Univerzita Karlova v Praze

1. lékařská fakulta

Autoreferát disertační práce

Vliv různých druhů antidiabetických intervencí na vývoj neurodegenerativních změn v mozku diabetických myší a potkanů

Impact of different types of antidiabetic interventions on the development of neurodegenerative changes in brains of diabetic mice and rats

Andrea Špolcová

Praha (2015)

Doktorské studijní programy v biomedicíně

Univerzita Karlova v Praze a Akademie věd České republiky

Obor:	**Biochemie a patobiochemie**
Předseda oborové rady:	**Prof. MUDr. Stanislav Štípek, DrSc.**
Školicí pracoviště:	**Ústav organické chemie a biochemie AV ČR, v.v.i.**
	& INSERM UMR-S-1172, Lille, France
Školitel:	**RNDr. Lenka Maletínská, CSc**.
Spoluškolitel:	**Dr. Marie-Christine Galas**
Konzultant:	**Prof. MUDr. Martin Haluzík, DrSc.**

Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

CONTENTS

ABBREVIATIONS

Aβ amyloid-beta peptide
AD Alzheimer's disease
APP amyloid precursor protein
ANOVA analysis of variance
Arc nucleus arcuatus
BBB blood brain barrier
cdk5 cyclin dependent kinase 5
CNS central nervous system
DIO diet-induced obesity
ELISA enzyme-linked immunosorbent assay
GLP-1 glucagon-like peptide 1
GSK-3 glycogen-synthase kinase 3
HFD high-fat diet
JNK c-Jun N-terminal kinase
MAPK/ERK1/2 mitogen-activated protein kinase/extracellular-regulated kinase 1/2
MSG monosodium glutamate
PP2A protein phosphatase 2A
PrRP prolactin-releasing peptide
RIA radioimmunoassay
SC subcutaneous
SEM standard error of mean
T2DM type 2 diabetes mellitus
Tau Tau protein
WAT white adipose tissue

ABSTRAKT

Alzheimerova nemoc (AN) je neurologické onemocnění způsobené dvěma patologickými znaky; extracelulárními plaky amyloidního beta peptidu a intracelulárními neurofibrilárními shluky tvořených hyperfosforylovaným proteinem Tau.

Jelikož diabetes mellitus druhého typu (DM2T) je rizikovým faktorem pro rozvoj AN, byl v první části mé disertační práce zkoumán možný vztah mezi hyperfosforylací proteinu Tau a centrální inzulínovou rezistencí, a to v hipokampech fa/fa potkanů a MSG myší, zvířecích modelů pre-diabetu, který je vyvolán obezitou. U 8 měsíců starých fa/fa potkanů a 6 měsíců starých MSG myší docházelo ke snížené fosforylaci inzulínové signalizační kaskády, která vedla ke zvýšené aktivaci glykogensynthasy kinázy-3Beta (GSK-3β), což je hlavní kináza způsobující hyperfosforylaci proteinu Tau. Následně byla pozorována zvýšená fosforylace proteinu Tau na Ser396 a Thr231. Tento jev nebyl pozorován u 2 měsíců starých MSG myší.

Druhá část mé práce byla zaměřena na nové analogy potenciálně neuroprotektivního anorexigenního neuropeptidu, peptidu uvolňujícího prolaktin (PrRP), které byly navrženy na ÚOCHB AV ČR. Palmitoylace umožnila přechod PrRP přes hematoencefalickou bariéru, čímž byl umožněn jeho centrální anorexigenní účinek.

Ve třetí části byl zkoumán vliv 14denního subkutánního (SC) podávání liraglutidu, nejpoužívanější látky k léčbě DM2T s centrálním anorexigenním účinkem, a palmitovaného PrRP31 na aktivaci inzulínové signalizační kaskády a hyperfosforylaci proteinu Tau v hipokampech 6 měsíců starých MSG myší. Obě látky zvýšily aktivaci inzulínové signalizační kaskády, a dále snížily hyperfosforylaci proteinu Tau na Thr212, Thr231 a Ser396. Vliv 2měsíčního SC podávání palmitovaného PrRP31 byl také zkoumán na Thy-Tau22 myších, modelu AN-podobné Tau patologie, které exprimují lidský mutovaný protein Tau. Léčba vyústila ve sníženou fosforylaci proteinu Tau na Thr231, Ser396 a Ser404.

Naše studie odhalily škodlivý vliv pre-diabetu na vývoj hyperfosforylace proteinu Tau, jednoho ze znaků AN, a její snížení po podání anorexigenních látek. Anorexigenní látky se tudíž jeví jako potenciální látky k léčbě neurodegenerativních onemocnění.

KLÍČOVÁ SLOVA:

Alzheimerova nemoc, inzulínová rezistence, obezita, glykogensyntáza kináza-3Beta, hyperfosforylace proteinu Tau, fa/fa potkani, MSG myši, Thy-Tau22 myši, peptid uvolňující prolaktin, liraglutid

ABSTRACT

Alzheimer's disease (AD) is neurological disorder characterized by extracellular beta amyloid plaques and intracellular neurofibrillary tangles formed by hyper-phosphorylated Tau protein.

Since type 2 diabetes mellitus (T2DM) is a risk factor of AD development, in the first part of the thesis, a potential relationship between hyper-phosphorylation of Tau protein and central insulin resistance was followed in hippocampi of two models of obesity-induced pre-diabetes, fa/fa rats, and mice with monosodium glutamate (MSG) induced obesity. In both 8-month-old fa/fa rats and 6-month-old MSG mice a decreased phosphorylation of insulin signaling cascade resulted in an increased activation of main Tau kinase glycogen-synthase kinase-3Beta (GSK-3β) and an increased Tau phosphorylation at epitopes Ser396 and Thr231. This phenomenon was less developed in 2-month-old animals.

The second part of the thesis was focused on a potential neuroprotective anorexigenic neuropeptide, prolactin-releasing peptide (PrRP), designed at our Institute. Palmitoylation enabled PrRP to cross the blood brain barrier and employ its central anorexigenic activity.

In the third part of the thesis, an effect of 14-day-long SC administration of liraglutide, the most used anti-T2DM drug with central anorexigenic effect, and palmitoylated PrRP31 on insulin signaling cascade and Tau hyper-phosphorylation was examined in the hippocampi of 6-month-old MSG mice. Both compounds streamlined insulin signaling cascade, and also attenuated Tau phosphorylation at Thr212, Thr231, and Ser396. The effect of 2-month-long SC administration of palmitoylated PrRP31 was examined also in a model of AD-like Tau pathology, Thy-Tau22 mice overexpressing mutated human Tau protein. The treatment resulted in decreased Tau protein phosphorylation at Thr231, Ser396 and Ser404.

Our study revealed a deleterious effect of obesity-related pre-diabetes on the development of Tau pathology, and the beneficial effect of anorexigenic compounds on the hyper-phosphorylation of Tau. Anorexigenic peptides thus showed potency for possible treatment of neurodegenerative disorders.

KEY WORDS:

Alzheimer's disease, insulin resistance, obesity, glycogen-synthase kinase – 3Beta, Tau protein hyper-phosphorylation, fa/fa rats, MSG mice, Thy-Tau22 mice, prolactin-releasing peptide, liraglutide

1 INTRODUCTION

Alzheimer's disease (AD), the most common type of dementia, is an age-associated neurological disorder Histopathologically, AD is characterized by two hallmarks: first, extracellular senile plaques, which are formed by amyloid-beta peptide (Aβ), and second, intracellular neurofibrillary tangles, formed by hyper-phosphorylated Tau protein (Tau) (Alzheimer's, 2014). In spite of immense research of AD, the exact mechanism leading to its development remains unknown. Consequently, there is no sufficient treatment of AD; only drugs slowing AD progression (State Institute for Drug Control, 2012).

