

Transcriptional interruption of cAMP response element binding protein modulates superoxide dismutase and neuropeptide Y-mediated feeding behavior in freely moving rats

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Abstract

The appetite-suppressing effect of phenylpropanolamine (PPA) has been attributed to its inhibitory action on neuropeptide Y (NPY), an appetite stimulant. However, molecular mechanisms underlying this effect are not clear. This study aimed to investigate if cAMP response element binding protein (CREB) signaling was involved. Moreover, possible role of superoxide dismutase-2 (SOD-2) during PPA treatment was also examined. Rats were daily treated with PPA for 4 days. Changes in hypothalamic NPY, protein kinase A, CREB, and SOD-2 mRNA contents were measured and compared. Results showed that protein kinase A, CREB, and SOD-2 mRNA levels increased during PPA treatment, which is concomitant with decreases in NPY and feeding. Moreover, CREB DNA binding activity detected by electromobility shift

assay increased during PPA treatment, revealing an involvement of CREB-dependent gene transcription. Furthermore, infusions of CREB antisense oligonucleotide (or missense control) into cerebroventricle were performed at 1 h before daily PPA treatment in free-moving rats, and results showed that CREB knockdown could block PPA-induced anorexia and modify NPY and SOD-2 mRNA content toward normal. It is suggested that CREB signaling may participate in the central regulation of PPA-mediated appetite suppression via the modulation of NPY gene expression and that an increase of SOD-2 may favor this modulation.

Keywords: brain, oxidative stress, phenylpropanolamine, signal transduction.

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Hypothalamic neuropeptide Y (NPY) is a powerful orexiogenic agent which plays critical roles in periodic eating behavior and maintenance of body weight (Wynne *et al.* 2005). Central administration of NPY can induce hyperphagia even under conditions of satiation, resulting in an increase of fat deposition, a decrease of energy expenditure, and a promotion of obesity (Williams *et al.* 2001). Phenylpropanolamine (PPA) is an over-the-counter anorectic drug that can be used in human dieters to reduce obesity (Schteingart 1992; Borovicka *et al.* 2002; Cooper *et al.* 2005). PPA is structurally and functionally related to amphetamine (AMPH)-like anorectic drugs, such as ephedrine, phentermine, diethylpropion, and methamphetamine, and is regarded as a sympathomimetic agent because of its

effect on the brain (Colman 2005). Evidence reveals that the mechanism underlying the appetite-suppressing effect of PPA is implicated in the central release of catecholamine

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Abbreviations used: AMPH, amphetamine; CART, cocaine- and amphetamine-regulated transcript; CREB, c-AMP response element binding protein; EMSA, electromobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; i.c.v., intracerebroventricular; NPY, neuropeptide Y; ODN, oligodeoxynucleotides; PKA, protein kinase A; PPA, phenylpropanolamine; SOD, superoxide dismutase.

(Wellman and Davies 1992; Cheng and Kuo 2003), which can exert its effect on hypothalamic NPY neurons to suppress appetite (Hsieh *et al.* 2004).

It is unclear whether the intracellular signaling of cAMP response element binding protein (CREB) is required for NPY gene expression during PPA treatment. CREB is a downstream nuclear transcription factor of protein kinase A (PKA), which can be elicited by various physiological ligands and is critically involved in the regulation of energy metabolism (Habener *et al.* 1995; Griffioen and Thevelein 2002). Given the role in neuronal plasticity, CREB has emerged as a molecule that is important in modulating behavioral responses (Nair and Vaidya 2006). In the brain, CREB is required for behavioral sensitization (McDaid *et al.* 2006) and self-administration behavior (Choi *et al.* 2006). Moreover, several studies demonstrate that cAMP is involved in the regulation of NPY-induced feeding behavior (Akabayashi *et al.* 1994; Konradi *et al.* 1994; Sheriff *et al.* 2003) and that CREB is required for dopamine-dependent gene expression (Das *et al.* 1997; Andersson *et al.* 2001) and can regulate the expression of NPY gene that is implicated in anxiety and alcohol drinking behaviors (Pandey 2003). Thus, we hypothesized that CRE-mediated gene induction might involve the regulation of NPY gene expression during PPA treatment.

