

Learning and memory functions were assessed using a spatial acquisition task in an MWM [36]. The apparatus consisted of a circular basin (diameter=180cm, height=50cm) filled with water (24±1°C) to a depth of 30cm, with a clear (invisible) escape platform (diameter=8cm) placed 1cm below the water surface. Rats (n=8 for each group) were trained for 4 consecutive

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days, with 4 trials on each day. During each trial, an individual rat was put in a randomly chosen quadrant in the pool with the head toward the pool wall. Each rat was given 60s to search for and locate the submerged platform. If a rat failed to locate the platform within 60s, it would be guided gently to the platform to stay on it for 30s. The latency time to find the platform was recorded and the average time on 4 trials represented the daily result for the rat. On the 5th day of the MWM, each rat was subjected to a probe test where no platform was present. The time of swimming in the former platform quadrant and the total time of swimming in all four quadrants were recorded for 60s. The percentage of swimming in the quadrant of the former platform was considered a measurement of spatial memory [36].

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2.6.2. Passive avoidance

Passive avoidance is a fear-motivated test classically used to assess long-term memory and learning. The passive avoidance paradigm requires the animal to behave contrary to their innate tendencies for preference of dark areas and avoidance of bright ones. One-trial passive avoidance test (Ugo Basile srl, Varese, Italy, model 40550) was performed, as previously described [37];[38]. Briefly, a single rat was introduced into the light compartment; during habituation, rats were allowed to freely explore the box for 5min with the sliding door between the light and dark compartments open; after that, the rats were returned to their home cage. For conditioning (acquisition), which was carried out 2h after habituation, the rats were introduced into the light compartment, the sliding door was closed when both hind limbs had entered into the dark box, and an electrical footshock (0.3 mA, 3 sec) was delivered via the floor grid in the dark compartment. In this trial, the initial latency (IL) of entrance into the dark chamber was recorded. Tests were carried out 24h after the conditioning by re-introducing the rats into the light compartment of the light–dark box. The interval between placement in the illuminated chamber and entry into the dark chamber was measured as step-

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through latency (STL, up to a maximum of 300s). Memory performance is positively correlated with the latency to escape from the white compartment; the latency time for rats to enter the dark compartment was measured (light-dark latency, with a 5min cut-off) as an index of memory consolidation.

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2.6.3. Open field test

The OF test was performed as previously described [39]. The test started by placing the animal in the center of the field. During the test, the time spent in the center and the number of center entries were analyzed. Lower activity in the OF test is usually taken as a measure of a higher level of anxiety and *vice versa* [39]. Mean velocity was also statistically analyzed for every experimental group. The "*effective*" time spent in the center was given by the difference between the total time spent in the center and the latency to first. The latency to first is the time taken to leave the center and go for the first time in the outer quadrants.

2.6.4. Elevated plus maze test

The EPM test was performed as previously described [36]. Briefly, the EPM consisted of two opposing open arms (50x10cm) and two opposing closed arms of the same size with 40cm high walls. The arms were connected by a central platform (10×10 cm) and elevated 80cm above the floor, surrounded by a black curtain and exposed to dim illumination (40W). The animal was placed in the center of the maze facing a closed arm. The number of entries into, time spent on each arm and central square were scored for 10min. Less time spent in open arm and in central square indicates anxious behavior and *vice versa* [40].

2.6.5. Forced swimming test

The FST has been previously used for measuring immobility time (IT) and assessing depressive-like behavior in rodents [40]. Rats were placed individually to swim for 6min into a clear plastic cylinder (47cm in height; 38cm in diameter) containing 38cm of water (25±1°C). The total duration of immobility, including passive swimming, was measured during the last 4min of the 6min testing period.

2.6.6. Rotarod test

The rotarod test can be used to assay the motor coordination of rodents. It requires rodents to balance on a rotating cylinder, the speed of which can be changed. The rotarod unit consists of a roller lane where 4 rats can be measured simultaneously, base unit dimensions: 362 (W) x 240 (D) x 400 (H) mm (Panlab s.l.u., LE8300, Spain). To evaluate endurance (riding time), the rotarod speed was set to 5 rpm simulating a treadmill test. For training, the rats *(n*=8 per group) were placed on the rotarod at 5 rpm on day 1 and subsequently tested to establish individual baseline levels of performance before the beginning of the experiment (drug administration). Rats were divided randomly in two groups CTRL and LIR-treated group (300 μg/kg; s.c.) and treated daily for a week, then they were placed on the rod at the preset speed (5 rpm), and the latency to fall off the rotarod device was assessed. We also measured the blood glucose levels both before and after testing to spot any possible hypoglycemic event that could affect rotarod performance.

2.7. Histology

After behavioral analysis, the CA1 area of the hippocampus of rats of the icvSTZ groups (STZ- vehicle, STZ-LIR, CTRL-vehicle *n*=5 per group) was studied for the evaluation of neuronal damage by cresyl violet staining. Rats of the icvSTZ groups were anesthetized as described previously (in Section *Drugs*) and killed by transcardiac perfusion with cold

phosphate buffer saline (PBS), pH 7.4 and subsequently with cold 4% paraformaldehyde, containing 0.2% saturated picric acid in PBS. Brains were removed, post-fixed overnight at 4°C in the same fixative solution. Paraffin embedded sections were cut in a coronal plane at a thickness of 4μm by a microtome. The de-paraffined sections were rinsed in PBS twice and immersed in 0.3% H_2O_2 in PBS for 10min followed by three rinses in PBS [41]. Paraffin-embedded brain sections were de-paraffined with xylene and rehydrated with ethanol at graded concentrations of 100-70% (v/v), followed by washing with distilled water. Sections were stained with 0.1% (w/v) cresyl violet and severity of neuronal damage was evaluated by the number of surviving neurons (i.e. conventionally intact cells when stained with cresyl violet) in the hippocampus. The mean number of morphologically intact neurons per 100μm length was calculated in the CA1 hippocampal area to accurately estimate the extent of neuronal damage. Cell counting was performed in six serial sections per animal using a light microscope equipped with a 25× objective, as previously described [42].

2.8. Western blotting

In order to study any direct effects of LIR on the mTOR signaling pathway, avoiding possible confounding factors linked to diabetes, these experiments were performed in a separate group of rats following the protocol for the STZ i.c.v. groups (STZ- vehicle, STZ-LIR, CTRL-vehicle; *n*=5 per group). Rats were decapitated, the brains were rapidly removed and immersed in ice-cold artificial cerebrospinal fluid. Blocks of tissue containing the hippocampus were cut using a vibratome (Vibratome 1500, Warner Instruments, CT, USA). A brain slice of 1.5mm was cut (interaural from +7.1mm to +5.6mm); after that, brain areas were isolated and dissected under an optical microscope (M650, Wild Heerbrugg, Switzerland), as previously described [34]. Frozen brain proteins were extracted using T-PER

buffer (Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. Protein concentration was determined with the Bradford assay (DC Protein Assay; Bio-Rad, Hercules, CA) according to the manufacturer's instructions (BioRad). Equal amounts of proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membranes were incubated with primary antibodies followed by incubation with peroxidase-conjugated secondary antibodies. Proteins were detected by using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and band densities were quantified by densitometry. To normalize the blots for protein levels, after being immunoblotted with antiphosphospecific antibodies, the same blots were stripped and re-probed with appropriate primary antibodies. The antibodies used were anti-p-AKT (S473), anti-AKT, anti-p-AMPK (T172), anti-AMPK, anti-p-P70S6K (T389) and anti-P70S6K (Cell Signaling Technology, Beverly, MA, USA) anti-ERK1/2, anti-p-ERK1/2 (Santa Cruz, CA, USA).

2.9. Statistical methods

All statistical procedures were performed using Graphpad prism software, version 6.0 (La Jolla, California, USA). The results are expressed as mean ± S.E.M. Student's t-test was used for analyzing the data between two groups (rotarod test) whereas one or two-way ANOVA followed by Bonferroni's post hoc test or Tukey's post hoc test, respectively, were employed if there were more than two groups. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Body weight and blood glucose levels

All LIR-treated groups (*i.e.* CTRL-LIR, ipSTZ-LIR and icvSTZ-LIR) starting from the 2nd-4th week, showed a significant (P<0.05) slowing in growth with decreased body weight in comparison to their respective control groups (Figure 2A). As expected, blood glucose levels in the ipSTZ-LIR group were significantly (P<0.05; 57%) reduced from the second week of treatment in comparison to the untreated diabetic ipSTZ-vehicle group. By contrast, LIR treatment alone in the CTRL group, did not modify blood glucose levels, confirming that LIR has antidiabetic effects only when blood glucose levels are elevated (Figure 2B). Blood glucose levels in the i.c.v.-treated groups were only measured up to the 5th week and since no alterations or LIR effects were observed, monitoring was suspended (data not shown).