Besides the ageing, type 2 diabetes mellitus (T2DM) was recently characterized as a risk factor for AD. T2DM, related to obesity, is characterized by insulin resistance in the periphery, and increased glucose level in the blood (Schrijvers et al., 2010). Moreover, it was discovered that T2DM and AD have several common features which are impaired glucose metabolism, insulin resistance, higher level of cholesterol, increased inflammation and oxidative stress, Aβ aggregation, increased Tau phosphorylation, and above all, both diseases are age-dependent (de la Monte and Wands, 2008; Doble and Woodgett, 2003; Gotz et al., 2009; Li and Holscher, 2007; Takeda et al., 2010). Therefore Suzanne de la Monte proposed the new term for AD: Type 3 diabetes mellitus (Steen et al., 2005). In brain of patients with AD and T2DM, the impaired activation of insulin signaling cascade manifested by decreased phosphorylation of implicated kinases and finally by a decreased phosphorylation of glycogen synthase kinase – 3Beta (GSK-3β) at Ser9 was observed; the decreased phosphorylation at Ser9 activates GSK-3β, one of the most important kinases implicated in Tau hyper-phosphorylation (Liu et al., 2011; Takashima, 2006). The hyper-phosphorylation of Tau is also affected either by increased activation of other kinases, such as cyclin dependent kinase 5 (cdk5) (Jicha et al., 1999), c-Jun N-terminal kinase (JNK) (Atzori et al., 2001), mitogen-activated protein kinase/extracellular-regulated kinase 1/2 (MAPK/ERK1/2) (Ekinci and Shea, 1999) or decreased activation of phosphatases, such as protein kinase 2A (PP2A) (Martin et al., 2013).

To study the pathological processes leading to AD development, different rodent models were designed. AD pathology is studied in APP transgenic mice with mutation in amyloid precursor protein (APP) characterized by increased level of Aβ (Irizarry et al., 1997), APP/PS1 mice that moreover have mutation in presenilin1 (PS1) which increased the production of Aβ, and have also memory impairment (Holcomb et al., 1998), or Thy-Tau22 mice, the model of AD-like Tau pathology, overexpressing human Tau protein with mutations

G272V and P301S characterized by impaired spatial memory, and hyper-phosphorylated Tau protein (Schindowski et al., 2006). Due to the relationship between T2DM and AD progression, models of T2DM became important tools for AD research. The rodents with spontaneous mutation in leptin receptor are common models of obesity and T2DM; these are db/db mice characterized by age-dependent hyper-phosphorylation of Tau protein (Kim et al., 2009), and impaired cognition (Stranahan et al., 2008), or fa/fa rats characterized by cognitive impairment (Winocur et al., 2005). Another models of obesity are MSG mice, where obesity is caused by specific lesions in nucleus arcuatus (Arc), the hypothalamic center of food intake regulation (Olney, 1969), or rodents with diet-induced obesity (DIO), fed by high fat diet (HFD), which is rich in fat (Buettner et al., 2007).

Because of newly discovered link between T2DM and AD, a possible new strategy for the prevention/treatment of neurodegenerative diseases has emerged; it is hypothesized that agents increasing insulin sensitivity could ameliorate insulin function in central nervous system (CNS), thus could be used for the AD treatment. Insulin itself enhances memory and learning ability when administered intranasaly (Banks et al., 2012), but because of the high incidence of insulin resistance in AD patients, it is not possible to use it for AD treatment. The drugs used as T2DM treatment, such as metformin or liraglutide were proven to have beneficial effect on AD pathology. Administration of metformin resulted in improvement of spatial memory owing to increased neurogenesis (Wang et al., 2012), or in attenuated Tau phosphorylation connected with increased activity of PP2A (Kickstein et al., 2010). Liraglutide, the lipidized stable analog of glucagon-like peptide – 1 (GLP-1) (Drucker and Nauck, 2006), was shown to increase neurogenesis, reduced the level of Aβ, enhance spatial memory (McClean et al., 2011), or decrease phosphorylation of Tau protein (Yang et al., 2013). Because the primary central effect of liraglutide is exclusively anorexigenic (food intake lowering), there is a possibility that Tau phosphorylation could be affected by other anorexigenic peptides produced and acting in the brain, such as prolactin-releasing peptide (PrRP).

2 AIMS OF THE THESIS

- **Characterization of Tau phosphorylation in the brains of obese pre-diabetic animal models**

Obesity and related T2DM, characterized by insulin resistance and high glucose levels, are considered as risk factors for AD development; in the brains of AD patients, the impaired insulin signaling cascade was observed, leading to increased phosphorylation of Tau protein, one of the hallmarks of AD. The first aim of the thesis was to examine the possible neuropathological changes in central insulin signaling cascade activation and phosphorylation of Tau protein caused by obesity and pre-diabetes, the state preceding T2DM in two rodent models of obesity – fa/fa rats and MSG mice.

- **Characterization of the new lipidized analogs of prolactin-releasing peptide in *in vitro* and *in vivo* experiments**

Anorexigenic neuropeptides are promising compounds in obesity treatment; however, their application is limited due to their low stability and inability to cross BBB and manifest their anorexigenic action when administered peripherally. In our laboratory at IOCB AS CR, new more stable lipidized analogs of anorexigenic neuropeptide PrRP were designed. The second aim of the thesis was to characterize lipidized PrRP regarding their affinity to PrRP receptor in *in vitro* binding experiments and acute anorexigenic effect when administered peripherally in *in vivo* experiments in mice.

- **Investigation of the effect of anorexigenic peptides on the development of Tau phosphorylation in hippocampi of obese MSG mice, and Thy-Tau22 mice with AD-like Tau pathology**

GLP-1 receptor agonists are frequently used anti-T2DM drugs and moreover, their neuroprotective properties were proven in several experiments. It was shown that in the CNS their effect is exclusively anorexigenic. The third aim of my thesis was to test the possible neuroprotective properties of stable anorexigenic analogs of PrRP and GLP-1 analog liraglutide after long-term intervention in MSG mice, model of obesity and pre-diabetes, and in Thy-Tau22 mice, model of AD-like Tau pathology.

3 MATERIALS & METHODS

Animal Models All animal experiments performed in Czech Republic followed the ethical guidelines for animal experiments and the Czech Republic Act No. 246/1992.

The experiments with fa/fa rats were performed in Poland (Jagellonian University in Krakow) in collaboration with Institute of Experimental Endocrinology, Bratislava, Slovak Academy of Sciences, and followed ethical standards of the Declaration of Helsinki. This study conformed to national and international guidelines and was approved by the authors' institutional review board.

fa/fa rats The rats obtained at the age of 9 weeks from Harlan (Udine, Italy) were housed at Jagellonian University in Krakow, Poland, and had free access to standard diet and water. For experiments, 12 weeks old and 33 weeks old obese male fa/fa rats and their age-matched lean controls were used. The overnight-fasted rats were sacrificed by decapitation. The blood glucose was measured at Synlab (Bratislava, Slovakia) using the multi-analyzer COBAS Integra 800 (Roche Diagnostics Ltd., Rotkreuz, Switzerland). Concentrations of serum leptin and insulin levels were determined using radioimmunoassay (RIA) kits (Millipore, USA) following the manufacturer's instructions (Spolcova et al., 2014).

NMRI, and C57BL/6 mice (both models obtained from Charles Rivers Laboratories, Sulzfeld, Germany) were housed at the certified animal facility of IOCB AS CR at the campus of Academy of Sciences in Krč, Prague, Czech Republic. The mice had free access to water and standard chow diet (Mlýn Kocanda, Jesenice, Czech Republic).

For MSG-induced obesity, the newborn NMRI males were SC administered with L-glutamic acid monosodium salt hydrate (Sigma, St. Louis, USA) at a dose 4 mg/g of body weight at postnatal days 2 – 8 as previously described (Maletínská et al., 2006). These MSG-obese mice were fed with the same standard diet as the untreated control group. For the study MSG and control mice at the age of 2 and 6 months were used.

Overnight fasted MSG-obese mice and their controls at the ages 2 and 6 months (n = 10 animals per group) were sacrificed by decapitation starting at 8:00 a.m. The trunk blood was collected, glucose was measured using glucometer Glucocard (Arkray, Tokyo, Japan), and the plasma was separated and stored at -20 °C. The white adipose tissue (WAT) (subcutaneous and visceral fat) was dissected, weighed, freezed in liquid nitrogen and stored in -80°C. The rate of adiposity is expressed as the ratio of the total adipose tissue weight to the total body weight. Concentrations of serum leptin was measured using ELISA kit (Millipore, St. Charles, MI, USA) and plasma insulin using RIA kits (Millipore, St. Charles, MI, USA and

Linco Research, St. Charles, MI, USA) following the manufacturer's instructions (Spolcova et al., 2015).

