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Dipeptidyl peptidase IV in the hypothalamus and hippocampus of monosodium glutamate obese and food-deprived rats

Rafaela Fadoni Alponti[a,b], Rodrigo Frezzatti[a], Juliana Marton Barone[a], Valter de Sousa Alegre[a], Paulo Flavio Silveira[a,*]

[a]*Laboratory of Pharmacology, Instituto Butantan, Av. Vital Brasil, 1500, 05503-900 São Paulo, SP, Brazil*
[b]*Department of Neurosciences and Behavior, Universidade de São Paulo, Av. Prof. Mello Moraes, 1721, 05508-030 São Paulo, SP, Brazil*

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Abstract

Proline-specific dipeptidyl peptidases are emerging as a protease family with important roles in the regulation of signaling by peptide hormones related to energy balance. The treatment of neonatal rats with monosodium glutamate (MSG) is known to produce a selective damage on the arcuate nucleus with development of obesity. This study investigates the relationship among dipeptidyl peptidase IV (DPPIV) hydrolyzing activity, CD26 protein, fasting, and MSG model of obesity in 2 areas of the central nervous system. Dipeptidyl peptidase IV and CD26 were, respectively, evaluated by fluorometry, and enzyme-linked immunosorbent assay and reverse transcriptase polymerase chain reaction in soluble (SF) and membrane-bound (MF) fractions from the hypothalamus and hippocampus of MSG-treated and normal rats, submitted or not to food deprivation (FD). Dipeptidyl peptidase IV in both areas was distinguished kinetically as insensitive (DI) and sensitive (DS) to diprotin A. Compared with the controls, MSG and/or FD decreased the activity of DPPIV-DI in the SF and MF from the hypothalamus, as well as the activity of DPPIV-DS in the SF from the hypothalamus and in the MF from the hippocampus. Monosodium glutamate and/or FD increased the activity of DPPIV-DI in the MF from the hippocampus. The monoclonal protein expression of membrane CD26 by enzyme-linked immunosorbent assay decreased in the hypothalamus and increased in the hippocampus of MSG and/or FD relative to the controls. The existence of DPPIV-like activity with different sensitivities to diprotin A and the identity of insensitive with CD26 were demonstrated for the first time in the central nervous system. Data also demonstrated the involvement of DPPIV-DI/CD26 hydrolyzing activity in the energy balance probably through the regulation of neuropeptide Y and β-endorphin levels in the hypothalamus and hippocampus.

1. Introduction

In obesity and fasting, there is an imbalance in energy homeostasis, altering the synthesis and release of a variety of peptides [1-3] susceptible of hydrolysis by dipeptidyl peptidase IV (DPPIV). The treatment of neonatal rats with monosodium glutamate (MSG) results in a selective damage to the arcuate nucleus and constitutes a model of obesity [4-7]. Dipeptidyl peptidase IV enzyme activity is widely expressed in most mammalian tissues. Canonical DPPIV has been considered the serine type protease activity in the extracellular region of the cell surface CD26 glycoprotein (EC 3.4.14.5) [8,9]. In addition to its membrane form, a soluble form of DPPIV/CD26 has been described in plasma and recognized as the extracellular domain of the molecule, thought to be cleaved from the cell surface [9,10]. Canonical DPPIV preferentially cleaves N-terminal dipeptides from polypeptides with proline or alanine in the penultimate position, thereby regulating the activities of a number of cytokines, chemokines, neuropeptides, and peptide hormones [11]. It is also known as *adenosine deaminase* (EC 3.5.4.4) binding protein [12]. Several biological functions have been attributed to DPPIV/CD26, including involvement in T-cell activation, cell adhesion, digestion of proline containing peptides in the kidney and intestines, its role in HIV infection and apoptosis, and regulation of tumorigenicity in certain melanoma cells [8,13]. The hydrolysis of the insulinotropic hormone, a glucagon-like peptide type 1 (GLP-1), has been recognized as one of its most important

* Corresponding author. Tel.: +55 11 7067 9058; fax: +55 11 3726 7222 2061.
E-mail address: pefesil@butantan.gov.br (P.F. Silveira).

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activities [14]. Over the past few years, several related enzymes with similar DPPIV activity have been discovered, raising questions on their molecular structure and functionality [15]. Growing evidence suggests that soluble and membrane-bound DPPIV activity has important physiopathologic functions [16-18]. However, the characterization and relationship between soluble and membrane-bound DPPIV in the central nervous system (CNS) with metabolic disorders have not yet been investigated. This study investigates the relationship among DPPIV hydrolyzing activity, CD26 protein, fasting, and MSG model of obesity in soluble (SF) and membrane-bound (MF) fractions of the hypothalamus and hippocampus.

2. Experimental procedures

2.1. Animals

Immediately after birth, male Wistar rats were housed with a lactant female in a polypropylene box (inside length × width × height, 56 cm × 35 cm × 19 cm), with food (commercially available ration; Nuvilab CR-1, Nuvital, Colombo, PR, Brazil; composed of 22% protein, 55% carbohydrate, 4% lipids, 8% fibers, 10% vitamins and minerals; total of 3 kcal/g) and tap water ad libitum, in a ventilated container (Alesco Ind Com, Monte Mor, SP, Brazil), with a controlled temperature (24°C ± 2°C), relative humidity (65% ± 1%), and 12:12-hour light/dark photoperiod (lights on at 6:00 AM). Twenty-four hours after birth, the animals received a daily subcutaneous bolus injection of L-glutamic acid monosodium salt (Sigma, St Louis, MO) in saline 0.9% (4 mg/g body weight) in the cervical region between 7:30 AM and 9:00 AM of the light period, at a maximum volume of 0.2 mL (MSG animals), until they were 10 days old. At 22 days, the animals were weaned; and the female was removed from the cage [19]. At 90 days, obesity was determined by the Lee index, calculated by body mass (in grams)$^{0.33}$/nasoanal length (in centimeters) [4,20]. These animals were then subdivided into 2 groups: MSG (obese = Lee index >0.300) and MSG-FD (obese fasted for 72 hours). Rats of the same age and strain, receiving 0.9% NaCl under the same experimental conditions, were subdivided into 2 groups: C (normal = Lee index ≤0.300 = control) and FD (normal = Lee index ≤0.300 animals and fasted for 72 hours). Food deprivation was performed by transferring pairs of animals, between 7:30 AM and 9:00 AM of the light period, into metabolic cages without food and with drinking water ad libitum for 72 hours. Except during this period, all experimental groups had access ad libitum to the same ration of Nuvilab CR-1.

