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Glucagon-like peptide-1 mediates effects of oral galactose in streptozotocin-induced rat model of sporadic Alzheimer's disease

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This paper is dedicated to the memory of Professor Werner Reutter, whose scientific curiosity and expertise in galactose research initiated our collaboration on the preliminary research of the beneficial effects of oral galactose treatment.

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ABSTRACT

Insulin resistance and metabolic dysfunction in the brain are considered to be the pathophysiological core of sporadic Alzheimer's disease (sAD). In line with that fact, nutrients that could have therapeutic effects at this level have been investigated as possible targets in AD therapy. Galactose, an epimer of glucose, may serve as an alternative source of energy, and given orally may stimulate secretion of the incretin hormone glucagon-like peptide-1 (GLP-1). Our preliminary research indicated that oral galactose might prevent development of memory impairment in a rat model of sAD generated by intracerebroventricular administration of streptozotocin (STZ-icv). Here, we explored whether chronic oral galactose treatment could have beneficial effects on cognitive deficits already manifested at the time of initiation of galactose treatment in adult STZ-icv rats (treatment initiated 1 month after STZ-icv injection). The results clearly show that a 2-month exposure to oral galactose (200 mg/kg/day administered in a drink ad libitum) normalises impaired learning and memory functions. Memory improvement was accompanied by an improvement in brain glucose hypometabolism measured by ^{18}fluorodeoxyglucose-positron emission tomography neuroimaging and by increments in active GLP-1 plasma levels as well as by an increased expression of GLP-1 receptors in the hippocampus and hypothalamus. Our findings provide strong evidence of beneficial effects of oral galactose treatment in the STZ-icv rat model of sAD and present possible underlying mechanisms including both direct effects of galactose within the brain and indirect GLP-1-induced neuroprotective effects that might open a new, dietary-based strategy in sAD treatment.

1. Introduction

Sporadic Alzheimer disease (sAD) is the most frequent human neurodegenerative disorder associated with progressive loss of cognitive function (Reitz and Mayeux, 2014). Recent studies proposed sAD to be a central insulin-resistant state (De Felice et al., 2014; De La Monte and Tong, 2014; Chen et al., 2014a; Osmanovic Barilar et al., 2015) accompanied by brain glucose hypometabolism already seen in the early stages of the disease (Cholerton et al., 2013), (Langbaum et al., 2013), (Baker et al., 2011), (Burns et al., 2013). Using ^{18}fluorodeoxyglucose (FDG) positron emission tomography (PET) measurements of regional cerebral metabolic rates for glucose (CMRgl), a significant relationship was

Abbreviations: APP, amyloid precursor protein; ATP, adenosine triphosphate; Aβ1-42, amyloid β 1-42; CMRgl, cerebral metabolic rate for glucose; CNS, central nervous system; CSF, cerebrospinal fluid; CTR, control; CTX, cortex; DPP-IV, dipeptidyl peptidase IV; FDG, fluorodeoxyglucose; GIP, gastric inhibitory polypeptide; GLP-1, glucagon like peptide-1; GLP-1R, glucagon like peptide-1 receptor; GLUT, glucose transporter; HPC, hippocampus; icv, intracerebroventricular; ip, intraperitoneal; MWM, Morris Water Maze Test; PA, Passive Avoidance Test; PET, positron emission tomography; po, per oral; PS1, presenilin 1; sAD, sporadic Alzheimer's disease; SGLT, sodium glucose cotransporter; STZ, streptozotocin.

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detected between higher disease severity and lower CMRgl in regions associated with learning and memory (Langbaum et al., 2009). Insulin resistance has been considered as one of the factors that could be involved in the reduction of CMRgl (Meguro et al., 1999; Magistretti and Pellerin, 1996; Salkovic-Petrisic and Hoyer, 2007). The causes of brain glucose hypometabolism in sAD as well as the development of brain insulin resistance are hard to investigate in humans during life and particularly in the preclinical phase of sAD, which is why representative animal models are needed.

Modelling of brain insulin resistance is performed by intracerebroventricular (icv) administration of streptozotocin (STZ; 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) in rat and mouse (Agrawal et al., 2011; Chen et al., 2014b; Yeo et al., 2015; Lee et al., 2014) as well as in monkey (Blondel and Portha, 1989; Szkudelski, 2001). STZ is a substance selectively toxic for insulin-secreting/producing cells (Blondel and Portha, 1989; Szkudelski, 2001) and its icv application also induces cholinergic deficits (Blokland and Jolles, 1993), oxidative stress (Sharma and Gupta, 2001), neuroinflammation (Prickaerts et al., 1999; Knezovic et al., 2017) and a reduction in cortical and hippocampal glucose/energy metabolism (Blondel and Portha, 1989; Lannert and Hoyer, 1998; Pathan et al., 2006; Plaschke and Hoyer, 1993; Saxena et al., 2008), followed by progressive deficits in learning and memory in rats and mice (Salkovic-Petrisic and Hoyer, 2007; Saxena et al., 2008). Other sAD-like features in the STZ-icv model are synaptic loss and neuronal damage as well as pathological amyloid β 1-42 (Aβ1-42) and tau accumulation (Salkovic-Petrisic et al., 2006, 2011; Knezovic et al., 2015). Based on all these findings, the STZ-icv model is considered to be a representative non-transgenic model for sAD (Osmanovic Barilar et al., 2015; Agrawal et al., 2011; Lee et al., 2014; Salkovic-Petrisic et al., 2013).

The STZ-icv model has also been widely used as a platform for testing the therapeutic potential of different compounds (Salkovic-Petrisic et al., 2013). There is an urgent need for novel anti-AD therapeutic strategies that can prevent, slow down or stop the progression of neurodegeneration and dementia (Mangialasche et al., 2010; Misra and Medhi, 2013). Our preliminary research demonstrated that a 1-month daily treatment with oral galactose (200 mg/kg) that was initiated immediately after the STZ-icv injection prevented STZ-icv-induced learning and memory deficits (Salkovic-Petrisic et al., 2014).

The exact mechanism by which orally given galactose affects the cognition is still unknown. As a C-4 epimer of glucose, galactose could serve as an alternative source of energy to the brain following its conversion to glucose in the Leloir pathway (Frey, 1996; Ross et al., 2004). Since galactose enters the cell via insulin-independent glucose transporter GLUT3 (Gould et al., 1991; Seatter et al., 1997), in the condition of glucose hypometabolism and insulin resistance in the brain such a galactose-induced and insulin-independent increment in intracellular glucose level might compensate for the reduced glucose and energy metabolism in the brain. To test this hypothesis, in this study we measured glucose metabolism in the brain by FDG-PET in a STZ-icv rat model following chronic oral galactose treatment.

Another factor that could account for the beneficial effects of oral galactose is the stimulation of glucagon-like peptide-1 (GLP-1) secretion (Baggio and Drucker, 2007) induced by oral (but not parenteral) galactose administration. GLP-1 is an incretin hormone secreted from the intestinal L-cells in response to food, which increases glucose-stimulated pancreatic insulin release (Perry and Greig, 2005), (Sandoval and D'Alessio, 2015). GLP-1 also acts as a neuropeptide in the brain where it has a neuroprotective and neurotrophic effect (Duarte et al., 2013; Salcedo et al., 2012) and can promote neurogenesis (Duarte et al., 2013; Hunter and Holscher, 2012). Treatment with GLP-1 analogues was found to significantly improve learning and memory in a STZ-icv rat model (Li et al., 2012). GLP-1 can also be produced centrally in the nucleus of the solitary tract (Sandoval and D'Alessio, 2015), from which proglucagon neurons project to the hypothalamus and hippocampus (Baggio and Drucker, 2007). GLP-1 binds to and activates its receptor (GLP-1R), the expression of which is also found in the brain, especially in the hypothalamus and hippocampus (Wei and Mojsov, 1995), (Göke et al., 1995). This study explored whether oral galactose treatment alters GLP-1 and insulin levels in the blood and cerebrospinal fluid (CSF), as well as the expression of GLP-1R in the brain of STZ-icv-treated rats. Metabolic parameters, as well as associated learning and memory functions, were measured after two months of oral galactose treatment which was initiated when cognitive deficits were already manifested in the STZ-icv rat model of sAD.

2. Material and methods

2.1. Animals

Adult (3-month old) male Wistar rats weighing 270–370 g (University of Zagreb, School of Medicine, Department of Pharmacology) were used in all experiments. The animals were kept on standardised food pellets and water ad libitum, in a room with a 12-h light/12-h dark cycle, at a constant temperature (21–23 °C) and humidity (40–70%). The animals were group-housed (2-3 per cage) after icv administration of STZ or citrate buffer (controls). During the chronic oral galactose treatment the animals were taken from the grouped cages and placed in individual cages, with a single bottle per animal, each day from 4 p.m. overnight to 8 a.m. the following day, after which they were returned to their previous grouped cages.

2.2. Materials

Streptozotocin (STZ), D-galactose, protease inhibitor cocktail, PhosStop phosphatase inhibitor tablets and DPP-IV inhibitor were acquired from Sigma-Aldrich Chemie (Munich, Germany). The glucose measuring kit (Greiner Diagnostic Glucose GOD-PAD-PAP) was acquired from Dijagnostika (Sisak, Croatia). The Amplex Red Galactose/Galactose Oxidase Kit was purchased from Invitrogen (Eugene, OR, USA). The ELISA Kits for rat/mouse insulin, active GLP-1 and total GLP-1 were acquired from Merck Millipore (Billerica, USA). The following antibodies were used: anti-GLP-1R/Santa Cruz Biotechnology (Santa Cruz, CA, USA), monoclonal anti-β-actin/Sigma-Aldrich (St. Louis, Missouri, USA) as well as anti-mouse IgG horseradish peroxidase-linked antibody and anti-rabbit IgG horseradish peroxidase-linked antibody acquired from CellSignaling (Beverly, MA, USA). The chemiluminescent Western blot detection kit (SuperSignal West Femto Chemiluminescent Substrate) was acquired from Thermo Scientific (Rockford, IL, USA). The Anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 555 was acquired from Thermo Fisher Scientific (Waltham, MA, USA) and donkey serum from Sigma-Aldrich Chemie (Munich, Germany). Colourimetric and fluorimetric measurements were performed on an Infinite F200 PRO multimodal microplate reader (Tecan, Switzerland).

