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Novel Lipidized Analog of Prolactin-Releasing Peptide Improves Memory Impairment and Attenuates Hyperphosphorylation of Tau Protein in a Mouse Model of Tauopathy

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Abstract. Obesity and type 2 diabetes mellitus (T2DM) were characterized as risk factors for Alzheimer's disease (AD) development. Subsequently, T2DM drugs, such as liraglutide, were proven to be neuroprotective compounds attenuating levels of amyloid deposits, and tau hyperphosphorylation, both hallmarks of AD. The central anorexigenic effects of liraglutide inspired us to examine the potential neuroprotective effects of palm^{11}-PrRP31, a strong anorexigenic analog with glucose-lowering properties, in THY-Tau22 mice overexpressing mutated human tau, a model of AD-like tau pathology. Seven-month-old THY-Tau22 mice were subcutaneously infused with palm^{11}-PrRP31 for 2 months. Spatial memory was tested before and after the treatment, using a Y-maze. At the end of the treatment, mice were sacrificed by decapitation and hippocampi were dissected and analyzed by immunoblotting with specific antibodies. Treatment with palm^{11}-PrRP31 resulted in significantly improved spatial memory. In the hippocampi of palm^{11}-PrRP31-treated THY-Tau22 mice, tau protein phosphorylation was attenuated at Thr231, Ser396, and Ser404, the epitopes linked to AD progression. The mechanism of this attenuation remains unclear, since the activation of those kinases most implicated in tau hyperphosphorylation, such as GSK-3β, JNK, or MAPK/ERK1/2, remained unchanged by palm^{11}-PrRP31 treatment. Furthermore, we observed a significant increase in the amount of postsynaptic density protein PSD95, and a non-significant increase of synaptophysin, both markers of increased synaptic plasticity, which could also result in improved spatial memory of THY-Tau22 mice treated with palm^{11}-PrRP31. Palm^{11}-PrRP31 seems to be a potential tool for the attenuation of neurodegenerative disorders in the brain. However, the exact mechanism of its action must be elucidated.

Keywords: Alzheimer's disease, palm^{11}-PrRP31, spatial memory, synaptic plasticity, tau hyperphosphorylation, THY-Tau22 mice

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the progressive development of memory deficits. AD is neuropathologically

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defined by the extracellular accumulation of amyloid-β (Aβ) peptides into amyloid plaques and intraneuronal fibrillar aggregates of hyperphosphorylated and abnormally phosphorylated tau proteins [1, 2]. Tau pathology is observed early in the brain stem and entorhinal cortex [3], and its progression through the cortex from the entorhinal cortex to the hippocampus and finally to the neocortex corresponds to the progression of the symptoms in AD [4–6].

Despite a large body of research on AD, currently, there is no sufficient treatment. Recently, agonists of glucagon-like peptide 1 (GLP-1) originally used as type 2 diabetes mellitus (T2DM) drugs, such as liraglutide, lixisenatide, or exenatide, showed neuroprotective properties in different AD mouse models. In transgenic APP/PS1 mice overexpressing amyloid-β protein precursor (AβPP) and presenilin-1 (PS1), liraglutide [7] and lixisenatide [8] reduced the amount of Aβ deposits, increased synaptic plasticity, neurogenesis, and enhanced memory, and exenatide improved spatial memory in the Morris water maze (MWM) [9]. In senescence-accelerated mouse prone 8 (SAMP8) mice, which have pathological tau phosphorylation [10], liraglutide enhanced spatial memory and increased the number of pyramidal neurons in the CA1 region of hippocampus. Moreover, in AD mouse models exhibiting tau hyperphosphorylation, where the hyperphosphorylation was induced by administration of $A\beta_{1\text{-}42}$ [11] or by administration of methylglyoxal [12], liraglutide improved spatial memory and decreased the tau phosphorylation through decreased kinase activity of glycogen synthase kinase 3β (GSK-3β) [11, 12]. In our previous study on monosodium glutamate (MSG) obese pre-diabetic mice, we also found that liraglutide increased activation of the insulin signaling cascade and decreased GSK-3β activity leading to reduced hippocampal tau hyperphosphorylation [13]. A similar effect on the activation of the insulin signaling cascade, and attenuation of tau hyperphosphorylation was observed using an analog of prolactin releasing peptide palmitoylated at the N-terminus ($palm^1$-PrRP) [13]. It seems that liraglutide and $palm^1$-PrRP have a similar mechanism of action and potential neuroprotective properties. Physiologically, PrRP is a neuropeptide implicated in regulation of food intake and energy balance [14]. However, natural PrRP is not able to cross the blood-brain barrier and if administered into the periphery, it shows no central effect. Palmitoylation enables PrRP to manifest its central effect after peripheral administration [15]. In the present study, we have evaluated the impact of our novel analog of PrRP palmitoylated in position 11 - $palm^{11}$-PrRP31 [16] with improved bioavailability, on the pathophysiological development in a mouse model of AD-like tau pathology, namely, the THY-Tau22 strain, which overexpresses human 4R tau with mutations G272V and P301S under a neuronal Thy1.2-promoter [17]. This mouse model displays progressive development of tau hyperphosphorylation/aggregation associated with a detrimental cognitive phenotype delayed learning from 3 months of age and reduced spatial memory at 10 months of age [18, 19].