2.2. Statement of ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research and that the animal care and handling procedures used were in accordance with the guidelines of the Brazilian College of Animal Experimentation (COBEA, Brazil) and approved by the Ethics Committee of the Butantan Institute (291/06).

2.3. Brain collection

The animals were anesthetized with a solution containing ketamine hydrochloride (König, Buenos Aires, BA, Argentina) (100 mg/mL) and xylazine chloridrate (Vetbrands, Paulínia, SP, Brazil) (100 mg/mL) by intraperitoneal injection (0.2 mL/100 g of body mass) between 4:00 AM and 6:30 AM during the light phase and subsequently received intracardiac bolus injection of 0.1 mL of sodium heparin solution (Roche, Rio de Janeiro, RJ, Brazil) (1000 IU/mL of saline). Cardiac perfusion was then performed with 0.9% NaCl plus 50 mmol/L phosphate buffer, pH 7.4, over a period of 5 to 10 minutes at a flow rate of 12 to 15 mL/min, with a circulatory circuit opened by an incision in the right atrium to ensure the elimination of blood and to avoid the interference of its enzyme activities. The descending aorta was then clamped to ensure better perfusion of brain structures. After perfusion, the animals were decapitated with a guillotine; and the brains were removed for immediate enzyme activity and protein content measurements, and enzyme-linked immunosorbent assay (ELISA) and reverse transcription and polymerase chain reaction (RT-PCR) procedures.

2.4. Preparation of soluble and solubilized membrane-bound fraction

After manual dissection, whole hypothalamic (HT) and hippocampal (HC) tissues (HT: 0.24-5.04 mm posterior to the bregma; HC: 1.56-6.84 mm posterior to the bregma) [21] were homogenized in 10 mmol/L Tris-HCl buffer (pH 7.4) (3 minutes, 800 rpm) (4 mL buffer per gram wet weight) with a pestle mixer (Tecnal model Te-099, Piracicaba, SP, Brazil) and ultracentrifuged (Hitachi model HIMAC CP60E, Tokyo, TYO, Japan) (100 000*g*, 35 minutes). The resulting supernatants correspond to SF and were used to measure the DPPIV activity, CD26 (ELISA), protein content, and markers for the fractionation procedure. To avoid contamination with SF, the resulting pellet was washed 3 times with the same buffer. The pellet was then homogenized (3 minutes, 800 rpm) in 10 mmol/L Tris-HCl buffer, pH 7.4, plus 0.1% Triton X-100 (Calbiochem, San Diego, CA) and ultracentrifuged (100 000*g*, 35 minutes). The supernatants corresponded to MF and were used to measure the DPPIV activity, CD26 (ELISA), protein content, and markers for the fractionation procedure. To evaluate the influence of Triton X-100 on the peptidase activity under study, MF pools of HT and HC of 4 control animals were divided into 2 parts: one that was submitted to incubation for 2 hours with Bio-beads (Bio-Rad Laboratories, Hercules, CA) (100-mg/mL sample) and subsequently submitted to enzyme assay, and another that was immediately used for enzyme assay.

Protein content was also measured in both parts. All steps were carried out at 4°C.

2.5. Markers for the fractionation procedure

For this purpose, lactate dehydrogenase (LDH) and puromycin-insensitive neutral aminopeptidase (APM) activities were measured. The LDH was determined at 340 nm with a spectrophotometer (Power Wave X, Bio-Tek, Winooski, VT) by the method of Bergmeyer and Brent [22], as follows. Samples of 3 μL of SF or MF, in triplicate, were incubated with 297 μL of 100 mmol/L phosphate buffer, pH 7.4, containing 200 mmol/L NaCl and 1.6 mmol/L sodium pyruvate solution, plus 0.2 mmol/L of β-nicotinamide adenine dinucleotide, reduced form (NADH). Values of LDH activity were obtained by subtracting the absorbance read at 0 time from that read at 10 minutes of incubation at 37°C and extrapolated by comparison with a standard curve of NADH. The LDH activity was expressed as millimoles NADH oxidized per minute per milligram of protein. The APM was determined at 460/40-nm emission wavelength and 360/40-nm excitation wavelength with a fluorometer (Bio-Tek FL600FA microplate fluorescence reader) as described by Zambotti-Villela et al [16]. Briefly, samples of 50 μL of SF or MF, in triplicate, were incubated with prewarmed solution of 0.125 mmol/L L-Ala-β-naphthylamide (Sigma) (solubilized in 0.012 N HCl) in 0.05 mol/L phosphate buffer, pH 7.4, containing 1 mmol/L DL-dithiothreitol (Sigma), 0.1 mg/mL bovine serum albumin (BSA), and 0.02 mmol/L puromycin (Sigma). Values of APM activity were obtained by subtracting the absorbance read at 0 time from that read at 30 minutes of incubation at 37°C, and extrapolated by comparison with a standard curve of β-naphthylamine (Sigma). The APM activity was expressed as picomoles of hydrolyzed substrate per minute per milligram protein.

2.6. Protein

Protein content was measured at 630 nm, in triplicates, using a Bio-Rad protein assay kit (Bio-Rad Laboratories) [23] in the Bio-Tek Power Wave X spectrophotometer absorbance reader. Protein contents were extrapolated by comparison with the standard curve of BSA (Sigma) in the same diluents as the samples.

2.7. Quantification of catalytic activity of DPPIV

The DPPIV activity was quantified based on the amount of 4-methoxy-β-naphthylamine released [24] as the result of incubation (30 minutes, 37° C), in 96-well flat-bottom microplates, of 10 to 100 μL of SF and MF of the HT and HC, in triplicate, with prewarmed solution of 0.2 mmol/L H-Gly-Pro-4-methoxy-β-naphthylamide (Peninsula Laboratories, San Carlos, CA) (solubilized in dimethyl-sulfoxide) (Sigma) in 0.05 mol/L Tris-HCl, pH 8.3, containing 0.1 mg/mL BSA, in the presence and absence of 0.1 mmol/L of diprotin A (H-Ile-Pro-Ile-OH) (Bachem Bioscience, Torrance, CA). The methoxy-β-naphthylamine content was estimated fluorometrically (with a Bio-Tek FL600FA microplate fluorescence reader) at 460/40-nm emission wavelength and 360/40-nm excitation wavelength. The fluorescence value obtained at zero time (blank) was subtracted, and the relative fluorescence was then converted to picomoles of methoxy-β-naphthylamine by comparison with the standard curve of methoxy-β-naphthylamine dissolved in the same diluent used in the incubation (Sigma). Dipeptidyl peptidase IV activity was expressed as picomoles of hydrolyzed substrate per minute (UP) per milligram of protein. The existence of a linear relationship between hydrolysis time and protein content in the fluorometric assay was a prerequisite.

