Dipeptidyl peptidase 4 inhibitor improves brain insulin sensitivity, but fails to prevent cognitive impairment in orchiectomy obese rats

Hiranya Pintana^{1,2}, Wanpitak Pongkan^{1,2}, Wasana Pratchayasakul^{1,2},
Nipon Chattipakorn^{1,2} and Siriporn C Chattipakorn^{1,3}

¹Neurophysiology Unit, Faculty of Medicine, Cardiac Electrophysiology Research and Training Center,

²Department of Physiology, Faculty of Medicine, and ³Department of Oral Biology and Diagnostic Science, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200, Thailand

Correspondence
should be addressed
to S C Chattipakorn

Email
scchattipakorn@gmail.com or
siriporn.c@cmu.ac.th

Abstract

It is unclear whether the dipeptidyl peptidase 4 (DPP4) inhibitor can counteract brain insulin resistance, brain mitochondrial dysfunction, impairment of hippocampal synaptic plasticity and cognitive decline in testosterone-deprived obese rats. We hypothesized that DPP4 inhibitor vildagliptin improves cognitive function in testosterone-deprived obese rats by restoring brain insulin sensitivity, brain mitochondrial function and hippocampal synaptic plasticity. Thirty male Wistar rats received either a sham-operated (S, $n=6$) or bilateral orchiectomy (ORX, $n=24$). ORX rats were divided into two groups and fed with either a normal diet (ND (NDO)) or a high-fat diet (HFO) for 12 weeks. Then, ORX rats in each dietary group were divided into two subgroups ($n=6$ /subgroup) to receive either a vehicle or vildagliptin (3 mg/kg per day, p.o.) for 4 weeks. After treatment, cognitive function, metabolic parameters, brain insulin sensitivity, hippocampal synaptic plasticity and brain mitochondrial function were determined in each rat. We found that HFO rats exhibited peripheral and brain insulin resistance, brain mitochondrial dysfunction, impaired hippocampal synaptic plasticity and cognitive decline. NDO rats did not develop peripheral and brain insulin resistance. However, impaired hippocampal synaptic plasticity and cognitive decline occurred. Vildagliptin significantly improved peripheral insulin sensitivity, restored brain insulin sensitivity and decreased brain mitochondrial reactive oxygen species production in HFO rats. However, vildagliptin did not restore hippocampal synaptic plasticity and cognitive function in both NDO and HFO rats. These findings suggest that vildagliptin could not counteract the impairment of hippocampal synaptic plasticity and cognitive decline in testosterone-deprived subjects, despite its effects on improved peripheral and brain insulin sensitivity as well as brain mitochondrial function.

Key Words

- ▶ vildagliptin
- ▶ obese-insulin resistance
- ▶ testosterone deprivation
- ▶ brain mitochondrial function
- ▶ hippocampal synaptic plasticity
- ▶ cognitive function

Journal of Endocrinology
(2015) 226, M1–M11

Introduction

Long-term high-calorie diets cause obesity, leading to the development of insulin resistance, metabolic syndrome and type 2 diabetes mellitus (Pandolfi *et al.* 1994, Kahn & Flier 2000). Moreover, several studies demonstrated that insulin resistance was also associated with cognitive decline (Craft 2005, Greenwood & Winocur 2005, Stranahan *et al.* 2008). Our studies also showed that 12-weeks of a high-fat diet (HFD) consumption in male rats induced not only obese-insulin resistance, but also impaired brain insulin sensitivity, brain mitochondrial dysfunction and cognitive decline (Pintana *et al.* 2012, 2013, Pipatpiboon *et al.* 2013).

Testosterone is an androgenic steroid hormone, which plays an important role in the regulation of male reproduction and behaviors, as well as in the maintenance of glucose and lipid metabolism (Aydilek & Aksakal 2005, Christoffersen *et al.* 2010). Several studies have shown that testosterone replacement exerted beneficial effects on brain function, including the prevention of neuronal cell death, the balancing of brain oxidative stress and antioxidant activity, improvement of synaptic plasticity and the involvement of cognitive formation in animal models (Janowsky 2006, Meydan *et al.* 2010, Filova *et al.* 2013). Interestingly, growing evidence demonstrated that testosterone deficiency is associated with obese insulin-resistance in men (Haffner *et al.* 1994, Stellato *et al.* 2000, Grossmann *et al.* 2008, Shi *et al.* 2013). All of those findings indicate that testosterone-deprivation can lead to obesity and cognitive decline. Therefore, it is possible that testosterone deprivation combined with obesity could lead to the worsening of cognitive decline.

The dipeptidyl peptidase 4 (DPP4) inhibitor, vildagliptin, is an anti-diabetic drug, which exerts beneficial effects in diabetic and insulin resistant models, such as the regulation of blood glucose levels without hypoglycemic incidence (Burkey *et al.* 2005), restoring pancreatic β -cells as well as preserving pancreatic function and improving insulin sensitivity (Ahren *et al.* 2004). Our previous study demonstrated that vildagliptin improved not only peripheral insulin sensitivity, but also increased brain insulin sensitivity in obese-insulin resistant rats, as indicated by improvement in brain insulin receptor (IR) function (Pipatpiboon *et al.* 2013). In addition, vildagliptin have been shown to enhance hippocampal neurogenesis, leading to improved learning and memory in HFD-fed rodents (Pintana *et al.* 2013, Pipatpiboon *et al.* 2013, Gault *et al.* 2015). All of these findings indicate the beneficial effects of vildagliptin on peripheral insulin sensitivity,

brain insulin signaling and cognitive function in obese-insulin resistant conditions. However, the effects of vildagliptin on brain insulin sensitivity, brain mitochondrial function, hippocampal synaptic plasticity and cognitive function in testosterone-deprived obese rats have never been investigated. We tested the hypothesis that testosterone-deprived rats with or without obesity have impaired brain IR function, decreased brain insulin signaling, impaired brain mitochondrial function and impaired hippocampal synaptic plasticity, thus leading to cognitive decline. We also hypothesized that vildagliptin could restore these impairments in testosterone-deprived conditions with or without obesity.