3.2. LIR effects on memory and learning

Memory/cognitive functions were tested in the MWM, which is commonly used for testing drug effects on memory and learning. The i.c.v. injection of STZ showed a persistent deficit in spatial learning and memory, as evidenced by a significant (P=0.023) increase in the latency time required to reach the platform, indicating poorer learning and memory performance, which is consistent with earlier findings [43]; STZ administered i.p. also increased the latency time to reach the platform, but not significantly (P=0.6). Administration of LIR to the STZ-treated groups (STZ-LIR: both i.p. and i.c.v.) produced a significant (P<0.05) improvement of learning and memory with latency to the platform being maintained nearly at CTRL level (Figure 3A). LIR treatment decreased the initial latency to find the platform (first 2 days) indicating a favorable effect on learning, moreover it increased the time spent in the former platform quadrant on day 5 showing an improvement in memory task (Figure 3B). The mean velocity did not differ between groups (on average about 50cm/s).

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Similar effects were observed in the passive avoidance test; in particular, STZ groups did not show a significant improvement in the latency time confirming a memory impairment, while LIR treatment significantly (P<0.05) increased the latency time for entering the dark compartment both during the acquisition trial and 24h later (Figure 3C), supporting a protective effect on learning and long-term memory.

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3.3. LIR effects in anxiety-like behavior paradigms

STZ treatment (either i.p. or i.c.v.), did not influence animal behavior in both tests (Figure 4A, B). In contrast, LIR treatment was significantly (P<0.05) efficacious in all groups increasing the "*effective*" time (time in the center excluding latency to first; see methods) spent in the center in the OF (Figure 4A) and the time spent in the central square (Figure 4B) and open arms in the EPM. This effect did not discriminate between groups being all equally affected. The necessity to measure the "*effective*" time spent in the center derived from the observation that LIR-treated groups presented a significantly (P<0.05) reduced mean velocity, which might underlie a locomotor impairment not observed in the MWM and might influence OF results. In fact, placing an animal in the center which then is unable to move, would increase the time spent in the center and might be interpreted as an anxiolytic effect. In the OF, the number of center entries did not significantly differ among groups (data not shown); likewise in the EPM the number of entries in both closed and open arms did not significantly change among experimental groups (data not shown).

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3.4. LIR effects in depressive-like behavior paradigms

STZ-treated groups (both i.p. and i.c.v.) had a significantly (P<0.05) increased immobility time (IT) in comparison to the CTRL group. LIR significantly (P=0.037; 63%) increased IT (sign of depressive-like behavior) in the CTRL-LIR group in comparison to the CTRL-vehicle

group, whereas no significant changes were observed on IT between all other groups treated with LIR (Figure 4C). Mean velocity was also decreased only in the CTRL-LIR group in comparison to the CTRL-vehicle group even if not significantly (data not shown). Considering that this alteration in mean velocity can mimic a pro-depressant effect and that it was not observed in other LIR-treated groups, we have analyzed the data at different time-windows. In the first three minutes of the six min test, there were no differences in mean velocity between the two groups (CTRL-vehicle=7.7cm/s; CTRL-LIR=5.0cm/s) (no motor impairment), while in the last two minutes of the test, the two groups differed significantly (CTRL=8.2cm/s; CTRL-LIR=4.2cm/s; *P*=0.03) showing that LIR-treated rats had some kind of motor impairment likely remaining motionless; no differences were observed in all other groups. We supposed that the observed pro-depressive effect could be due to poor endurance (also see rotarod test results) and not directly related to depressive behavior.

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3.5. LIR effects on locomotor activity

In order to determine whether LIR had significant effects on motor function that might have influenced performance in the OF and FST experiments, we measured the rotarod performance with the aim of evaluating endurance rather than coordination. We analyzed time spent on the rotarod, showing that the LIR-treated group walked for a significantly shorter total time in comparison to the CTRL-vehicle group (CTRL-vehicle=2508.5s; CTRL-LIR= 859s; *P*=0.019) showing a decreased endurance on the rotarod test (Figure 4D). Moreover, we also measured blood glucose levels both before and after the test (immediately when the animal fell from the rotarod) to avoid any possible hypoglycemic event that could eventually affect rotarod performance. We found no significant alteration in blood glucose levels: pre-test blood glucose levels: CTRL-vehicle=138±5.4 mg/dL; CTRL-LIR=116.25±2.78 mg/dL;

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blood glucose levels at fall: CTRL-vehicle=186.5±6.02 mg/dL; CTRL-LIR=181±16.38 mg/dL.

3.6. Histology

Cresyl violet staining showed neuronal alterations in the hippocampal CA1 region of i.c.v. STZ-treated rats, characterized by pronounced shrinkage of the neuronal bodies with loss of nuclei and pyknotic pyramidal cells, whereas in the CTRL-vehicle group, neurons were large, conical shaped cells with well demarcated amphophillic cytoplasm and round vesicular nuclei with prominent nucleoli (Figure 5A). Chronic treatment (4 weeks) with LIR in icvSTZ-treated rats reduced the STZ-induced cell loss and pyknotic cells, but some degenerating cells with changed morphology were still observed (Figure 5B,C); therefore, LIR treatment largely prevented neuronal cell loss induced by i.c.v. STZ in the hippocampal CA1 region, in a significant (P=0.035) manner, showing protection against icvSTZ-induced neurotoxicity (Figure 5D).

3.7. LIR effects on mTOR signaling: AKT, AMPK, ERK and p70S6K phosphorylation

To test the potential role of LIR in the activation of the mTOR signaling pathway, we quantified, by Western blot analysis, the phosphorylation levels of three activated upstream regulators of mTOR activity, namely, AKT, AMPK and ERK in addition to the p70 ribosomal protein S6 kinase (p70S6K), the best characterized downstream kinase target of mTOR activity. At a cellular/molecular level, i.c.v. infusion of STZ induced hippocampal neurodegeneration accompanied by a significantly (P<0.05) decreased phosphorylation of AKT, AMPK, and p70S6K but not ERK1/2. LIR was able to reduce hippocampal neuronal death and to prevent the decrease in p70S6K and AKT phosphorylation (*i.e.* both maintained at control level) (Figure 6A,B); however, rather interestingly, AMPK was *hyper-*

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phosphorylated by LIR treatment in comparison to the CTRL group (Figure 6C), while LIR had no obvious effects on ERK1/2 phosphorylation (data not shown).

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4. Discussion

Recent epidemiological data have associated T2DM with an increased risk of developing mild cognitive impairment as well as AD [44]. Therefore, the possibility of preventing the development of such comorbidity in diabetic patients is a great research and therapeutic challenge. In this light, the increasing knowledge about the role of GLP-1 and its receptors in the CNS is very interesting, where they seem to have a key role in promoting cellular growth and reducing apoptosis besides other effects including a role in cognitive decline. For example, pre-clinical data have demonstrated significant neuroprotective effects of GLP-1 agonists in models of AD, by reducing β–amyloid plaques, preventing loss of synapses and memory impairment, and reducing oxidative stress [12, 19, 22, 24, 27] and GLP-1 receptor knockout mice show an impairment of synaptic plasticity and memory formation [14]. Indeed, one intriguing and attractive possibility would be that these stable GLP-1 agonists (*e.g.* exenatide, LIR, lixisenatide) currently used to treat diabetes would also be able to prevent the development of cognitive decline.

Our results demonstrate that LIR, a long-lasting GLP-1 analog, has protective effects against the development of cognitive decline in diabetic rats by preventing the impairment of learning (measured as the time to reach the platform in the MWM test) and memory (probe day; time latency entering the dark compartment by the passive avoidance). This effect was only limited to STZ diabetic rats and not control rats and was independent from LIR effects on blood glucose levels since the same effect was observed in the icvSTZ group. These data are in agreement with a recent study that demonstrated that LIR (200 μg/kg, i.p., for 4 days before the MWM) and sitagliptin (6 mg/kg, i.p., for 4 days before the MWM) ameliorate PTZ

induced cognitive impairment in EPM and scopolamine induced cognitive impairment in MWM test in a dose-dependent manner [45].