2.8. ELISA of CD26

CD26 content was spectrophotometrically measured at 450 nm, in triplicate of SF and MF pools of HT and HC of 4 animals per treated group, using the Human sCD26 Elisa kit (Bender MedSystems, Vienna, VIE, Austria) with anti-CD26 (EC 3.4.14.5) monoclonal antibody. CD26 contents were extrapolated by comparison with the standard curve of purified human CD26 resuspended in the reaction buffer, both as part of the commercial kit.

2.9. RT-PCR assay for CD26

2.9.1. Extraction of total RNA

Immediately after removal, HT and HC were macerated on ice and homogenized at room temperature (20°C) with Trizol (Invitrogen Life Technologies, San Diego, CA) at a ratio of 100:1 (milligram of tissue:milliliter of Trizol) (pestle mixer Tecnal model Te-099) and subsequently transferred to a polypropylene tube. Afterward, 1 mL of chloroform (Synth, Diadema, SP, Brazil) was added for each 0.5 mL of homogenate and mixed 20 times by inversion. This suspension was cooled for 8 minutes at 4°C and then centrifuged (microcentrifuge CT-14000R, Cientec, Piracicaba, SP, Brazil) at 12 000*g* for 20 minutes at 4°C. After centrifugation, 3 layers were formed: the top, or aqueous phase, composed of RNA; the intermediate phase, composed of DNA; and the lower, or phenol, phase. The aqueous phase was transferred to a new tube, mixed with 250 μL of isopropyl alcohol (Merck, Darmstadt, HES, Germany), and then placed at −80°C for 1 hour to precipitate the RNA. The sedimentation was performed at 12 000*g* for 15 minutes; and the resulting pellet was dissolved in 0.5 mL 75% ethanol, homogenized, and centrifuged at 8500*g* for 5 minutes at 4°C. The resulting supernatant was discarded, and the pellet was dried at 20°C and resuspended in 0.015 mL autoclaved water. The suspension was homogenized, heated to 55°C for 5 minutes, and stored at −80°C.

2.9.2. Analysis of total RNA

The total RNA isolated was quantified spectrophotometrically (Bio-Tek Power Wave X spectrophotometer), and its purity was evaluated by the ratio between the absorbance at 260 and 280 nm. The adequate quality of total RNA was checked by the existence of bands corresponding to 25S and

Table 1
Effect of diprotin A on the hydrolysis of synthetic substrate of DPPIV (H-Gly-Pro-4-methoxy-*b*-naphthylamide) by SF and MF fractions of hypothalamus and hippocampus of rats

Hypothalamus		Hippocampus	
SF	MF	SF	MF
52.51 ± 14.45† (6)	73.67 ± 11.28† (6)	59.97 ± 24.47* (6)	36.1 ± 25.4† (7)

*P< .05 and †P < .01 in relation to values obtained for the same sample without diprotin A (paired 2-side Student t test).
Values are mean ± SEM. Values are percentage relative to activity without diprotin A = 100%. Final concentration of diprotin A in incubates = 0.016 mmol/L. Number of animals in parenthesis.

18S ribosomal RNA, obtained in 1% agarose (Amersham Bioscience, São Paulo, SP, Brazil) gel electrophoresis (wt/vol), prepared in Tris/sodium acetate/EDTA 1× buffer, pH 8.0, under constant voltage (80 V), and stained with ethidium bromide solution (0.5 μg/mL) under ultraviolet light.

2.9.3. RT and PCR

Reverse transcription and PCR were performed using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen Life Technologies). Complementary DNA synthesis was achieved by converting total (500 ng) (Thermocycler PerkinElmer 2400, PerkinElmer, Waltham, MA), in cycles of 55°C for 20 minutes and 94°C for 2 minutes, for an initial complementary DNA denaturation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (control) was amplified by PCR with 30 cycles of 15 seconds each at 94°C, 30 seconds at 55°C, and 1 minute at 72°C; subsequently, a cycle of 7 minutes at 72°C was performed. CD26 was amplified with 30 cycles of 15 seconds at 94°C, 30 seconds at 50°C, and 1 minute at 72°C, and a final cycle of 7 minutes at 72°C. The following primers were used: Rat CD26 (EC 3.4.14.5) CD26 sense: 5′-GCACAAGAGCTAATAACACCACT'-3′, CD26 anti-sense: 5′-TGAAGGAGAACTATGATGCCCAGAATG-3′ (GenBank access no. NM_012789); GAPDH was used as positive control, and the following primers were adopted: GAPDH sense: 5′-TTCAACGGCACAGTCAAGGC-3′, GAPDH anti-sense: 5′-CACCAGCATCACCCCATTTG-3′ [25] (GenBank access no. NM_017008).

Band densities were determined by a GS 700 Densitometer (Bio-Rad Laboratories) using the image analysis software Molecular Analyst (Bio-Rad Laboratories) and standardized with the GAPDH band densities.

2.10. Statistics

The quantitative data were presented as mean ± SEM and statistically analyzed using the GraphPad Instat, GraphPad Prism, and Prism 3.0 software packages (GraphPad Software, La Jolla, CA). Regression analysis was performed to obtain standard curves of albumin and methoxy-β-naphthylamine. The Student t test was used to compare pairs of values. Because the t test assumes that the means come from populations with equal standard deviations, the F test was applied to verify that assumption. In all the calculations, a minimum critical level of $P < .05$ was set.

3. Results

3.1. Efficiency of the fractionation procedure

The LDH activity was higher in SF than in MF of HT (SF: 1.1655 ± 0.16, MF: 0.6185 ± 0.08) and of HC (SF: 1.706 ± 0.4, MF: 0.6407 ± 0.1). The APM activity exists in MF but was absent in SF of HT (MF: 670.7 ± 131.52) and of HC (MF: 615.41 ± 262.31).