2.3. Drug treatments

Streptozotocin (STZ) treatment. The rats were subjected to general anaesthesia (ketamine 50 mg/kg/xylazine 5 mg/kg ip) and given STZ bilaterally into each lateral ventricle (2 μL/ventricle) in a total dose of 3 mg/kg (dissolved in 0.05 M citrate buffer, pH 4.5, split

in two doses on day 1 and 3), according to the procedure first described by Noble et al. (1967) and used afterwards in our experiments (Osmanovic Barilar et al., 2015; Grünblatt et al., 2007; Knezovic et al., 2015; Salkovic-Petrisic et al., 2015). The control animals received an equal volume of vehicle icv (0.05 M citrate buffer, pH 4.5) by the same procedure.

Galactose treatment. Single oral doses (200 mg/kg dissolved in 1 mL of water/6%/) given via gastric tube and single intraperitoneal (ip) doses (200 mg/kg dissolved in 1 mL saline/6%/) were both administered at the required concentrations adjusted according to the rats' body weight in a volume of 1 mL per rat, in order to explore the effect of single galactose dose on GLP-1, insulin, glucose and galactose levels in the plasma and cerebrospinal fluid (CSF) of non-anaesthetised animals. Two-month oral administration of galactose (200 mg/kg/day) dissolved in tap water was given as a drink ad libitum (final daily administration of 20 mL galactose solution per rat placed alone in a cage, corresponding to 0.3% galactose solution; the required concentration was adjusted to the rat body weight, measured on a weekly basis). The respective controls (placed in individual cages from 4 p.m. overnight to 8 a.m. the following day) received equal amounts of tap water for drinking in similar bottles. A daily water intake of 20 mL per rat was chosen based on previous observations (Salkovic-Petrisic et al., 2014), and a titration period during the first 5 days when the volumes of 10–25 mL of galactose solution were put in the bottle and the retained amount checked the following morning. After smaller volumes of galactose solution the animals appeared thirsty on the following morning, whereas with large volumes small amounts remained in the bottle, which were then given by oral gastric tube to assure that all animals received the desired daily galactose dose. The volume of 20 mL per rat was found to be optimal, with no remnants of liquid in the bottles and no thirst in the animals. After checking that no galactose solution was retained in the bottles in the morning (solution remnants were not found with the volume of 20 mL), the animals were placed back and kept group-housed (2-3 per cage), supplied with tap water bottles until 4 p.m. in the afternoon.

2.4. Experimental design

1) Assessment and comparison of the effects of a single galactose dose given orally and peritoneally in STZ-icv pretreated rats (1 month before galactose load). The animals were divided into 4 groups (10 animals per group): CTR, control group (vehicle-icv treated); STZ, STZ-icv group; STZ+200 po GAL, STZ-icv group treated with 1 × 200 mg/kg galactose po (gastric tube); STZ+200 ip GAL, STZ-icv group treated with 1 × 200 mg/kg galactose ip. A time-point of 15 min after galactose load was chosen for blood/CSF measurements based on literature data of rapid conversion of galactose to glucose in the blood measured by (C14)-detection 15 min after 14C-galactose-oral ingestion in rats (Kliegman and Morton, 1988). The rats were anaesthetised using thiopental 50 mg/kg/diazepam 5 mg/kg ip; blood samples were taken from the retro-orbital sinus and CSF samples from the cistern magna. DPP-IV inhibitor (1:100) was added immediately to each retrieved sample and the blood was centrifuged for 10 min at 3600 rpm. Afterwards, the animals were decapitated, the brains quickly removed, and the hippocampus dissected out, frozen in liquid nitrogen, and stored at −80 °C. Cognitive deficits in STZ-icv rats were confirmed before single galactose load by Passive Avoidance (PA) test.

2) Determination of possible therapeutic effects of 2-month oral galactose treatment (drink) on cognitive deficits and brain glucose hypometabolism in rats pretreated with STZ-icv 1 month before galactose treatment initiation. The animals were divided into 4 groups (10 animals per group): CTR, control group (vehicle-icv treated); STZ, STZ-icv group; CTR+GAL, control group treated daily with galactose 200 mg/kg po; STZ+GAL, STZ-icv group treated daily with galactose 200 mg/kg po. Cognitive performance was assessed by the Morris Water Maze Swimming Test (MWM) before the initiation of galactose treatment to confirm the presence of cognitive deficits in STZ-icv rats and to equally distribute the animals based on their cognitive performance. After the completion of galactose treatment, cognitive performance was assessed by the MWM and PA tests. Following cognitive testing and FDG-PET scan and blood/CSF sample retrieval, 6 animals per group were euthanised in general anaesthesia (thiopental 50 mg/kg/diazepam 5 mg/kg ip) followed by decapitation; the brains were quickly removed and the hippocampus dissected out, frozen in liquid nitrogen and stored at −80 °C. The remaining 4 animals per group were also subjected to deep anaesthesia (thiopental 50 mg/kg/diazepam 5 mg/kg ip) and then transcardially perfused with 250 mL of saline followed by 250 mL of 4% paraformaldehyde, pH 7.4. The brains were quickly removed and cryoprotected with sucrose (through series of sucrose 15% and 30%) and then stored at −80 °C for further histology analysis.

The galactose dose of 200 mg/kg was chosen based on the results of our preliminary research demonstrating that this dose successfully prevented the development of cognitive deficits in the STZ-icv rat model (Salkovic-Petrisic et al., 2014).

2.5. Cognitive testing

Morris Water Maze Swimming Test (MWM). The procedure of MWM (Vorhees and Williams, 2006; Morris, 1981) consisted of 5-day learning and memory training trials, and a probe trial on day 6, all performed in a 180- cm-diameter round pool, 60 cm deep, with water temperature set at 25±1 °C. On days 1–5, the rats were trained to escape the water by finding a hidden glass platform (15-cm diameter) submerged about 2 cm below the water surface and placed at the edge of the NorthWest/NW quadrant. Staying on the platform in order to memorise its location was allowed for 15 s. Four consecutive trials were performed per day, each from a different starting position (SouthWest/SW, South/S, East/E and NorthEast/NE), separated by a 30-min rest period. The rats were released into the pool facing its wall. The platform was kept at the same position during the training period, but the starting position schedule changed. The escape latency (time needed to find the platform after being released into the pool), was recorded during training trials which tested the capability of learning memory. A probe trial which tested memory retention was performed (from quadrant SouthEast/SE) with the platform removed from the pool, where the time spent in search for the platform within the NW quadrant was recorded. The cut-off time was 1 min. The rats with preserved spatial memory (controls) were supposed to remember that the platform had previously been there, and, consequently, to spend a considerable period of time swimming within the NW quadrant. The rats with damaged memory were supposed to have diminished memory of the platform being in the NW quadrant, and to spend less time in search of the platform within this quadrant, in comparison with the control rats. The number of entries into non-target zones (i.e. quadrants other than NW where the platform had been located, expressed as "number of mistakes") and velocity (Suppl 1) were recorded in the training and probe trials. The data was recorded by camera (Basler AG) and tracked and analysed using EthoVision XT video tracking software (Noldus Information Technology).

Passive avoidance test (PA). Passive avoidance behaviour was studied with a step-through type passive avoidance test (Ugo Basile, Comerio, Italy) utilising the natural preference of rats for dark environments (Walters and Abel, 1971). The PA test is a fear-motivated avoidance task where rats learn to avoid stepping

through a door to an apparently safer but previously punishment-related dark compartment. The latency to avoid crossing into the punishment compartment serves as an index of the ability to avoid, and allows memory to be evaluated. On the test day, the animals with no alterations in memory functions (vehicle-icv treated, controls) were supposed to remember receiving a foot shock after entering the dark compartment and, therefore, to stay in the light compartment longer. The animals with damaged memory were supposed to have less memory of the received foot shock in the dark compartment, and thus to spend less time in the light compartment and enter the dark area more rapidly in comparison with the control rats. The three-day PA test performance started with a habituation day to make the rats familiar with the environment (no foot shock, i.e. pre-shock latency), followed by a training day in which a foot shock (0.3–0.5 mA, depending on the rat weight, duration 2 s) was delivered, and, lastly, a testing day (without foot shock, i.e. post-shock latency). The time required to enter the dark compartment was measured with a cut-off time of 5 min. The PA test was performed at the end of the 2-month oral galactose treatment.

2.6. FDG-PET imaging

For FDG-PET imaging, the animals were anaesthetised in an induction chamber with 4% isoflurane (Forane, Abbott laboratories, UK) in 100% oxygen with a delivery rate of 0.6 l/min, and intravenously injected with 30 MBq of PET radiotracer [18F]Fluoro-2-deoxy-2-D-glucose (^{18}FDG). After 30 min of uptake time, the animals were anaesthetised in the same manner and placed into a ClearPET high-performance small animal PET scanner. The performances of the commercial ClearPET scanner were described by Roldan et al. (Sempere Roldan et al., 2007). During the 30-min scanning, anaesthesia was maintained with 2% isoflurane in 100% oxygen with a delivery rate of 0.6 l/min. The obtained PET scan images were analysed in PMOD software for biomedical analysis. For brain analysis, the template of the rat brain atlas available in PMOD software was used. For this study, nine regions (CTX frontal/orbito frontal, frontal and medial prefrontal/, CTX parietal/parietal, cingulate, motor, retrosplenial, somatosensory and visual/, CTX temporal/auditory, entorhinal and insular/, hippocampus, amygdala, hypothalamus, thalamus, brainstem and cerebellum) were selected as regions of interest and underwent further analysis. The results were given as FDG activity per tissue volume [kBq/cc]. Food and tap water/galactose solution were available to the rats until the beginning of anaesthesia and after FDG-PET imaging.