Thy-Tau22 female mice and their age-matched controls were a kind gift from INSERM Laboratory U-1172, Lille, France, the research group "Alzheimer & Tauopathies". Mice arrived to Prague at the age of 7 months and were housed 3-4 per cage in the certified animal facility of the Institute of Physiology AS CR, Prague, Czech Republic, with free access to water and Altromin diet (Altromin, Eastern Westphalia, Germany). Mice were sacrificed at the age of 9 months; decapitaion and metabolic parameters measurement was performed following the procedure described for MSG mice.

Method of Western blot (WB) Hippocampi were dissected and homogenized in cold lysis buffer (62.5 mM Tris-HCl buffer with pH 6.8, 1% deoxycholate, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4 and complete protease inhibitor (Roche Applied Science, Mannheim, Germany)) using a Bullet Blender homogenizer (Next Advance, Inc., Averill Park, NY, USA). The lysates were sonicated for 1 min, the protein concentration was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following manufacturer's instructions and the lysates were diluted to a final concentration 1 μg/μl in Laemmli sample buffer (62.5 mM Tris-HCl with pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol, 50 mM NaF and 1 mM Na_3VO_4). The samples aliquots were stored at -20 °C. Before the WB, samples were sonicated for 1 min and boiled at 100 °C for 2 min. Then, 10 μg/10 μl of each sample was resolved using 5/10% SDS-PAGE electrophoresis. The proteins were transferred onto a nitrocellulose membrane, blocked in 5% non-fat milk or BSA in TBS/Tween-20 buffer (20 mM Tris, 136 mM NaCl, 0.1% Tween-20, 50 mM NaF, and 5 mM Na3VO4) and incubated overnight in the corresponding antibody diluted in 5% non-fat milk or 5% BSA in TBS/Tween-20 buffer at 4 °C. After incubation for 1 h with a HRP-linked secondary antibody at room temperature, the membranes were developed using Luminata Classico/Crescendo Western HRP Substrates (Merck Millipore, Darmstadt, Germany), visualized in a ChemiDoc^{TM} System (Bio-Rad, Hercules, CA, USA) and quantified using Image Lab Software (Bio-Rad, Hercules, CA, USA). The protein level was normalized to β-actin as a housekeeping protein (Spolcova et al., 2015).

Binding studies of prolactin-releasing peptide CHO-K1 cells overexpressing GPR10 receptor (Perkin Elmer, Waltham, MA, USA) were grown following the manufacturer's instruction. Competitive binding experiments were performed according to Motulsky and Neubig (Motulsky and Neubig, 2002). CHO-K1 cells were incubated with 0.03 nM ^{125}I-human PrRP31 and 10^{-11}–10^{-5} M non-radioactive lipidized analog of PrRP31 in competitive

binding experiments on 24-well plates incubated for 60min at 25°C. Non-specific binding was determined using 10^{-5}M PrRP31 (Maletinska et al., 2015).

Acute food intake was tested in lean overnight (17h) fasted C57BL/6 mice. For the experiment mice were SC injected with saline or lipidized PrRP analogs at a dose 5mg/kg (all dissolved in saline) (n = 6-8 animals per group). Fifteen minutes after injection, the mice were given weighed food pellets which were subsequently weighed every 30min for at least 6h (Maletinska et al., 2015).

Long-term treatment with lipidized prolactin-releasing peptide or liraglutide

MSG mice were SC injected for 14 days twice per day at 8:00 a.m. and 18:00 p.m. with saline, palm-PrRP31 at a dose 5 mg/kg, liraglutide at a dose 0.2 mg/kg of body weight, both peptide were dissolved in saline; saline-treated group was used as a control group (Spolcova et al. 2015).

Thy-Tau22 mice, 3 - 4 per cage, were infused for 2 months with palm^{11}-PrRP31 at a dose 5 mg/kg of body weight per day dissolved in phosphate-buffered saline (PBS)/5% Tween 80 pH 6 (vehicle), using SC Alzet® osmotic pumps (Alzet, Cupertino, CA, USA). Control Thy-Tau22 mice as well as WT mice were infused with vehicle. Spatial memory was tested before the beginning of the treatment with palm^{11}-PrRP31, and after 2 months of treatment using Y-maze following the protocol described in the article of Belarbi et al (Belarbi et al., 2011). The time spent in every arm was measured using software created in Development Workshops of IOCB AS CR, Prague where also the Y-maze system was constructed.

Statistical analyses The data are presented as the means ± SEM, and were analyzed in Graph-Pad Software (San Diego, CA, USA) using a two-way analysis of variance (ANOVA), followed by a Bonferroni post-hoc test, or using an one-way ANOVA, followed by a Dunnett's post-hoc test, or a Student’s t-test, as stated in the Figure and Table legends. $P < 0.05$ was considered statistically significant.

4 RESULTS

Metabolic parameters and hyper-phosphorylation of Tau protein in pre-diabetic rodent models

fa/fa rats The results obtained from experiments performed in fa/fa rats presented in following chapters were published in BMC Neuroscience (Spolcova et al., 2014).

Due to non-functional leptin receptor fa/fa rats developed sever obesity; the body weight was significantly higher in fa/fa rats of both ages (young 386 ± 14 g, old 683 ± 48 g) compared to the age-matched controls (young 257 ± 14 g, old 457 ± 21 g). The extreme levels of hyperleptinemia in fa/fa rats resulted from both the excess of adipose tissue and lack of leptin receptor; significantly increased level of leptin in blood was observed already in young fa/fa rats (36.72 ± 5.20 ng/ml) compared to their-age matched controls (2.02 ± 1.23 ng/ml), and was more pronounced in old fa/fa rat (88.66 ± 32.71 ng/ml; old controls 6.33 ± 1.72 ng/ml). Obesity was accompanied by hyperinsulinemia; significant hyperinsulinemia in fa/fa rats was represented by extreme insulin levels that reached 12-fold in young animals (controls 0.50 ± 0.24 ng/ml, fa/fa 6.26 ± 2.14 ng/ml) and 9-fold in old animals (controls 1.43 ± 0.38 ng/ml, fa/fa 12.96 ± 4.50 ng/ml) compared to age-matched controls. However, the level of glucose rest unchanged in fa/fa rats of both ages (young 6.27 ± 0.63 mmol/l, old 6.80 ± 0.51 mmol/l) compared to their age-matched controls (young 6.00 ± 0.42 mmol/l, old 6.38 ± 0.43 mmol/l).

The effect of fa/fa genotype, and ageing on the activation of GSK-3β, one of the most important kinase implicated in Tau hyper-phosphorylation, and consequent hyper-phosphorylation of Tau was examined in hippocampi of fa/fa rats and their age-matched controls using the method of WB. Both, the ageing and fa/fa genotype, caused the significant lowering of phosphorylation of GSK-3β at the inhibition epitope Ser9 (Fig. 1A). The phosphorylation of Ser396 was significantly increased in fa/fa rats, compared to their controls, also the significant increase in phosphorylation was observed due to ageing (Fig. 1B). Higher phosphorylation of Thr231 was observed in fa/fa rats already at the young age, however, there was no effect of ageing on phosphorylation of this epitope (Fig. 1C).

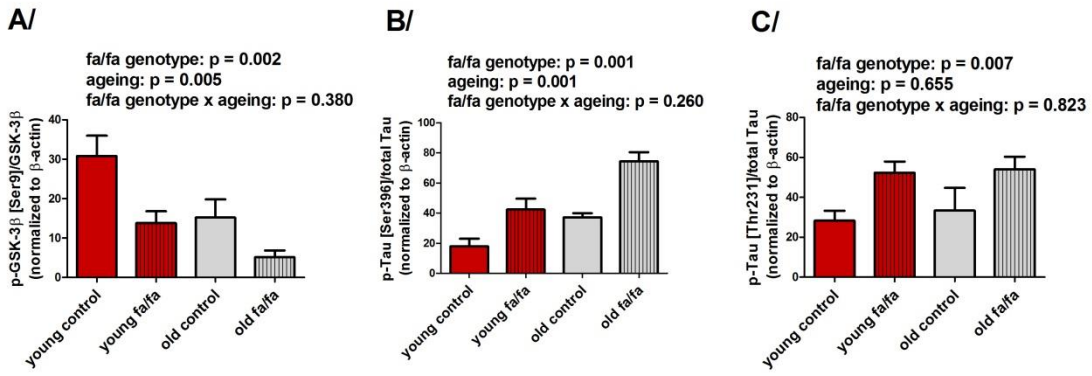

Fig. 1 Phosphorylation of GSK-3β and Tau protein in hippocampi of 12-week, and 33-week-old fa/fa rats and their age-matched control

Phosphorylation of GSK-3β and Tau protein was determined by method of western blot (WB). Data are mean ± SEM, n = 6 animals per group. Statistical analysis was calculated by 2-way ANOVA with Bonferroni post-hoc test.