Materials and methods

Animal models and experimental protocols

Thirty male Wistar rats, weighing 180–200 g (aged ~5–6 weeks old) obtained from the National Animal Center, Salaya Campus, Mahidol University, Bangkok, Thailand were used. All experiments were conducted in accordance with the approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. All animals were housed in environmentally controlled conditions ($25 \pm 0.5^\circ\text{C}$ and a 12 h light:12 h darkness cycle) and allowed to acclimate for 1 week. Rats were then divided into two groups as either control sham-operated (S, $n=6$) or bilateral orchiectomized (ORX, $n=24$) rats as described previously (Dulisch 1976). Rats in the sham-operated group were fed with a ND for 12 weeks and were the control group (NDS); after that rats received vehicle until the end of experiment. Rats in ORX group were randomized and fed with either a normal diet (ND: 19.77% E fat, NDO) or a HFD (59.28% E fat, HFO) for 12 weeks (Pratchayasakul *et al.* 2011). At the end of 12 weeks, the rats in each group were divided into two subgroups ($n=6$ /subgroup) and received either the vehicle (normal saline solution; 2 ml/kg per day) (NDO rats treated with vehicle (NDOV) and HFO rats treated with vehicle (HFOV)) or vildagliptin (Gulvus, Novartis, Bangkok, Thailand; 3 mg/kg per day) (NDO rats treated with vildagliptin (NDOVil) and HFO rats treated with vildagliptin (HFOVil)), via gavage feeding for 4 weeks (Burkey *et al.* 2005, Pintana *et al.* 2013). The cognitive function was subsequently determined by the Morris water maze (MWM) test. Blood samples were collected from a tail

vein at week 12, and week 15 for further plasma analysis. At the end of the experimental period, rats were deeply anesthetized with isoflurane and killed by decapitation. The brain of each rat was removed as quickly as possible after death and carefully sliced in preparation for extracellular recording (insulin-induced long-term depression (LTD) and hippocampal synaptic long-term potentiation (LTP)), immunoblot and brain mitochondrial functions. The study protocol is shown in Fig. 1.

Blood sample assays

Plasma glucose and cholesterol levels were determined via colorimetric assay (Biotech, Bangkok, Thailand). Plasma HDL and LDL levels were determined using a commercial colorimetric assay kit (Biovision, CA, USA). Plasma insulin levels were determined using the Sandwich ELISA Kit (Linco Research, St Charles, MO, USA). Peripheral insulin resistance was assessed using the homeostasis model assessment (HOMA), which was calculated by the

following formula: (fasting plasma insulin ($\mu\text{U/ml}$)) $\times$ (fasting plasma glucose (mmol/l))/22.5 as previously described (Pipatpiboon *et al.* 2012). Plasma testosterone levels were measured using the electrochemiluminescence immunoassay (ECLIA) technique (Roche Diagnostics).

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed as described previously (Pipatpiboon *et al.* 2013). Briefly, rats were starved overnight before the test and received 2 g/kg glucose solution via oral gavage. Blood samples were collected from the tail vein at 0, 15, 30, 60, 90 and 120 min after glucose administration. Areas under the curves (AUC) were calculated to evaluate glucose tolerance.

Brain slice preparation

At the end of experiment, animals were anesthetized with isoflurane and decapitated. The brain slice preparation was

Figure 1

The experimental protocol of the present study.

performed as described previously (Pratchayasakul *et al.* 2011, Pipatpiboon *et al.* 2012, 2013).

Extracellular recordings of hippocampal slices for insulin-induced LTD

An extracellular recording of hippocampal slices for insulin-induced LTD was performed as described in our previous studies (Pratchayasakul *et al.* 2011, Pipatpiboon *et al.* 2012, 2013). Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulating the Schaffer collateral–commissural pathway with a bipolar tungsten electrode, while recordings were taken from the stratum radiatum of the hippocampal CA1 region with micropipettes (3 Mohm) filled with 2 M NaCl. Stimulus frequency was 0.033 Hz. The stimulus intensity of fEPSPs was adjusted to 0.8–1.0 mV in amplitude. Hippocampal slices were perfused with aCSF (as baseline condition) for 10 min, and then perfused with aCSF plus 500 nM insulin (as insulin-induced LTD) for an additional 10 min. Afterward, the slices were perfused with aCSF for an additional 50 min and readings recorded. Data was filtered at 3 kHz, digitized at 10 kHz, and stored in a computer using pClamp 9.2 Software (Axon Instruments, Union City, CA, USA). The initial slope of the fEPSP was measured and plotted against time.

Extracellular recordings of hippocampal slices for synaptic LTP

The examination of electrical-induced hippocampal synaptic LTP was performed as described in our previous study (Sripetchwandee *et al.* 2014). fEPSPs were evoked by stimulating the Schaffer collateral–commissural pathway with a bipolar tungsten electrode, while the fEPSPs recordings were taken from the stratum radiatum of the hippocampal CA1 region with micropipettes (3 Mohm) filled with 2 M NaCl. LTP was induced by delivering high-frequency stimulation (HFS; four trains at 100 Hz; 0.5 s duration; 20 s interval). Experiments were performed for at least 40 min after HFS. The amount of potentiation was calculated at 40 min after tetanus. All data were filtered at 3 kHz, digitized at 10 kHz, and stored in a computer using pClamp 9.2 Software (Axon Instruments). The initial slopes of the fEPSPs were measured and plotted against time.

Immunoblotting for brain insulin signaling

To investigate the expression of IR phosphorylation (p-IR) and IRs, homogenate brain slices from each subgroup were used as described in our previous study (Pratchayasakul

et al. 2011, Pipatpiboon *et al.* 2012, 2013). IR tyrosine phosphorylation were electrophoresed and immunoblotted with rabbit antibodies for IR at tyrosine respectively. Examination of the level of IR protein expression was conducted with homogenates prepared from another set of four whole brain slices. These proteins were resolved by an immunoblot assay conducted with rabbit anti-IR at tyrosine phosphorylation (p-IR^{Tyr1162/1163}; 1:1000; sc-25103-R; Santa Cruz Biotechnology, Dallas, TX, USA), IR (1:1000; sc-711; Santa Cruz Biotechnology). For a loading control, immunoblotting for each membrane was incubated with anti- β -actin (1:4000; #4967; Cell Signaling Technology, Danvers, MA, USA). All membranes for visualizing the phosphorylation and the protein levels of IR expression were incubated with secondary goat anti-rabbit antibody conjugated with HRP (1:2000; #7074; Cell Signaling Technology). The protein bands were visualized on Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) using Amersham ECL Western Blot Detection Reagents (GE Healthcare). Band densities of phosphorylated IR were represented as a ratio of insulin stimulation (+) to no insulin stimulation (–) and were normalized to total IR. In addition, band intensities were quantified by Scion Image (Scion Corporation, National Institute of Health, Frederick, MD, USA) and the results were shown as average signal intensity (arbitrary) units.