A preventive effect, more than an improving effect, is also supported by the ability of LIR to reduce hippocampal neurodegeneration following i.c.v. STZ administration. However, it remains to be determined whether LIR treatment might reverse cognitive impairment in these models as well as the exact mechanism(s) underlying this action. A possible explanation for the latter might be found in the ability of LIR to act as a growth factor in the brain, increasing cell growth, proliferation, and inhibiting apoptosis [46].

In an attempt to better understand the mechanism of action of LIR, we analyzed its effects on the mTOR signaling pathway, which is known to be involved in the CNS in synaptic plasticity and memory function, as well as neuronal repair mechanisms after injury [29]. The hippocampus requires p70S6K activation for the acquisition of conditioned place preference, inhibitory avoidance and both the formation and retrieval of contextual fear memories [47]. Furthermore, mice deficient in p70S6K show impairments in hippocampal-dependent memory tasks (MWM, contextual fear conditioning, conditioned taste aversion) confirming that the p70S6K is essential for normal learning and memory formation [48]. However, some controversial results have also been reported; for example, it has been demonstrated that partial inhibition of mTOR by chronic oral rapamycin improves learning and memory in young adult mice and blocks cognitive decline in older mice probably by stimulating major monoamine pathways in the brain [49].

Previous studies have demonstrated that GLP-1 improves signaling through the PI3K/AKT/mTOR pathway, which results in the inhibition of GSK3β activity and might be relevant for the neuroprotective function of GLP-1 analogs [50]. Recent studies have demonstrated that lixisenatide, another GLP-1 agonist, increases hippocampal expression of

mTOR [51] and rescues spatial memory and synaptic plasticity from Aβ protein-induced impairments in rats [52].

A recent study reported that the mTOR/p70S6K signaling pathway is hyperactive in the hippocampus of STZ-induced diabetic mice (200mg/kg; i.p.) and inhibiting mTOR signaling by rapamycin (2.24mg/kg for 45 days) prevented cognitive deficits related to diabetes partly by reduction of tau hyperphosphorylation [53]. At odds, we found that the mTOR/p70S6K signaling pathway in the icvSTZ group (rats with pure cognitive decline and not diabetes) was hypoactive at a time point where cognitive function was indeed impaired and LIR treatment prevented the inactivation of this pathway, maintaining p70S6K phosphorylation at control levels. The inhibition of mTOR activity by STZ was accompanied by a reduced phosphorylation of two well-known upstream regulators of mTOR, namely AKT and AMPK while ERK1/2 was not significantly affected. LIR treatment also maintained the AKT phosphorylation level to that of control while AMPK was actually *hyper*-phosphorylated. Indeed, AKT and p70S6K are essential for hippocampal-dependent memory formation [47]; however, contrasting data are present in the literature on the role of AMPK in the pathogenesis of neurodegenerative disorders. AMPK is a key energy modulator that is activated in response to alterations in cellular energy levels. Our results are in agreement with data obtained in a recent study demonstrating that AMPK activation ameliorates spatial memory impairment in a STZ (3 mg/kg once i.c.v.)-induced AD model in rats via repair of mitochondrial functions [54]. Furthermore, several studies have demonstrated that LIR can activate AMPK in other tissues such as heart, pancreatic islets [55] and liver but not skeletal muscles [56]. Therefore, the effects of LIR seem to be due to its ability to inhibit the negative effects of STZ on this pathway; however, other mechanisms might also be involved. In fact, it is known that GLP-1 can activate the PI3K/PKB pathway, thereby improving neuronal function [57] and the cAMP/PKA/CREB pathway, which plays a significant role in learning,

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memory, and synaptic plasticity [19, 58]. Finally, in our study, a neuroprotective effect of LIR was also confirmed by histological analysis of the hippocampal CA1 region, where the neuronal density was significantly lower in the icvSTZ group in comparison to icvSTZ-LIR treated groups, showing LIR protection against STZ-induced neurotoxicity. With regard to the mechanism underlying this toxicity, it has been suggested that i.c.v. STZ in rats may affect neuronal function by disrupting central glucose metabolism [59], leading to neurodegeneration, although the involvement of nitric oxide synthase (NOS) *per se* in this process was discounted [60]. Evidence for microglial activation and release of pro-inflammatory mediators (*e.g*. tumor necrosis factor-alpha (TNF-α)) following by i.c.v. STZ in rats has also been reported [61] which could contribute to our observed neurodegenerative changes. Further experiments using TNF-α antagonists might therefore be useful in this respect [62]. Our results are therefore in agreement with previous studies demonstrating neuroprotection and memory improvement in various animal models of neurodegenerative disorders treated with GLP-1 agonists [19, 23]. LIR effects on the mTOR pathway and neurodegeneration were only studied in the icvSTZ group in order to avoid the influence of diabetes in the evaluation of LIR effects on the brain.

Besides LIR effects on cognitive function, we also studied its effects on both depressive- and anxious-like behavior. According to previous data [63] our results confirmed that STZ, both i.p. and i.c.v., was only able to increase the IT in the FST, suggesting a *pro*-depressant effect, while, in contrast to evidence that suggests anxiety-like behavior induced by STZ [64], no effects were observed on anxiety in the two models considered (*i.e.* OF and EPM). In these latter tests, LIR showed consistent anxiolytic effects, which has never been described before, this effect was significant for all groups and therefore can be considered a non-specific effect under diseased conditions, as it is for the prevention of cognitive decline. Indeed, this effect needs to be further studied in order to confirm this action and to better characterize the

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mechanism underlying it. Our experiments exclude the possibility that the observed effects depend on possible negative LIR effects on locomotor activity. However, this is in contrast with recent data showing an anxiogenic effect of LIR (200 µg/kg, i.p.), even if substantial differences in the dose, route of administration and treatment duration used (200 µg/kg i.p., administration 1 h before the EPM and 30 minutes before the OF) in comparison to our protocol may justify different results [45].

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On the other hand, LIR treatment significantly increased IT in the FST (pro-depressant effects) in the control group only but not in the other groups, where an increase was due to STZ treatment. Based on the present data, we cannot completely exclude the possibility that LIR *per se*, has *pro*-depressant effects; indeed, the observed increase in IT is more convincingly related to an effect of LIR on animal endurance rather than effort as confirmed by our rotarod results. Furthermore, it is in contrast with previous data showing that GLP-1 and exendin-4 stimulate release of serotonin from rat hypothalamus synaptosomes *in vitro* [65] and long-term administration of exendin-4 had an *anti*depressant-like effect [66]; while a recent study demonstrated that acutely administered LIR at doses of 100 µg/kg and 200 µg/kg i.p. has no effects on IT in the FST [45]. Finally, if LIR had a real pro-depressant effect this should have been observed in all groups as in the case of its anxiolytic effects. Instead, the decreased endurance might be due to a direct negative effect of LIR on muscles, and this point deserves further appropriate studies, which could not be observed in the other groups in the FST considering that IT was already high and therefore rats could not worsen according to the lack of possible accumulating fatigue during the test. The action of GLP-1 in muscles is likely exerted through a signaling pathway different from the pancreatic effects [67].

In this light, the effects of LIR on the mTOR pathway also go against the possibility of real pro-depressant effects, considering that the function of this pathway is disrupted and not maintained in major depression [68].

5. Conclusion

Our results support the important possibility that LIR and maybe other GLP-1 agonists might be able to prevent the development of long-term cognitive decline in patients with T2DM and this effect might be accompanied by beneficial anxiolytic effects in addition to its favorable effects on peripheral blood glucose levels. However, of relevance might also be the negative effect of LIR on endurance. Indeed, the possibility to prevent cognitive decline in diabetic patients with the same drug used for the treatment of diabetes would highly impact on clinical practice. Our data therefore warrant further research both pre-clinically for the identification of the exact central mechanism of action of LIR but also clinically to confirm these effects in patients with chronic diabetes.

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Conflict of Interests

There are no conflicts of interest to be disclosed.