3.2. DPPIV activity in the MF adsorbed in Bio-beads

The activity of total DPPIV in the MF of HT adsorbed (103.40 ± 3.5) in Bio-beads was lower than that of the

Table 2
Kinetic parameters of DPPIV-DS and DPPIV-DI activities obtained by fluorometric measurements of hydrolysis of synthetic substrate H-Gly-Pro-4-methoxy-β-naphthylamide by SF and MF fractions of HT and HC of rats

Tissue	Fraction	Enzyme	V_{max} (pmol min^{-1} mg^{-1} protein)	K_{cat} (s^{-1})	$K_m \times 10^{-5}$ (mol/L)	$K_{cat}/K_m \times 10^{-5}$ ([mol/L]$^{-1}$ s^{-1})
HT	SF	DPPIV-DS	72 ± 3	1.2	1.5 ± 1.2	0.8
		DPPIV-DI	3330 ± 153‡	55.5‡	25.2 ± 2.2†	2.2‡
	MF	DPPIV-DS	1720 ± 112	28.7	0.9 ± 0.3	31.9
		DPPIV-DI	5026 ± 560†	83.8‡	13.0 ± 2.8*	6.4‡
HC	SF	DPPIV-DS	16 ± 5	0.3	1.0 ± 0.3	0.3
		DPPIV-DI	243 ± 17†	4.1‡	7.0 ± 0.6†	0.6‡
	MF	DPPIV-DS	141 ± 20	2.4	3.8 ± 0.4	0.6
		DPPIV-DI	445 ± 185	7.4‡	1.7 ± 0.2*	4.4‡

Values are mean ± SEM. Number of animals = 3. Values for K_{cat} (maximum amount of substrate [picomoles] converted per second per enzyme unit [UI]) were calculated considering 1 UI = amount of enzyme, in 1 mg of protein, which hydrolyzes 1 pmol of substrate per second. Kinetic analysis was performed by Michaelis-Menten methodology, using GraphPad Prism software program. SEM = 0 for K_{cat} and K_{cat}/K_m.
Comparisons of hydrolyzing activities on the same substrate by the same tissue and fraction with or without inhibitor (unpaired 2-side Student t test):
* P< .05.
† P< .005.
‡ P< .0001.

Table 3
Effect of obesity and food deprivation on DPPIV-DS and DPPIV-DI activities (picomoles of substrate hydrolyzed per minute per milligram protein) in SF and MF fractions from the HT and HC

Treatments	Fractions	DPPIV-DS		DPPIV-DI	
		HT	HC	HT	HC
C	SF	55.81 ± 9.07 (5)	23.47 ± 8.73 (4)	39.88 ± 8.38 (5)	37.51 ± 8.73 (4)
	MF	103.23 ± 23.7 (5)	86.07 ± 23.54 (5)	288.78 ± 44.23 (6)	7.96 ± 5.09 (6)
FD	SF	47.02 ± 10.91 (4)	51.77 ± 10.79 (6)	28.73 ± 6.2 (5)	32.60 ± 11.41 (4)
	MF	174.33 ± 29.64 (5)	21 ± 9.81* (4)	311.23 ± 90.78 (5)	34.61 ± 10.75* (4)
MSG	SF	29.02 ± 8.19 (4)	30 ± 5.16 (5)	19.74 ± 2.75* (6)	25.5 ± 5.7 (4)
	MF	61.53 ± 14.36 (6)	6.15 ± 1.47* (4)	49.88 ± 11.13* (4)	131.48 ± 33.15† (4)
MSG-FD	SF	21.91 ± 10.68* (4)	15.13 ± 1 (4)	11.75 ± 2.49* (5)	24.88 ± 4.07 (4)
	MF	55.38 ± 12.74 (4)	51.03 ± 20.64 (4)	142.02 ± 40.74* (4)	43.75 ± 6.25† (4)

Values are mean ± SEM. Number of animals in parenthesis. Comparisons between DPPIV-DS vs DPPIV-DI in C animals: HT-SF ($P = .2331$), HC-SF ($P = .2988$), HT-MF ($P < .005$), HC-MF ($P < .01$) (unpaired 2-side Student t test).

* $P < .05$ and $^{\dagger}P < .003$ in relation to C in the same fraction (unpaired 2-side Student t test).

nonadsorbed (226.72 ± 14.7). In HC, there was no influence of adsorption on DPPIV activity (adsorbed: 31.15 ± 1.7, nonadsorbed: 92.88 ± 28.28).

Table 1 shows the effect of diprotin A on hydrolytic activity on H-Gly-Pro-4-methoxy-β-naphthylamide substrate (total DPPIV). In SF and MF of HT and HC, the diprotin A decreased the hydrolysis of the substrate. Based on the results of the substrate hydrolysis in the presence of diprotin, the following nomenclature was adopted in the present study: DPPIV-DS for diprotin A–sensitive DPPIV activity and DPPIV-DI for diprotin A–insensitive DPPIV activity.

The kinetic features distinguishing these peptidases activities are shown in Table 2.

Table 3 shows that, in HT and HC, the DPPIV-DS and -DI activities in the SF did not differ in C. Table 3 also shows that DPPIV-DS activity was about 3 times lower than DPPIV-DI in HT-MF of C, whereas in HC-MF of C, the inverse occurred; that is, DPPIV-DS activity was about 10 times higher than DPPIV-DI. Furthermore, Table 3 shows