MATERIALS AND METHODS

Animals

THY-Tau22 female mice and wild-type (WT) female littermate controls were obtained from Dr. Luc Buée (INSERM Laboratory UMR-S-1172, Lille) and were housed in the animal facility of the Institute of Physiology AS CR, Prague, Czech Republic with a 12-h light/dark cycle and temperature set at $23 \pm 2°C$. Mice were housed 3-4 per cage with free access to water and an Altromin diet (Altromin, Eastern Westphalia, Germany). All animal experiments followed the ethical guidelines for animal experiments and the Czech Republic Act No. 246/1992 and were approved by the Committee for Experiments with Laboratory Animals of the Academy of Sciences of the Czech Republic.

Study design and treatment of THY-Tau22 mice with $palm^{11}$-PrRP31

$Palm^{11}$-PrRP31, the palmitoylated analog of prolactin releasing peptide 31 (PrRP31), with the structure: SRTHRHSMEI K γ-E (N-palmitoyl) TPDINPAWYASRGIRPVGRF-NH2, was synthesized at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic (IOCB AS CR) as described by Pražienková et al. [16]. THY-Tau22 mice were infused from 7 months of age for 2 months with $palm^{11}$-PrRP31 (THY-Tau22 $palm^{11}$-PrRP31 group, $n = 10$ mice) at a dose of 5 mg/kg of body weight per day using subcutaneous (SC) Alzet® osmotic pumps (Alzet, Cupertino, CA, USA), which are certified to infuse 6 μl of solution daily. Control THY-Tau22 mice

(THY-Tau22 vehicle group, $n = 10$ mice) and WT littermate mice (WT vehicle group, $n = 10$ mice) were infused with phosphate-buffered saline (PBS)/5% Tween 80 pH 6, which was used as a solvent for palm^{11}-PrRP31. Alzet® osmotic pumps were implanted subcutaneously following short-term ether anesthesia, and changed after one month of the experiment. The body weight and food intake were measured three times per week. At the end of the experimental procedure, overnight fasted mice were sacrificed by decapitation. The trunk blood was collected, glucose was measured using a glucometer Glucocard (Arkray, Tokyo, Japan), and plasma was separated and stored at –20°C. The white adipose tissue (WAT, subcutaneous and visceral fat) was dissected and weighed. Adiposity was estimated as the ratio of the total adipose tissue weight to the total body weight. The brains were maintained on ice to prevent tissue degradation. From each group of mice, 7 brains were used for immunoblotting, and 3 brains were used for immunohistochemistry, as described below. Concentration of serum leptin was measured using the ELISA kit (Millipore, St. Charles, MI, USA) and plasma insulin was determined by RIA kits (Millipore, St. Charles, MI, USA) following the manufacturer's instructions. The concentration of palm^{11}-PrRP31 infused by Alzet® osmotic pumps in blood plasma collected from a tail vein was determined by PrRP(1-31) EIA high-sensitivity kit (Peninsula Laboratories International, Inc., San Carlos, CA, USA).

Y-maze test

Short-term spatial memory was tested before the beginning of the treatment with palm^{11}-PrRP31 and before the completion of the 2 months of treatment as previously described for this model ($n = 6$ mice per group) [20, 21]. The Y-maze consisted of three identical arms (28 cm long, 8 cm width, and walls 15 cm high). On the walls of the maze, clues were placed to help the mouse to orient in the maze. In the first session, mice were placed through the start arm for 5 min into the maze where only two arms, the start and other arm, were opened. Then, the mice were taken out of the maze for 2 min; meanwhile the floor of the maze was cleaned with 70% ethanol to remove the odor, and the third new arm was open. In the second session, the mice were placed through the start arm into the maze with all arms open for 1 min. The time spent in every arm was measured using the software created in the Development Workshops of IOCB AS CR, Prague where the Y-maze system was also constructed.

Immunoblotting

The method of immunoblotting was performed as described in Špolcová et al. [13]. Briefly, homogenized hippocampi ($n = 7$ mice per group) were diluted in Laemmli sample buffer to a final concentration 1 μg/μl and resolved using 10% SDS-PAGE electrophoretic Mini-PROTEAN Tetra Cell module (Bio-Rad, Hercules, CA, USA) at a constant voltage of 200 V. After proteins were transferred onto nitrocellulose membranes, the membranes were blocked in 5% non-fat milk or 5% BSA, according to the manufacturer's instructions for each antibody, for 1 h at room temperature, and incubated overnight with primary antibody at 4°C. A list of antibodies used and their appropriate dilutions is shown in Table 1. Further, the membranes were incubated for 1 h at room temperature in an appropriate anti-mouse or anti-rabbit IgG, HRP-linked secondary antibody and developed using ECL solution Luminata Classico/Crescendo Western HRP Substrates (Merck Millipore, Darmstadt, Germany). Chemiluminescence was visualized in a ChemiDocTM System (Bio-Rad, Hercules, CA, USA) and quantified using Image Lab Software (Bio-Rad, Hercules, CA, USA). The exact protein level on each membrane was normalized to β-actin as an internal loading control.