2.7. Biochemical analysis

Tissue preparation for Western blot analysis. Fresh frozen hippocampal tissue samples were thawed and homogenised with three volumes of lysis buffer containing 10 mM HEPES, 1 mM EDTA, 100 mM KCl, 1% Triton X-100, pH 7.5, and a protease inhibitor cocktail (1:100). The resulting homogenates were centrifuged at 12000 rpm (13700 × g) for 10 min at 4 °C and the supernatants frozen and stored at −80 °C. Protein concentration was measured by the Lowry protein assay (Lowry et al., 1951).

CSF sampling. Withdrawal of CSF was carried out in deeply anaesthetised animals before scarification (non-recovery procedure). The animals were subjected to general anaesthesia (thiopental 50 mg/kg/diazepam 5 mg/kg ip). The heads were flexed downwards at an angle of approximately 45°. A small-gauge needle (29 G) was inserted into the cisterna magna by using the occipital bone as a landmark, and CSF was sampled by syringe aspiration until its colour changed from transparent to reddish (approximately 200 μL per animal).

Glucose measurements. Blood/CSF glucose concentration was measured spectrophotometrically (by the method first described by Trinder (1969)) using a commercial kit. The measurement was conducted in strict compliance with the manufacturer's protocol. Absorbance was measured at 500 nm. The concentration of glucose was expressed in mmol/L (mM).

Galactose measurements. Blood/CSF galactose concentration was measured spectrophotometrically using a commercial kit. The measurement was conducted by strictly following the manufacturer's protocol. Absorbance was measured using a microplate reader at 570 nm. The concentration of galactose was expressed in mmol/L (mM).

Insulin measurements. Blood/CSF insulin levels were measured using a commercial insulin ELISA kit. The measurement was conducted by strictly adhering to the manufacturer's protocol. Absorbance was measured at 450 nm and 590 nm using a microplate reader. The concentration of insulin was expressed in ng/mL.

Total GLP-1 measurements. Blood/CSF levels of total GLP-1 (GLP1 7-36 and 9-36) were measured using a commercial ELISA kit. The measurement was conducted in strict accordance with the manufacturer's protocol. Absorbance was measured at 450 nm and 590 nm using a microplate reader. The concentration of total GLP-1 was expressed in pmol/L (pM).

Active GLP-1 measurements. Active GLP-1 (GLP-1 7-36 and 7-37) levels in blood and CSF samples were measured using a commercial ELISA kit. The measurement was conducted by strictly following the manufacturer's protocol. The plate was read on a fluorescence plate reader at an excitation/emission wavelength of 355/460 nm. The concentration of active GLP-1 was expressed in pmol/L (pM). The GLP-1 active ELISA kit quantifies active forms of GLP-1 (7-36 amide and 7-37) and does not detect other forms, while the GLP-1 total ELISA kit is intended for quantification of both active (7-36) and non-active (9-36; degraded by dipeptidyl peptidase IV) forms.

Western blot analysis (GLP-1R expression). Equal amounts of total protein in the hippocampus (35 μg per sample) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using 9% polyacrylamide gels, and transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked for 1 h at RT in 5% non-fat milk, added to low-salt washing buffer (LSWB) containing 10 mM Tris, 150 mM NaCl, pH 7.5 and 0.5% Tween 20. The blocked blots were incubated with primary anti-GLP-1R antibody (1:500) overnight at 4 °C. After incubation, the membranes were washed 3x with LSWB and incubated for 1 h at RT with secondary antibody solution (anti-rabbit IgG, 1:2000). After 3x washing in LSWB, immunoreactive signals were visualised using a chemiluminescence Western blotting detection reagent. The signals were captured and visualised with a MicroChemi video camera system (DNR Bio-Imaging Systems). The membranes were washed 3x with LSWB, blocked the same way, and incubated overnight with loading control β-actin (1:2000) at 4 °C. The membranes were then washed and incubated for 1 h at RT with a secondary antibody solution (anti-mouse IgG, 1:2000), washed in LSWB, and immunostained using a chemiluminescence reagent. The signals were captured and visualised with a MicroChemi video camera system.

Immunohistochemistry. Frozen (previously fixative-perfused) brain samples (4 per group) were cut using a cryostat (Leica) into 35-μm sections (6 per animal; AP plane: Bregma −1.20 mm to −3.12 mm) at −25 °C. The sections were washed 3x with PBS-T buffer (0.025% Triton X-100 in PBS), incubated with 200 μL of 10x normal donkey serum for 1 h at RT and incubated with primary anti-GLP-1R antibody (1:500, diluted in 1x normal donkey serum) or normal donkey serum (negative controls) overnight at 4 °C. After incubation, the sections were washed 3x with PBS-T and incubated for 2 h at RT in the dark with anti-rabbit IgG secondary antibody, Alexa Fluor 555. Finally, the sections were washed 3x with PBS-T

and mounted on slides, viewed and analysed using an Olympus BX51 microscope and CellSense Dimension software.

2.8. Ethics

The animal procedures were in compliance with current institutional (University of Zagreb School of Medicine), national (Animal Protection Act, NN135/2006; NN 47/2011) and international (Directive 2010/63/EU) guidelines on the use of experimental animals. The experiments were approved by the national regulatory body, the Croatian Ministry of Agriculture (licence number 525-10/0255-15-5).

2.9. Statistical analysis

Data were expressed as mean ± SEM with the significance of between-group differences in all cognitive and biochemical analyses tested by Kruskall-Wallis one-way ANOVA analysis of variance, followed by Mann-Whitney *U* test, with $p < 0.05$ considered statistically significant using GraphPad Prism 5 statistical software. The Morris Water Maze test results (learning trials) were analysed by Two-way ANOVA for repeated-measures with Bonferroni post-hoc test on ln-transformed data to meet the condition of normality.

3. Theory

The protective role of diet has gained considerable attention in the research of novel AD therapeutic strategies, but has been generally focused on diets rich in antioxidants and polyunsaturated fatty acids (Barnard et al., 2014; Cooper, 2014). This study combines two current trends, a nutrient as a possible therapeutic agent and brain glucose hypometabolism as the therapeutic target, both explored here following the administration of a single agent which might have direct (glucose/energy replenishing) and indirect (neuroprotective factor stimulating) effects in the STZ-icv rat model of sAD. The hypothesis proposed here complies with the current theories that multimodal compounds/drugs which combine more than one mechanism of action and/or have more than one target might be more efficient than single-target drugs in AD treatment (Mangialasche et al., 2010; Weinreb et al., 2016). It is important to mention that oral galactose might have additional effects, like stimulation of the secretion of another incretin, glucose-dependent insulinotropic polypeptide (GIP), in the gut (Flatt et al., 1989). GIP has properties similar to GLP-1 in protecting neurons from toxic effects, and reversing the detrimental effects that beta-amyloid fragments have on synaptic plasticity (Holscher, 2010). GIP analogues have been shown to facilitate synaptic plasticity and reduce plaque load in aged wild-type mice and in a transgenic AD mouse model (Faivre and Hölscher, 2013; Duffy and Hölscher, 2013), while a dual GLP-1/GIP receptor agonist recently demonstrated a potential neuroprotective effect in a STZ-icv rat model (Shi et al., 2017). Further research in animal AD models is needed to explore whether long-term administration of a nutrient like oral galactose, which acts synergistically on different targets, might replace therapy with GLP-1 and GIP agonists or dual GLP-1/GIP agonists, or even be more efficient due to its additional glucose-replenishing effects. It should also be kept in mind that a nutrient might generally have a more acceptable safety profile than a drug. Therefore, knowing the detrimental effects of high galactose levels (Zhang et al., 2007; Budni et al., 2016; Ho et al., 2003; Wei et al., 2005; Cui et al., 2006; Kumar et al., 2011), the safety profile of oral galactose should be well characterised, taking into account its blood level and the appropriate pathophysiological state which might be responsive to such a therapy.

4. Results

4.1. Chronic oral galactose treatment improves cognitive deficits previously developed after STZ-icv administration in rats

Three months after STZ-icv administration, cognitive deficits were found in the animals which were not subjected to chronic oral galactose treatment as demonstrated by MWM and PA tests (Fig. 1). In the MWM training (learning) trials STZ rats needed more time to find the hidden platform in comparison with the control vehicle-icv treated rats (CTR), which was demonstrated by a significantly longer escape latency ($p < 0.001$ on days 3–5; 133%-140%-107%) and a higher number of mistakes ($p < 0.05$ on days 2–5; 175%-233%-397%–458%), indicating impaired spatial memory and learning ability (Fig. 1a). The deficits in retention of spatial memory were manifested as less time spent in search for the removed platform (−56%, $p = 0.0014$) in the MWM probe trial, while no difference was found in the number of mistakes between the STZ and CTR groups (Fig. 1b). However, the STZ group demonstrated impairment of fear-motivated retention memory in the PA test, manifested as a shorter post-shock latency (−86%, $p = 0{,}0002$) in comparison with the CTR rats (Fig. 1c).

The beneficial effects of 2-month oral galactose treatment on spatial memory in the MWM test were demonstrated both in the learning ability and memory retention, but more pronounced in the former (Fig. 1a and b). They were manifested as a significantly shorter time to find the platform ($p < 0.05$ on day 4/-49%/and 5/-41%/) and a lesser number of mistakes ($p < 0.01$ on day 4/-72%) in STZ+GAL compared to STZ rats in the MWM training trials (Fig. 1a). The tendency of the increment of the time spent in the target quadrant (+40%) in the probe trial did not reach statistical significance ($p = 0.09$ vs STZ) in the galactose-treated STZ group (Fig. 1b). The velocity of swimming in the MWM learning and probe trials was not affected by any of the treatments, as presented in Suppl. 1.