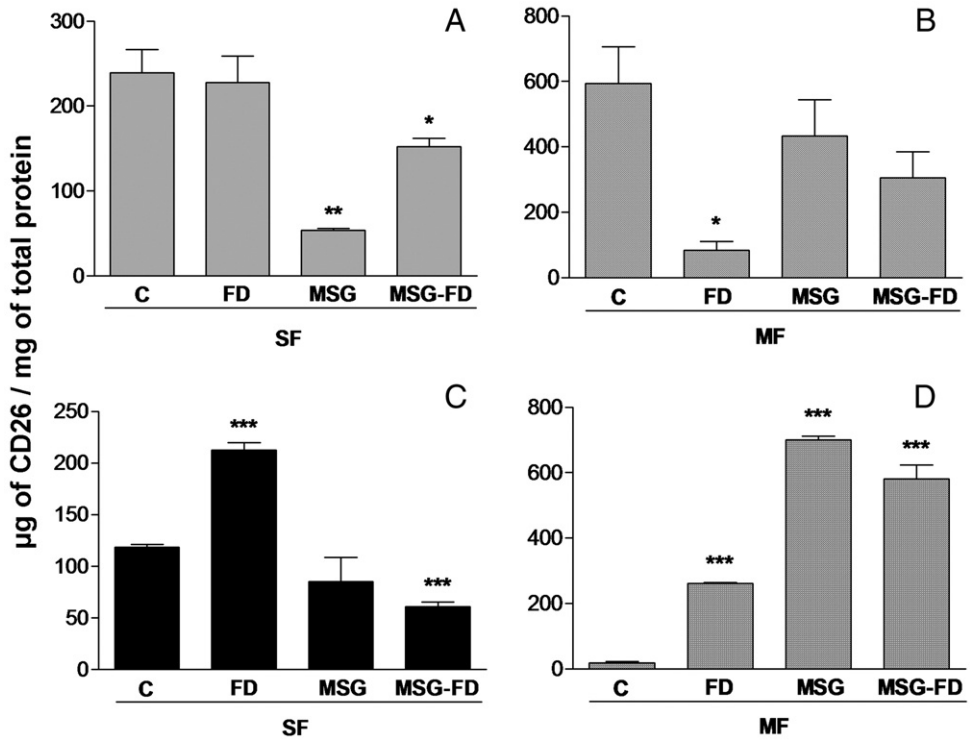

Fig. 1. CD26 (microgram per milligram protein) (mean ± SEM) quantified by ELISA in triplicates of pools of SF and MF fractions from the hypothalamus (A and B) and hippocampus (C and D) of 4 animals of each of the following groups: normal control (C), normal submitted to food deprivation (FD), MSG treated (MSG), and MSG treated and food deprived (MSG-FD). *$P < .04$, **$P < .003$, and ***$P < .0005$ relative to C in the same fraction (unpaired 2-side Student t test). Comparisons between SF vs MF in C animals: HT ($P < .05$), HC ($P < .0001$) (unpaired 2-side Student t test).

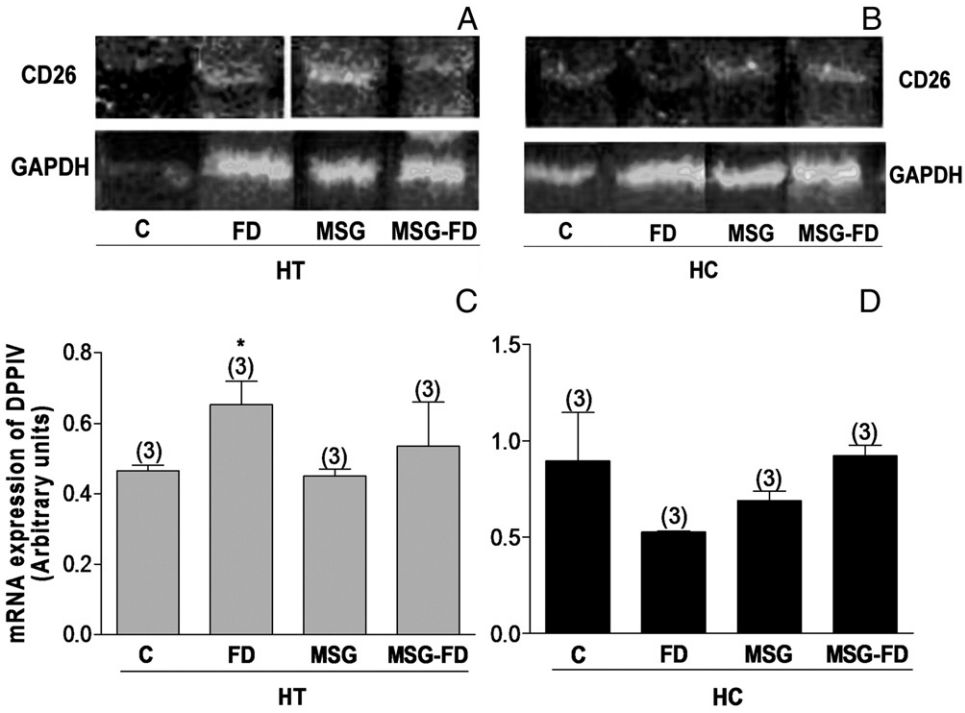

Fig. 2. Expression of CD26 mRNA detected by RT-PCR in the HT and HC of normal control animals (C), normal animals submitted to food deprivation (FD), MSG-treated animals (MSG), and MSG-treated and food-deprived animals (MSG-FD). A and B, Agarose gel electrophoresis of the products of RT-PCR. The products obtained by PCR were viewed by incorporation with ethidium bromide. C and D, Quantification by densitometry of the bands of products of the expression of mRNA, standardized from the band of GAPDH. Number of animals in parenthesis. *$P < .05$ relative to C in the same brain area (unpaired, 2-side Student t test). Comparisons between HT vs HC in C animals ($P = .1637$) (unpaired 2-side Student t test).

that MSG-FD animals had significant reduction of DPPIV-DS activity in HT-SF, compared with C. The DPPIV-DI activity was lower in SF and MF in HT of MSG and MSG-FD in relation to C. In MF, DPPIV-DS activity was lower in FD and MSG in relation to C. The DPPIV-DI activity increased in HC-MF of FD, MSG, and MSG-FD in relation to C.

3.3. ELISA of CD26

Fig. 1 shows that CD26 content (microgram of CD26 per milligram of total protein) in HT of C was 3 times higher in MF than in SF and that in HC of C was about 7 times higher in SF than in MF. Fig. 1 also shows the reduced content of CD26 in MSG (54 ± 2.08) and MSG-FD (152.12 ± 10.08) in HT-SF in relation to C (239.46 ± 27), whereas in HT-MF, CD26 content was reduced in FD (83.48 ± 27.11) in relation to C (594.10 ± 111.82). Fig. 1 also shows that CD26 content increased in HC-SF of FD (212.44 ± 7.14) and decreased in MSG-FD (61 ± 4.32) in relation to C (118.40 ± 2.87), whereas in MF, the CD26 content increased in FD (260.88 ± 3.01), MSG (701.06 ± 11.40), and MSG-FD (581.82 ± 41.64) in relation to C (17.96 ± 4.44).

3.4. Gene expression of CD26

Fig. 2 shows that the CD26 gene expression did not differ in HT and HC of C. The bands of messenger RNA (mRNA) expression of DPPIV in HT and HC are illustrated in Figs. 2A and B, respectively. As illustrated in Fig. 2C, densitometric analysis of these bands shows the increase of mRNA expression of CD26 in HT of FD (0.653 ± 0.066) in relation to C (0.466 ± 0.015). Compared with C (0.896 ± 0.252), there was no statistical difference in mRNA expression of CD26 in HC for all the groups of animals examined (Fig. 2D).