Immunohistochemistry

Brains of mice used for immunohistochemistry ($n = 3$ mice per group) were fixed for 24 h in 4% paraformaldehyde and transferred to 70% ethanol. Brains were embedded in paraffin at the histology laboratory of the Faculty of Medicine, Lille, France (Laboratoire d'histologie, Faculté de Médecine, Lille, France). Five-micrometer thick paraffin-embedded sagittal brain slices were deparaffinized by washing three times in toluene, rehydrated in ethanol range (100, 95, and 70%) and unmasked by boiling for 10 min in citrate buffer pH 6 (3.75 mM acid citrate, 2.5 mM disodium phosphate). After 1 h of blocking in 1% horse serum in PBS buffer (Sigma-Aldrich, St. Louis, MO, USA), the slices were incubated overnight at 4°C with the appropriate antibody (AT180, ThermoScientific, Rockford, IL, USA; pSer396, ThermoScientific, Rockford, IL, USA; Tau 5, Invitrogen Grand Island, NY, USA) diluted in

Table 1
List of primary antibodies and their appropriate dilution used for immunoblotting

Antibody	Manufacturer	Immunoblotting dilution
AD2 rabbit pAb	Produced in Lille (INSERM Laboratory UMR-S-1172)	1 : 5 000 5% milk TBS/tween-20
AT8 mouse mAb	Thermo Scientific Rockford, IL, USA	1 : 1 000 5% BSA TBS/tween-20
Phospho-Akt (Ser473) rabbit mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 1 000 5% BSA TBS/tween-20
Phospho -Akt (Thr308) rabbit mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 1 000 5% BSA TBS/tween-20
Akt (pan) rabbit mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 1 000 5% BSA TBS/tween-20
Phospho -GSK-3β (Ser9) rabbit mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 1 000 5% BSA TBS/tween-20
GSK-3β rabbit mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 1 000 5% BSA TBS/tween-20
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mouse mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 2 000 5% milk TBS/tween-20
p44/42 MAPK (Erk1/2) mouse mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 2 000 5% milk TBS/tween-20
PP2A C subunit rabbit mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 1 000 5% BSA TBS/tween-20
Methyl-PP2A C subunit mouse mAb	Sigma, St. Louis, MO, USA	1 : 1 000 5% BSA TBS/tween-20
PSD95 rabbit pAb	Cell Signaling Technology, Beverly, MA, USA	1 : 1 000 5% BSA TBS/tween-20
Phospho-SAPK/JNK (Thr183/Tyr185) rabbit mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 1 000 5% BSA TBS/tween-20
SAPK/JNK rabbit mAb	Cell Signaling Technology, Beverly, MA, USA	1 : 1 000 5% BSA TBS/tween-20
Synaptophysin rabbit pAb	Santa Cruz Biotechnology, Dallas, TX, USA	1 : 1 000 5% milk TBS/tween-20
anti-Tau [pT231] rabbit pAb	Invitrogen Grand Island, NY, USA	1 : 10 000 5% BSA TBS/tween-20
Tau5 mouse mAb	Invitrogen Grand Island, NY, USA	1 : 10 000 5% milk TBS/tween-20
β-actin mouse mAb	Sigma, St. Louis, MO, USA	1 : 10 000 5% milk TBS/tween-20

PBS/0.2% Triton X-100 buffer. Then, the slices were incubated for 1 h with secondary antibody Alexa 488 (#A11001) or Alexa 568 (#A11011) (ThermoFisher Scientific Inc., Waltham, MA, USA). The nuclei were stained with Vectashield/DAPI (4,6-diamidino-2-phenylindole, Vector Laboratories, Burlingame, CA, USA). Images were acquired on a Zeiss confocal laser-scanning microscope LSM 710 using a 488 nm Argon laser and a 405 nm ultraviolet laser with the same laser intensities to compare the images at Lille 2 University (Plate-forme d'Imagerie Moléculaire et Cellulaire).

Statistical analyses

The data are presented as the means ± SEM and were analyzed with Graph-Pad Software (San Diego, CA, USA) using a two-way ANOVA followed by Bonferroni *post hoc* test, 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.

RESULTS

Palm^{11}-PrRP31 did not influence the metabolic parameters of THY-Tau22 mice or their age-matched WT controls

Throughout the 2-month treatment, the cumulative food intake (Fig. 1A) and body weights (Fig. 1B) were measured ($n = 10$ mice per group). As expected, the body weight of THY-Tau22 vehicle mice was significantly decreased compared to the WT vehicle treated group, in accordance with previous data [17, 22]. From day 49 of the treatment,

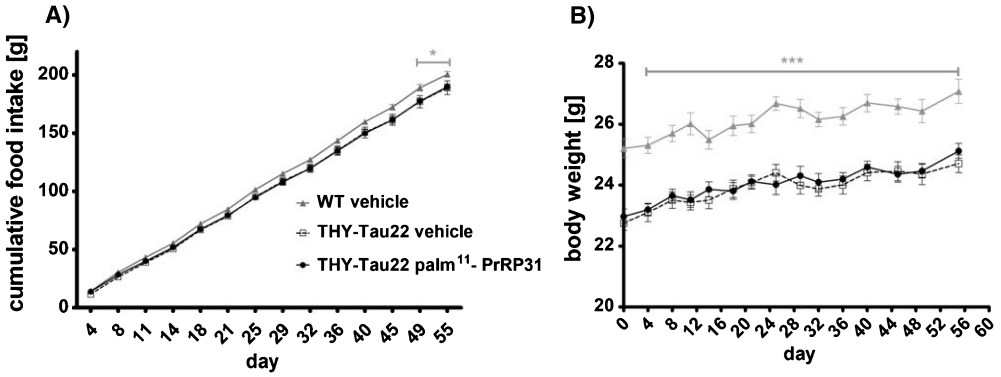

Fig. 1. Cumulative food intake (A) and body weight change (B) in THY-Tau22 mice and their WT controls during 2-month-long palm^{11}-PrRP31 treatment. Body weight change and cumulative food intake were measured three times per week during the 2-month-long treatment with palm^{11}-PrRP31 at a dose of 5 mg/kg/day, or with vehicle administered by SC Alzet® osmotic pump. Data are the means ± SEM, $n = 10$ mice per group. Statistical analysis was calculated by 2-way ANOVA with Bonferroni *post hoc* test. $^{*}p < 0.05$, and $^{***}p < 0.001$ versus THY-Tau22 vehicle.