Preparation of brain mitochondria

Brain mitochondria were isolated using the method described previously (Pipatpiboon *et al.* 2013). Mitochondrial protein concentration was measured using BCA assay (Pipatpiboon *et al.* 2013). Brain mitochondrial functions including brain mitochondrial reactive oxygen species (ROS), mitochondrial membrane potential change ($\Delta\Psi$ m) and mitochondrial swelling were also determined.

Brain mitochondrial ROS assay Brain mitochondrial ROS were measured using dichloro-hydrofluoresceindiacetate (DCFHDA) fluorescent dye. Brain mitochondria (0.4 mg/ml) were incubated with 2- μ M DCFHDA at 25 °C for 20 min. The fluorescence was determined using a fluorescent microplate reader at the excitation wavelength of 485 nm and emission wavelength of 530 nm (Pipatpiboon *et al.* 2013). Increased fluorescence intensity indicates increased ROS production.

Brain $\Delta\Psi$ m assay The change in $\Delta\Psi$ m was measured using the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolcarbocyanine iodide (JC-1).

The JC-1 monomer form (green fluorescence) was excited at a wavelength of 485 nm and detected at the emission wavelength of 590 nm. JC-1 aggregate form (red fluorescence) was excited at a wavelength of 485 nm and detected at the emission wavelength of 530 nm. Brain mitochondria (0.4 mg/ml) were incubated with JC-1 dye at 37 °C for 15 min. $\Delta\Psi_m$ changes were determined by measuring the fluorescence intensity using a fluorescent microplate reader. The change in $\Delta\Psi_m$ was calculated as the ratio of red to green fluorescent intensity (Pipatpiboon *et al.* 2013). Decreased red/green fluorescence intensity ratio indicates mitochondrial depolarization.

Brain mitochondrial swelling assay Brain mitochondrial swelling was determined by measuring the change in the absorbance of brain mitochondrial suspension. Brain mitochondria (0.4 mg/ml) were incubated in 2 ml of respiration buffer. The suspension was read at 540 nm using a microplate reader. Mitochondrial swelling was indicated by a decrease in the absorbance (Pipatpiboon *et al.* 2013).

Cognitive function test

The open-field test (OFT) was used to screen locomotor activity via counting the number of lines that the rats crossed during the test, as described in previous studies (Arakawa 2005, Pintana *et al.* 2013). The assessment of cognitive function was performed by using MWM with two assessments including the acquisition test which was carried out for 5 consecutive days, and the probe test which was performed on day 6 (Vorhees & Williams 2006, Pintana *et al.* 2013). Data analysis of MWM test was done manually from video tape recordings by the investigators, who were blinded to experimental groups.

Statistical analysis

Data were expressed as mean $\pm$ S.E.M. For all comparisons, the significance of the differences in peripheral

biochemical parameters was calculated using the Mann-Whitney *U* test. The comparisons in the percentage of insulin-induced LTD, the percentage of LTP, brain mitochondrial function, immunoblot, the OFTs and the MWM tests for the probe test among groups were performed using the one-way ANOVA test, followed by *post-hoc* Fisher's least significant difference (LSD) analysis. Comparisons among groups in MWM tests for the acquisition test were performed using a two-way ANOVA, followed by *post-hoc* LSD analysis. $P < 0.05$ was considered statistically significant.

Results

Obesity, but not testosterone deprivation, caused peripheral insulin resistance, which was attenuated by vildagliptin treatment

At the baseline levels (before ORX), the metabolic parameters were not significantly different between the sham-operated group and the ORX group (Table 1). After 12 weeks of diet regimens, HFO rats had significantly increased plasma insulin, HOMA index and total cholesterol levels, when compared with NDS and NDO rats. Plasma glucose levels were not significantly different among groups. However, the body weight of NDO had significantly decreased, when compared with NDS (Table 1). These findings suggested that long-term HFD consumption caused peripheral insulin resistance as indicated by hyperinsulinemia with euglycemia and increased HOMA index. However, testosterone deprivation alone did not impair peripheral insulin sensitivity.

After 4 weeks of vildagliptin treatment, testosterone deprivation by ORX was confirmed by reduced plasma testosterone levels (<0.025 ng/ml) in all ORX groups (NDOV, NDOVil, HFOV and HFOVil) compared to NDS group (0.75 ± 0.06 ng/ml). NDOV and NDOVil had significantly decreased body weight and visceral fat, without any significant alteration in plasma insulin, HOMA index,

Table 1 Metabolic parameters at baseline and the end of week 12 (before treatment)

Metabolic parameters	Baseline		Week 12		
	Sham	ORX	NDS	NDO	HFO
Body weight (g)	192.5 $\pm$ 3.7	197.5 $\pm$ 3.7	466.6 $\pm$ 4.9	409.2 $\pm$ 14.1 ^{a,b}	472.0 $\pm$ 8.4
Plasma glucose (mg/dl)	106.8 $\pm$ 4.5	111.8 $\pm$ 4.3	133.8 $\pm$ 4.9	140.0 $\pm$ 5.5	139.9 $\pm$ 7.1
Plasma insulin (ng/ml)	2.1 $\pm$ 0.3	2.2 $\pm$ 0.4	2.0 $\pm$ 0.2	2.1 $\pm$ 0.2 ^b	3.7 $\pm$ 0.3 ^a
HOMA index	13.4 $\pm$ 1.2	14.0 $\pm$ 2.2	15.3 $\pm$ 2.0	17.7 $\pm$ 2.1 ^b	27.6 $\pm$ 3.4 ^a
Plasma total cholesterol (mg/dl)	45.5 $\pm$ 2.2	47.7 $\pm$ 1.7	48.5 $\pm$ 2.7	52.1 $\pm$ 2.0 ^b	75.0 $\pm$ 6.6 ^a

^aCompared to NDS.