MSG mice The results from experiments with MSG mice were published in Journal of Alzhiemer's disease (Spolcova et al., 2015).

For the study, male MSG mice were used at the age of 2 and 6 months, as were NMRI control mice. The MSG treatment of newborn male NMRI mice resulted in significantly increased amount of WAT in MSG mice of both ages (2 month-old 12.04 ± 0.63 % of body weight, 6-month-old 11.46 ± 0.48 % of body weight) mice compared to age-matched controls (2-month-old 4.54 ± 0.47 % of body weight, 6-month-old 6.88 ± 0.47 % of body weight). However, the body weight of the MSG-obese mice did not significantly differ, either in 2-month-old (controls 40.29 ± 0.94 g, MSG 42.50 ± 0.59 g) or in 6-month-old animals (controls 53.73 ± 1.84 g, MSG 57.18 ± 1.26 g). An increased WAT weight and significant increase in leptin blood level was observed in MSG mice of both ages (2-month-old 27.38 ± 4.14 ng/ml, 6-month-old 18.11 ± 2.91 ng/ml), compared to their age-matched controls (2-month-old 2.07 ± 0.43 ng/ml, 6-month-old 4.03 ± 1.55 ng/ml). Fasting glucose was not significantly increased in MSG mice either in 2-month-old (8.55 ± 0.34 mmol/l) or in 6-month-old (5.83 ± 0.45 mmol/l) compared to their age matched controls (2-month-old 6.63 ± 0.46 mmol/l, 6-month-old 6.43 ± 0.52 mmol/l), whereas the blood insulin levels were significantly higher in MSG mice, both 2-month-old (3.48 ± 0.57 ng/ml) and 6-month-old (3.64 ± 0.99 ng/ml), compared to their controls (2-month-old 0.96 ± 0.15 ng/ml, 6-month-old 0.83 ± 0.27 ng/ml). Thus, MSG mice exhibit obesity manifested by WAT accumulation and higher levels of leptin, and they

are in a pre-diabetic state demonstrated by an increased insulin level, but nearly normal glucose level.

By the method of Western blot using specific antibodies, phosphorylation at Ser9 of GSK-3β, and phosphorylation of Tau protein at epitopes Ser396 and Thr231, which are directly phosphorylated by GSK-3β, were determined in hippocampus. Significant decreased phosphorylation at inhibitory Ser9 of GSK-3β leading to higher kinase activity was observed in 6-month-old control animals (Fig. 2A). Significantly increased phosphorylation of Tau protein at epitopes Ser396 (Fig. 2B) and Thr231 (Fig. 2C) was observed in hippocampi of 6-month-old MSG mice.

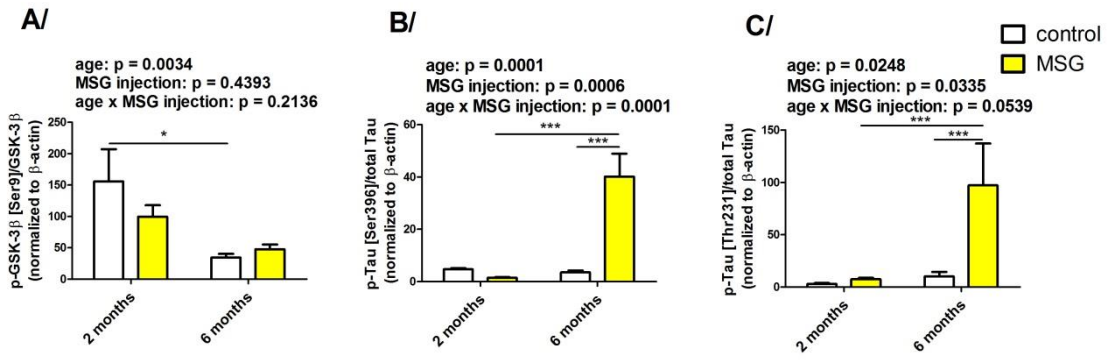

Fig. 2 Phosphorylation of GSK-3β at Ser9, and Tau protein at epitopes Ser396 and Thr231 in hippocampi of 2- and 6-month-old MSG obese mice and their control

*Phosphorylation of GSK-3β, and of Tau protein at different epitopes was determined by method of western blot (WB). Data are mean ± SEM, n = 10 animals per group. Statistical analysis was calculated by 2-way ANOVA with Bonferroni post-hoc test. Significance is * P < 0.05 and *** P < 0.001.*

Testing of lipidized analogs of PrRP31 on affinity to the receptor and anorexigenic effect

The results were published in International Journal of Obesity (Maletinska et al., 2015).

New lipidized analogs were designed and synthesized at the IOCB AS CR in Prague. Structure of natural PrRP31 (SRAHQHSMETRTPDINPAWYTGRGIRPVGRF-NH_2) was modified; lipidized PrRP31 analogs were modified with norleucine (Nle) to avoid oxidation of the original Met. The affinity of new lipidized analogs of PrRP31 was tested in binding studies. PrRP analogs with palmitic acid bound at N-terminus of the peptide (palm-PrRP31), or at Lys^{11} throug γ-glutamic acid as a linker (palm^{11}-PrRP31), as well as natural PrRP31, showed high binding affinity in competitive binding experiments with human ^{125}I-PrRP31 to CHO cells overexpressing the human PrRP receptor GPR10. K_i values were in a nanomolar range; K_i of PrRP31 was 3.91 ± 0.21 nM, palm-PrRP31 2.94 ± 0.33 nM and palm^{11}-PrRP31

7.96 ± 1.47 nM. It means that lipidization of its peptide chain did not affect the affinity of PrRP to GPR10 receptor.

Further, food intake after acute peripheral SC administration of palmitoylated analogs of PrRP31 was measured in lean fasted C57Bl/6 mice. As shown in Fig. 3 the significant food intake lowering was observed after administration of palmitoylated analogs of PrRP31, even 7 hours after the administration, showing that palmitoylated analogs are able to cross BBB and act in the hypothalamic nuclei regulating food intake where the GPR10 receptors are presented.

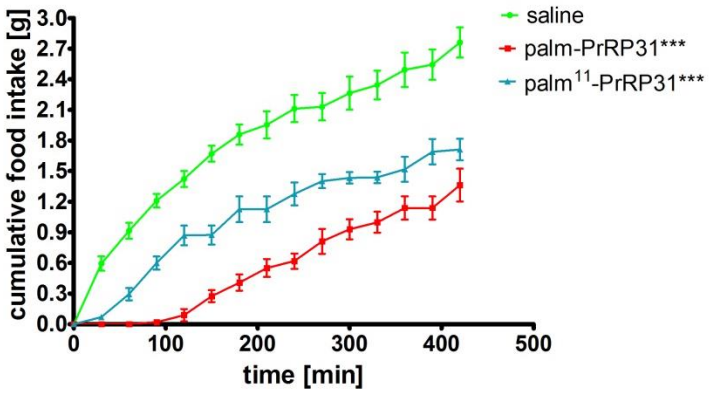

Fig. 3 Food intake after the peripheral administration of palmitoylated analogs of PrRP31

*Food intake was measured in lean fasted C57Bl/6 mice after SC injection with palmitoylated analogs of PrRP31 at a dose 5 mg/kg. Data are mean ± SEM (n = 6 – 8 animals per group). Statistical analysis was calculated by 1-way ANOVA with Dunnett post-hoc test. Significance is *** P < 0.001.*

Long-term treatment with palmitoylated PrRP31 or liraglutide

6-month-old MSG mice were injected SC for 14 days either with liraglutide at a dose 0.2 mg/kg, or with our palm-PrRP31 at a dose 5 mg/kg, the doses significantly lowering food intake. Control mice were injected with saline. After the treatment, the body weight (saline 53.08 ± 2.22 g, palm-PrRP31 50.07 ± 1.68 g, liraglutide 48.23 ± 1.70 g), the amount of WAT (saline 6.37 ± 0.84 % of body weight, palm-PrRP31 4.91 ± 0.43 % of body weight, liraglutide 5.38 ± 0.67 % of body weight), and leptin level (saline 23.10 ± 3.85 ng/ml, palm-PrRP31 21.20 ± 2.62 ng/ml, liraglutide 18.16 ± 2.41 ng/ml) tended to decrease. The fasting glucose (saline 6.48 ± 0.42 mmol/l, palm-PrRP31 6.49 ± 0.32 mmol/l, liraglutide 5.63 ± 0.34 mmol/l) and plasma insulin levels (saline 1.53 ± 0.20 ng/ml, palm-PrRP31 1.57 ± 0.13 ng/ml, liraglutide 1.91 ± 0.27 ng/ml) did not significantly differ compared to the saline treated group.