Table 2
Metabolic parameters of THY-Tau22 mice after the treatment with palm^{11}-PrRP31 and their age-matched WT controls

mice	body weight [g]	white adipose tissue [% body weight]	leptin [ng/ml]	glucose [mmol/l]	insulin [ng/ml]
WT vehicle	$23.89 \pm 0.37^{***}$	2.16 ± 0.11	0.51 ± 0.12	4.59 ± 0.30	0.19 ± 0.04
THY-Tau22 vehicle	21.49 ± 0.25	2.47 ± 0.14	0.37 ± 0.04	3.94 ± 0.37	0.19 ± 0.01
THY-Tau22 palm^{11}-PrRP31	21.82 ± 0.28	2.77 ± 0.23	0.52 ± 0.07	4.72 ± 0.33	0.21 ± 0.02

Data are mean ± SEM ($n = 10$ animals per group). Data were analyzed by 1-way ANOVA with Dunnett's *post hoc* test. Significance is $^{***}p < 0.001$ compared to THY-Tau22 vehicle mice.

the cumulative food intake was significantly reduced in THY-Tau22 vehicle compared to WT vehicle. Palm^{11}-PrRP31 treatment of THY-Tau22 mice did not affect their body weight or cumulative food intake. At the end of the experiment, the metabolic parameters in blood plasma were measured. The results are shown in Table 2. The body weight of palm^{11}-PrRP31-treated THY-Tau22 mice remained unchanged. No significant effect of palm^{11}-PrRP31 on WAT amount, level of leptin, insulin, or glucose was observed. It is noteworthy that the concentration of palm^{11}-PrRP31 infused by Alzet® osmotic pumps in blood plasma was determined by EIA kit. The average concentration of palm^{11}-PrRP31 was 20.4 ± 7.5ng/ml.

Palm^{11}-PrRP31 improved short-term spatial memory in the Y-maze test

Short-term spatial memory was tested in THY-Tau22 mice and their WT controls using the Y-maze test before starting the treatment and after two months of SC treatment with palm^{11}-PrRP31 ($n = 6$ mice per group). As expected [20, 21, 23], memory was impaired in THY-Tau22 mice compared to WT control mice (Fig. 2A). Such impaired memory was manifested by a significantly shorter time spent in the new arm of the Y-maze and longer time spent in the start arm and other arm. Interestingly, following two months of treatment, memory was improved in THY-Tau22 mice treated with palm^{11}-PrRP31 compared to THY-Tau22 vehicle mice, as shown in Fig. 2B. Indeed, compared to THY-Tau22 vehicle mice, mice treated with palm^{11}-PrRP31 spent significantly more time in the new arm of the Y-maze.

Palm^{11}-PrRP31 mitigated tau hyperphosphorylation in the hippocampi of THY-Tau22 mice

Immunoblotting was performed using the hippocampi of THY-Tau22 mice treated with vehicle or palm^{11}-PrRP31 ($n = 7$ mice per group). A significant attenuation of tau phosphorylation at Ser396 and Ser404 (AD2 antibody) and Thr231 was observed in THY-Tau22 palm^{11}-PrRP31 mice compared to the control THY-Tau 22 mice (Fig. 3). Non-significant attenuation of tau phosphorylation was observed in

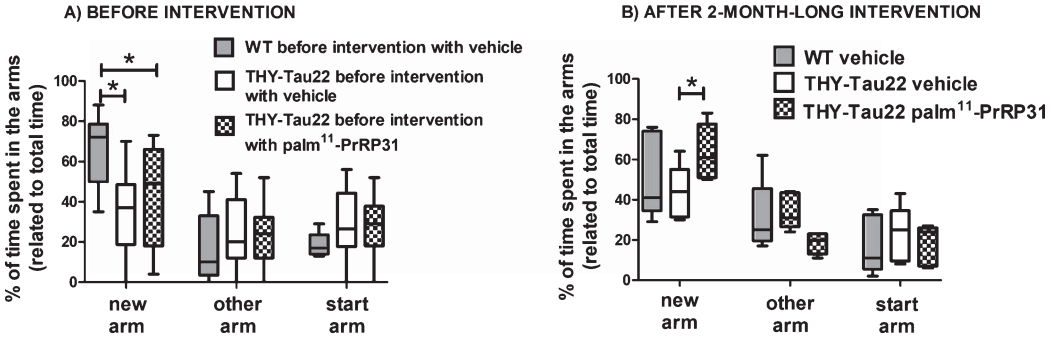

Fig. 2. Short-term spatial memory testing of THY-Tau22 mice and their age-matched WT controls in the Y-maze: before the beginning of the experiment (A) and after treatment with palm^{11}-PrRP31 (B). Short-term spatial memory was tested in Y-maze. In first session, two arms (start and other arm) were opened and mice spent in the maze 5 min. After 2 min-long relaxing time out of the maze, all three arms were opened and mice spent in the maze 1 min. Results show total time spend in the arms. Data are presented using Box and Whisker Plot, $n = 6$ mice per group. Statistical analysis was calculated by Student's t-test. $^{*}p < 0.05$ versus THY-Tau22 vehicle [A) WT before intervention with vehicle versus THY-Tau22 before interventions with vehicle $p = 0.0191$ and WT before intervention with vehicle versus THY-Tau22 before intervention with palm^{11}-PrRP31, $p = 0.0337$; B) THY-Tau22 vehicle versus THY-Tau22 palm^{11}-PrRP31 $p = 0.045$].