The enzyme superoxide dismutase (SOD), including CuZn-SOD (SOD-1) and Mn-SOD (SOD-2), is essential in destroying oxygen-based radicals and reported to play a role in the reduction of methamphetamine-induced neurotoxicity (Sheng *et al.* 1996). Moreover, SOD is activated during AMPH treatment, which is associated with the normalization of AMPH-induced appetite suppression (Hsieh *et al.* 2006). Thus, we hypothesized that SOD gene might be activated for the decrease of the oxidative stress and thus help to normalize the feeding behavior during PPA treatment. The enzyme SOD-2, a mitochondrial SOD, was detected as it was reported to implicate in the neuroprotective action in the brain (Hu *et al.* 2007).

Daily application of CREB antisense oligodeoxynucleotides (ODN) into brain was employed in the present study to disrupt CREB-dependent gene transcription or CREB translation in free-moving rats. Antisense technique was recently applied to study the effect of drug on behavioral response as it was preferentially taken up by neurons in the rodent brain after intracerebral administration (Yee *et al.* 1994; Ogawa *et al.* 1995). Moreover, antisense had been used to interrupt specific gene expression in the brain (Chiasson *et al.* 1992; Ghosh and Cohen 1992) or in the hypothalamus (Hulsey *et al.* 1995). Therefore, we chose antisense, which had been previously used to specifically decrease CREB translation in the brain (Chance *et al.* 2000), to examine its effect on PPA anorexia following central ventricular administration.

Experimental procedures

Animals

Male Wistar rats (with a weight of 200–300 g, Animal Center of National Cheng Kung University Medical College, Tainan, Taiwan) were housed individually in transparent plastic cages with stainless steel covering and hardwood bedding. Food and tap water were provided *ad libitum*. Animals were maintained at $22 \pm 2^\circ\text{C}$ according to a 12 h light : 12 h dark cycle (light on at 6:00 AM) and habituated to frequent handling. Drug administration and food intake assessment were performed daily at the beginning of dark phase (6:00 PM). This study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Drug treatments

To examine the effect of PPA ([\pm]-PPA, a hydrochloride salt; Sigma-Aldrich, St Louis, MO, USA) on feeding behavior, rats were given the PPA (0, 75, or 115 mg/kg, i.p.; $n = 6$ –8 each group) daily for 4 days at the beginning of dark phase (at 6:00 PM). Feeding behavior was examined at 24 h after daily drug treatment. The first injection of PPA was conducted at the end of day 0 (at 6:00 PM) and the intake data were calculated as the total amount of food during the previous day. The drug PPA is dissolved in sterile distilled water. The control vehicle is normal saline.

To assess the effect of PPA on NPY, CREB, PKA, and SOD-2 mRNA levels, rats ($n = 5$ –6 each group) were given the PPA (0 or 75 mg/kg, i.p.) daily for 1, 2, 3, or 4 days, and then were killed. Rats receiving PPA at 40 min before killing were anesthetized with pentobarbital (30 mg/kg, i.p.) and then decapitated. The hypothalamus was removed from the brain immediately and subjected to determinations of mRNA levels, or stored at -80°C until the day to use.

To determine the effect of PPA on CREB DNA binding activity, rats were given the PPA (75 mg/kg; i.p.; $n = 4$ –6 each group) daily for 4 days at the beginning of dark phase (at 6:00 PM). At 40 min after daily PPA treatment, the hypothalamus was removed to determine CREB DNA binding activity by a technique of electromobility shift assay (EMSA) daily for 4 days.

To detect the time course of a 24-h pattern of feeding behavior induced by a single treatment of PPA (75 mg/kg; i.p.; $n = 6$ –8 per group) in normal and PPA-tolerant rats, the amounts of food intake were measured every 6-h intervals, including at 6:00 PM, 12:00 PM, 6:00 AM, 12:00 AM, and 6:00 PM (the next day), over a 24-h period after PPA treatment.