4. Discussion

4.1. Validation of the procedure to obtain the soluble and solubilized membrane fractions and interference of Triton X-100 in the quantification of DPPIV activity

Lactate dehydrogenase is a recognized cytosolic marker [26,27]. Therefore, its higher activity in soluble than in membrane-bound fraction of both structures under study reflects the efficiency of the fractionation procedure adopted. Another important indicator of the efficiency of fractionation was the existence of detectable APM only in membrane-bound fraction because this enzyme is known to be restricted to the membrane fraction of brain tissue [28-30]. Given that, after reducing the presence of Triton X-100 by adsorption in Bio-beads, a significant decrease of DPPIV activity was detected in the hypothalamus, this procedure was not adopted throughout this study. This reduction of enzyme

activity can be attributed to denaturation and/or proteolysis of DPPIV during the additional time required by the adsorption protocol (2 hours), which retarded the storage of the membrane fraction at −80°C.

4.2. Characterization of the DPPIV activity and its hypothalamic and hippocampal levels in soluble and membrane-bound fractions

The use of inhibitors to distinguish enzymes with similar catalytic activity is a standard practice. For example, the neutral aminopeptidase insensitive to puromycin (APM), the neutral aminopeptidase sensitive to puromycin, and Ap200 were distinguished using inhibitors [31-33]. Diprotin A has been regarded as the canonical inhibitor of DPPIV activity, which was the condition to choose this inhibitor in the present study. Nowadays, however, it is known that there are several proteins with DPPIV catalytic activity [15] that are also inhibited by diprotin A, such as DPP8 and DPP9 [34]. The existence of the activities of DPPIV sensitive (DPPIV-DS) and insensitive (DPPIV-DI) to diprotin A was recently reported in rat plasma [33]. In the present study, the sensitivity to diprotin A and different kinetic properties also distinguished, for the first time in the hypothalamus and the hippocampus of rats, 2 DPPIV activities: DPPIV-DS and DPPIV-DI. In general, DPPIV-DI was more active than DPPIV-DS (as shown by the V_{max} values) and the affinity of DPPIV-DS was higher (lower K_m) than that of DPPIV-DI (except in the membrane-bound fraction of the hippocampus). Furthermore, the K_{cat} of DPPIV-DI was higher than that of DPPIV-DS. However, in general, the catalytic efficiency (K_{cat}/K_m) of DPPIV-DI was higher than that of DPPIV-DS (except in the membrane-bound fraction of the hypothalamus). In humans, the CD26 gene is located in chromosome 2 (2q24.2), whereas the DPP8 gene is located in chromosome 15 (15q22) [35]; and thus, the present data are related to the gene expression of CD26 (NM_012789.1). The CD26 protein (EC 3.4.14.5) is known to be structurally homologous with DPP8 and DPP9 (not classified by the Enzyme Nomenclature Commission) and with the fibroblast activation protein-α (EC 3.4.21.B28) [10,35]. However, only the DPP8 has been found in CNS [35]. In the present study, the anti-CD26 (anti EC 3.4.14.5) used in ELISA is a monoclonal antibody that should react with the canonical CD26.

In the control animals, the CD26 protein content (ELISA) and DPPIV catalytic activity in the membrane fraction were higher in the hypothalamus than in the hippocampus. Furthermore, in the hypothalamus, the CD26 (ELISA) levels in membrane-bound fraction were higher than in the soluble fraction, whereas the opposite occurred in the hippocampus. Therefore, the CD26 gene expression does not correspond exactly to its complete protein expression in the hypothalamus. In the control animals, the DPPIV-DI catalytic activity was predominant in relation to DPPIV-DS in the hypothalamus and hippocampus. In addition to suggesting different prevalences for each form of DPPIV catalytic activity (soluble, membrane, diprotin A sensitive and insensitive), these data also strongly suggest that CD26 protein in these 2 brain areas is responsible for DPPIV-DI catalytic activity. Some divergences regarding the changes between gene and protein expressions of CD26 and DPPIV catalytic activity observed here in the hypothalamus and hippocampus from different treated animals are in agreement with the known receptor intracellular signal transduction function of proteins homologous to CD26 [36-38]. For example, the differences between the protein content of CD26 and DPPIV-DI activity in soluble fraction of the hippocampus and in membrane-bound fraction of the hypothalamus in food-deprived rats did not change compared with the control group; but an increased amount of CD26 protein without a necessary increase of DPPIV catalytic activity could be due to this aforementioned receptor function of CD26 protein and/or to different sensitivities between the measurement techniques used; that is, the ELISA is more sensitive than fluorometric detection of enzymatic activity.

4.3. Hypothalamic changes of DPPIV in MSG obesity and food deprivation

The DPPIV activity levels in the hypothalamus decreased in MSG obese animals (in both forms, soluble and membrane), with similar changes in the CD26 protein detectable in soluble fraction of MSG and fasted MSG, as well as in membrane-bound fraction of normal fasted animals compared with the controls. Changes in DPPIV-DS in relation to the controls were much less significant, with a decrease occurring only in the soluble fraction in animals submitted to 2 of the stimuli under study (fasted MSG). The decrease in catalytic activity of DPPIV (mainly of the DI type) in the hypothalamus should promote changes in the levels of neuropeptide Y (NPY), GLP-1, and β-endorphin [10,35,39]. It is noteworthy that DPPIV-DI also decreases in the plasma of fasted MSG [33]. Neuropeptide Y and β-endorphin are known to increase in obese individuals [40-43], whereas hydrolysis of NPY 1-36 by DPPIV generates the product NPY 3-36, which has higher affinity to the Y2 receptor [44] and mediates the inhibition of NPY 1-36 release [45,46]. Thus, decreased DPPIV activity in the hypothalamus may contribute to disrupting the energy balance by promoting increased levels of NPY and β-endorphin.