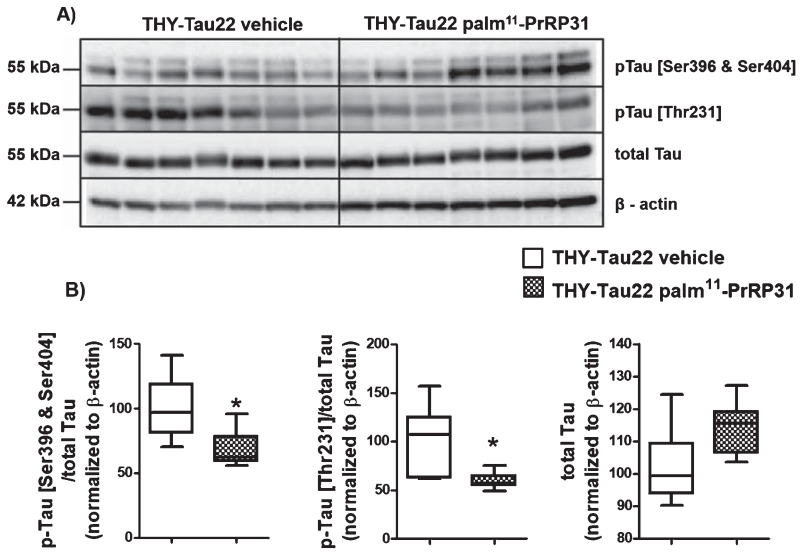

Fig. 3. Tau phosphorylation at different epitopes in hippocampi of THY-Tau22 mice after 2-month-long intervention with palm^{11}-PrRP31: immunoblots of tau hyperphosphorylation (A) and quantification of western blots (B). Phosphorylation of tau protein in hippocampi of THY-Tau22 mice treated for 2 months with vehicle or palm^{11}-PrRP31 was determined by immunoblotting using specific antibodies. Data are presented using Box and Whisker Plot, $n = 7$ mice per group. Statistical analysis was calculated by Student's t-test. $^{*}p < 0.05$ versus THY-Tau22 vehicle (pTau Ser396&Ser404: THY-Tau22 vehicle versus THY-Tau22 palm^{11}-PrRP31, $p = 0.0128$; pTau Thr231: THY-Tau22 vehicle versus THY-Tau22 palm^{11}-PrRP31, $p = 0.0145$).

THY-Tau22 palm^{11}-PrRP31 mice using AT8 antibody (Supplementary Figure 1). The immunohistochemical analysis of the hippocampi ($n = 3$ mice per group) supported the results of immunoblotting. In the CA1 region of the hippocampus, the phosphorylation of tau protein at Thr231 (AT180), as well as at Ser396, decreased after palm^{11}-PrRP31 treatment compared with that of the control group treated with vehicle (Fig. 4).

Treatment with palm^{11}-PrRP31 did not affect activation of tau kinases but slightly increased activation of PP2A

The tau phosphorylation state is controlled by a balance between kinases and phosphatases [2]. We then evaluated whether the activity of kinases targeting tau at Thr231 and Ser396/404 could be modulated by palm^{11}-PrRP31. In the hippocampi