The beneficial effect of chronic oral galactose treatment in the STZ rats was also demonstrated by the fear-conditioned escape memory in the PA test, manifested by a much longer post-shock latency time (+349%, $p = 0.001$) compared to the galactose-untreated STZ rats (Fig. 1c).

To summarise, chronic oral galactose treatment had no effect on the spatial and fear-motivated escape memory functions (either beneficial or detrimental ones) in the control vehicle-icv treated rats, but was successful in the normalisation of previously induced cognitive deficits in the STZ rats, in both the MWM and PA tests ($p < 0.05$) (Fig. 1).

4.2. Galactose and glucose concentrations in rat plasma and CSF after galactose treatment

Plasma galactose concentrations were found increased 15 min after a single galactose load (200 mg/kg) in the STZ pre-treated rats (galactose load performed 1 month after the STZ-icv injection) (Table 1). The increment was 2x higher after parenteral (+208%) than after oral (+111%) galactose administration in STZ rats in comparison to the CTR ($p = 0.0152$ and $p = 0.0007$, respectively) and galactose-untreated STZ rats ($p = 0.0152$ and $p = 0.0009$, respectively) (Table 1). However, after a 2-month oral galactose treatment no alterations were seen in the plasma galactose concentrations of the STZ rats (Table 1).

There were no significant changes in CSF galactose levels of the STZ rats following a single galactose load, although a tendency for a mild increment was observed (+27% to +39%) (Table 1). The CSF galactose levels remained unchanged after chronic oral galactose treatment (Table 1).

A very mild but statistically significant (+13%, $p < 0.0126$) rise in

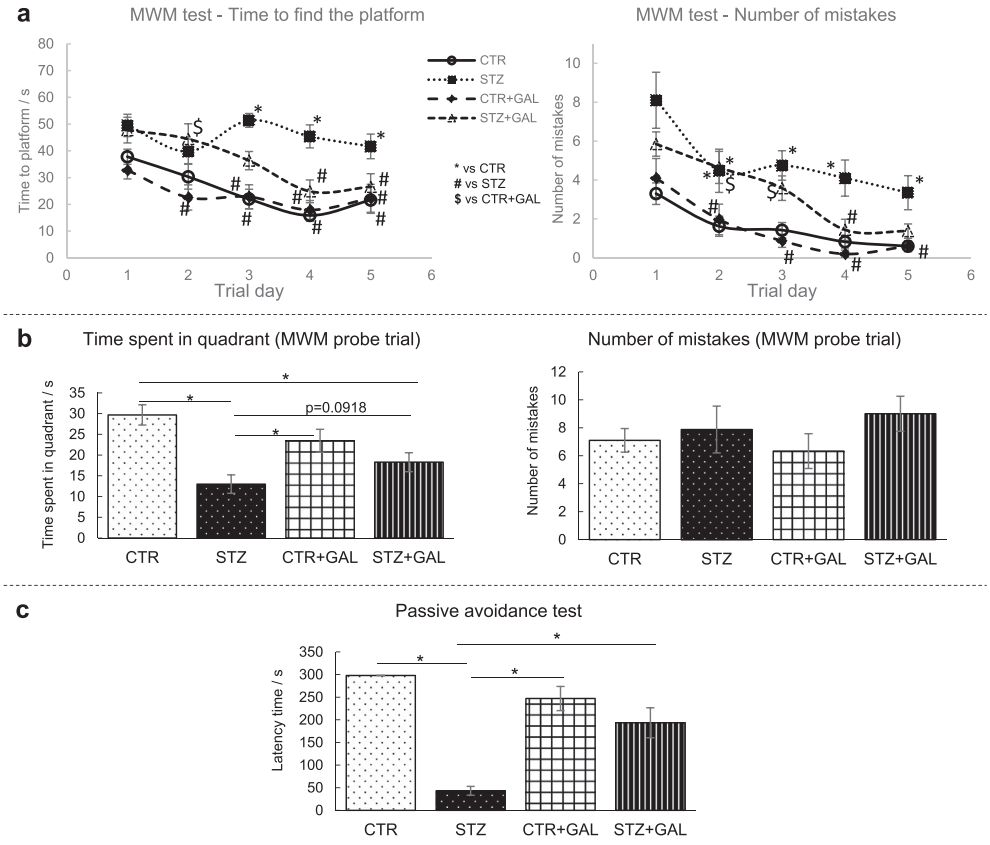

Fig. 1. Oral galactose treatment normalises cognitive deficits induced by intracerebroventricular administration of streptozotocin. Animals were euthanised 3 months after intracerebroventricular (icv) treatment with streptozotocin (STZ-icv) (3 mg/kg) or vehicle (CTR). Oral galactose treatment (200 mg/kg/day in a drink ad libitum) started 1 month after the STZ-icv injection and lasted 2 months. Cognitive performance was measured at the end of galactose treatment. **(a)** Each dot represents a group value (time to find the platform or number of mistakes) expressed as mean ± SEM of learning trials (days 1–5) in Morris Water Maze (MWM) test. Statistical significance for a particular group at the particular time-point is expressed by different marks depending on the group used as comparator: *p < 0.05 compared to the control (CTR); #p < 0.05 compared to the STZ-icv group (STZ); $ p < 0.05 compared to the galactose-treated controls (CTR+GAL). STZ+GAL, galactose-treated STZ-icv rats N = 10 for CTR, STZ, CTR+GAL, N = 9 for STZ+GAL. **(b)** Each bar represents mean ± SEM of the time spent in target quadrant and number of mistakes in the probe trial of MWM test (day 6) (*p < 0.05) N = 10 per group. **(c)** Each bar represents post-shock latency time measured by Passive Avoidance (PA) test. Values are expressed as mean ± SEM. N = 10 per group. Data were analysed by non-parametric Kruskal-Wallis one-way ANOVA test followed by a Mann Whitney *U* test (*p < 0.05). Group comparisons during the learning trials were analysed by Two-way ANOVA for repeated-measures with Bonferroni post-hoc test on ln transformed data to meet the condition of normality.

Table 1
Galactose and glucose concentrations in plasma and cerebrospinal fluid of streptozotocin-intracerebroventricularly treated rats measured following a single dose and chronic galactose treatment.

	GLUCOSE CONCENTRATION ± %SEM (% above or below the respective control)		GALACTOSE CONCENTRATION± %SEM (% above or below the respective control)	
	PLASMA	CSF	PLASMA	CSF
Measured 15 min after a single galactose dose				
STZ	+3% ± 5.13 (N = 10)	+10% ± 8.06 (N = 9)	+3% ± 24.48 (N = 9)	−2%±23.28 (N = 9)
STZ + single 200 po GAL	13% ± 3.32[a] (N = 10)	+10% ± 6.38 (N = 7)	+111% ± 12.60[a,b] (N = 9)	+39% ± 25.59 (N = 8)
STZ + single 200 ip GAL	+7% ± 2.96 (N = 9)	+22% ± 3.62[a] (N = 8)	+208% ± 3.10[a,b,c] (N = 9)	+27% ± 30.47 (N = 9)
Measured after 2 months of oral galactose treatment				
STZ	−5%±8.10 (N = 10)	+4% ± 2.53 (N = 10)	+2% ± 11.68 (N = 10)	−8%±17.77 (N = 10)
CTR + chronic 200 po GAL	−26% ± 5.36[a] (N = 10)	+8% ± 3.49 (N = 10)	−4%±6.70 (N = 10)	−28% ± 15.51 (N = 8)
STZ + chronic 200 po GAL	−29% ± 8.63[a,b] (N = 10)	+15% ± 3.44[a,b] (N = 8)	−16% ± 5.35 (N = 10)	−31% ± 19.48 (N = 7)

CSF, cerebrospinal fluid; GAL, galactose, CTR, control rats; STZ, rats treated intracerebroventricularly with streptozotocin (3 mg/kg); STZ + single 200 po GAL, rats treated orally (gastric tube) with a single galactose dose (200 mg/kg) given 1 month after STZ; STZ + 200 ip GAL, rats treated intraperitoneally with a single galactose dose (200 mg/kg) given 1 month after STZ; CTR/STZ + chronic 200 po GAL, rats treated 2 months with galactose (200 mg/kg/day in drinking water); %SEM, percent of standard error of mean.

[a] p<0.05 vs respective control.

[b] p < 0.05 vs STZ.

[c] p < 0.05 vs STZ + 200 po GAL. N - number of animals per group (N = 10 for CTR groups in both experiments).

glucose levels was observed in the STZ rats after a single oral load of 200 mg/kg dose in the plasma, and after a single parenteral load of the same dose in the CSF (+22%, $p < 0.0208$) (Table 1). In contrast, chronic oral galactose treatment was associated with a smaller but significant decrease in glucose plasma concentrations both in the CTR (−26%; $p = 0.0025$) and STZ (−29%; $p = 0.0343$) rats compared to their galactose-untreated respective controls (Table 1). The opposite was found in the glucose CSF levels after chronic oral galactose administration, i.e. a mild but statistically significant increase (+15%, $p = 0.0112$) in STZ+GAL group (Table 1).

4.3. FDG uptake in the rat brain following chronic oral galactose treatment

The glucose uptake was measured by FDG-PET scan in 2 animals per group chosen as group representatives based on their cognitive results. The representative images demonstrated a generally lower intensity of FDG uptake in the brain of the STZ rats (3 months after STZ-icv) compared to the CTRs (Fig. 2a). Quantification of FDG uptake (Fig. 2b) clearly follows the pattern of glucose metabolism presented in the brain images by showing an increment (+14%) of glucose metabolism in the total brain after chronic oral galactose treatment (STZ+GAL) compared to the galactose-untreated STZ group (Fig. 2b). Across the brain regions, this effect was most pronounced in the brainstem (+32%) and hypothalamus (+29%), but was also seen in the amygdala (+23%), thalamus (+21%) and, to a lesser extent, in the frontal cortex, hippocampus and cerebellum (Fig. 2b). Reduction in glucose metabolism in the STZ group compared with the CTR group was very mild (−3%) at the level of total brain due to region-dependent differences: a reduction in the hypothalamus (−15%), cerebellum (−7%), hippocampus (−6%) and brainstem, but a slight increment in the frontal cortex (+7%) and no change in the parietal and temporal cortices (Fig. 2b). In the CTR rats chronic oral galactose treatment induced no changes in glucose metabolism at the level of total brain, but some region-specific differences were observed in this group as well: an increment in the thalamus (+17%) and, to a lesser extent (+6%), in the amygdala, hippocampus and temporal cortex, with no changes in other regions (Fig. 2b).