4.4. Hippocampal changes of DPPIV in MSG obesity and food deprivation

Whereas the hypothalamus is the brain structure related to the sense of satiety, adiposity, and hunger, the final processing of these signals depends on the regions of the brain involved with behavioral control, such as the hippocampus [47]. The hippocampus receives signals of the energy status from the periphery via the vagus nerve [47] and has a high density of NPY [48] and opioid [49,50]

receptors. It should be noted that changes in DPPIV-DI catalytic activity and membrane CD26 protein were fully coincident in the hippocampus and in the opposite direction to that observed in the hypothalamus, which showed a significant increase in normal fasted and fasted and nonfasted MSG animals. However, DPPIV-DS catalytic activity decreased in these situations, mainly in the membrane-bound fraction, together with a drop of CD26 only in the soluble fraction of fasted MSG animals. Therefore, the pathophysiologic significance of changes in DPPIV-DS activity levels in the hippocampus seems to be difficult to interpret; but these changes serve to reinforce the hypothesis that DPPIV-DS activity does not correspond to CD26. For the same reason, considering the identity of the DPPIV-DI with CD26 and the correspondence with their known natural substrates, the increase of DPPIV-DI catalytic activity should induce decreased levels of NPY in the hippocampus. The NPY has an important anxiolytic role in the limbic structures, and stress increases its expression and release [42,51,52]. The increase of hippocampal DPPIV-DI catalytic activity could be related to increased anxiety in the situations studied here, which are readily recognized as stressful [53,54]. The apparent paradox is that the membrane fraction from the hippocampus changes to the opposite direction under each treatment, depending on the presence of diprotin; that is, hippocampal membrane-bound DPPIV-DI increased in normal fasted, MSG, and fasted MSG animals, whereas DPPIV-DS decreased in normal fasted and MSG (compared with control) animals. Besides corroborating the fact that these 2 enzyme activities should be attributed to different proteins, this finding also suggests a substitutive physiologic relationship of these hippocampal enzymes in certain situations; that is, increased DPPIV-DI activity offsets the reduction in DPPIV-DS activity, and vice versa. This intriguing possibility may be related to the need for a refined and redundant 2-way metabolic control of DPPIV substrates in this critical area of the CNS. In this sense, recent report has demonstrated that the phenotype of neurogenin 3–specific DPP2 knockdown mice is opposite that of DPP4 knockout mice, respectively, with regard to the presence of normal GLP-1 with glucose intolerance and the increased GLP-1 with increased glucose tolerance [55].

5. Conclusions

This study evidenced the existence of DPPIV catalytic activity with distinct sensitivities to diprotin A in the CNS (the insensitive identified as the CD26 protein), as well as the involvement of CD26 catalytic activity of DPPIV in the endocrine regulation of the energy balance in the hypothalamus (in the soluble and membrane-bound forms), and probably in anxiety in the hippocampus (in the membrane-bound form), through its action on β-endorphin and NPY, respectively.

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References

[1] Zammaretti F, Panzica G, Eva C. Fasting, leptin treatment and glucose administration differentially regulate Y(1) receptor gene expression in the hypothalamus of transgenic mice. Endocrinology 2001;142:3774-82.
[2] Chelikani PK, Ambrose JD, Keisler DH, et al. Effect of short-fasting on plasma concentrations of leptin and other hormones and metabolites in dairy cattle. Domest Anim Endocrinol 2004;26:33-48.
[3] Crane C, Akhter N, Johnson BW, et al. Fasting and glucose effects on pituitary leptin expression: is leptin a local signal for nutrient status? J Histochem Cytochem 2007;55:1059-73.
[4] Bernardis LL, Patterson BD. Correlation between “Lee index” and carcass fat content in weanling and adult female rats with hypothalamic lesions. J Endocrinol 1968;40:527-8.
[5] Leigh FS, Kaufman LN, Young JB. Diminished epinephrine excretion in genetically obese (*ob/ob*) mice and monosodium glutamate-treated rats. Int J Obes Relat Metab Dis 1992;16:597-604.
[6] Nakagawa T, Ukai K, Ohyama T, et al. Effects of chronic administration of sibutramine on body weight, food intake and motor activity in neonatally monosodium glutamate-treated obese female rats: relationship of antiobesity effect with monoamines. Exp Anim 2000;49:239-49.
[7] Dolnikoff M, Martin-Hidalgo A, Machado UF, et al. Decreased lipolysis and enhanced glycerol and glucose utilization by adipose tissue prior to development of obesity in monosodium glutamate (MSG) treated-rats. Int J Obes Relat Metab Dis 2001;25:426-33.
[8] Pethiyagoda CL, Welch DR, Fleming TP. Dipeptidyl peptidase IV (DPPIV) inhibits cellular invasion of melanoma cells. Clin Exp Metastasis 2000;18:391-400.
[9] Gorrell MD, Gysbers V, McCaughan GW. CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. Scand J Immunol 2001;54:249-64.
[10] Gorrell MD. Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. Clin Sci 2005;108:277-92.
[11] Boonacker E, Van Noorden CJ. The multifunctional or moonlighting protein CD26/DPPIV. Eur J Cell Biol 2003;82:53-73.
[12] Sharoyan SG, Antonyan AA, Mardanyan SS, et al. Complex of dipeptidyl peptidase II with adenosine deaminase. Biochemistry (Mosc) 2008;73:943-9.
[13] Hildebrandt M, Reutter W, Arck P, et al. A guardian angel: the involvement of dipeptidyl peptidase IV in psychoneuroendocrine function, nutrition and immune defence. Clin Sci 2000;99:93-104.
[14] Ranganath LR. Incretins: pathophysiological and therapeutic implications of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1. J Clin Pathol 2008;61:401-9.
[15] Maes MB, Dubois V, Brandt I, et al. Dipeptidyl peptidase 8/9-like activity in human leukocytes. J Leukoc Biol 2007;81:1252-7.
[16] Zambotti-Villela L, Yamasaki SC, Villarroel JS, et al. Prolyl, cystyl and pyroglutamyl peptidase activities in the hippocampus and hypothalamus of streptozotocin-induced diabetic rats. Peptides 2007;28:1586-95.
[17] Zambotti-Villela L, Yamasaki SC, Villarroel JS, et al. Prospective evaluation of aminopeptidase activities in plasma and peripheral organs of streptozotocin-induced diabetic rats. J Endocrinol Invest 2008;31:492-8.