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Figure legends

Figure 1. Experimental protocol. Graph branches specify the experimental sequence followed and the number of rats used in every test. CTRL, controls; LIR, Liraglutide; STZ, streptozotocin; i.p., intraperitoneal administration; i.c.v., intracerebroventricular administration; s.c. ,subcutaneously; FST, forced swimming test; EPM, elevated plus maze; OF, open field test; MWM, morris water maze test; PA, passive avoidance.

Figure 2. (A) Body weight (grams) and (B) blood glucose levels (mg/dl) were monitored in different rat groups during 6 weeks of LIR treatment (300 µg/kgper day). LIR reduced blood glucose levels only in diabetic rats while all LIR treated rats showed a significant slowing in growth with decreased body weight in comparison to their respective control groups. Values are means ± S.E.M.; data marked with * are significantly different (P<0.05) from control untreated animals in the same week. The arrow indicates the start of LIR treatment.CTRL-vehicle = control rats; CTRL-LIR = liraglutide (alone)-treated rats; ipSTZ-vehicle = intraperitoneally streptozotocin-administered rats; ipSTZ-LIR = intraperitoneally streptozotocin-administered rats treated with liraglutide; icvSTZ-vehicle = intracerebroventricular streptozotocin-administered rats; icvSTZ-LIR = intracerebroventricular streptozotocin-administered rats treated with liraglutide.

Figure 3. Effects of LIR on: (A) learning curve (latency time to reach platform) over 4 consecutive days in the Morris water maze test; (B) time spent in the target quadrant during the probe test on the fifth day; (C) performance in the passive avoidance. LIR improved learning and memory parameters in both models only in STZ-treated rats and not in controls. Values are means ± S.E.M.; data marked with * are significantly different (P<0.05) from

Eliminato: 1
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Eliminato: -1
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control untreated animals. CTRL-vehicle = control rats; CTRL-LIR = liraglutide-treated rats; ipSTZ-vehicle = intraperitoneally streptozotocin-administered rats; ipSTZ-LIR = intraperitoneally streptozotocin-administered rats treated with liraglutide; icvSTZ-vehicle = intracerebroventricular streptozotocin-administered rats; icvSTZ-LIR = intracerebroventricular streptozotocin-administered rats treated with liraglutide; IL=initial latency; STL=step-through latency.

Figure 4. LIR effects on anxiety- and depressive-like behavior. (A) LIR increased time spent in the center (seconds) in the open-field test in all groups; (B) LIR increased time spent in the open arms in the elevated plus maze test in all groups; (C) LIR increased immobility time (IT) in CTRL group only in the forced swimming test; (D) LIR reduced the time spent on the rod in the rotarod test. Values are means ± S.E.M.; data marked with * are significantly different ($P< 0.05$) from control untreated animals. CTRL-vehicle = control rats; CTRL-LIR = liraglutide treated rats; ipSTZ-vehicle = intraperitoneally streptozotocin-administered rats; ipSTZ-LIR = intraperitoneally administered streptozotocin rats treated with liraglutide; icvSTZ-vehicle = intracerebroventricular streptozotocin-administered rats; icvSTZ-LIR = intracerebroventricular administered-streptozotocin rats treated with liraglutide.

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Figure 5. Histological analysis of the effects of LIR on neuronal injury induced by i.c.v. injection of streptozotocin (STZ) in rats. Cresyl violet staining was performed on sections from the hippocampal CA1 region. Magnification 20 x. (A) Control vehicle-injected rat shows large conical shaped pyramidal neurons, with well demarcated amphophillic cytoplasm and round vesicular nuclei with prominent nucleoli, and no signs of degeneration; (B) CA1 region of rat following injury induced by i.c.v. injection of STZ, shows distinct neuronal alterations characterized by pronounced shrinkage of the neuronal bodies with loss of nuclei

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and pyknotic pyramidal cells; (C) CA1 region of rat following injury induced by i.c.v. injection of STZ and subsequent treatment with LIR shows some protection against STZ-induced cell loss and pyknotic cells but some degenerating cells with changed morphology are still observed. (D) LIR treatment significantly prevented neuronal cell loss in the hippocampal CA1 region. Values are means ± S.E.M.; data marked with * are significantly different ($P< 0.05$) from control untreated animals; # significantly different ($P< 0.05$) from icvSTZ-vehicle group. icvSTZ-vehicle = intracerebroventricular streptozotocin-administered rats; icvSTZ-LIR = intracerebroventricular streptozotocin-administered rats treated with liraglutide.

Figure 6. Effect of i.c.v. STZ with or without LIR treatment on the expression of mTOR signaling targets P70S6K (A), AKT (B) and AMPK (C) in the rat brain. The columns represent mean relative protein levels normalized to control. Loading was normalized according to the total protein levels. I.c.v. infusion of STZ induced hippocampal neurodegeneration accompanied by a significantly ($P<0.05$) decreased phosphorylation of p70S6K, AKT and AMPK relative to control. Administration of LIR after STZ reduced hippocampal neuronal death and prevented the decrease in p70S6K and AKT phosphorylation (*i.e.* both maintained at control level). Note in contrast, that AMPK phosphorylation was *increased* relative to control, by LIR treatment. (A) Significantly different *$P<0.001$ *vs*. icvSTZ-vehicle, **$P<0.003$ *vs.* CTRL-vehicle; (B) *$P<0.01$ *vs*. CTRL-vehicle, **$P<0.05$ *vs.* icvSTZ-vehicle; (C) *$P<0.003$ *vs*. CTRL-vehicle; **$P<0.001$ *vs*. icvSTZ-vehicle; & $P<0.002$ *vs*. CTRL-vehicle. icvSTZ-vehicle = intracerebroventricular streptozotocin-administered rats; icvSTZ-LIR = intracerebroventricular streptozotocin-administered rats treated with liraglutide.