4.4. Insulin concentrations in plasma and CSF after galactose treatment

The decrease in plasma insulin concentrations found in the STZ

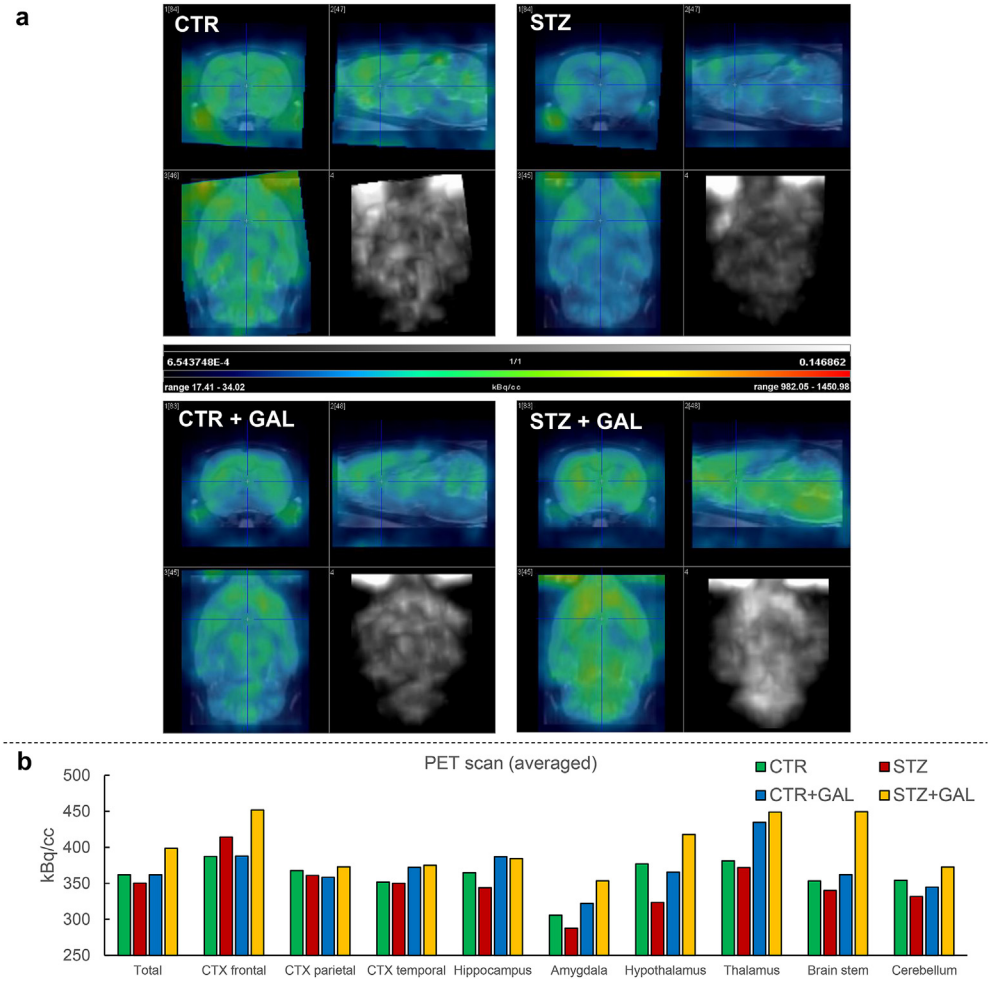

Fig. 2. Glucose uptake using FDG as a radiotracer in streptozotocin-treated animals subjected to chronic oral galactose treatment. Oral galactose treatment (200 mg/kg/day in a drink ad libitum) started 1 month after intracerebroventricular (icv) treatment with streptozotocin (STZ-icv) (3 mg/kg) or vehicle (CTR) and lasted for 2 months, after which two animals per group were subjected to PET scanning. **(a)** Representative images of one animal per group are presented. **(b)** Regions of interest were selected and analysed by PMOD software for indication of glucose uptake/metabolism rate. The bars represent calculated kBq/cc for each region and total brain. CTR, control vehicle-icv treated rats who drank tap water; CTR+GAL, control vehicle-icv treated rats who drank galactose for 2 months; STZ, STZ-icv treated rats who drank tap water; STZ+GAL, STZ-icv treated rats who drank galactose; CTX, cortex.

rats measured 1 month after icv treatment (−45.2%, p = 0.0092) was normalised to the control level by a single galactose dose of 200 mg/kg given either orally or parenterally (Fig. 3a). Although insulin levels were higher after an oral (+161%) than after a parenteral (+96.5%) bolus dose compared to the STZ rats, the difference between the groups did not reach statistical significance (p = 0.24) (Fig. 3a). No significant changes in CSF insulin levels were observed after administration of a single galactose dose (Fig. 3a), and neither after chronic oral galactose treatment in both plasma and CSF of the STZ rats (Fig. 3b).

4.5. GLP-1 levels in plasma and CSF after galactose treatment

Both total and active GLP-1 levels were measured in the plasma, while due to an unmeasurably low level of its active form, it was only possible to measure total GLP-1 in the CSF after single or chronic galactose treatments (Fig. 4). The most marked change was found in the plasma concentration of active GLP-1 both after a single dose and following chronic oral galactose treatment (Fig. 4, grey-coloured area). No change was found between the galactose-untreated STZ and CTR groups, but a single galactose dose induced a huge increase in active GLP-1 levels in the STZ-icv rats only after an oral bolus dose (5-fold change) but not after a parenteral one, compared to the CTR group (p < 0.05) (Fig. 4a). However, due to high intragroup (STZ+200 po GAL) variability, this difference did not reach the significance level, although it was nearly significant compared to the STZ (3.8-fold increment; p = 0.09) and STZ+200 ip GAL (2.9-fold increment; p = 0.07) groups (Fig. 4a). Although no significant changes in total GLP-1 levels were found in the plasma of the STZ-icv rats after a single galactose load, a 78%-rise was found following the oral bolus with a nearly significant difference (p = 0.08) compared to the STZ group (Fig. 4a). The CSF levels of total GLP-1 were found unchanged after a single galactose dose in all groups (Fig. 4a).

In contrast to the unchanged plasma levels of active GLP-1 in the STZ rats compared to the respective CTR group that were recorded 1 month after icv drug treatment (Fig. 4a), 3 months after STZ-icv treatment these levels were found significantly decreased (−51.2%, p = 0.0045) (Fig. 4b). Chronic oral galactose treatment increased active GLP-1 concentrations in the plasma of the STZ rats (+70.2%, p = 0.0275 vs STZ group), close to the levels in the CTR group (p = 0.21 vs CTR) (Fig. 4b).

The plasma levels of total GLP-1 were unchanged in the STZ group measured either 1 (Fig. 4a) or 3 (Fig. 4b) months after icv treatment in comparison to the respective CTR groups. Oral galactose treatment demonstrated a tendency to increase total GLP-1 levels in the plasma of the STZ-rats both after a single dose (+78.5%, p = 0.08 vs STZ) and after chronic treatment (+58.5%, p = 0.06 vs CTR+GAL) (Fig. 4a and b). No changes in total GLP-1 levels were found in the CSF of the STZ-icv rats 3 months after icv treatment, regardless of the galactose treatment (Fig. 4b). However, decreased total GLP-1 levels in the CSF (−30.7%; p = 0.01) were found in the galactose-treated CTRs in comparison to the untreated CTR group (Fig. 4b), which was also accompanied by decreased levels of active GLP-1 in the plasma of the galactose-treated CTRs (−79.6%, p = 0.0008 vs CTR) (Fig. 4b).

4.6. GLP-1R expression in the brain of galactose-treated rats

We wanted to find out whether the increment in active plasma GLP-1 affected the expression of the GLP-1 receptor (GLP-1R) in the hippocampus, dissected out as a region of interest related to memory functions. As demonstrated by Western blot analysis, no significant changes were observed in the GLP-1R expression in the