14-day-long treatment with palm-PrRP31 or with liraglutide resulted in increased phosphorylation of GSK-3β at inhibitory Ser9 in hippocampi of 6-month-old MSG mice (Fig.

4A), thus decreased kinase activity. Consequently, significantly attenuated phosphorylation at Tau epitopes Ser396 (Fig. 4B), Thr212 (Fig. 4C), and Thr231 (Fig. 4D) was observed.

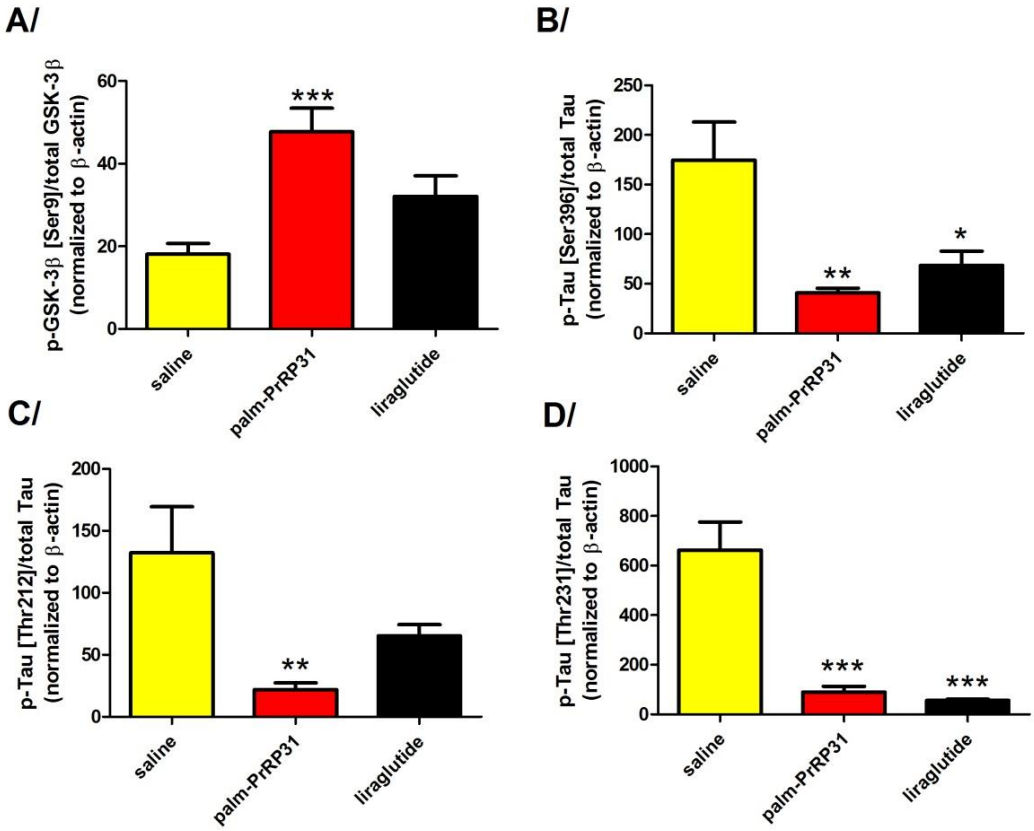

Fig. 4 Western blot analysis of phosphorylation of GSK-3β and Tau phosphorylation at different epitopes in hippocampi of 6-month-old MSG obese mice after 14-day-long intervention with palm-PrRP31 or liraglutide

*Phosphorylation of Tau after treatment with anorexigenic peptides was determined by method of western blot (WB). Data are mean ± SEM, n = 10 animals per group. Statistical analysis was calculated by 1-way ANOVA with Dunnett post-hoc test. Significance is * P < 0.05, **P < 0.01 and *** P < 0.001.*

Thy-Tau22 mice Spatial memory was tested using Y-maze test, before starting the treatment, and after the 2-month-long treatment with palm^{11}-PrRP31. Compared to WT control mice the spatial memory was impaired in Thy-Tau22 mice before the beginning of experiment, see Fig. 5A. The impaired memory is manifested by significantly decreased time spent in the new arm of the Y-maze, and increased time spent in the start arm and other arm. After 2-month-long treatment the memory was improved in Thy-Tau22 mice treated with palm^{11}-PrRP31 compared to vehicle-treated Thy-Tau22mice (Fig. 5B); compared to vehicle-treated Thy-Tau22 mice, mice treated with palm^{11}-PrRP31 spent significantly increased time in the new arm of the Y-maze.

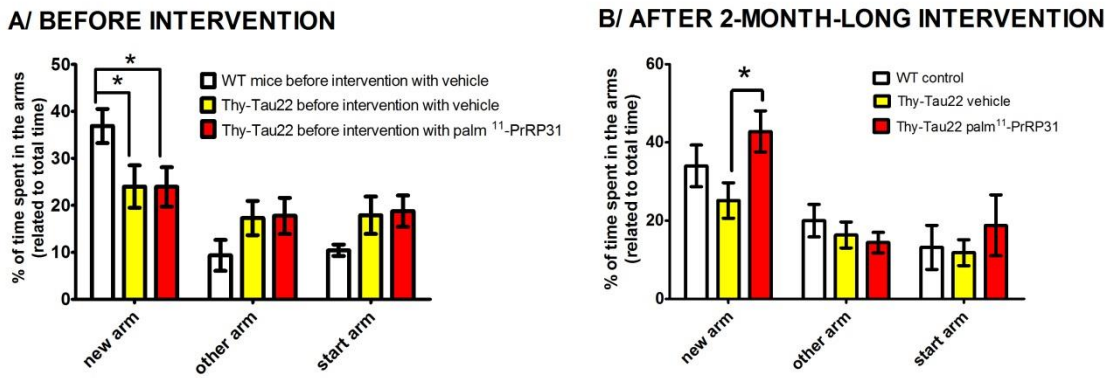

Fig. 5 Spatial memory testing of Thy-Tau22 mice and their age-matched WT controls in Y-maze: A/before the beginning of experiment and B/after treatment with palm^{11}-PrRP31

*Spatial memory was tested in Y-maze described in Materials and Methods. Data are mean ± SEM, n = 12 animals per group. Statistical analysis was calculated by Student's t-test. Significance is * P < 0.05.*

After the treatment the metabolic parameters were measured. Compared to Thy-Tau22-vehicle group, the WT-vehicle controls had significantly increased body weight (WT vehicle 23.86 ± 0.36 g, Thy-Tau22 vehicle 21.47 ± 0.29 g); the body weight of palm^{11}-PrRP31-treated Thy-Tau22 mice stayed unchanged (21.84 ± 0.24 g). No significant effect on WAT amount (WT vehicle 2.17 ± 0.13 % of body weight, Thy-Tau22 vehicle 2.48 ± 0.13 % of body weight, Thy-Tau22 palm^{11}-PrRP31 2.76 ± 0.19 % of body weight), level of leptin (WT vehicle 0.59 ± 0.13 ng/ml, Thy-Tau22 vehicle 0.38 ± 0.04 ng/ml, Thy-Tau22 palm^{11}-PrRP31 0.52 ± 0.05 ng/ml), insulin (WT vehicle 0.20 ± 0.03 ng/ml, Thy-Tau22 vehicle 0.19 ± 0.01 ng/ml, Thy-Tau22 palm^{11}-PrRP31 0.21 ± 0.02 ng/ml) or glucose (WT vehicle 4.55 ± 0.32 mmol/l, Thy-Tau22 vehicle 3.88 ± 0.38 mmol/l, Thy-Tau22 palm^{11}-PrRP31 4.72 ± 0.39 mmol/l) was observed.