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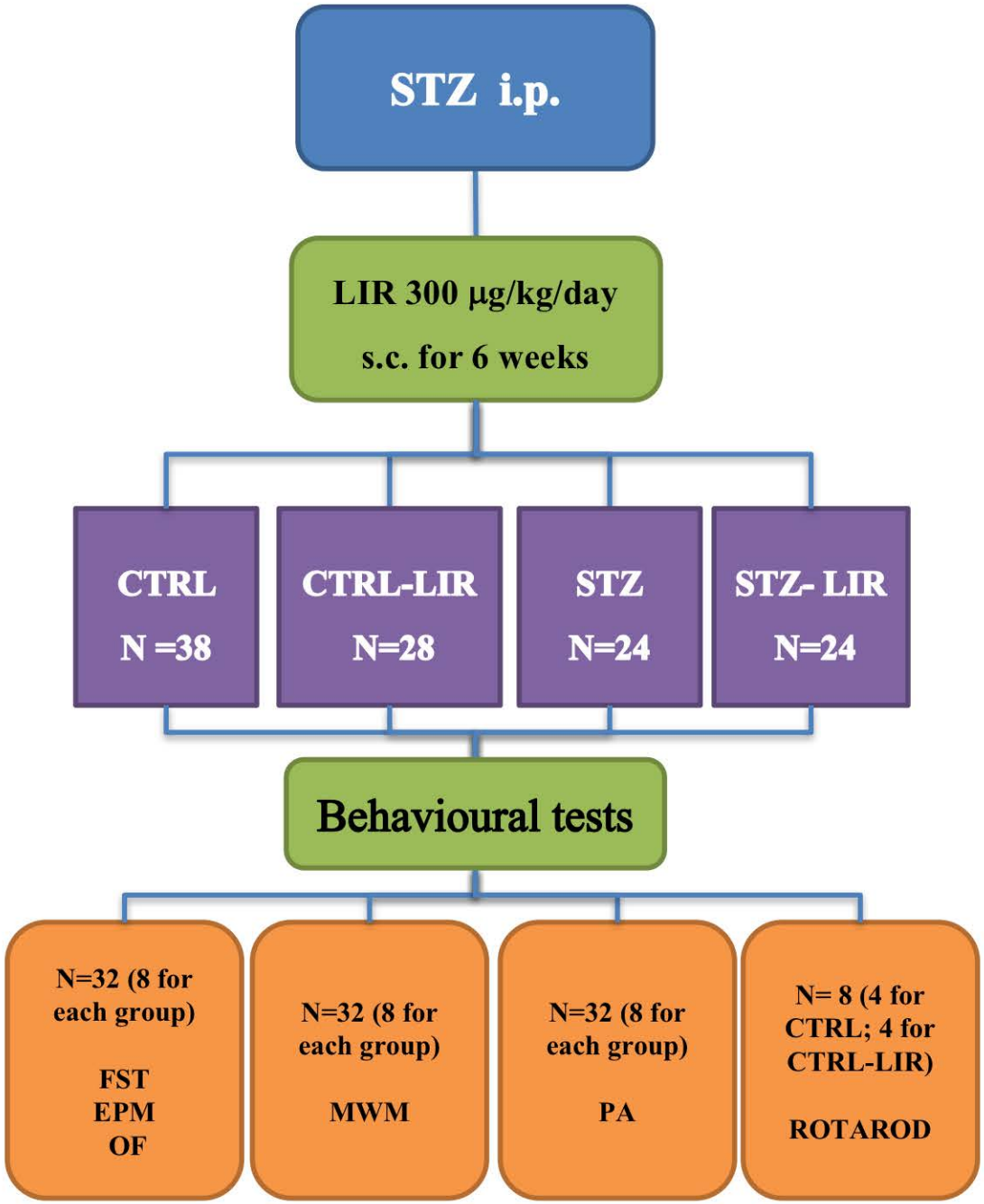
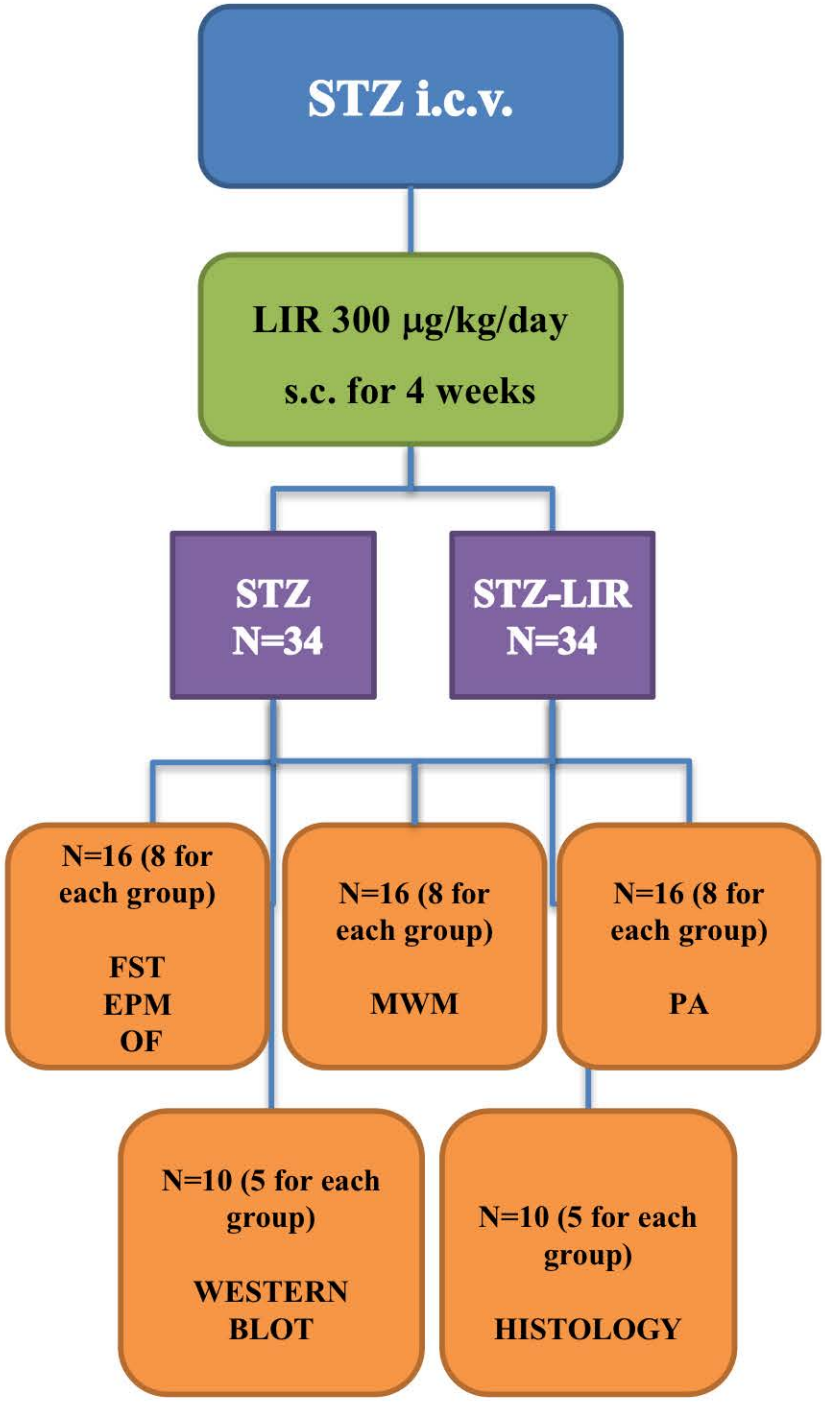