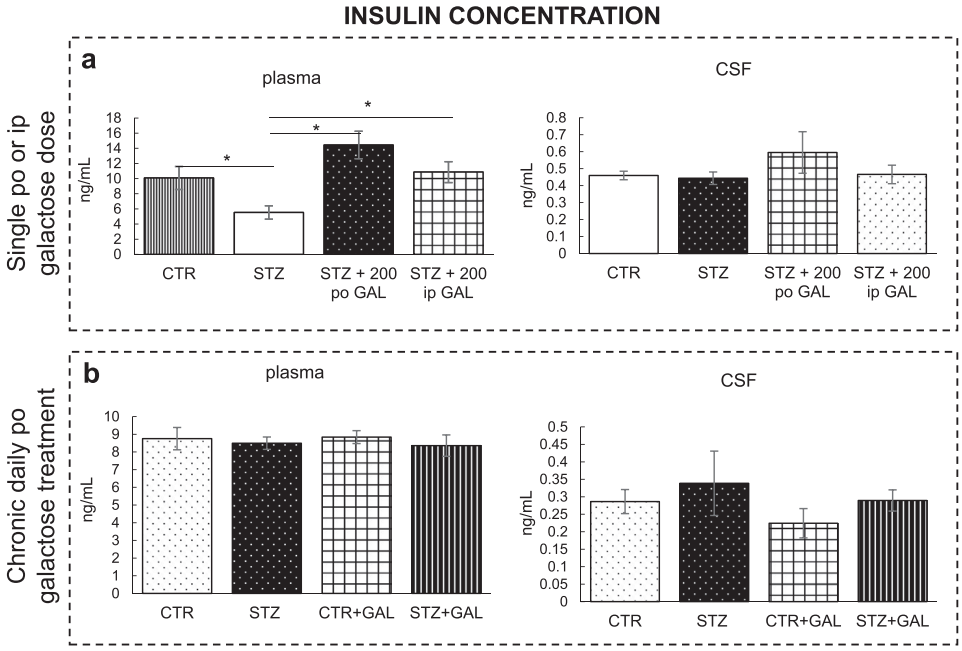

Fig. 3. Insulin concentrations in plasma and cerebrospinal fluid after a single galactose dose and after chronic galactose treatment. (a) Insulin concentration in plasma and cerebrospinal fluid (CSF) after a single galactose bolus dose (200 mg/kg) given orally or intraperitoneally 1 month after intracerebroventricular streptozotocin (3 mg/kg) treatment (STZ + 200 po GAL and STZ + 200 ip GAL, respectively). N (insulin/plasma) = 9 per group. N (insulin/CSF): N = 9 for control (CTR) and STZ, N = 7 for STZ + 200 po GAL, N = 8 for STZ + 200 ip GAL. **(b)** Insulin concentration in plasma and CSF of controls (CTR) and STZ-icv rats measured after 2-month oral galactose treatment (200 mg/kg/day) given as a drink ad libitum (CTR+GAL, STZ+GAL) initiated 1 month after STZ-icv injection. Each bar represents mean ± SEM. N (insulin/plasma) = 10 per group. N (insulin/CSF): N = 9 for CTR, N = 10 for STZ, CTR+GAL, N = 8 for STZ+GAL. Data were analysed by non-parametric Kruskal-Wallis one-way ANOVA test followed by Mann Whitney *U* test (*p < 0.05).

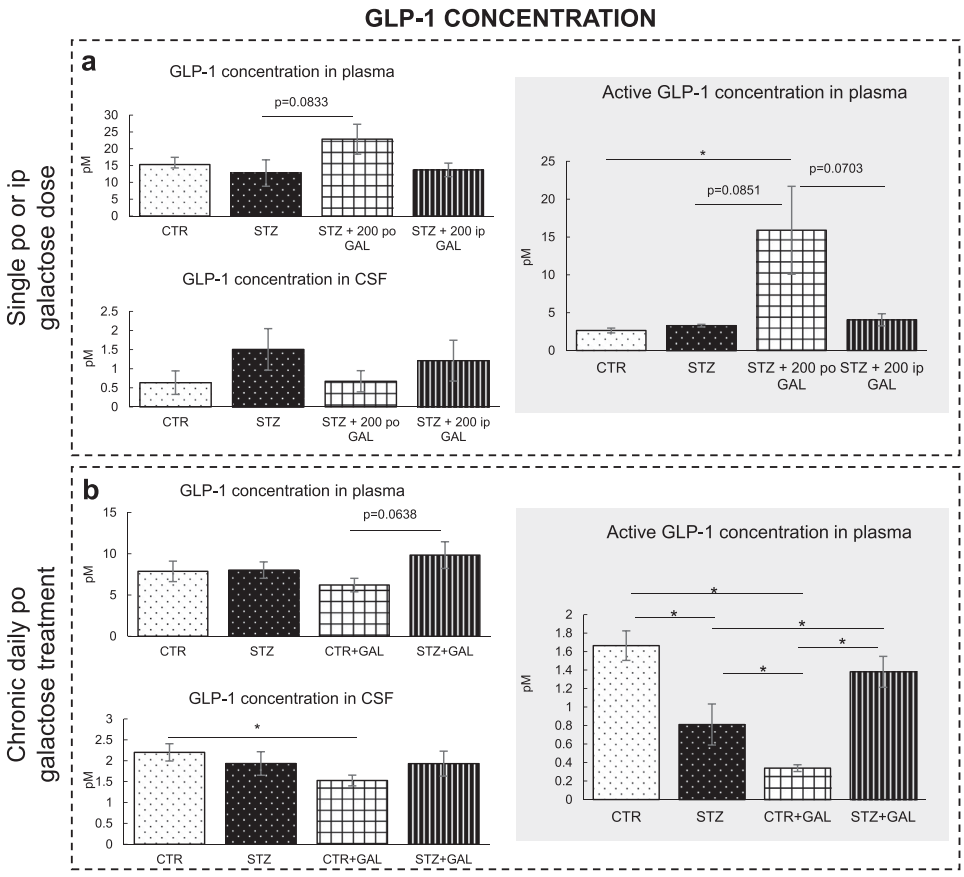

Fig. 4. Glucagon-like peptide-1 concentration in plasma and cerebrospinal fluid after a single galactose dose and chronic galactose treatment. (a) Total glucagon-like peptide 1 (GLP-1) concentration in plasma and cerebrospinal fluid (CSF) and active GLP-1 concentration in plasma after a single galactose bolus dose (200 mg/kg) given orally or intraperitoneally 1 month after intracerebroventricular streptozotocin (3 mg/kg) treatment (STZ + 200 po GAL and STZ + 200 ip GAL, respectively). N (GLP-1/plasma) = 8 per group, N (active GLP-1/plasma) = 9 per group, N (GLP-1/CSF): N = 6 for control (CTR), N = 9 for STZ, N = 7 for STZ + 200 po GAL, STZ + 200 ip GAL. **(b)** Total GLP-1 concentration in plasma and CSF and active GLP1 concentration in plasma of CTR and STZ-icv rats after a 2-month galactose treatment (200 mg/kg/day) given as a drink (CTR+GAL, STZ+GAL) that started 1 month after STZ-icv injection. N (GLP-1/plasma = 10 per group, N (GLP-1/CSF): N = 10 for CTR, STZ, CTR+GAL, N = 8 for STZ+GAL. N (active GLP-1/plasma): N = 10 for STZ; N = 8 for CTR, CTR+GAL; N = 9 for STZ+GAL. Grey-coloured graphs represent significant changes of active GLP1 concentration found in plasma. Each bar represents mean ± SEM. Data were analysed by non-parametric Kruskal-Wallis one-way ANOVA test followed by Mann Whitney *U* test (*p < 0.05).

hippocampus after single oral and parenteral galactose doses administered to the STZ rats (Fig. 5a). No changes in the hippocampal expression of GLP-1R were observed either 1 (Fig. 5a) or 3 (Fig. 5b) months after STZ-icv administration compared to their respective controls. However, in the STZ rats chronic oral galactose treatment induced a huge rise in hippocampal GLP-1R expression (up to +80%, p = 0.0022 vs all other treatments), while no effects were seen in the chronically galactose-treated CTR group (Fig. 5b).

Considering the pronounced increase in FDG uptake/glucose metabolism found in the hypothalamus after chronic oral galactose treatment of the STZ rats compared to the untreated STZ group (Fig. 2b), we wanted to verify if the GLP-1R expression was also affected in this region. Since only the hippocampal tissue was dissected out in accordance with the determined protocol, and the total brain was resected from four animals for histological analysis, GLP-1R expression in the hypothalamus was further explored at the histological level (Fig. 6). Immunofluorescence staining revealed a positive red signal in the hypothalamus which was more intense and extensive after chronic oral galactose treatment both in the CTR and STZ rats, indicating increased oral galactose-induced expression of GLP-1R in this region (Fig. 6). No changes in GLP-1R immunofluorescence staining were observed between the galactose-untreated CTR and STZ rats, respectively.

5. Discussion

The results presented here clearly show that a 2-month oral galactose treatment in doses of 200 mg/kg normalised cognitive deficits in a STZ-icv rat model of sAD (Fig. 1). This new evidence strongly supports our recent finding that a 1-month oral galactose treatment successfully prevented the development of cognitive deficits in the same model when initiated on the day of the STZ-icv injection (Salkovic-Petrisic et al., 2014).

D-galactose is a C-4 epimer of D-glucose normally found only in very small quantities in eukaryotic systems. At high levels, which can be induced by exogenous galactose load or endogenously due to its dysfunctional metabolism, galactose reacts with the free amines of amino acids in proteins and peptides and consequently forms advanced glycation end products which cause oxidative damage in the body (Zhang et al., 2007). Chronic exposure of mice or rats to parenteral galactose has been widely used as a model for age-related development of brain oxidative stress and cognitive impairment (Budni et al., 2016; Ho et al., 2003; Wei et al., 2005; Cui et al., 2006; Kumar et al., 2011; Wang et al., 2009). At first glance, these parenteral galactose-induced effects might seem contradictory to the findings presented in this study. However, a major difference in the route of galactose administration between these studies and our research (parenteral *versus* oral) may provide a