^bCompared to HFO.

Table 2 Metabolic parameters after vildagliptin treatment for 4 weeks

Metabolic parameters	Groups				
	NDS	NDOV	NDOVil	HFOV	HFOVil
Body weight (g)	496.5 ± 10.7	423.3 ± 8.4 ^{a,b}	417.2 ± 17.6 ^{a,b}	506.6 ± 17.8	496.6 ± 16.4
Visceral fat (g)	26.8 ± 1.6	16.0 ± 1.9 ^{a,b}	15.7 ± 1.4 ^{a,b}	31.3 ± 3.4	30.3 ± 3.3
Plasma glucose (mg/dl)	131.5 ± 10.6	129.0 ± 13.1	137.3 ± 3.1	134.1 ± 8.9	131.7 ± 6.3
Plasma insulin (ng/ml)	2.53 ± 0.34	2.78 ± 0.18	2.71 ± 0.36	5.07 ± 1.16 ^a	2.37 ± 0.37 ^b
HOMA index	21.3 ± 2.9	24.4 ± 2.8	21.5 ± 3.4	44.7 ± 13.8 ^a	20.6 ± 3.8 ^b
Plasma glucose AUC (AUCg) (mg/dl × min × 10 ⁴)	4.47 ± 0.20	5.01 ± 0.33	4.48 ± 0.23	5.92 ± 0.25 ^a	4.97 ± 0.26 ^b
Plasma total cholesterol (mg/dl)	68.0 ± 5.5	63.8 ± 2.6	73.3 ± 5.8	110.8 ± 14.9 ^a	101.7 ± 8.7 ^a
Plasma triglyceride (mg/dl)	51.9 ± 2.2	55.3 ± 9.9	57.4 ± 6.1	49.8 ± 4.1	50.2 ± 7.5
HDL cholesterol (mg/dl)	6.7 ± 0.4	7.2 ± 0.3	7.5 ± 0.4	4.9 ± 0.6 ^a	6.9 ± 0.4 ^b
LDL cholesterol (mg/dl)	60.6 ± 7.5	52.9 ± 3.6	56.9 ± 4.0	116.4 ± 15.7 ^a	81.5 ± 8.1 ^b

^aCompared to NDS.^bCompared to HFOV.

plasma glucose AUC of OGTT (AUCg), total cholesterol, plasma LDL cholesterol levels as well as plasma HDL cholesterol levels, when compared with those of NDS rats (Table 2). These findings indicated that peripheral insulin resistance was not observed in the NDOV and NDOVil rats. In contrast, HFOV showed the characteristics of peripheral insulin resistance, including increased plasma insulin, HOMA index, plasma AUCg, total cholesterol, plasma LDL cholesterol levels as well as decreased plasma HDL cholesterol levels, when compared with those of NDS rats. However, plasma glucose and plasma triglyceride levels showed no significant difference among all groups. Interestingly, HFOVil had significantly decreased plasma insulin, HOMA index, plasma AUCg and plasma LDL cholesterol levels as well as increased plasma HDL cholesterol levels, when compared with those of HFOV rats. All of these findings suggested that vildagliptin attenuated peripheral insulin resistance via increased peripheral insulin sensitivity and improved lipid profiles in testosterone-deprived obese rats.

Obesity, but not testosterone deprivation, caused the impairment of brain IR function, which was attenuated by vildagliptin treatment

Brain IR function was tested by measuring insulin-induced LTD and brain insulin signaling. For the insulin-induced LTD study, the degrees of insulin-mediated LTD in ND-fed rats (NDS, NDOV and NDOVil) were not significantly different among groups ($n=2-3$ independent slices/animal, $n=6$ animals/group, Fig. 2A). However, the degree of insulin-induced LTD was significantly reduced in HFOV rats, when compared with that of NDS rats ($n=2-3$ independent slices/animal, $n=6$ animals/group, Fig. 2A).

After 4 weeks of vildagliptin administration, the degrees of insulin-induced LTD in HFOVil was significantly increased compared with that of HFOV rats ($n=2-3$ independent slices/animal, $n=6$ animals/group, Fig. 2A).

For the brain insulin signaling study, the expression of IRs in all groups showed no significant difference among them (Fig. 2C). The p-IRs at tyrosine site was not significantly different among groups of ND-fed rats (NDS, NDOV and NDOVil; Fig. 2B). However, levels of p-IR of HFOV rats were significantly decreased, when compared with those of NDOV rats (Fig. 2B). Furthermore, vildagliptin treatment significantly increased p-IR levels in HFO rats (Fig. 2B). All of these findings suggested that the obese insulin-resistant condition, but not testosterone deprivation, caused the impairment of brain IR function and brain insulin signaling. In addition, vildagliptin restored the impairment of brain IR function as well as brain insulin signaling in the testosterone-deprived obese condition.

Obesity, but not testosterone deprivation, caused brain mitochondrial dysfunction and vildagliptin attenuated that impairment

Brain mitochondrial function was determined by assessing changes in brain mitochondrial ROS production and brain $\Delta\Psi_m$ change after H_2O_2 stimulation, and brain mitochondrial swelling. The results demonstrated that brain mitochondrial function, including brain mitochondrial ROS level, brain $\Delta\Psi_m$ changes and brain mitochondrial swelling, was not significantly different among groups of ND-fed rats (NDS, NDOV and NDOVil; Fig. 3A, B and C). These findings suggested that testosterone deprivation did not impair brain mitochondrial function. Furthermore, HFOV rats had a significant increase in brain

Figure 2

The effects of vildagliptin on brain insulin receptor (IR) function, including insulin-induced LTD, the phosphorylation of IR (p-IR) and the expression of IRs in testosterone-deprived rats with or without obesity. Insulin-induced LTD in the CA1 hippocampus and the p-IR significantly decreased in HFOV rats, when compared with that of NDS rats (A and B). The administration of vildagliptin for 4 weeks significantly improved the ability of insulin-induced LTD and the p-IR in HFOVil rats, when compared with that of HFOV rats (A and B). No differences in the expression of IR among all groups were found (C). NDS, sham-operated rats with normal diet feeding; NDOV, orchiectomized-operated rats with normal diet feeding and treated with vehicle; NDOVil, orchiectomized-operated rats with normal diet feeding and treated with vildagliptin; HFOV, orchiectomized-operated rats with high-fat diet feeding and treated with vehicle; HFOVil, orchiectomized-operated rats fed with a high-fat diet and treated with vildagliptin.