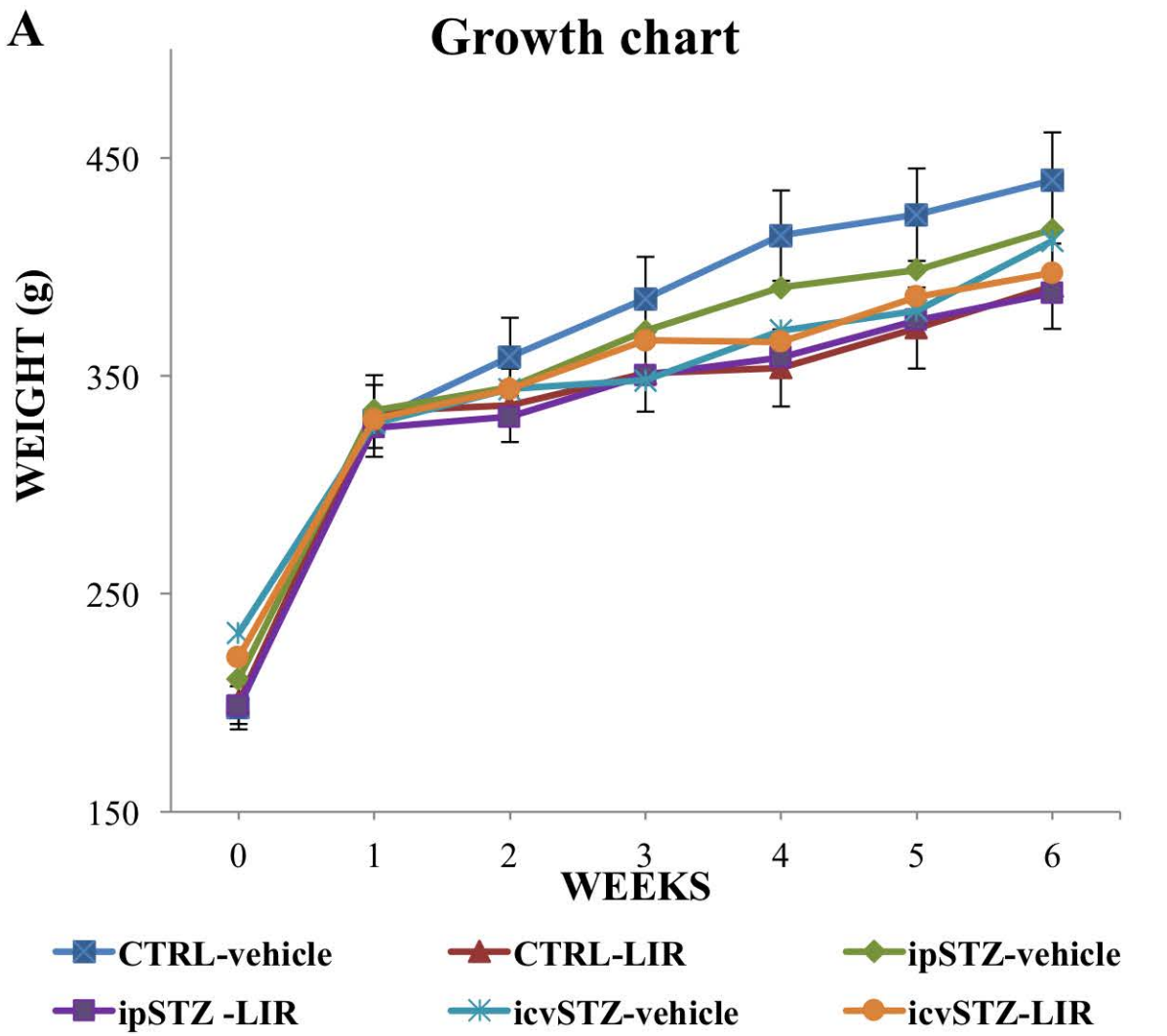
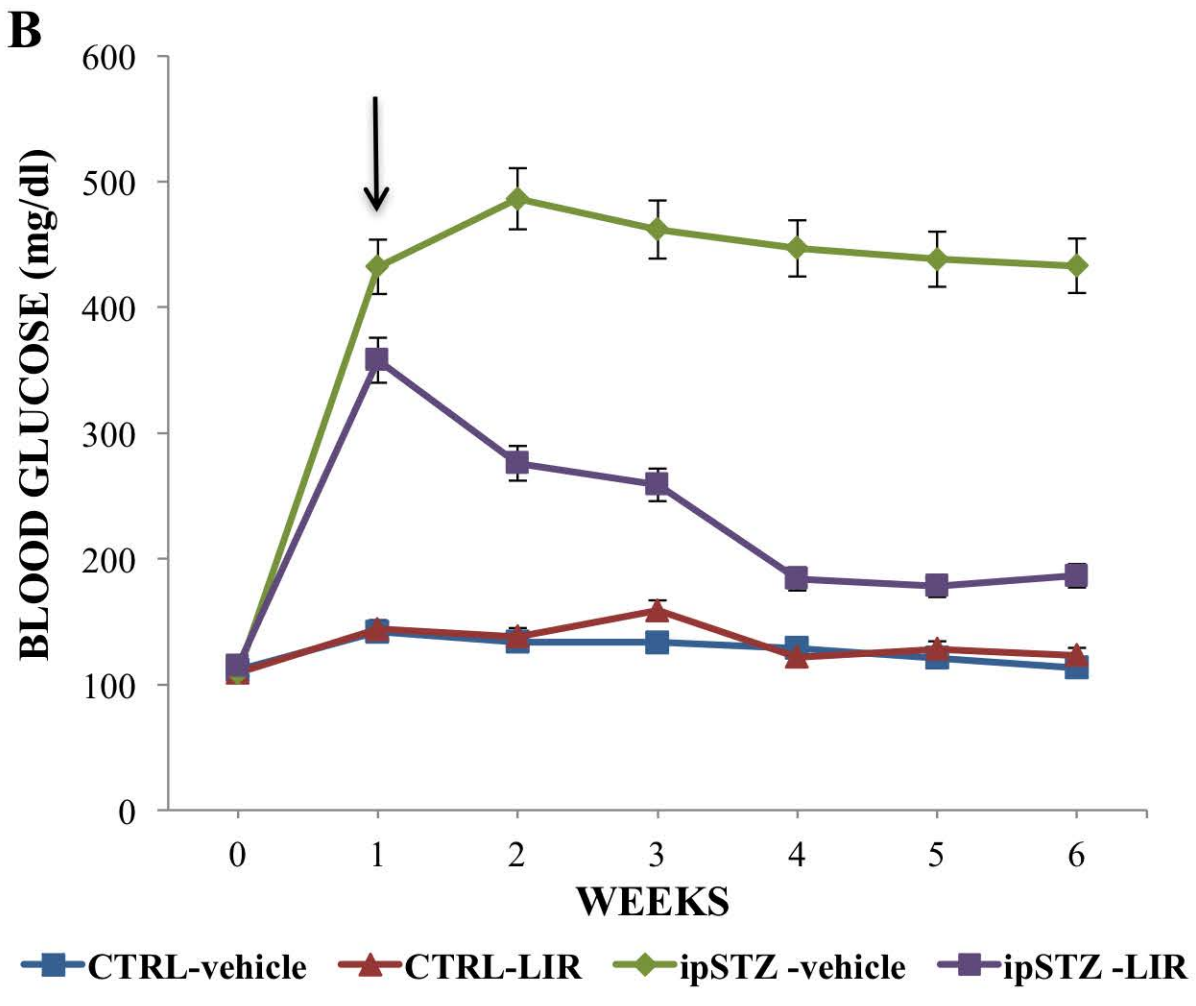
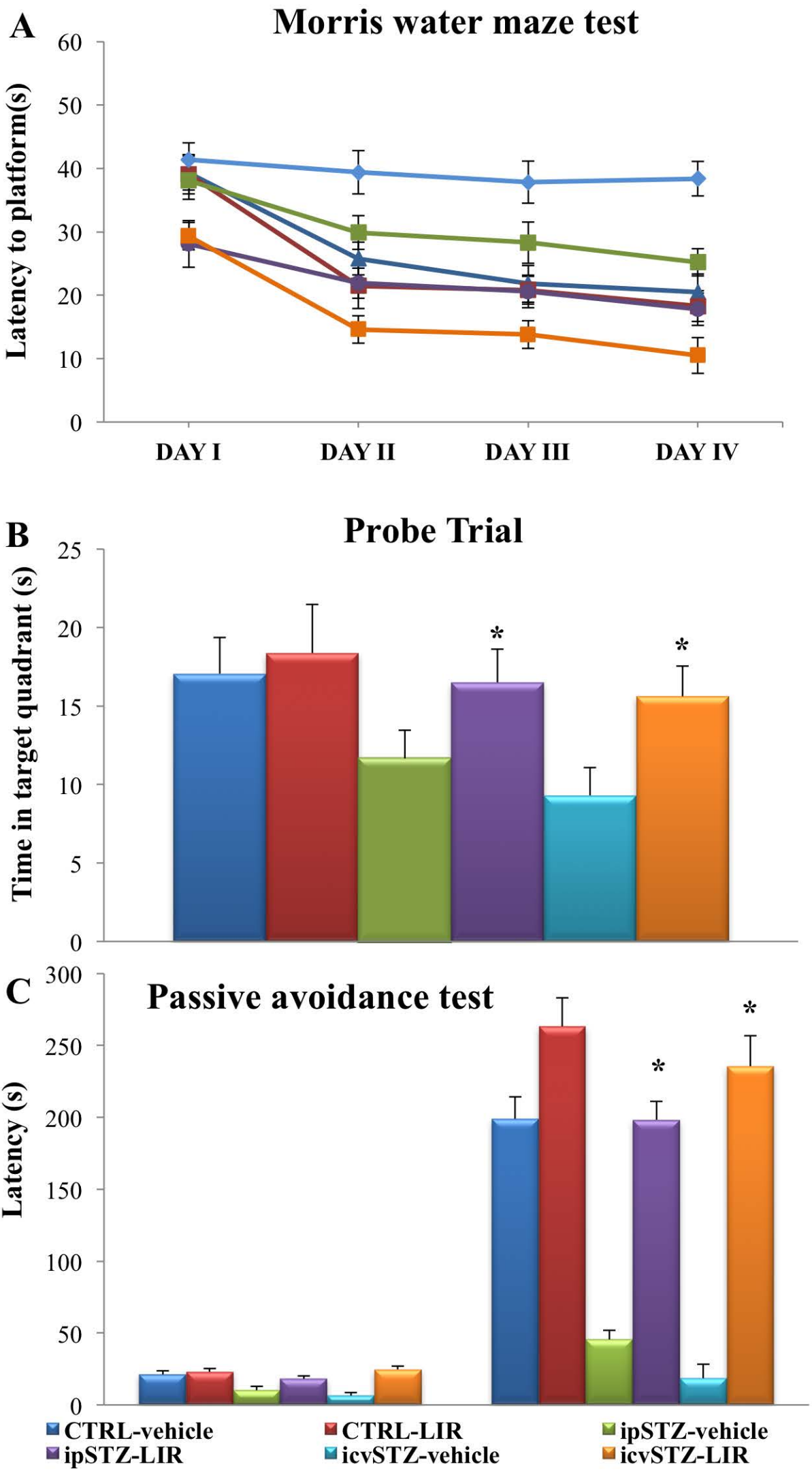
Experimental Protocol