To assess the effect of PPA on NPY, CREB, and SOD-2 protein contents, rats ($n = 5$ –6 each group) were given the PPA (0 or 75 mg/kg, i.p.) daily for 1, 2, 3, or 4 days, and then were killed. Rats receiving PPA at 40 min before killing were anesthetized with pentobarbital (30 mg/kg, i.p.) and then decapitated. The hypothalamus was removed from the brain immediately and subjected to determinations of protein contents, or stored at -80°C until the day to use.

To determine the effect of intracerebroventricular (i.c.v.) injection of CREB antisense (or missense) on CREB protein and mRNA levels, rats ($n = 4$ –6 for each group) were daily infused with antisense or missense (20 μg in a 10- μL vehicle; i.c.v.) for 2–3 days until the response of feeding behavior was slightly reduced in

antisense group when compared with missense group. At 40 min after the last treatment of antisense or missense, the hypothalamus was removed from the brain and its CREB protein content and mRNA level were determined by western blot and RT-PCR, respectively.

RNA extraction

Hypothalamic NPY mRNA levels in a block of mediobasal hypothalamic tissue were measured as described previously (Morris 1989). The block of mediobasal hypothalamic tissue was dissected rostral-caudally from the optic chiasma to the mammillary body, and extended laterally from the midline of hypothalamus to the perihypothalamic nucleus and superiorly to the anterior commissure. The weight of each dissected hypothalamic tissue is about 0.053 ± 0.012 g. Total RNA was isolated from this block using the modified guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). Each hypothalamic block was homogenized in 1 mL of TRIZOL reagent (Life Technologies Inc., Grand Island, NY, USA) using an Ultrasonic Processor (Vibra Cell, Model CV17; Sonics & Materials Inc., Danbury, CT, USA). After an incubation at 22°C for 5 min, 0.2 mL of chloroform was added to each sample, shaken vigorously for 15 s, incubated at 22°C for 3 min, and then centrifuged at 12 000 *g* for 15 min at 4°C. After removal of aqueous phase and precipitation with 0.5 mL isopropanol, samples were incubated at 22°C for 10 min and centrifuged at 12 000 *g* for 15 min at 4°C. The gel-like RNA pellets were washed with 75% ethanol by vortexing and centrifugation at 7500 *g* for 5 min at 4°C. Thereafter, RNA pellets were dried briefly, dissolved in RNase-free water, and stored at -80°C. The content of RNA was determined spectrophotometrically at 260 nm (Hitachi U-3210, Tokyo, Japan).

RT-PCR

Using the first Strand cDNA Synthesis Kit (Boehringer Mannheim GmbH, Ingelheim, Germany), RNA was reversely transcribed into single-stranded cDNA. For each sample, 8 μ L of sterile diethyl pyrocarbonate water containing 2 μ g of RNA were added to oligo-p(dT)15 primer (0.8 μ g/ μ L) followed by a heating at 65°C for 15 min, a cooling at 25°C for 10 min, and then added to a reaction mixture consisting of 10x reaction buffer (100 mM Tris and

500 mM KCl, pH 8.3), deoxynucleotide mix (10 mM each), MgCl₂ (25 mM), RNase inhibitor (40 U/ μ L), and avian myeloblastosis virus reverse transcriptase (25 U/ μ L). Reaction mixtures were incubated at 42°C for 2 h and then brought to 95°C for 5 min to terminate the reaction followed by soaking at 16°C. PCR was subsequently carried out by mixing 3 μ L of cDNA product with master-mix solution consisting of diethyl pyrocarbonate water, 10x reaction buffer, MgCl₂ (25 mM), deoxynucleotide mix (10 mM each), P1 and P2 primers (1 μ g/ μ L each), and Taq polymerase (5 U/ μ L). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal standard calibrator. PCR reactions for NPY were carried out on a PCR thermocycler (Perkin-Elmer GeneAmp 2400, Applied Biosystems, Foster City, CA, USA) for 28 cycles with the following steps: 91°C for 1 min (denaturing), 60°C for 1 min (annealing), and 72°C for 30 s (extension), followed by a final elongation step at 72°C for 7 min, and finally the PCR products were soaked at 16°C. PCR reactions for the other molecules analyzed were carried out in steps similar to those described above except the changes of two steps (annealing and cycles) that were described as follows: CREB (60°C, 35 cycles); PKA (60°C, 28 cycles); SOD-2 (60°C, 25 cycles); cocaine- and amphetamine-regulated transcript (CART) (60°C, 28 cycles); and GAPDH (52°C, 25 cycles). All PCR products were measured during the exponential phase of the DNA amplification in the present study. The sequences of primers used in RT-PCR were shown in Table 1.