* $P < 0.05$ vs NDS and $^{\dagger}P < 0.05$ vs HFOV. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-15-0099>.

mitochondrial ROS production, brain mitochondrial depolarization and brain mitochondrial swelling, when compared with those of NDOV rats (Fig. 3A, B and C). In addition, vildagliptin treatment significantly decreased brain ROS production in HFOVil rats, when compared with that of HFOV rats (Fig. 3A). However, vildagliptin did not attenuate brain mitochondrial depolarization and brain mitochondrial swelling in HFO rats (Fig. 3B and C). The morphological changes of brain mitochondria from all groups were shown in Fig. 4. Intact brain mitochondrial morphology with apparent folded cristae was observed in

all ND-fed rats (Fig. 4A, B and C). Brain mitochondrial swelling was observed in both the HFOV and HFOVil groups as indicated by markedly unfolded cristae (Fig. 4D and E). All of these findings suggested that the obese

Figure 3

The effects of vildagliptin on brain mitochondrial function in testosterone deprived rats with or without obesity. HFOV rats demonstrated brain mitochondrial dysfunction, as indicated by increased brain mitochondrial ROS production following H_2O_2 application (A), increased brain mitochondrial membrane potential ($\Delta\Psi_m$) change following H_2O_2 application (B) and decreased absorbance values, indicating brain mitochondrial swelling (C). The administration of vildagliptin for 4 weeks significantly decreased brain mitochondrial ROS production in HFOVil rats, when compared with that of HFOV rats (A). However, vildagliptin did not improve brain $\Delta\Psi_m$ change and brain mitochondrial swelling in HFOVil rats (B and C). NDS, sham-operated rats with normal diet feeding; NDOV, orchiectomized-operated rats with normal diet feeding and treated with vehicle; NDOVil, orchiectomized-operated rats with normal diet feeding and treated with vildagliptin; HFOV, orchiectomized-operated rats with high-fat diet feeding and treated with vehicle; HFOVil, orchiectomized-operated rats with high-fat diet feeding and treated with vildagliptin.

* $P < 0.05$ vs NDOV and $^{\dagger}P < 0.05$ vs HFOV.

Figure 4

Representative images of brain mitochondrial morphology by transmission electron microscopy (JEM-2200FS field emission electron microscope, original magnification $\times 20\,000$) after 4 weeks of vildagliptin treatment. Normal folding of cristae in brain mitochondrial morphology was shown among all normal diet-fed rats (A, B and C). However, brain mitochondrial swelling, as indicated by unfolded cristae, of both HFOV and HFOVil groups was observed (D and E). NDS, sham-operated rats with normal diet feeding;

NDOV, orchietomized-operated rats with normal diet feeding and treated with vehicle; NDOVil, orchietomized-operated rats with normal diet feeding and treated with vildagliptin; HFOV, orchietomized-operated rats with high-fat diet feeding and treated with vehicle; HFOVil, orchietomized-operated rats with high-fat diet feeding and treated with vildagliptin.

insulin-resistant condition, but not testosterone deprivation, caused the impairment of brain mitochondrial function. In addition, vildagliptin attenuated brain mitochondrial dysfunction by reducing brain ROS production in the testosterone-deprived obese condition.

Testosterone deprivation with or without obesity caused impaired hippocampal synaptic plasticity and cognitive decline, and vildagliptin failed to restore those impairments

To study hippocampal synaptic plasticity, electrical-induced LTP was determined. The results demonstrated that the degrees of electrical-induced LTP significantly decreased in both NDOV and HFOV rats, when compared with that of NDS rats. The reduction of electrical-induced LTP in both NDOV and HFOV rats ($n=2-3$ independent slices/animal, $n=6$ animals/group, Fig. 5A) was not significantly different. In addition, vildagliptin administration did not improve the degree of electrical-induced LTP in both NDO and HFO rats. These findings indicated that testosterone deprivation with or without obesity caused the impairment of hippocampal synaptic plasticity. However, vildagliptin treatment could not attenuate that impairment.

Furthermore, the OFT was used to screen locomotor activity. The results showed that it was not significantly different among all groups, indicating that the locomotor activity did not differ in all groups. In addition, the present study determined cognitive function by using the MWM test. For the acquisition test, the time to reach the platform was not significantly different among ND-fed rats (NDS, NDOV and NDOVil rats; Fig. 5B). However, we

found that HFOV and HFOVil rats took a longer time to reach the platform, when compared with that of NDS rats (Fig. 5B). For the probe test, the results showed that time spent in the target quadrant of the NDOV, NDOVil, HFOV and HFOVil rats was significantly decreased compared to NDS rats (Fig. 5C). These findings suggested that testosterone deprivation with or without obesity caused cognitive decline, and vildagliptin failed to restore cognitive function in the testosterone-deprived rats.

Discussion

The major findings of the present study are as follows: i) the condition of obesity in testosterone-deprived rats leads to the development of peripheral insulin resistance, impairment of brain insulin sensitivity, brain mitochondrial dysfunction and impaired hippocampal synaptic plasticity, resulting in cognitive decline; ii) vildagliptin improves peripheral insulin sensitivity, brain insulin sensitivity and brain mitochondrial function in testosterone-deprived obese rats; and iii) vildagliptin fails to restore hippocampal synaptic plasticity and cognitive function in testosterone deprived rats.