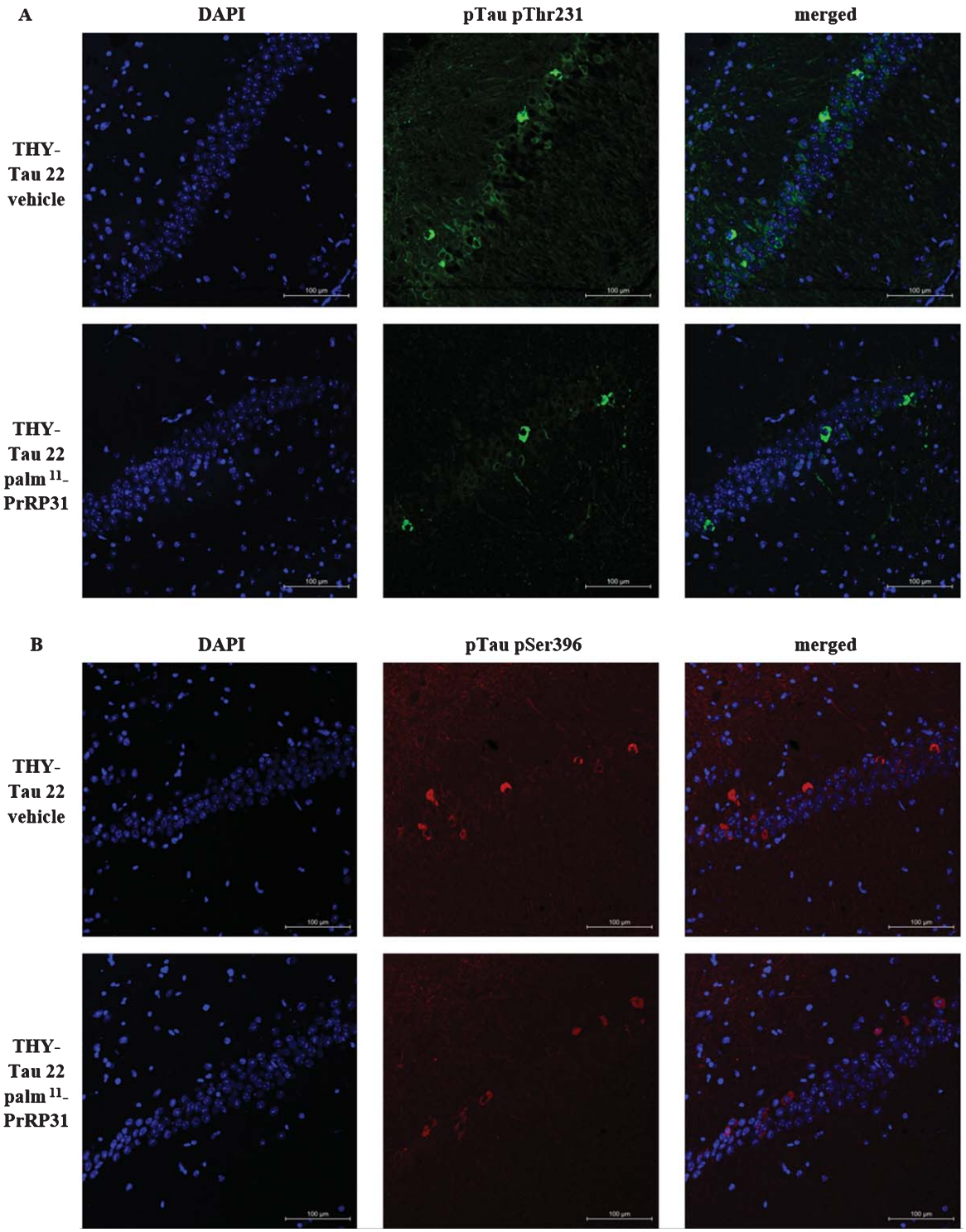

Fig. 4. Immunohistochemical staining of tau phosphorylation at different epitopes in the CA1 region of hippocampus of THY-Tau22 mice after 2-month-long intervention with palm^{11}-PrRP31. Phosphorylation of tau protein in CA1 region of hippocampus after 2-month-long treatment with palm^{11}-PrRP31 was determined by immunohistochemical staining of paraffin-embedded sagittal brain slices of THY-Tau22 mice using specific antibodies. $n = 3$.

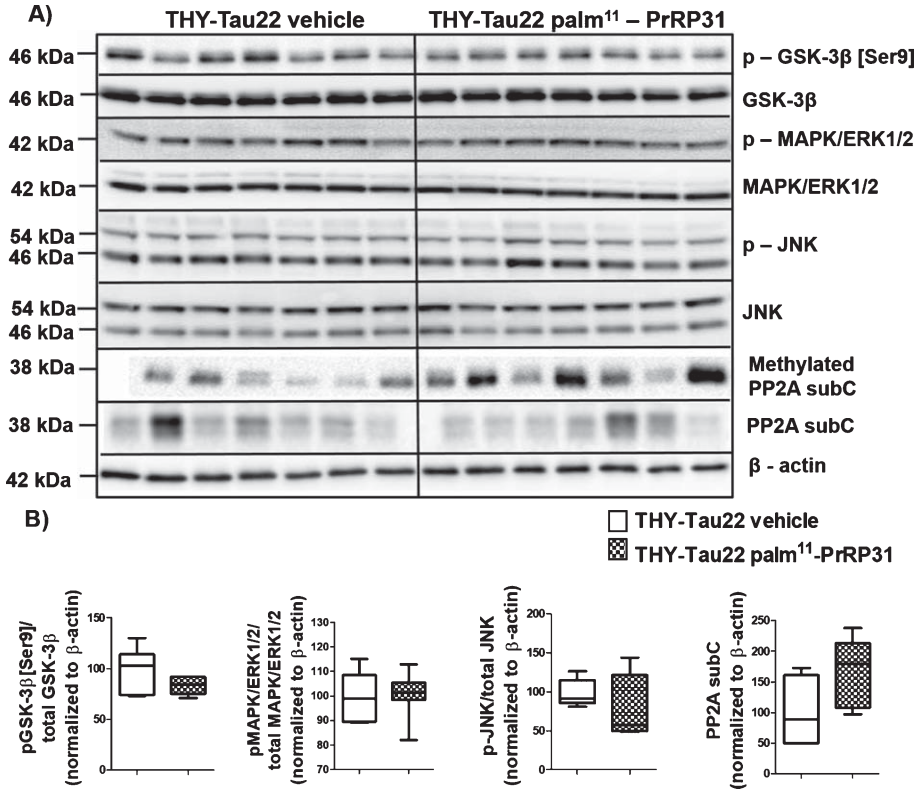

Fig. 5. Activation of kinases implicated in tau hyperphosphorylation and PP2A in hippocampi THY-Tau22 mice after 2-month-long intervention with palm^{11}-PrRP31: immunoblots of kinases and PP2A (A) and quantification of western blots (B). Phosphorylation of tau protein kinases in hippocampi of THY-Tau22 mice treated for 2 months with vehicle or palm^{11}-PrRP31 was determined by immunoblotting using specific antibodies. Data are presented using Box and Whisker Plot, $n = 6$ - 7 mice per group. Statistical analysis was calculated by Student's *t*-test.

of the THY-Tau22 palm^{11}-PrRP31 group compared to THY-Tau22 vehicle mice, there was no statistically significant effect on activation of GSK-3β at Ser9, MAPK/ERK1/2, or JNK, kinases implicated in tau phosphorylation, as shown in Fig. 5. We also evaluated the expression of the main tau phosphatase PP2A. Only the activation of PP2A at subunit C, which is the main tau phosphatase, was non-significantly increased.