Gel electrophoresis

At the completion of RT-PCR, 8 μ L of each PCR product was subsequently separated by flat-bed gel electrophoresis on a 3% agarose gel. Gels stained by ethidium bromide (0.5 μ g/mL; Sigma-Aldrich Co.) were visualized under UV light, photographed, and then scanned densitometrically. Ratios of NPY and GAPDH mRNA were calculated to determine relative NPY mRNA levels. Contents of NPY mRNA in PPA-treated group were indicated as the percentage of control group. The ratio of NPY/GAPDH mRNA was measured by digital densitometry (Kodak Gel Logic 100 Imaging System; Eastman Kodak Company, Rochester, NY, USA). Similar steps were used to determine the contents of CREB, PKA, CART, and SOD-2 mRNA.

	Primer	Sequence 5' → 3'	Size of product (bp)
NPY	Forward	GGGCTGTGTGGACTGACC	264
	Reverse	GGAAGGGTCTTCAAGCCT	
CREB	Forward	GAAAGCAGTGACTGAGGAGCTTGTA	616
	Reverse	GGGCTAAGCAGTTGGTGGTGCAGGATGCA	
PKA	Forward	AGAGTGAATCGGACTCGGACG	383
	Reverse	GCCACGGTTTGCATACTGACC	
CART	Forward	CTCCTGGGCGCCGCCCTGCTGC	252
	Reverse	CATGGGGACTTGGCCGTACTTC	
SOD-2	Forward	CTTCAGCCTGCACTGAAGTTCAAT	327
	Reverse	CTGAAGATAGTAAGCGTGCTCCC	
GAPDH	Forward	TCCCTCAAGATTGTCTAGCAA	309
	Reverse	AGATCCACAACGGATACATT	

Table 1 Sequences of primers used in the experiment of RT-PCR

NPY, neuropeptide Y; CREB, c-AMP response element binding protein; PKA, protein kinase A; CART, cocaine- and amphetamine-regulated transcript; SOD, superoxide dismutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

CREB binding assay

Binding of CREB in nuclear extracts was assessed by EMSA with double-stranded deoxyoligonucleotides specific for CREB consensus sequence 5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3' which was labeled on the 3'-end with biotin. EMSA was carried out using the Lightshift kit (Promega Life Science, Madison, WI, USA). Briefly, 10 µg of nuclear protein was pre-incubated with 10 mM Tris, 50 mM KCl, 1 mM dithiothreitol, 5 mM MgCl₂, 2 µg poly (dI × dC), and 2 pmol of oligonucleotide probe for 20 min at 25°C. Specific binding was confirmed by using a 200-fold excess of unlabeled probes as specific competitor. Protein–DNA complexes were separated by a 6% non-denaturing acrylamide gel electrophoresis. Complexes were transferred to positively charged nylon membranes and UV-cross-linked in a cross-linker. Gel shifts were visualized with a streptavidin–horseradish peroxidase followed by chemiluminescent detection (Chen *et al.* 2006).