The present study demonstrates that peripheral insulin resistance, as indicated by increased HOMA index and impaired OGTT as well as impaired lipid profiles, developed in testosterone-deprived rats with obesity. Consistent with these findings, previous studies demonstrated that HFD-induced obesity caused the development of peripheral insulin resistance in male rats (Akiyama *et al.* 1996, Stranahan *et al.* 2008, Pintana *et al.* 2013, Pipatpiboon *et al.* 2013). The development of peripheral insulin resistance in the present study

Figure 5

The effects of vildagliptin on hippocampal synaptic long-term potentiation (LTP) and the cognitive function determined by Morris water maze (MWM) test in testosterone-deprived rats with or without obesity. Both orchietomized-operated groups (NDOV and HFOV rats) significantly reduced the percentage of normalized fEPSPs, when compared with that of NDS rats (A). The administration of vildagliptin for 4 weeks did not improve the percentage of normalized fEPSPs in both orchietomized-operated groups (NDOVil and HFOVil), when compared with that of HFOV rats (A). HFOV rats and HFOVil rats significantly increased time to reach the platform in the acquisition test as well as had a decreased time spent in target quadrant in the probe test, when compared with that of NDS rats (B and C). NDOV rats had a significantly decreased time spent in the target quadrant in the probe test, but there was no change in time to reach the platform in the acquisition test (B and C). The administration of vildagliptin for 4 weeks did not restore the time taken to reach the platform in HFOVil rats, and the time spent in the target quadrant in both NDOVil and HFOVil rats (B and C). NDS, sham-operated rats with normal diet feeding; NDOV, orchietomized-operated rats with normal diet feeding and treated with vehicle; NDOVil, orchietomized-operated rats with normal diet feeding and treated with vildagliptin; HFOV, orchietomized-operated rats with high-fat diet feeding and treated with vehicle; HFOVil, orchietomized-operated rats with high-fat diet feeding and treated with vildagliptin; HFS, high frequency stimulation. * $P < 0.05$ vs NDS.

depended on HFD-induced obesity, but not testosterone-deprivation, as confirmed by no change in insulin sensitivity in NDOV rats. Our findings are also consistent with a previous report by Christoffersen *et al.* (2006).

In addition, the present study demonstrated that ORX led to a loss of body weight. The reduction of daily food intake in the ORX group could be the explanation of weight loss in these rats. These findings are consistent with several previous studies, which showed that the reduction of body weight in ORX rats was caused by the reduction of daily food intake (Gentry & Wade 1976, Borst & Conover 2006) and muscle atrophy (Gao *et al.* 2005, Axell *et al.* 2006, Borst & Conover 2006). In contrast to our findings, the studies of Xia *et al.* (2013) found that ORX-induced testosterone deficiency increased the fasting blood glucose in mature adult (10 weeks old) Sprague–Dawley male rats. Jayaraman *et al.* (2014) demonstrated that ORX adult mice (3 months of age) had impaired insulin sensitivity. Since young adult male Wistar rats were used and were ORX at 5–6 weeks of age in the present study, the differences in species and age at the time of ORX procedure could be responsible for these inconsistent findings.

Interestingly, we found that HFOV rats, but not NDOV rats, developed brain insulin resistance as indicated by a reduction of insulin induced-LTD and decreased p-IR, as well as brain mitochondrial dysfunction through increased brain ROS production, brain mitochondrial depolarization and brain mitochondrial swelling. These findings and other previous studies indicated that obesity caused impaired brain mitochondrial function, resulting in brain insulin resistance (Pipatpiboon *et al.* 2012, 2013). Furthermore, our results demonstrated that testosterone deficiency alone had no effects on peripheral insulin resistance, brain insulin sensitivity as well as brain mitochondrial function. These findings suggest that testosterone deficiency is not required for the development of HFD-induced insulin resistance, and that factors contributing to peripheral and brain insulin resistance as well as brain mitochondrial dysfunction in this model could be mainly due to obesity.

Growing evidence demonstrated that testosterone deprivation is associated with cognitive decline in both men and animal models (Sakata *et al.* 2000, Hogervorst *et al.* 2001, 2004, Spritzer *et al.* 2011). We also found that testosterone-deprived rats impaired cognition and impaired hippocampal synaptic plasticity, but HFD-induced obesity did not aggravate those impairments in the testosterone-deprived condition. The present findings and other previous studies (Sakata *et al.* 2000, Naghdi *et al.* 2005, Sandstrom *et al.* 2006, Spritzer *et al.* 2011) indicated that testosterone plays an important role in cognitive function. Since brain mitochondrial function and brain insulin sensitivity were intact in NDO rats, these results indicate that they were not mainly contributed to

impaired synaptic plasticity and cognitive decline in testosterone-deprived model.

In the present study, vildagliptin treatment improved peripheral insulin resistance, attenuated brain insulin resistance and partially improved brain mitochondrial dysfunction in testosterone-deprived obese rats. The possible underlying mechanisms of vildagliptin, which improve both peripheral and brain insulin sensitivity, could be due to its ability to i) inhibit the DPP4 activity, resulting in an increase in plasma active GLP1 levels, leading to improve insulin sensitivity and ii) act as an anti-oxidant agent. Although the level of GLP1 was not measured in the present study, our previous study already demonstrated that vildagliptin increased plasma and brain active GLP1 levels, leading to increased peripheral and brain insulin sensitivity in obese male rats (Burkey *et al.* 2005, Pipatpiboon *et al.* 2013, Gault *et al.* 2015). In addition, an anti-oxidant effect of vildagliptin was already demonstrated via reduced malondialdehyde (MDA) levels in the circulation and the brain in our previous report (Pintana *et al.* 2013). Although vildagliptin increased brain insulin sensitivity and decreased brain mitochondrial dysfunction, it could not restore hippocampal synaptic plasticity and cognitive function in testosterone-deprived rats with or without obesity. Furthermore, findings from previous studies (Sandstrom *et al.* 2006, Spritzer *et al.* 2011, McConnell *et al.* 2012) and the present study (Fig. 5B and C) suggest that testosterone plays a critical role in maintaining the normal function of cognition. These findings also indicate that the impairment of synaptic plasticity that leads to cognitive decline in testosterone-deprived model was not mainly influenced by brain insulin resistance.