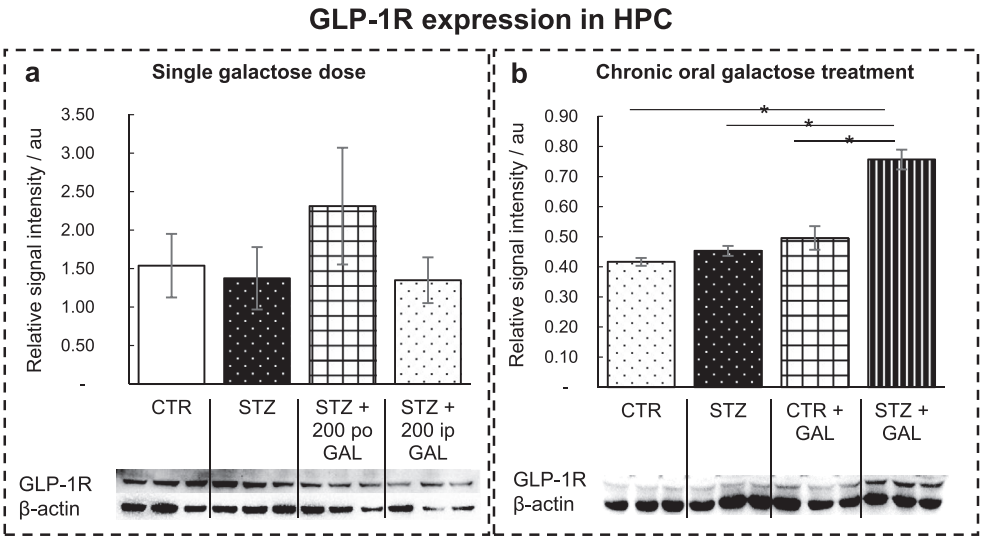

Fig. 5. Glucagon-like peptide-1 receptor expression in hippocampus after a single galactose dose and chronic galactose treatment. Glucagon-like peptide-1 receptor (GLP-1R, 53 kDa) expression in the hippocampus (HPC) was measured by Western blot analysis with the representative blots presented beneath the graphs. Anti-β-actin (42 kDa) was used as a loading control. **(a)** GLP-1R expression in HPC after a single galactose bolus dose (200 mg/kg) given orally or intraperitoneally 1 month after intracerebroventricular streptozotocin (3 mg/kg) treatment (STZ + 200 po GAL and STZ + 200 ip GAL, respectively) N = 6 per group. **(b)** GLP-1R expression in HPC of controls (CTR) and STZ-icv rats after a 2-month galactose treatment (200 mg/kg/day) given as a drink ad libitum (CTR + GAL, STZ + GAL) that started 1 month after STZ-icv injection N = 6 per group. Values are expressed as mean ± SEM and data were analysed by non-parametric Kruskal-Wallis one-way ANOVA test followed by Mann Whitney *U* test (*p < 0.05).

plausible explanation for the observed opposite effects of chronic galactose treatment on cognition in animals. There are several factors along the path of ingested galactose which differ from those induced by its parenteral administration, and thus might account for the beneficial effects induced by oral galactose administration. According to the results presented, it could be speculated that oral galactose may have direct and indirect effects (Fig. 7).

Direct effect. After ingestion, most of the galactose absorbed in circulation from the gut is cleared by the liver, but the remaining small quantities reach other organs, including the brain, and may thus directly act on the brain cells (Fig. 7). Literature data indicate that the transport of galactose into neurons is mediated by the insulin-independent glucose transporter GLUT3 (Gould et al., 1991; Seatter et al., 1997), whose density is reduced in the brain of sAD patients (Kalaria and Harik, 1989; Simpson et al., 1994). Our preliminary research has shown that 1-month oral galactose treatment prevents reduction in the expression of GLUT3 in the hippocampus of STZ-icv rats (Salkovic-Petrisic et al., 2014). Following the intracellular uptake, which occurs in a concentration-dependent manner (Mueckler, 1994), galactose is quickly metabolised to glucose via the Leloir pathway (Cohn and Segal, 1973). Recent data indicate that the capacity of the brain to take up and metabolise galactose is similar to that of the liver (Roser et al., 2009), suggesting that under pathological conditions with a decreased intracellular glucose level/metabolism (like in sAD) galactose may serve as an alternative source of glucose/energy within the neurons. Considering the co-localisation of GLUT3 and insulin-dependent GLUT4 found in neurons of the rat cerebral cortex and hippocampus (Apelt et al., 1999), continuous exposure to galactose could compensate for GLUT4 dysfunction in the insulin-resistant brain state in AD condition (Pearson-Leary, 2012) and thus improve glucose hypometabolism in the brain, supported by our results of the FDG-PET scan analysis (Fig. 3).

Our additional analysis of hippocampal GLUT3 density in the same animals whose cognitive results have been presented here provides evidence for a pronounced effect of oral galactose on GLUT3, which might be its primary target in STZ-icv-treated rats, whereas the less affected GLUT4 might be involved in the compensatory response (Suppl 2). At this stage of STZ-icv-induced pathology (Knezovic et al., 2015), when the first signs of early neurofibrillary changes and intraneuronal accumulation of Aβ1-42 are just becoming manifested in the parietotemporal cortical region, hippocampal metabolic parameters seem to be more affected by oral galactose treatment in the STZ-icv rat model of sAD (Suppl 2) than the advanced AD-stage hallmarks.

Galactose can reach the brain via circulation after both oral and parenteral administration, but considering the harmful effects of high galactose concentrations (Chiu et al., 2011) and due to the high hepatic clearance, such amounts would be much lower after oral galactose administration (Henderson et al., 1982). This has been clearly demonstrated by our results of galactose levels following the administration of oral and parenteral bolus doses of 200 mg/kg to STZ-icv rats (Table 1). Since this dose is generally within the range of parenteral doses used by others to generate neurodegeneration and aging models in mice and rats (Wei et al., 2005; Cui et al., 2006; Kumar et al., 2011; Wang et al., 2009), the effects on cognition are likely to differ significantly between our and their results. Moreover, the results of others indicate that the harmful effects of parenteral galactose on cognition in healthy rats may sometimes be lacking, depending on other factors (Cardoso et al., 2015), (Hao et al., 2014).

Indirect effects. Galactose may also act in a paracrine fashion in the gut and, in contrast to its parenteral administration, strongly stimulate adjacent endocrine L-cells to secrete GLP-1 (Ritzel et al., 1997; Shima et al., 1990) (Herrmann et al., 1995) (Fig. 7). Intestinal L-cells have direct contact with orally given nutrients at their luminal surface, and with vascular tissue and parenterally given compounds through their basolateral surface (Candeias et al., 2015). Different structures/signaling mechanisms and their regulations are present at the enterocyte luminal and basolateral membranes, respectively, which could also account for different responses following oral and parenteral galactose treatment. Intestinally-derived (and, to a smaller extent, pancreatic alpha-cell-derived) GLP-1 acts as an incretin which dose-dependently

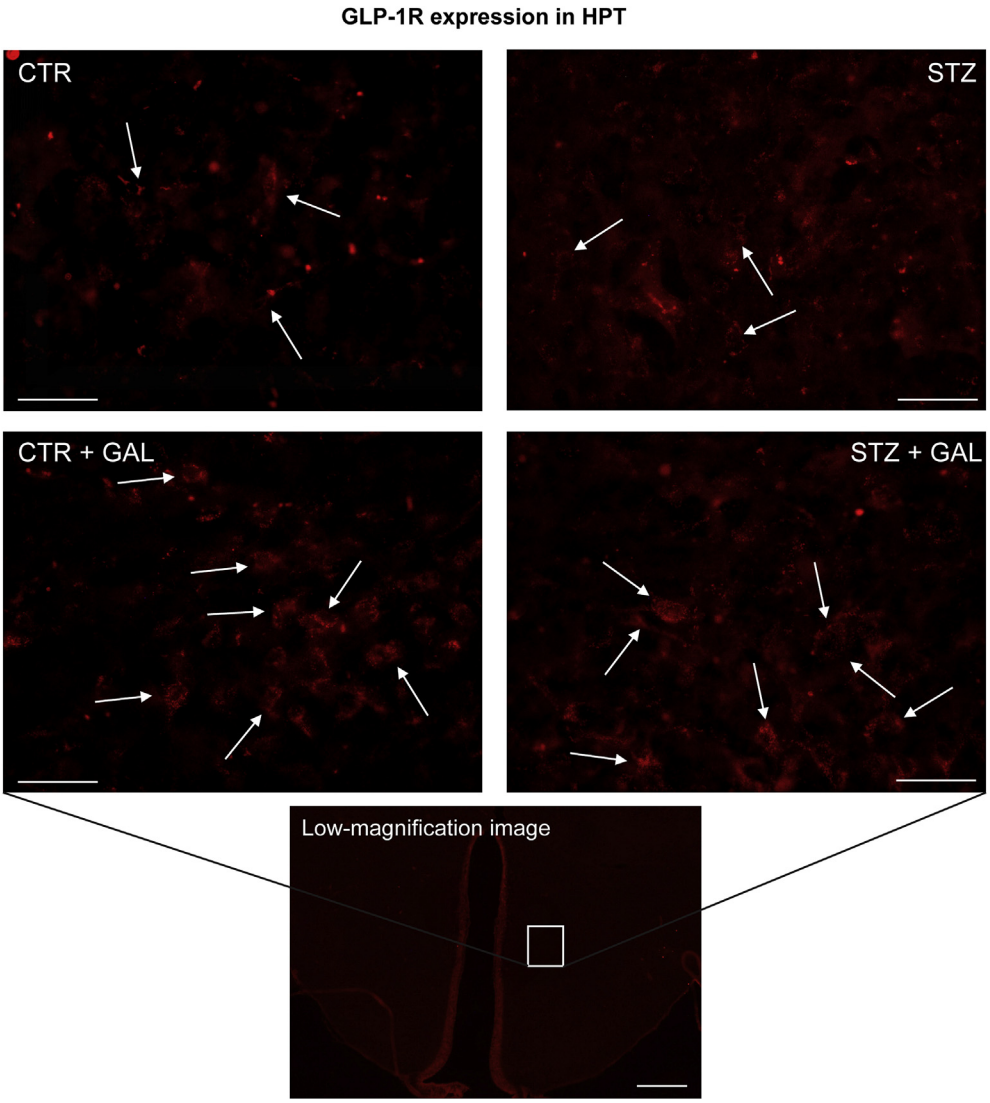

Fig. 6. Immunofluorescence staining of glucagon-like peptide-1 receptor in rat hypothalamus following chronic oral galactose treatment. Animals were euthanised 3 months after intracerebroventricular (icv) treatment with streptozotocin (STZ-icv) (3 mg/kg) or vehicle (CTR). Oral galactose treatment (200 mg/kg/day in a drink ad libitum) started 1 month after STZ-icv injection and lasted for 2 months until sacrifice. Brain slices (35 μm) of 4 rats per group were subjected to immunofluorescence staining with glucagon-like peptide-1 receptor (GLP-1R) antibody. Representative photomicrographs show a positive GLP-1R signal in the medial preoptic area of the anterior hypothalamus seen as red staining. The low-magnification image (Scale bar = 500 μm) shows the area where high-magnification photomicrographs have been recorded. Cells with a positive signal are indicated by the white arrows. Scale bar = 50 μm.

promotes insulin secretion from the pancreatic beta cells (Holst, 2007). In line with GLP-1-induced stimulation of insulin, in our experiments the single galactose doses demonstrated a potential to normalise the decrease in blood insulin levels seen transiently 1 month after STZ-icv treatment, with no effect on normal insulin homeostasis observed in the chronic experiment. This finding may allow the speculation that galactose-induced insulin increase contributes to the beneficial effects of oral galactose only when plasma insulin levels are altered.