WB analysis was performed in hippocampi of vehicle-treated Thy-Tau22 mice, and mice treated with palm^{11}-PrRP31. After 2-month-long treatment, the significant attenuation of Tau phosphorylation Thr231 (Fig. 6A), and at Ser396 and Ser404 (AD2 antibody) (Fig. 6B) was observed in mice treated with palm^{11}-PrRP31 compared to vehicle treated group. However, no statistically significant effect on phosphorylation of GSK-3β at its inhibitory Ser9 (Fig. 6C), MAPK/ERK1/2 (Fig. 6D) or JNK (Fig. 6E), several kinases implicated in Tau phosphorylation, was observed.

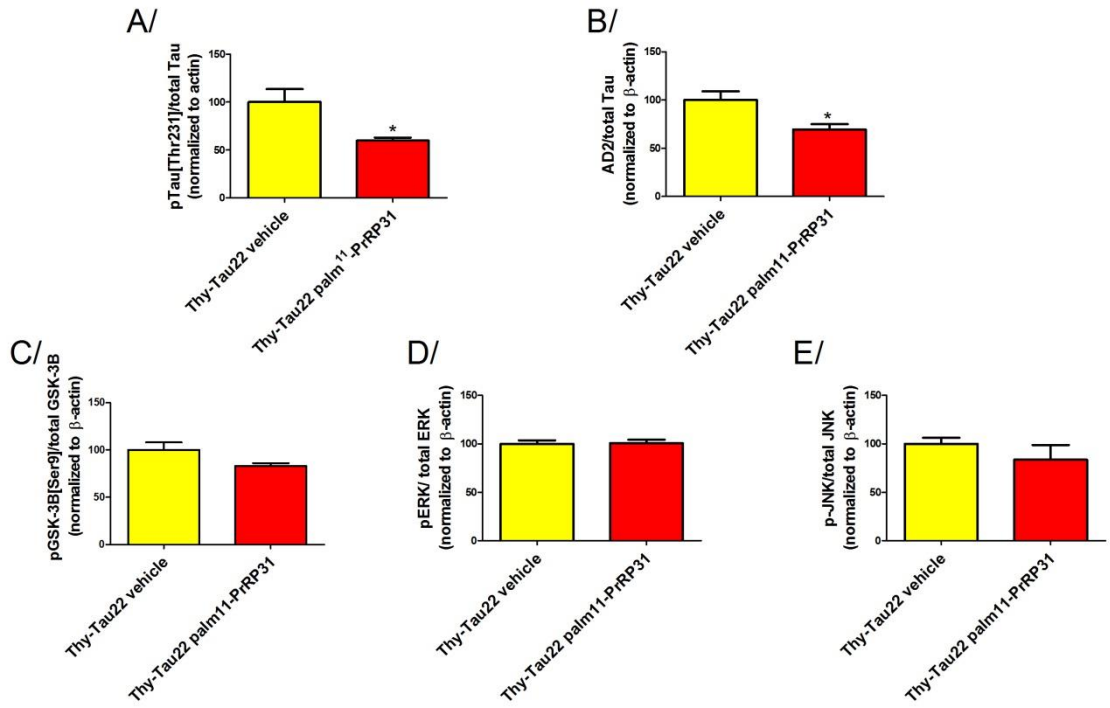

Fig. 6 Western blot analysis of Tau phosphorylation at different epitopes in hippocampi of 9-month-old Thy-Tau22 mice after 2-month-long intervention with palm^{11}-PrRP31

*Phosphorylation of Tau phosphorylation after the palm^{11}-PrRP31 treatment was determined by method of western blot (WB). Data are mean ± SEM, n = 12 animals per group. Statistical analysis was calculated by Student t-test. Significance is * P < 0.05.*

5 DISCUSSION

In the first part of my thesis, the effect of pre-diabetes, a state preceding T2DM when insulin resistance is presented but the blood glucose level remains normal, on Tau hyper-phosphorylation was determined. Both used rodent models of obesity and pre-diabetes, the fa/fa rats with mutation in leptin receptor, and MSG mice with obesity caused by lesions in Arc, developed obesity, hyperleptinemia, and hyperinsulinemia, similarly as db/db mice, another model of obesity and T2DM (Kim et al., 2009). However, their glucose level stayed unchanged even at a higher age; 33 weeks in fa/fa rats and 6 months in MSG mice. According to Suzanne de la Monte, peripheral insulin resistance results in central insulin resistance (de la Monte, 2009). GSK-3β, a potent kinase implicated in Tau hyper-phosphorylation, is a part of insulin signaling cascade; insulin dysfunction causes impaired activation of implicated kinases, and leads to a decreased phosphorylation of GSK-3β at inhibitory Ser9, thus to its activation. Its increased kinase activity was shown to be the cause of Tau hyper-phosphorylation in different T2DM rodent models (Schubert et al., 2004; Yang et al., 2013), as well as in human study, where the hyper-phosphorylation of Tau protein was more pronounced in patients with combined T2DM and AD than those with one disease mentioned (Liu et al., 2011). Also in the hippocampi of fa/fa rats and MSG mice, the significantly decreased phosphorylation at Ser9 of GSK-3β was observed. Disturbances in glucose or glucosamine availability are considered as one of the possible mechanisms of Tau hyper-phosphorylation, since it was discovered that decreased GlcNAcylation of Tau protein correlated with increased phosphorylation at serines and threonines (Gong et al., 2006; Liu et al., 2009a; Liu et al., 2009b). Despite this hypothesis, in both used insulin resistant normoglycaemic rodent models, fa/fa rats, even only 12-week-old, and MSG mice at the age of 6 months, an increased phosphorylation of Tau protein was observed at Ser396 and Thr231. This supports a hypothesis that insulin resistance rather than increased plasma glucose levels, which is a sign of T2DM, leads to Tau hyper-phosphorylation. Beside the insulin signaling, leptin dysregulation was suggested to have a detrimental impact on AD development (Bonda et al., 2014), and long-lasting hyperleptinemia was shown to accelerate Tau hyper-phosphorylation in hippocampi (Koga et al., 2014). Both obese fa/fa rats and MSG obese mice exhibit hyperleptinemia. Moreover, recently obesity rather than insulin resistance was proposed to exacerbate detrimental effect of DIO on the development of Tau pathology (Leboucher et al., 2013). But the precise role of leptin signaling and obesity on the Tau pathology, and AD development should be elucidated.

In the second part of my thesis, the new lipidized analogs of PrRP were characterized. PrRP is centrally acting anorexigenic neuropeptide. Anorexigenic neuropeptides are promising anti-obesity agents due to their specificity, low toxicity, and few side effects. However, their application as an efficient treatment is problematic since they have low stability, and most of them are unable to cross BBB. It was shown in our previous study (Maletinska et al., 2011) that for the proper PrRP biological function the C-terminal part of the molecule is crucial. Therefore, the lipidization with palmitic acid was performed at the N-terminal part of PrRP31 molecule, namely at Ser^{1} or Lys^{11}, where γ-glutamic acid was used as a linker. Palmitoylated analogs showed high binding affinity to PrRP receptor, and moreover significantly lowered food intake in lean over-night fasted mice after peripheral administration. This anorexigenic effect indicates that peripherally administered palmitoylated PrRP analogs are probably able to cross the BBB, and act centrally. Considering the fact that central effect of anti-T2DM drug liraglutide, whose beneficial effect on AD hallmarks was proven in several mouse models of AD and/or T2DM (Han et al., 2013; Holscher, 2014; McClean et al., 2011; McClean and Holscher, 2014; Porter et al., 2010), is exclusively anorexigenic, brings us to assumption that anorexigenic neuropeptide PrRP could have similar neuroprotective properties.