Conclusion

Testosterone deprivation can lead to cognitive decline via impaired hippocampal synaptic plasticity. However, testosterone-deprived obese condition leads to not only the development of cognitive decline and impaired hippocampal synaptic plasticity, but also brain insulin resistance and brain mitochondrial dysfunction. These findings suggest that testosterone-deprivation with obese insulin-resistant condition causes severe brain dysfunction. Although DPP4 inhibitor, vildagliptin, improves peripheral and brain insulin sensitivity as well as decreases brain mitochondrial ROS production, it fails to restore hippocampal synaptic plasticity and cognitive function. Therefore, vildagliptin does not provide benefits on preventing cognitive decline in subjects under testosterone-deprived obese condition.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from the Thailand Research Fund TRF-BRG5780016 (S C C), the Royal Golden Jubilee PhD program (PHD/0025/2555, H P and S C C), National Research Council of Thailand (S C C), a NSTDA Research Chair Grant from the National Science and Technology Development Agency (N C) and Chiang Mai University Excellent Center Award (N C).

Author contribution statement

H P performed the experiments, analyzed the data and wrote the manuscript. W P performed the experiments and analyzed the data. W P performed the experiments and analyzed the data. N C designed the study, analyzed the data and wrote the manuscript. S C C designed the study, performed the experiments, analyzed the data and wrote the manuscript.

References

- Ahren B, Landin-Olsson M, Jansson PA, Svensson M, Holmes D & Schweizer A 2004 Inhibition of dipeptidyl peptidase-4 reduces glycemia, sustains insulin levels, and reduces glucagon levels in type 2 diabetes. *Journal of Clinical Endocrinology and Metabolism* **89** 2078–2084. (doi:10.1210/jc.2003-031907)
- Akiyama T, Tachibana I, Shirohara H, Watanabe N & Otsuki M 1996 High-fat hypercaloric diet induces obesity, glucose intolerance and hyperlipidemia in normal adult male Wistar rat. *Diabetes Research and Clinical Practice* **31** 27–35. (doi:10.1016/0168-8227(96)01205-3)
- Arakawa H 2005 Age dependent effects of space limitation and social tension on open-field behavior in male rats. *Physiology & Behavior* **84** 429–436. (doi:10.1016/j.physbeh.2005.01.008)
- Axell AM, MacLean HE, Plant DR, Harcourt LJ, Davis JA, Jimenez M, Handelsman DJ, Lynch GS & Zajac JD 2006 Continuous testosterone administration prevents skeletal muscle atrophy and enhances resistance to fatigue in orchidectomized male mice. *American Journal of Physiology. Endocrinology and Metabolism* **291** E506–E516. (doi:10.1152/ajpendo.00058.2006)
- Aydilek N & Aksakal M 2005 Effects of testosterone on lipid peroxidation, lipid profiles and some coagulation parameters in rabbits. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine* **52** 436–439. (doi:10.1111/j.1439-0442.2005.00764.x)
- Borst SE & Conover CF 2006 Orchietomized Fischer 344 male rat models body composition in hypogonadal state. *Life Sciences* **79** 411–415. (doi:10.1016/j.lfs.2006.01.035)
- Burkey BF, Li X, Bolognese L, Balkan B, Mone M, Russell M, Hughes TE & Wang PR 2005 Acute and chronic effects of the incretin enhancer vildagliptin in insulin-resistant rats. *Journal of Pharmacology and Experimental Therapeutics* **315** 688–695. (doi:10.1124/jpet.105.087064)
- Christoffersen B, Raun K, Svendsen O, Fledelius C & Golozoubova V 2006 Evaluation of the castrated male Sprague–Dawley rat as a model of the metabolic syndrome and type 2 diabetes. *International Journal of Obesity* **30** 1288–1297. (doi:10.1038/sj.ijo.0803261)
- Christoffersen BO, Gade LP, Golozoubova V, Svendsen O & Raun K 2010 Influence of castration-induced testosterone and estradiol deficiency on obesity and glucose metabolism in male Gottingen minipigs. *Steroids* **75** 676–684. (doi:10.1016/j.steroids.2010.04.004)
- Craft S 2005 Insulin resistance syndrome and Alzheimer's disease: age- and obesity-related effects on memory, amyloid, and inflammation.