Lateral ventricular cannulation

Stereotaxic surgery (Kopf Model 900, Tujunga, CA, USA) of rats was performed under anesthesia with pentobarbital (30 mg/kg, i.p.). The target of cannulation was close to the junction between the right lateral ventricle and the third ventricle (coordinates: 0.8 mm posterior to Bregma, 1.5 mm from the midline, and 3.5–4.0 mm below the dura) (Paxinos and Watson 1986). A 23-g stainless steel guide cannula was implanted and secured to the skull using stainless steel screws and dental cement. The accuracy of placement was confirmed by observing a transient and rapid inflow of vehicle in polyethylene tube connected with a 28-g injector cannula. The cannula was then occluded with a 28-g stylet. For i.c.v. infusion of NPY antisense, the stylet was replaced with a 28-g injector cannula extending 0.5 mm below the tip of guide cannula. The injector cannula was connected via polyethylene tube to a 10-µL Hamilton microsyringe (Reno, NV, USA) driven by an infusion pump (KD scientific, model 100, Holliston, MA, USA). Antisense was dissolved in artificial CSF solution at concentration of 20 µg/10 µL that was infused over a 2-min period. After the infusion, the injector was left inside the cannula for an additional 30 s to allow for diffusion of solution away from the tip. Behavioral testing began at 1 week after the surgery. The right placement of the cannula was verified by the injection of dye at the end of the experiment. Moreover, for all experiments, verification of cannula placement was performed by the administration of angiotensin II (100 ng/rat; Sigma-Aldrich) before drug treatment. Angiotensin II reliably induced a transient response of water drinking in non-deprived rats when administered into the ventricles (Ritter *et al.* 1981). Only data from rats drinking more than 10 mL within 30 min were included in this study. Moreover, only rats restoring normal drinking and resting at least 2 day after the testing of angiotensin II were taken into the experiment of antisense infusion.

Intracerebroventricular administration of CREB antisense

To determine the effect of CREB antisense on the anorectic response of PPA, rats ($n = 6–8$ for each group) were given with antisense (20 µg in a 10-µL vehicle; i.c.v.) at 1 h before PPA (115 mg/kg, i.p.) daily for 4 days. Before PPA treatment, rats were i.c.v. injected with similar dose of antisense daily for about 3 days (from day –2 to 0) until the response of feeding behavior was slightly reduced. This is due to the fact that either continuous or repeated i.c.v. injections of

antisense may be necessary to maximize behavioral effect and especially to block the synthesis of constitutively active gene product (Zhang and Creese 1993; Ogawa and Pfaff 1998). The sequences of the CREB antisense and missense ODN were 5'-TGGTCATCTAGTCACCGGTG-3' and 5'-GTCTGCAGTCGATC-TACGGT-3', respectively. As expected, the CREB antisense sequence showed a perfect match (as the reverse complement) with the rat CREB gene corresponding to nucleotides 27–46 (GenBank accession no. X14788); this sequence overlaps the initiation codon used by all known mRNA splice variants of CREB and has been used in other studies (Widnell *et al.* 1996). The missense sequence did not show significant matches in the database. The sequence of CREB antisense used in this study can specifically reduce with the expression of both CREB mRNA level and protein content. Both antisense and missense phosphorothioate-ODN were dissolved in artificial CSF containing 140 mM NaCl, 3.35 mM KCl, 1.15 mM MgCl₂, 1.26 mM CaCl₂, 1.2 mM Na₂HPO₄, and 0.3 mM NaH₂PO₄, pH 7.4.

Another control experiment was designed to determine the effect of CREB antisense pre-treatment on NPY and SOD-2 mRNA levels in PPA-treated rats. Rats ($n = 5–6$ each group) were injected daily with antisense or missense (20 µg in a 10-µL vehicle; i.c.v.) at 1 h before daily PPA (115 mg/kg, i.p.) for 4 days. Before PPA treatment, rats were i.c.v. injected with similar dose of antisense daily for 2–3 days until the response of feeding behavior was slightly reduced. At 40 min after daily PPA treatment, the hypothalamus was removed daily to determine the NPY and SOD-2 mRNA contents.

Western blotting

Protein samples extracted from hypothalamus tissue were separated in a 12.5% polyacrylamide gel, transferred onto a nitrocellulose membrane, and then incubated separately with specific SOD-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or CREB antibodies (Cell Signaling Technology, Beverly, MA, USA) and α -tubulin antibodies (Sigma-Aldrich). After incubation with horseradish peroxidase goat anti-rabbit IgG, the color signal was developed by 4-chloro-1-naphthol/3,3'-diaminobenzidine and 0.9% (w/v) NaCl in Tris–HCl (Sigma Chemical Co., St Louis, MO, USA). Relative photographic density was quantified by scanning the photographic negative film on a Gel Documentation and Analysis System (AlphaImager 2000, Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis

Data were presented as the mean \pm SEM. A *t*-test or one- or two-way ANOVA followed by Dunnett's test was used to detect significances of difference among groups. A value of $p < 0.05$ was considered to be statistically significant.