Palm^{11}-PrRP31 increased level of markers of synaptic plasticity

Markers of synaptic plasticity were measured in hippocampi by immunoblotting and are shown in Fig. 6. In comparison with THY-Tau22 vehicle group, the level of phospho-Akt(Ser473) was significantly increased in the THY-Tau22 palm^{11}-PrRP31 group, while the level of phospho-Akt(Thr308) remained similar. In addition, the post-synaptic marker PSD95 was significantly increased in the THY-Tau22 palm^{11}-PrRP31 group, and the level of the pre-synaptic marker synaptophysin was non-significantly increased. The level of BDNF was not affected by palm^{11}-PrRP31 treatment (data not shown).

DISCUSSION

The mechanism of AD progression still remains unknown. Recently, metabolic disturbances, such as obesity [24] or T2DM [25], were connected to a higher incidence of dementia, mainly AD. These findings repurposed the role of neuropeptides with glucose-lowering properties as potentially neuroprotective compounds. In this study, PrRP analog palm^{11}-PrRP31, primarily with an anorexigenic and glucose-lowering properties was administered SC for 2 months to THY-Tau22 mice, a reliable model of AD-like tau pathology. It resulted in improved spatial memory in the Y-maze test,

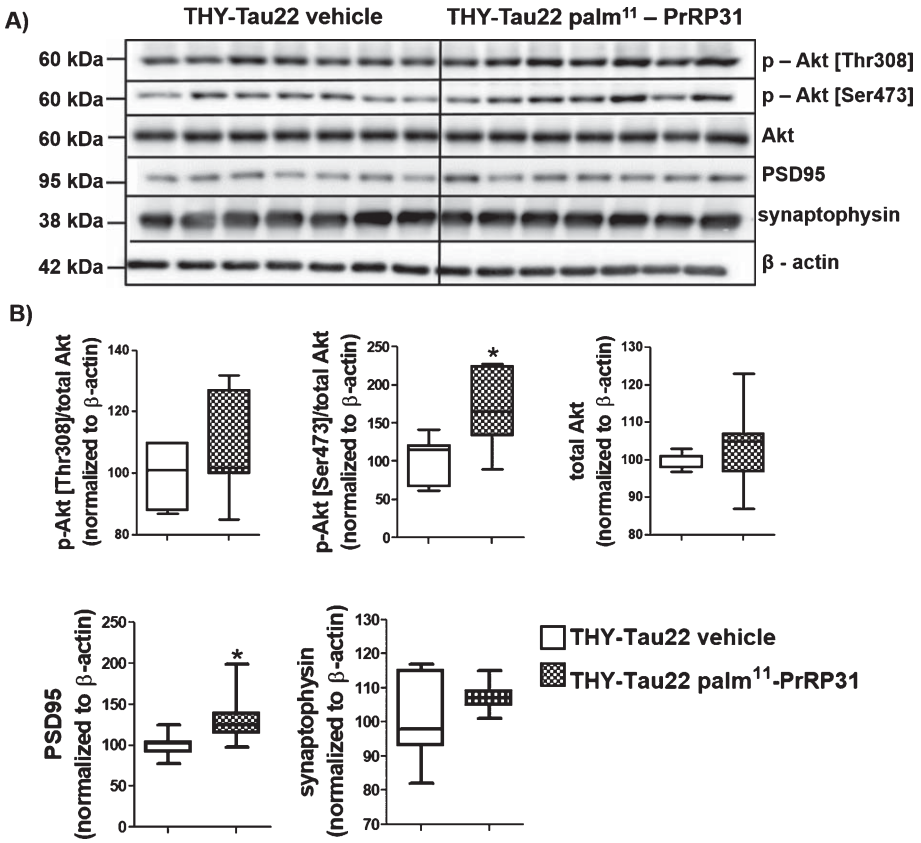

Fig. 6. Markers of synaptic plasticity and activation of Akt kinase in hippocampi of THY-Tau22 mice after 2-month-long intervention with palm^{11}-PrRP31: immunoblots of Akt and synaptic markers (A) and quantification of western blots (B). Markers of synaptic plasticity and Akt kinase activation in hippocampi of THY-Tau22 mice treated for 2 months with vehicle or palm^{11}-PrRP31 were determined by immublotting using specific antibodies. Data are presented using Box and Whisker Plot, $n = 7$ mice per group. Statistical analysis was calculated by Student's *t*-test. $^{*}p < 0.05$ versus THY-Tau22 vehicle (pAkt Ser473: THY-Tau22 vehicle versus THY-Tau22 palm^{11}-PrRP31, $p = 0.0124$; PSD 95: THY-Tau22 vehicle versus THY-Tau22 palm^{11}-PrRP31, $p = 0.0419$).

attenuated tau hyperphosphorylation at different epitopes involved in early stages of the etiology of AD, and increased hippocampal markers of synaptic plasticity. We previously demonstrated the beneficial effect of palm^{1}-PrRP31 on the attenuation of hyperphosphorylation of tau protein in the hippocampus of MSG obese mice [13]. In the present study, we evaluated the effects of a new, more stable and potent analog palm^{11}-PrRP with better bioavailability compared with palm^{1}-PrRP31 [16] in a model of AD-like tau pathology, namely, THY-Tau22 mice to evaluate the potential of this class of molecules toward the process of AD [17].