- Neurobiology of Aging* **26**(Suppl 1) 65–69. (doi:10.1016/j.neurobiolaging.2005.08.021)
- Dulisch ML 1976 A castration procedure for the rabbit, rat, hamster, and guinea pig. *Journal of Zoo Animal Medicine* **7** 8–11. (doi:10.2307/20094380)
- Filova B, Ostatnikova D, Celec P & Hodosy J 2013 The effect of testosterone on the formation of brain structures. *Cells, Tissues, Organs* **197** 169–177. (doi:10.1159/000345567)
- Gao W, Reiser PJ, Coss CC, Phelps MA, Kearbey JD, Miller DD & Dalton JT 2005 Selective androgen receptor modulator treatment improves muscle strength and body composition and prevents bone loss in orchidectomized rats. *Endocrinology* **146** 4887–4897. (doi:10.1210/en.2005-0572)
- Gault VA, Lennox R & Flatt PR 2015 Sitagliptin, a dipeptidyl peptidase-4 inhibitor, improves recognition memory, oxidative stress and hippocampal neurogenesis and upregulates key genes involved in cognitive decline. *Diabetes, Obesity & Metabolism* **17** 403–413. (doi:10.1111/dom.12432)
- Gentry RT & Wade GN 1976 Androgenic control of food intake and body weight in male rats. *Journal of Comparative and Physiological Psychology* **90** 18–25. (doi:10.1037/h0077264)
- Greenwood CE & Winocur G 2005 High-fat diets, insulin resistance and declining cognitive function. *Neurobiology of Aging* **26**(Suppl 1) 42–45. (doi:10.1016/j.neurobiolaging.2005.08.017)
- Grossmann M, Thomas MC, Panagiotopoulos S, Sharpe K, Macisaac RJ, Clarke S, Zajac JD & Jerums G 2008 Low testosterone levels are common and associated with insulin resistance in men with diabetes. *Journal of Clinical Endocrinology and Metabolism* **93** 1834–1840. (doi:10.1210/jc.2007-2177)
- Haffner SM, Karhapaa P, Mykkanen L & Laakso M 1994 Insulin resistance, body fat distribution, and sex hormones in men. *Diabetes* **43** 212–219. (doi:10.2337/diab.43.2.212)
- Hogervorst E, Williams J, Budge M, Barnetson L, Combrinck M & Smith AD 2001 Serum total testosterone is lower in men with Alzheimer's disease. *Neuro Endocrinology Letters* **22** 163–168.
- Hogervorst E, Bandelow S, Combrinck M & Smith AD 2004 Low free testosterone is an independent risk factor for Alzheimer's disease. *Experimental Gerontology* **39** 1633–1639. (doi:10.1016/j.exger.2004.06.019)
- Janowsky JS 2006 Thinking with your gonads: testosterone and cognition. *Trends in Cognitive Sciences* **10** 77–82. (doi:10.1016/j.tics.2005.12.010)
- Jayaraman A, Lent-Schochet D & Pike CJ 2014 Diet-induced obesity and low testosterone increase neuroinflammation and impair neural function. *Journal of Neuroinflammation* **11** 162. (doi:10.1186/s12974-014-0162-y)
- Kahn BB & Flier JS 2000 Obesity and insulin resistance. *Journal of Clinical Investigation* **106** 473–481. (doi:10.1172/JCI10842)
- McConnell SE, Alla J, Wheat E, Romeo RD, McEwen B & Thornton JE 2012 The role of testicular hormones and luteinizing hormone in spatial memory in adult male rats. *Hormones and Behavior* **61** 479–486. (doi:10.1016/j.yhbeh.2012.01.003)
- Meydan S, Kus I, Tas U, Ogeturk M, Sancakdar E, Dabak DO, Zarsarsiz I & Sarsilmaz M 2010 Effects of testosterone on orchietomy-induced oxidative damage in the rat hippocampus. *Journal of Chemical Neuroanatomy* **40** 281–285. (doi:10.1016/j.jchemneu.2010.07.006)
- Naghdi N, Majlessi N & Bozorgmehr T 2005 The effect of intrahippocampal injection of testosterone enanthate (an androgen receptor agonist) and anisomycin (protein synthesis inhibitor) on spatial learning and memory in adult, male rats. *Behavioural Brain Research* **156** 263–268. (doi:10.1016/j.bbr.2004.05.032)
- Pandolfi C, Pellegrini L, Sbalzarini G & Mercantini F 1994 Obesity and insulin resistance. *Minerva Medica* **85** 167–171.
- Pintana H, Apaijai N, Prachayasakul W, Chattipakorn N & Chattipakorn SC 2012 Effects of metformin on learning and memory behaviors and brain mitochondrial functions in high fat diet induced insulin resistant rats. *Life Sciences* **91** 409–414. (doi:10.1016/j.lfs.2012.08.017)
- Pintana H, Apaijai N, Chattipakorn N & Chattipakorn SC 2013 DPP-4 inhibitors improve cognition and brain mitochondrial function of insulin resistant rats. *Journal of Endocrinology* **218** 1–11. (doi:10.1530/JOE-12-0521)
- Pipatpiboon N, Prachayasakul W, Chattipakorn N & Chattipakorn SC 2012 PPAR γ agonist improves neuronal insulin receptor function in hippocampus and brain mitochondria function in rats with insulin resistance induced by long term high-fat diets. *Endocrinology* **153** 329–338. (doi:10.1210/en.2011-1502)
- Pipatpiboon N, Pintana H, Prachayasakul W, Chattipakorn N & Chattipakorn SC 2013 DPP4-inhibitor improves neuronal insulin receptor function, brain mitochondrial function and cognitive function in rats with insulin resistance induced by high-fat diet consumption. *European Journal of Neuroscience* **37** 839–849. (doi:10.1111/ejn.12088)
- Prachayasakul W, Kerdphoo S, Petsophonakul P, Pongchaidecha A, Chattipakorn N & Chattipakorn SC 2011 Effects of high-fat diet on insulin receptor function in rat hippocampus and the level of neuronal corticosterone. *Life Sciences* **88** 619–627. (doi:10.1016/j.lfs.2011.02.003)
- Sakata K, Tokue A & Kawai N 2000 Altered synaptic transmission in the hippocampus of the castrated male mouse is reversed by testosterone replacement. *Journal of Urology* **163** 1333–1338. (doi:10.1016/S0022-5347(05)67773-7)
- Sandstrom NJ, Kim JH & Wasserman MA 2006 Testosterone modulates performance on a spatial working memory task in male rats. *Hormones and Behavior* **50** 18–26. (doi:10.1016/j.yhbeh.2005.09.008)
- Shi Z, Araujo AB, Martin S, O'Loughlin P & Witter GA 2013 Longitudinal changes in testosterone over five years in community-dwelling men. *Journal of Clinical Endocrinology and Metabolism* **98** 3289–3297. (doi:10.1210/jc.2012-3842)
- Spritzer MD, Daviau ED, Coneeny MK, Engelman SM, Prince WT & Rodriguez-Wisdom KN 2011 Effects of testosterone on spatial learning and memory in adult male rats. *Hormones and Behavior* **59** 484–496. (doi:10.1016/j.yhbeh.2011.01.009)
- Sripetchwandee J, Pipatpiboon N, Chattipakorn N & Chattipakorn S 2014 Combined therapy of iron chelator and antioxidant completely restores brain dysfunction induced by iron toxicity. *PLoS ONE* **9** e85115. (doi:10.1371/journal.pone.0085115)
- Stellato RK, Feldman HA, Hamdy O, Horton ES & McKinlay JB 2000 Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts male aging study. *Diabetes Care* **23** 490–494. (doi:10.2337/diacare.23.4.490)
- Stranahan AM, Norman ED, Lee K, Cutler RG, Telljohann RS, Egan JM & Mattson MP 2008 Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats. *Hippocampus* **18** 1085–1088. (doi:10.1002/hipo.20470)
- Vorhees CV & Williams MT 2006 Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nature Protocols* **1** 848–858. (doi:10.1038/nprot.2006.116)
- Xia F, Xu X, Zhai H, Meng Y, Zhang H, Du S, Xu H, Wu H & Lu Y 2013 Castration-induced testosterone deficiency increases fasting glucose associated with hepatic and extra-hepatic insulin resistance in adult male rats. *Reproductive Biology and Endocrinology* **11** 106. (doi:10.1186/1477-7827-11-106)

Received in final form 17 May 2015

Accepted 26 May 2015

Accepted Preprint published online 27 May 2015