In the third part of my PhD thesis, the effect of anorexigenic liraglutide, and PrRP31 analogs on the development of Tau pathology was examined. In 6-month-old MSG mice, the 14-day-long treatment either with liraglutide, or with palm-PrRP31 resulted in increased phosphorylation of GSK-3β at inhibitory Ser9, thus decrease in its kinase activity. A similar trend was observed in rats with T2DM where the 4-week-long liraglutide treatment also reduced GSK-3β activity (Yang et al., 2013). Probably due to decreased activity of GSK-3β, the treatment with both peptides attenuated phosphorylation of Tau at epitopes Thr231 and Ser396 in hippocampi; palm-PrRP31 also attenuated phosphorylation at Thr212. The effect of $palm^{11}$-PrRP31 was observed in Thy-Tau22 mice, the model of AD-like Tau pathology. After 2-month-long treatment with $palm^{11}$-PrRP31, significantly attenuated Tau phosphorylation at epitopes Thr231, Ser396 and Ser404 was observed. Moreover, Thy Tau22 mice were shown to have impaired spatial memory (Van der Jeugd et al., 2013); the treatment with $palm^{11}$-PrRP31 resulted in enhanced spatial memory tested in Y-maze. However, the mechanism of action of $palm^{11}$-PrRP31 remains to be clarified, because no significant differences were observed in phosphorylation state of the most important kinases implicated in Tau phosphorylation, such as GSK-3β, JNK, or MAPK/ERK1/2.

6 CONCLUSIONS

- In both models of obesity and pre-diabetes, fa/fa rats and MSG mice, examined in my PhD thesis, decreased phosphorylation of the insulin signaling cascade was observed, resulting in decreased phosphorylation, and thus increased kinase activity, of GSK-3β at inhibitory Ser9. Consequently, increased phosphorylation of Tau protein at different epitopes, such as Ser396, or Thr231, the epitopes phosphorylated by GSK-3β, were detected. Considering the normoglycemic state of the animals and pre-diabetes with increased insulin levels, we can conclude that insulin resistance, rather than increased glucose blood level, is associated with hyper-phosphorylation of Tau protein, the hallmark of AD.
- We designed and tested lipidized prolactin releasing peptide 31 (PrRP31) analogs. Lipidization of PrRP31 did not influence its affinity to its receptor in competitive binding experiments. Moreover, the food intake after acute peripheral SC injection of lipidized analogs of PrRP31 was significantly decreased which indicates that lipidization enabled PrRP31 to cross the blood-brain barrier, and thus to exert its central anorexigenic effect. These findings suggest the possible utilization of lipidized analogs of PrRP31 as effective anti-obesity therapeutics.
- Fourteen-day-long treatment of 6-months-old MSG obese mice with palmitoylated analog of PrRP31 and liraglutide resulted in enhanced activation of the central insulin signaling cascade, decreased activation of GSK-3β, as well as MAPK/ERK1/2 and JNK, other potent kinases implicated in Tau hyper-phosphorylation, and attenuated phosphorylation of Tau protein at different epitopes. Attenuated phosphorylation of the Tau protein was also observed in the Thy-Tau22 mice, model of AD-like Tau pathology, after 2-month-long treatment with another potent analog, palm^{11}-PrRP31. The exact mechanism of PrRP31 action has to be elucidated; nevertheless, our findings indicate that anorexigenic compounds have potential neuroprotective properties and could be used in the future as a possible treatment of neurological disorders.

7 REFERENCES

Alzheimer's, A., 2014. 2014 Alzheimer's disease facts and figures. Alzheimers Dement. 10, e47-92.

Atzori, C., et al., 2001. Activation of the JNK/p38 pathway occurs in diseases characterized by tau protein pathology and is related to tau phosphorylation but not to apoptosis. J Neuropathol Exp Neurol. 60, 1190-7.

Banks, W.A., Owen, J.B., Erickson, M.A., 2012. Insulin in the brain: there and back again. Pharmacol Ther. 136, 82-93.

Belarbi, K., et al., 2011. Beneficial effects of exercise in a transgenic mouse model of Alzheimer's disease-like Tau pathology. Neurobiol Dis. 43, 486-94.

Bonda, D.J., et al., 2014. Dysregulation of leptin signaling in Alzheimer disease: evidence for neuronal leptin resistance. J Neurochem. 128, 162-72.

Buettner, R., Scholmerich, J., Bollheimer, L.C., 2007. High-fat diets: modeling the metabolic disorders of human obesity in rodents. Obesity (Silver Spring). 15, 798-808.

de la Monte, S.M., Wands, J.R., 2008. Alzheimer's disease is type 3 diabetes-evidence reviewed. J Diabetes Sci Technol. 2, 1101-13.

de la Monte, S.M., 2009. Insulin resistance and Alzheimer's disease. BMB Rep. 42, 475-81.

Doble, B.W., Woodgett, J.R., 2003. GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci. 116, 1175-86.

Drucker, D.J., Nauck, M.A., 2006. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet. 368, 1696-705.

Ekinci, F.J., Shea, T.B., 1999. Hyperactivation of mitogen-activated protein kinase increases phospho-tau immunoreactivity within human neuroblastoma: additive and synergistic influence of alteration of additional kinase activities. Cell Mol Neurobiol. 19, 249-60.

Gong, C.X., et al., 2006. Impaired brain glucose metabolism leads to Alzheimer neurofibrillary degeneration through a decrease in tau O-GlcNAcylation. J Alzheimers Dis. 9, 1-12.

Gotz, J., Ittner, L.M., Lim, Y.A., 2009. Common features between diabetes mellitus and Alzheimer's disease. Cell Mol Life Sci. 66, 1321-5.

Han, W.N., et al., 2013. Liraglutide protects against amyloid-beta protein-induced impairment of spatial learning and memory in rats. Neurobiol Aging. 34, 576-88.

Holcomb, L., et al., 1998. Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. Nat Med. 4, 97-100.

Holscher, C., 2014. Central effects of GLP-1: new opportunities for treatments of neurodegenerative diseases. J Endocrinol. 221, T31-41.

Irizarry, M.C., et al., 1997. APPSw transgenic mice develop age-related A beta deposits and neuropil abnormalities, but no neuronal loss in CA1. J Neuropathol Exp Neurol. 56, 965-73.

Jicha, G.A., et al., 1999. cAMP-dependent protein kinase phosphorylations on tau in Alzheimer's disease. J Neurosci. 19, 7486-94.

Kickstein, E., et al., 2010. Biguanide metformin acts on tau phosphorylation via mTOR/protein phosphatase 2A (PP2A) signaling. Proc Natl Acad Sci U S A. 107, 21830-5.

Kim, B., et al., 2009. Increased tau phosphorylation and cleavage in mouse models of type 1 and type 2 diabetes. Endocrinology. 150, 5294-301.

Koga, S., et al., 2014. Immunohistochemical analysis of tau phosphorylation and astroglial activation with enhanced leptin receptor expression in diet-induced obesity mouse hippocampus. Neurosci Lett. 571, 11-6.

Leboucher, A., et al., 2013. Detrimental effects of diet-induced obesity on tau pathology are independent of insulin resistance in tau transgenic mice. Diabetes. 62, 1681-8.

Li, L., Holscher, C., 2007. Common pathological processes in Alzheimer disease and type 2 diabetes: a review. Brain Res Rev. 56, 384-402.

Liu, F., et al., 2009a. Reduced O-GlcNAcylation links lower brain glucose metabolism and tau pathology in Alzheimer's disease. Brain. 132, 1820-32.

Liu, Y., et al., 2009b. Brain glucose transporters, O-GlcNAcylation and phosphorylation of tau in diabetes and Alzheimer's disease. J Neurochem. 111, 242-9.

Liu, Y., et al., 2011. Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes. J Pathol. 225, 54-62.

Maletinska, L., et al., 2011. Biological properties of prolactin-releasing peptide analogs with a modified aromatic ring of a C-terminal phenylalanine amide. Peptides. 32, 1887-92.

Maletinska, L., et al., 2015. Novel lipidized analogs of prolactin-releasing peptide have prolonged half-lives and exert anti-obesity effects after peripheral administration. Int J Obes (Lond).