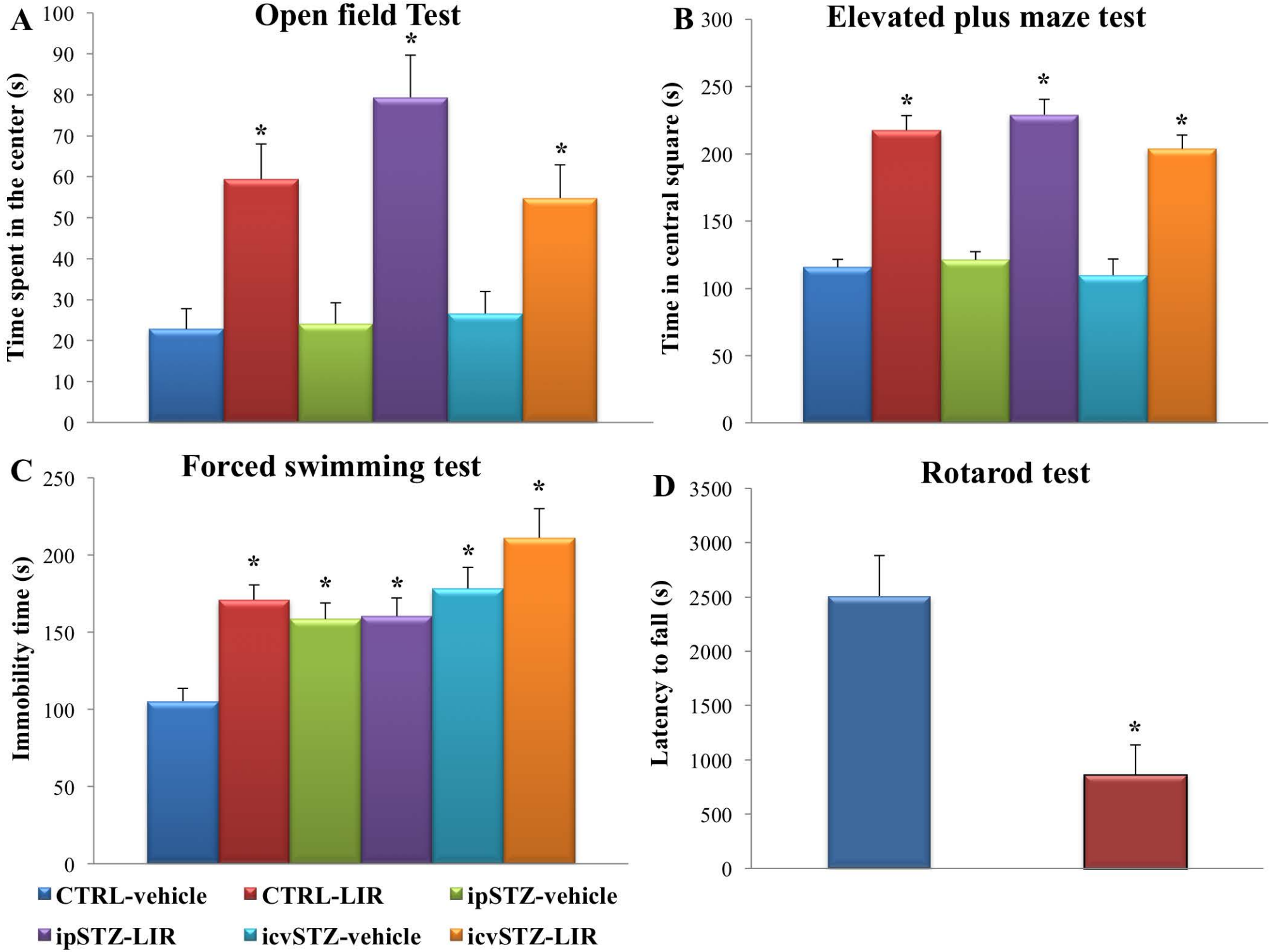




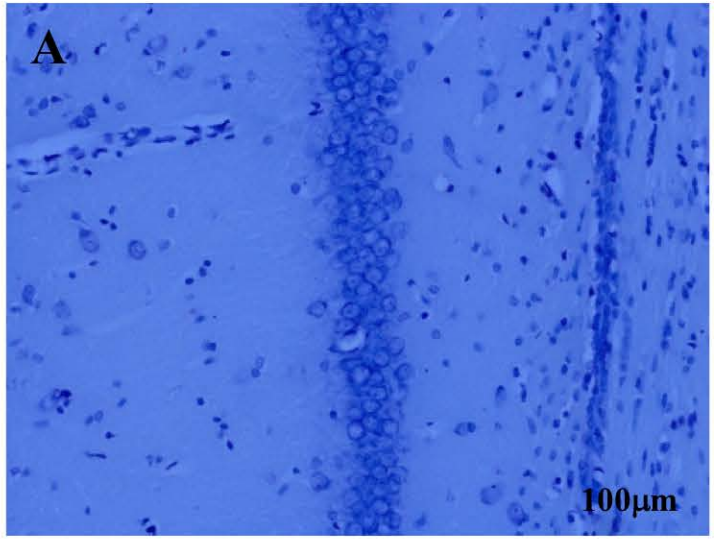

A
Growth chart
WEIGHT (g)
450
350
250
150
0
1
2
3
4
5
6
WEEKS
CTRL-vehicle
CTRL-LIR
ipSTZ-vehicle
ipSTZ -LIR
icvSTZ-vehicle
icvSTZ-LIR


B
BLOOD GLUCOSE (mg/dl)
600
500
400
300
200
100
0
0
1
2
3
4
5
6
WEEKS
CTRL-vehicle
CTRL-LIR
ipSTZ -vehicle
ipSTZ -LIR

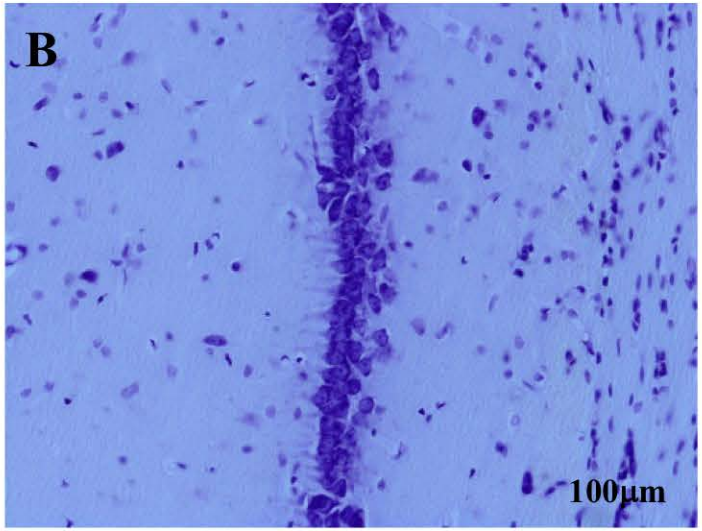

A
Morris water maze test
Latency to platform(s)
60
50
40
30
20
10
0
DAY I
DAY II
DAY III
DAY IV
B
Probe Trial
Time in target quadrant (s)
25
20
15
10
5
0
*
*
C
Passive avoidance test
Latency (s)
300
250
200
150
100
50
0
*
*
CTRL-vehicle
CTRL-LIR
ipSTZ-vehicle
ipSTZ-LIR
icvSTZ-vehicle
icvSTZ-LIR


A
Open field Test
Time spent in the center (s)
100
90
80
70
60
50
40
30
20
10
0
B
Elevated plus maze test
Time in central square (s)
300
250
200
150
100
50
0
C
Forced swimming test
Immobility time (s)
250
200
150
100
50
0
D
Rotarod test
Latency to fall (s)
3500
3000
2500
2000
1500
1000
500
0
CTRL-vehicle
CTRL-LIR
ipSTZ-vehicle
ipSTZ-LIR
icvSTZ-vehicle
icvSTZ-LIR