GLP-1 released from L-cells is extremely rapidly metabolised and inactivated by dipeptidyl peptidase IV (DPP-IV) (Mulvihill and Drucker, 2014), an enzyme expressed in enterocytes and endothelial cells (Hansen et al., 1999) (but also in the hypothalamus, hippocampus, circumventricular organs and choroid plexus (Candeias et al., 2015)), so that only <25% of newly secreted GLP-1 seems to leave the gut in its active form (Holst, 2007). In our experiments, oral (but not parenteral) administration of a single galactose dose (200 mg/kg) induced an increment (+500%) in active GLP-1 blood levels only in the STZ-icv and not in the control rats (Fig. 5). In the galactose-untreated STZ-icv rats active GLP-1 plasma levels were unchanged after 1 month, but were significantly decreased 3 months after STZ-icv injection, indicating a possible increment in GLP-1 degradation in the course of STZ-icv-induced pathology progression (Fig. 5). These results are in line with literature data on the long-term inhibition of DPP-IV in Alzheimer-prone mice (D'Amico et al., 2010), as well as on the therapeutic effects of DPP-IV inhibitors on cognitive impairment and brain mitochondrial dysfunction in insulin-resistant rats (Pintana et al., 2013) and in diabetic patients with or without AD (Isik et al., 2017). However, in the absence of pathology, long-term oral galactose load might have different, possibly undesirable effects on active GLP-1 in plasma, seen in our study as a decrease of active GLP-1 plasma levels in the galactose-treated control rats.

Intestinally produced GLP-1 and its active remnant in the circulation can gain access to the brain nuclei after simple diffusion across the blood-brain barrier (Kastin et al., 2002) but also at sites

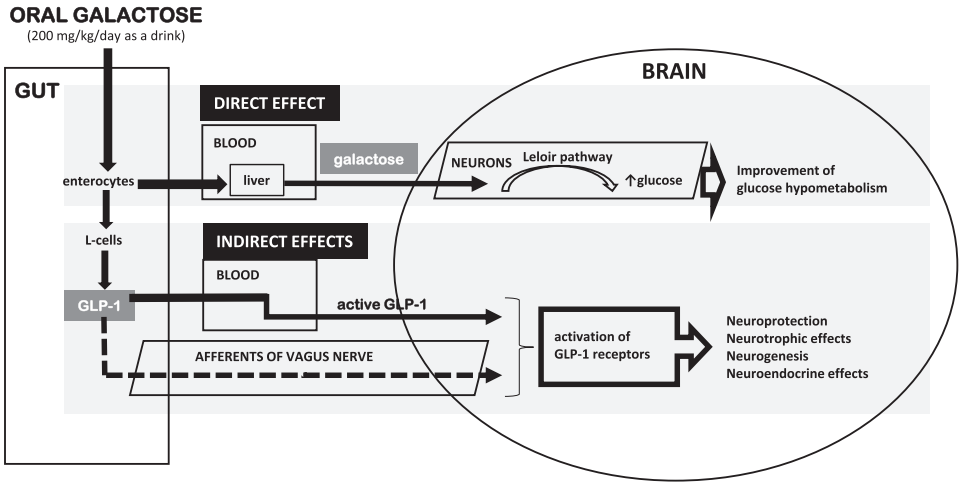

Fig. 7. Proposed mechanisms of the therapeutic effects of oral galactose treatment in a streptozotocin-induced rat model of sporadic Alzheimer's disease. Daily oral intake (drink) of galactose (200 mg/kg) may improve cognitive deficits and elicit beneficial effects in the brain of a streptozotocin intracerebroventricularly-treated rat model (STZ-icv model) of sporadic Alzheimer's disease (sAD) through the proposed direct and indirect effects. **Direct effect.** After ingestion and intake into/release from the gut enterocytes, galactose is absorbed into the circulation. A large proportion of the galactose undergoes hepatic clearance, but the small remnant amounts reach other organs including the brain, where galactose is taken up by the neurons and converted to glucose by the Leloir pathway. This may consequently increase intracellular glucose levels and improve previously developed glucose hypometabolism, as evidenced here by the FDG-PET scan images of galactose-untreated and galactose-treated STZ-icv rat model. **Indirect effects**. After being released from the gut enterocytes, galactose stimulates adjacent L-cells to secrete glucagon-like peptide 1 (GLP-1). Gut-born GLP-1 may reach the brain via circulation (active GLP-1 form) and directly stimulate the GLP-1 receptors in the brain, but can also additionally stimulate sensory afferents of the vagus nerve in the gut which project to the solitary tract nucleus with a widespread projection pattern in the brainstem, hypothalamus and forebrain. Upon stimulation, these nerves may also produce GLP-1, which can then stimulate GLP-1 receptors in the brain. Stimulation of GLP-1 receptors has been shown in the literature to be involved in neuroprotection, neurotrophic effects and neurogenesis, which may account for the improved cognition observed here, as well as for neuroendocrine effects. The contribution of direct and/or indirect effects on the therapeutic potential of oral galactose in a STZ-icv rat model needs to be further elucidated.

lacking this barrier and thus exerting a direct activation of certain neurons (Alvarez et al., 2005). Alternatively, intestinally released GLP-1 may bind to and activate sensory afferents of the vagus nerve. In turn, the vagus may activate neurons of the solitary tract nucleus with a widespread projection pattern in the brainstem, hypothalamus and forebrain (Jin et al., 1988), which consequently may also produce GLP-1 themselves (Jin et al., 1988; Renner et al., 2012). Memory improvement in STZ-icv rats treated orally with galactose for 2 months accompanied by normalised plasma levels of active GLP-1 and an increased expression of GLP-1R in the hippocampus is in line with literature data on the enhancement of associative and spatial learning in mice following increments in GLP-1R expression in the hippocampus, as well as following intracerebroventricular GLP-1 administration abolished by pre-treatment with a GLP-1 antagonist (During et al., 2003).

The level of active GLP-1 was below the detectable limit in our experiments on Wistar rats, although detectable amounts of active GLP-1 in the CSF were reported in Sprague-Dawley rats (Hsu et al., 2014). GLP-1 in the CSF could be of gut origin or/and produced in the nucleus of the solitary tract and released into the CSF (Alhadeff et al., 2012), (Gu et al., 2013), (Cork et al., 2015), but the relative contribution of peripherally-versus centrally-derived GLP-1 in affecting the brain functions is still unclear.

Another brain region that may be significantly affected by GLP-1 is the hypothalamus (Holst, 2007), which has a widespread distribution of GLP-1R (Renner et al., 2012). GLP-1R in the hypothalamus has been proposed to be involved in neuroendocrine responses and the regulation of energy balance (Schick et al., 2003). Not only does the hypothalamic projection regulated by metabolic hormones affect hippocampal activity, but the same is true in vice versa order (Davidson et al., 2007). Our results demonstrate that chronic oral galactose treatment induces a marked increment in hypothalamic GLP-1R expression. Considering that sAD is accompanied by a central glucose hypometabolism and insulin resistance (Baker et al., 2011), our recent data on the STZ-icv induced alteration of hypothalamic GLUT2 and insulin receptor expression (Knezovic et al., 2017), as well as the here detected galactose-induced increment in glucose metabolism in the hypothalamus and brainstem, suggest that chronic oral galactose treatment might also improve the hypothalamic metabolic dysfunction.

To summarise, various factors might affect the direction in which chronic oral galactose-induced effects will be manifested, depending on: (i) galactose dose, since diet highly rich (<30%) in galactose induced hypergalactosemia and symptoms of diabetes (in contrast to the beneficial effects of 15%-dry matter galactose) in rats (Niewoehner and Neil, 1992); (ii) duration of oral galactose treatment, in line with literature data suggesting that the beneficial effects could be nullified or turned to harmful in a long-term treatment (Budni et al., 2016); (iii) the presence of pathological condition and its stage, since effects of oral galactose could be manifested as beneficial only within a particular pathological background, as seen here in the STZ-icv rat model of early-stage sAD (Knezovic et al., 2015) or reported in patients with multiple sclerosis (Panfoli et al., 2016) and congenital prosopagnosia (Esins et al., 2014).

6. Conclusions

A two-month oral galactose treatment (200 mg/kg/day) normalised previously developed cognitive deficits in a STZ-icv rat model of early-stage sAD, associated with a galactose-induced improvement in the brain glucose metabolism (direct effect) and

stimulation of GLP-1-mediated effects (indirect effects) by increments in plasma levels of active GLP-1 accompanied by an increased expression of GLP-1R in the brain. In line with that it might be speculated that the therapeutic effects of oral galactose could be associated with the neuroprotective and neurotrophic, as well as regulatory, neuroendocrine role of GLP-1 in the brain (Duarte et al., 2013; Salcedo et al., 2012). Considering the clinical and non-clinical trials investigating GLP-1 analogues as new anti-AD drugs (Duarte et al., 2013; Hunter and Holscher, 2012; Talbot, 2014; Perry and Greig, 2004) and galactose as a mediator of endogenous GLP-1-induced effects as well as an alternative source of energy, the results presented here provide strong evidence of beneficial oral galactose effects and its interplay with GLP-1 in the reduction of cognitive deficits in a STZ-icv rat model of early sAD. Further research is needed to clarify the precise mechanism(s) involved, in order to possibly make way for new dietary-based strategies in AD treatment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2018.02.027.

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