[18] Olivo R do A, Teixeira C de F, Silveira PF. Representative aminopeptidases and prolyl endopeptidase from murine macrophages: comparative activity levels in resident and elicited cells. Biochem Pharmacol 2005;69:1441-50.
[19] Kaufhold A, Nigam PK, Dhir RN, et al. Prevention of latently expressed CYP2C11, CYP3A2, and growth hormone defects in neonatally monosodium glutamate-treated male rats by the n-methyl-d-aspartate receptor antagonist dizocilpine maleate. JPET 2002;302: 490-6.
[20] Rogers P, Webb GP. Estimation of body fat in normal and obese mice. Br J Nutr 1980;43:83-6.
[21] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 6th ed. Amsterdam: Academic Press; 2007.
[22] Bergmeyer HU, Brent E. Assay with pyruvate and NADH. Methods in enzymatic analysis, 2nd edLondon: Academic Press Inc.; 1972. p. 574-7.
[23] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
[24] Silveira PF, Gil J, Casis L, et al. Peptide metabolism and the control of body fluid homeostasis. Curr Med Chem Cardiovasc Hematol Agents 2004;2:219-38.
[25] Alonso E, Garrido E, Díez-Fernández C, et al. Yohimbine prevents morphine-induced changes of glial fibrillary acidic protein in brainstem and α_2-adrenoceptor gene expression in hippocampus. Neurosci Lett 2007;412:163-7.
[26] Hosokawa R, Ohashi-Takeuchi H, Yamada N, et al. Lactate dehydrogenase isoenzymes in matrix vesicles (review). Bone Miner 1992;17:177-81.
[27] Noraberg J, Jensen CV, Bonde C, et al. The developmental expression of fluorescent proteins in organotypic hippocampal slice cultures from transgenic mice and its use in the determination of excitotoxic neurodegeneration. Altern Lab Anim 2007;35:61-70.
[28] Gillespie L, Konings PN, Merril BJ, et al. A specific enzyme assay for aminopeptidase M in rat brain. Life Sci 1992;51:2091-106.
[29] Irazusta J, Larrinaga G, Agirregoitia N, et al. Effects of morphine administration and its withdrawal on rat brain aminopeptidase activities. Regul Pept 2003;110:225-30.
[30] Larrinaga G, Gil J, Meana JJ, et al. Aminopeptidase activity in the postmortem brain of human heroin addicts. Neurochem Int 2005;46: 213-9.
[31] Erbeznik H, Hersh LB. A novel mammalian high-molecular-weight aminopeptidase. Arch Biochem Biophys 1997;344:228-34.
[32] Thompson MW, Tobler A, Fontana A, Hersh LB. Cloning and analysis of the gene for the human puromycin-sensitive aminopeptidase. Biochem Biophys Res Commun 1999;258:234-40.
[33 Alponti RF, Silveira PF. Neutral aminopeptidase and dipeptidyl deptidase IV activities in plasma of monosodium glutamate obese and food-deprived rats. Obesity in press; doi:10.1038/oby.2009.378.
[34] Qi SY, Riviere PJ, Trojnar J, et al. Cloning and characterization of dipeptidyl peptidase 10, a new member of an emerging subgroup of serine proteases. Biochem J 2003;373:179-89.
[35] Frerker N, Wagner L, Wolf R, et al. Neuropeptide Y (NPY) cleaving enzymes: structural and functional homologues of dipeptidyl peptidase 4. Peptides 2007;28:257-68.
[36] Mentlein R. Cell-surface peptidases. Int Rev Cytol 2004;235:165-213.
[37] Pacheco R, Martinez-Navio JM, Lejeune M, et al. CD26, adenosine deaminase, and adenosine receptors mediate costimulatory signals in the immunological synapse. Proc Natl Acad Sci USA 2005;102: 9583-8.
[38] Havre PA, Abe M, Urasaki Y, et al. The role of CD26/dipeptidyl peptidase IV in cancer. Front Biosci 2008;13:1634-45.
[39] Bjelke JR, Christensen J, Nielsen PF, et al. Dipeptidyl peptidases 8 and 9: specificity and molecular characterization compared with dipeptidyl peptidase IV. Biochem J 2006;396:391-9.
[40] Dhillo WS, Bloom SR. Hypothalamic peptides as drugs targets for obesity. Curr Opin Pharmacol 2001;1:651-5.
[41] Volkoff H, Canosa LF, Unniappan S, et al. Neuropeptides and the control of food intake in fish. Gen Comp Endocrinol 2005;142:3-19.
[42] Arora S, Anubhuti. Role of neuropeptides in appetite regulation and obesity—a review. Neuropeptides 2006;40:375-401.
[43] Schwartz MW, Porte D. Diabetes, obesity, and the brain. Science 2005; 307:375-9.
[44] Mentlein R. Dipeptidyl-peptidase IV (CD26)—role in the inactivation of regulatory 3 peptides. Regul Pept 1999;85:9-24.
[45] Berglund MM, Hipskind PA, Gelert DR. Recent developments in our understanding of the physiological role of PP-fold peptide receptor subtypes. Exp Biol Med 2003;228:217-44.
[46] Kamiji MM, Inui A. NPY Y2 and Y4 receptors selective ligands: promising anti-obesity drugs? Curr Top Med Chem 2007;7:1734-42.
[47] Davidson TL, Kanoski SE, Schier LA, et al. A potential role for the hippocampus in energy intake and body weight regulation. Curr Opin Pharmacol 2007;7:613-6.
[48] Xapelli S, Agasse F, Ferreira R, et al. Neuropeptide Y as an endogenous antiepileptic, neuroprotective and pro-neurogenic peptide. Recent Patents CNS Drug Discov 2006;1:315-24.
[49] Simmons ML, Chavkin C. Endogenous opioid regulation of hippocampal function. Int Rev Neurobiol 1996;39:145-96.
[50] Eva C, Serra M, Mele P, et al. Physiology and gene regulation of the brain NPY Y1 receptor. Front Neuroendocrinol 2006;27:308-39.
51] Sajdyk TJ, Shekhar A, Gehlert DR. Interaction between NPY and CRF in the amygdale to regulate emotionality. Neuropeptides 2004;38: 225-34.
[52] Carvajal C, Dumont Y, Quirion R. Neuropeptide Y: role in emotion and alcohol dependence. CNS Neurol Disord Drug Targets 2006;5: 181-95.
[53] Scott KM, Bruffaerts R, Simon GE, et al. Obesity and mental disorders in adult general population: results from the world mental health surveys. Int J Obes 2008;32:192-200.
[54] Scott KM, McGee MA, Wells E, et al. Obesity and mental disorders in adult general population. J Pshyc Res 2008;64:94-105.
[55] Danilova OV, Tai AK, Mele DA, et al. Neurogenin 3-specific dipeptidyl peptidase-2 deficiency causes impaired glucose tolerance, insulin resistance, and visceral obesity. Endocrinology 2009;150:5240-8.