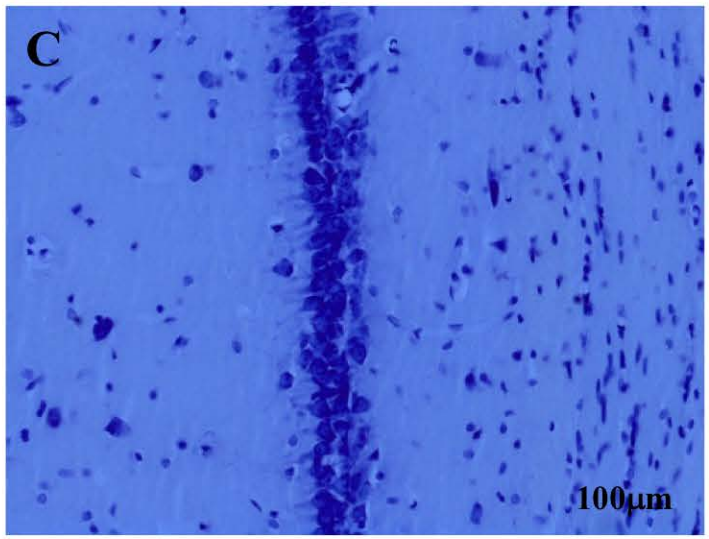
Hippocampal CA1 region Histology



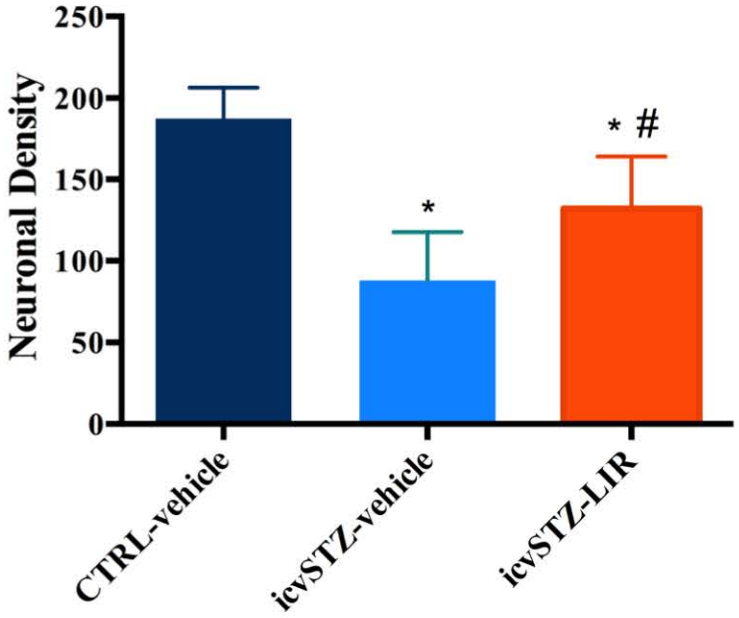
CTRL-vehicle

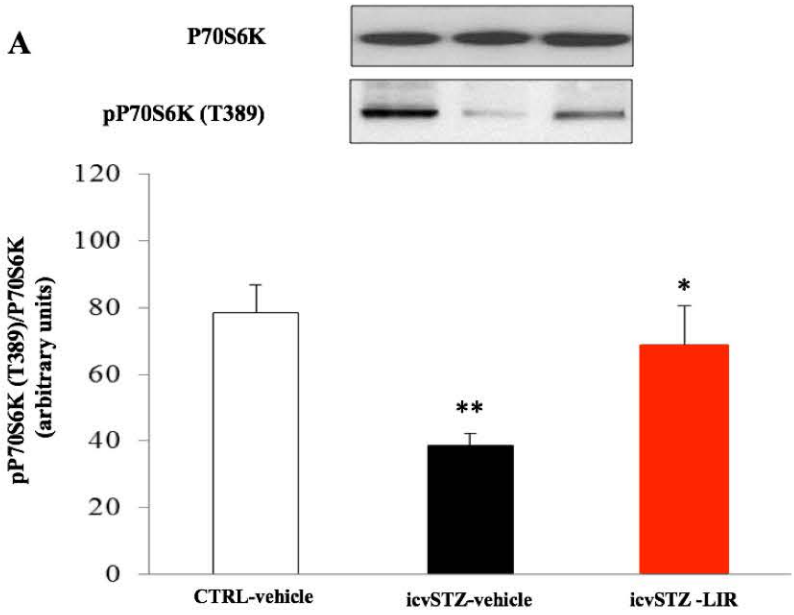


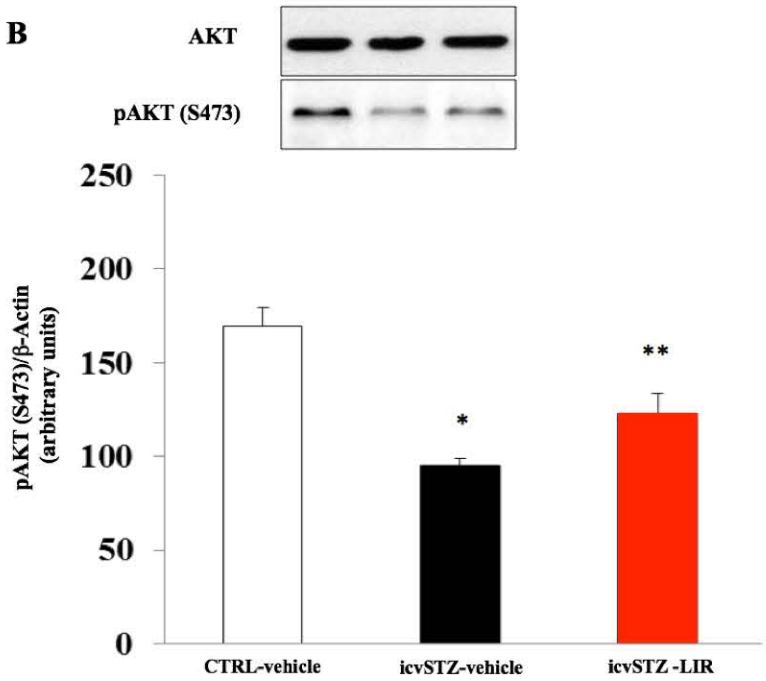
icvSTZ-vehicle

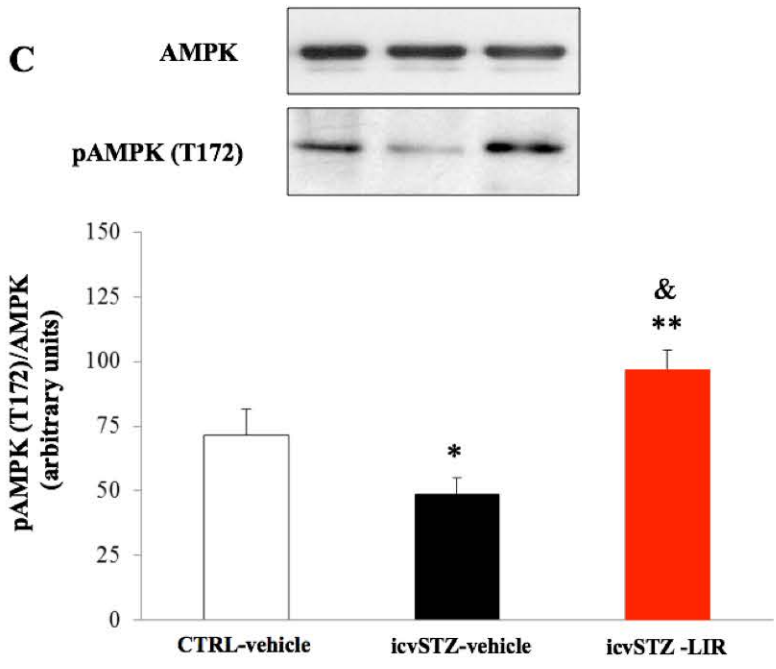


icvSTZ-LIR




A
P70S6K
pP70S6K (T389)
pP70S6K (T389)/P70S6K
(arbitrary units)
120
100
80
60
40
20
0
**
*
CTRL-vehicle
icvSTZ-vehicle
icvSTZ -LIR


B
AKT
pAKT (S473)
pAKT (S473)/β-Actin
(arbitrary units)
250
200
150
100
50
0
*
**
CTRL-vehicle
icvSTZ-vehicle
icvSTZ -LIR


C
AMPK
pAMPK (T172)
pAMPK (T172)/AMPK
(arbitrary units)
150
125
100
75
50
25
0
*
&
**
CTRL-vehicle
icvSTZ-vehicle
icvSTZ -LIR