Maletínská, L., et al., 2006. Effect of cholecystokinin on feeding is attenuated in monosodium glutamate obese mice. Regul Pept. 136**,** 58-63.

Martin, L., et al., 2013. Tau protein phosphatases in Alzheimer's disease: the leading role of PP2A. Ageing Res Rev. 12**,** 39-49.

McClean, P.L., et al., 2011. The diabetes drug liraglutide prevents degenerative processes in a mouse model of Alzheimer's disease. J Neurosci. 31**,** 6587-94.

McClean, P.L., Holscher, C., 2014. Liraglutide can reverse memory impairment, synaptic loss and reduce plaque load in aged APP/PS1 mice, a model of Alzheimer's disease. Neuropharmacology. 76 Pt A**,** 57-67.

Motulsky, H., Neubig, R., 2002. Analyzing radioligand binding data. Curr Protoc Neurosci. Chapter 7**,** Unit 7 5.

Olney, J.W., 1969. Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. Science. 164**,** 719-21.

Porter, D.W., et al., 2010. Four weeks administration of Liraglutide improves memory and learning as well as glycaemic control in mice with high fat dietary-induced obesity and insulin resistance. Diabetes Obes Metab. 12**,** 891-9.

Schindowski, K., et al., 2006. Alzheimer's disease-like tau neuropathology leads to memory deficits and loss of functional synapses in a novel mutated tau transgenic mouse without any motor deficits. Am J Pathol. 169**,** 599-616.

Schrijvers, E.M., et al., 2010. Insulin metabolism and the risk of Alzheimer disease: the Rotterdam Study. Neurology. 75**,** 1982-7.

Schubert, M., et al., 2004. Role for neuronal insulin resistance in neurodegenerative diseases. Proc Natl Acad Sci U S A. 101**,** 3100-5.

Spolcova, A., et al., 2014. Deficient hippocampal insulin signaling and augmented Tau phosphorylation is related to obesity- and age-induced peripheral insulin resistance: a study in Zucker rats. BMC Neurosci. 15**,** 111.

Spolcova, A., et al., 2015. Anorexigenic lipopeptides ameliorate central insulin signaling and attenuate tau phosphorylation in hippocampi of mice with monosodium glutamate-induced obesity. J Alzheimers Dis. 45**,** 823-35.

Steen, E., et al., 2005. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? J Alzheimers Dis. 7**,** 63-80.

Stranahan, A.M., et al., 2008. Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. Nat Neurosci. 11**,** 309-17.

Takashima, A., 2006. GSK-3 is essential in the pathogenesis of Alzheimer's disease. J Alzheimers Dis. 9**,** 309-17.

Takeda, S., et al., 2010. Diabetes-accelerated memory dysfunction via cerebrovascular inflammation and Abeta deposition in an Alzheimer mouse model with diabetes. Proc Natl Acad Sci U S A. 107**,** 7036-41.

Van der Jeugd, A., et al., 2013. Progressive age-related cognitive decline in tau mice. J Alzheimers Dis. 37**,** 777-88.

Wang, J., et al., 2012. Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation. Cell Stem Cell. 11**,** 23-35.

Winocur, G., et al., 2005. Memory impairment in obese Zucker rats: an investigation of cognitive function in an animal model of insulin resistance and obesity. Behav Neurosci. 119**,** 1389-95.

Yang, Y., et al., 2013. Subcutaneous administration of liraglutide ameliorates Alzheimer-associated tau hyperphosphorylation in rats with type 2 diabetes. J Alzheimers Dis. 37**,** 637-48.

LIST OF MY PUBLICATIONS

Publications related to PhD thesis:

1. **Spolcova, A.**, Mikulaskova, B., Krskova, K., Gajdosechova, L., Zorad, S., Olszanecki, R., Suski, M., Bujak-Gizycka, B., Zelezna, B., Maletinska, L. (2014). Deficient hippocampal insulin signaling and augmented Tau phosphorylation is related to obesity- and age-induced peripheral insulin resistance: a study in Zucker rats. *BMC Neuroscience* 15**,** 111. **IF = 2.85**
2. Maletinska, L., Nagelova, V., Ticha, A., Zemenova, J., Pirnik, Z., Holubova, M., **Spolcova, A.**, Mikulaskova, B., Blechova, M., Sykora, D., Lacinova, Z., Haluzik, M., Zelezna, B., Kunes, J. (2015). Novel lipidized analogs of prolactin-releasing peptide have prolonged half-lives and exert anti-obesity effects after peripheral administration. *International Journal of Obesity* (Lond). **IF = 5.38**
3. **Spolcova, A.**, Mikulaskova, B., Holubova, M., Nagelova, V., Pirnik, Z., Zemenova, J., Haluzik, M., Zelezna, B., Galas, M.C., Maletinska, L. (2015). Anorexigenic lipopeptides ameliorate central insulin signaling and attenuate tau phosphorylation in hippocampi of mice with monosodium glutamate-induced obesity. *Journal of Alzheimers Disease.* 45**,** 823-35. **IF = 3.61**

Publications not related to PhD thesis:

1. Maixnerova, J., **Spolcova, A.**, Pychova, M., Blechova, M., Elbert, T., Rezacova, M., Zelezna, B., Maletinska, L. (2011). Characterization of prolactin-releasing peptide: binding, signaling and hormone secretion in rodent pituitary cell lines endogenously expressing its receptor. *Peptides* 32**,** 811-7. **IF = 2.61**
2. Maletinska, L., **Spolcova, A.**, Maixnerova, J., Blechova, M., Zelezna, B. (2011). Biological properties of prolactin-releasing peptide analogs with a modified aromatic ring of a C-terminal phenylalanine amide. *Peptides* 32**,** 1887-92. **IF = 2.61**
3. Maletinska, L., Matyskova, R., Maixnerova, J., Sykora, D., Pychova, M., **Spolcova, A.**, Blechova, M., Drapalova, J., Lacinova, Z., Haluzik, M., Zelezna, B. (2011). The Peptidic GHS-R antagonist [D-Lys(3)]GHRP-6 markedly improves adiposity and related metabolic abnormalities in a mouse model of postmenopausal obesity. *Molecular and Cellular Endocrinology* 343**,** 55-62. **IF = 4.24**
4. Maletinska, L., Ticha, A., Nagelova, V., **Spolcova, A.**, Blechova, M., Elbert, T., Zelezna, B., (2013). Neuropeptide FF analog RF9 is not an antagonist of NPFF receptor and decreases food intake in mice after its central and peripheral administration. *Brain Research* 1498**,** 33-40. **IF = 2.82**
5. Holubova, M., **Spolcova, A.**, Demianova, Z., Sykora, D., Fehrentz, J.A., Martinez, J., Stofkova, A., Jurcovicova, J., Drapalova, J., Lacinova, Z., Haluzik, M., Zelezna, B., Maletinska, L. (2013). Ghrelin agonist JMV 1843 increases food intake, body weight and expression of orexigenic neuropeptides in mice. *Physiological Research* 62**,** 435-44. **IF = 1.48**
6. **Spolcova, A.**, Holubova, M., Mikulaskova, B., Nagelova, V., Stofkova, A., Lacinova, Z., Jurcovicova, J., Haluzik, M., Maletinska, L., Zelezna, B. (2014). Changes in FGF21 serum concentrations and liver mRNA expression in an experimental model of complete lipodystrophy and insulin-resistant diabetes. *Physiol Research* 63**,** 483-90. **IF = 1.48**
7. Gajdosechova, L., Krskova, K., Segarra, A.B., **Spolcova, A.**, Suski, M., Olszanecki, R., Zorad, S. (2014). Hypooxytocinaemia in obese Zucker rats relates to oxytocin degradation in liver and adipose tissue. *Journal of Endocrinology* 220**,** 333-43. **IF = 3.58**

Patents:

PCT/US13/50146. Lipidated peptides as anti-obesity agents. Patent pending to IOCB AS CR (Maletínská, L., Železná, B., Blechová, M., **Špolcová, A.**). Applied the 11th July 2013.

PCT/CZ2015/000047 Lipidated ppetides as neuroprotective agents. Patent pending to IOCB AS CR (Maletínská, L., Železná, B., Blechová, M., **Špolcová, A.**, Mikulášková, B., Kuneš, J.). Applied the 20th May 2015