Palm^{11}-PrRP31 is primarily a peptide with anorexigenic properties that are manifested in obese mice where palmitoylated analogs of PrRP31 decreased the amount of white adipose tissue, thus the body weight, and decreased the food intake [15]. In lean mice, the anorexigenic effect of palm^{11}-PrRP31 is not manifested in long-term administration; it is similar to the effect of liraglutide, which affects only food intake and body weight in obese animals [26] but not in the lean ones [10]. We first determined the metabolic parameters of THY-Tau22 mice and their age-matched WT controls following chronic subcutaneous application. Metabolic parameters observed in THY-Tau22 mice versus littermate WT animals were in accordance with the data by Leboucher [22], with tau transgenic mice exhibiting body weight loss. Additionally, we did not observe any significant differences regarding WAT, glucose, leptin, or insulin levels in the THY-Tau22 treated with palm^{11}-PrRP31. The latter data were not surprising since there was no effect on the amount of WAT, because THY-Tau22 are lean mice and palm^{11}-PrRP31 was found to only decrease the level of fat and not the level of lean mass

[16], so there was no effect on the level of leptin, or other metabolic parameters. Even in the case of long-term administration of palm^{11}-PrRP31 to male lean WT littermates of THY-Tau22 mice no effect of palm^{11}-PrRP31 on food intake or body weight loss was observed (data not shown).

Moreover, THY-Tau22 mice are not known to have any metabolic disturbances, such as hyperleptinemia, hyperglycemia, or hyperinsulinemia [22]; therefore, the palm^{11}-PrRP31 treatment was not expected to influence the physiological levels of these metabolic parameters. Before beginning the interventions, the hippocampal short-term spatial memory of THY-Tau22 mice and their WT littermates was tested using the Y-maze. In previously published studies, impaired spatial memory in the MWM or Y-maze was observed in the THY-Tau22 mice [17, 19]. In our experiment, 7-month-old THY-Tau22 females also had significantly impaired working memory in the Y-maze compared to the control WT group. The 2 months of treatment with palm^{11}-PrRP31 resulted in spatial memory improvement compared to the THY-Tau22-vehicle group. Similar results were obtained in mouse models of AD where mice were treated with GLP-1 analogs with anorexigenic, and potential neuroprotective properties. Eight weeks of liraglutide administration resulted in memory improvement using the Y-maze test in a model of A$\beta_{1\text{-}42}$-induced tau phosphorylation [11], or using the MWM in an AD-like model of tau pathology, where hyperphosphorylation is induced by administration of methylglyoxal [12], as well as in APP/PS1/Tau triple transgenic mouse model combining tau pathology and Aβ pathology [27]. Exenatide, another potent GLP-1 analog, ameliorates spatial memory in the MWM in a rat AD model induced by intracerebroventricular injection of streptozotocin. The memory impairment could be partly caused by the increase of hippocampal tau hyperphosphorylation seen in treated THY-Tau22 mice. Indeed, the treatment with palm^{11}-PrRP31 resulted in significantly attenuated tau phosphorylation at several epitopes that are important in AD progression [28–30]. Namely, the phosphorylation was attenuated at Thr231 which is one of the first epitopes hyperphosphorylated in AD [31] and whose hyperphosphorylation impairs stabilization of microtubules [28]. Further, the attenuation was observed at Ser396 and 404, which are known to favor depolymerization of tubulin and impaired axonal transport [32]. However, the mechanism of action of palm^{11}-PrRP31 remains to be clarified because no significant differences were observed in the activity of the main kinases targeting the modified site of tau phosphorylation, such as GSK-3β, JNK, or MAPK/ERK1/2.

GLP-1 analogs were demonstrated to improve spatial memory in APP/PS1 mice by an increased hippocampal synaptic plasticity [7, 33]. Similarly, in our study, we observed the activation of Akt, manifested by phosphorylation at epitope Ser473, in THY-Tau22 mice treated with palm^{11}-PrRP31. Upregulation of Akt is necessary for PSD95 activation [34], which is further important for synaptic stability, thus for proper memory function [35], and moreover, it is important factor for synaptic plasticity [36, 37]. In accordance, THY-Tau22 palm^{11}-PrRP31 exhibited increased level of PSD95. The level of synaptophysin, which is increased significantly by liraglutide [38], was increased by palm^{11}-PrRP31 only non-significantly.

A decline in synaptic plasticity and transmission, thus memory impairment, could be partly caused by hyperphosphorylation of tau protein [39, 40]. Increased phosphorylation at epitope Ser396 destabilizes the function of tau protein as a scaffolding protein of a post synaptic density (PSD) complex involving PSD95, which further results in impaired synaptic plasticity [39]. Therefore, the attenuation of tau phosphorylation at Ser396 observed in THY-Tau22 mice treated with palm^{11}-PrRP31 could stabilize the PSD95-related complex, thus increase the markers of synaptic plasticity, and probably improve memory formation.

The level of palm^{11}-PrRP31 infused into blood plasma by Alzet osmotic minipumps was measured using EIA kit. Alzet osmotic minipumps were chosen for palm^{11}-PrRP31 administration to avoid the everyday manipulation, which has been associated with stress in animals. It seems that for more robust effects of palm^{11}-PrRP31, twice daily injections are necessary. In spite of a relatively low plasma concentration of palm^{11}-PrRP31, there was a significant effect on memory formation and attenuation of tau phosphorylation at several epitopes, thus it seems that even a low dose of palm^{11}-PrRP31 is effective, and thus palm^{11}-PrRP31 is potentially a neuroprotective agent. However, the exact molecular mechanism of action is not still clear and must be further studied.

In conclusion, our findings confirm the hypothesis that originally anorexigenic compounds improved spatial memory, attenuated tau hyperphosphorylation, and increased markers of synaptic plasticity; thus, they could be used in the future as a possible treatment for neurological disorders.

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SUPPLEMENTARY MATERIAL

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