Results

The effect of PPA on feeding behavior

Changes of daily food intake in rats receiving PPA were shown in Fig. 1. Statistical analysis (two-way ANOVA) revealed significant dose-dependent [$F(3,28) = 8.22$,

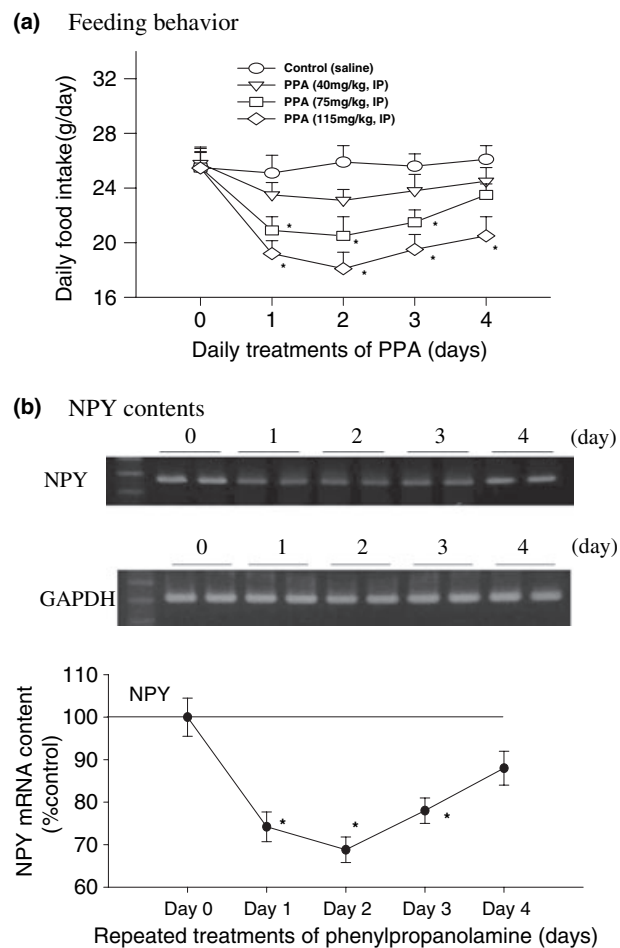


Fig. 1 (a) The effect of daily treatment of phenylpropanolamine (PPA) on feeding behavior. PPA (0, 40, 75, or 115 mg/kg, i.p.) was administered to rats once a day (at 6:00 PM of each day) for 4 days. The first injection of PPA was conducted at the end of day 0. Each point represents the mean \pm SEM of six to eight rats. (b) Upper panel: the effect of daily treatment of PPA (75 mg/kg) on neuropeptide Y (NPY) mRNA levels over a 4-day period. Lower panel: relative densitometric values for RT-PCR products of NPY mRNA between PPA and control groups. Content of each mRNA in PPA-treated group was indicated as the percentage of control. Bars are mean \pm SEM. $n = 5-6$ per group. * $p < 0.05$ versus control group of each treatment day.

$p < 0.05$] and time-dependent effects [$F(4,35) = 4.15$, $p < 0.05$]; however, the interaction effect failed to achieve significance. It revealed that a treatment with 75 mg/kg PPA reduced the food intake during days 1–3, and a treatment with 115 mg/kg PPA reduced food intake during days 1–4 when compared with controls. This result suggested that daily PPA (75 mg/kg) could produce an anorectic response on the first 3 days (from day 1 to 3) and a gradual return to normal intake (tolerant effect) on the following days (day 4), but daily PPA (115 mg/kg) produced a continuous anorectic response during a 4-day period of time. PPA at dose of

75 mg/kg was employed for subsequent measures since after 2 days there was tolerance. Moreover, PPA (115 mg/kg) was used for behavioral studies (antisense study) as it can exert a more significant effect than PPA (75 mg/kg) on the decrease of NPY expression and feeding response, which was a better situation for detecting the attenuating effect of antisense pre-treatment on PPA-induced responses.

Effects of PPA on NPY, CREB, PKA, CART, and SOD-2 mRNA levels

Results shown in Figs 1b and 2 revealed that daily PPA decreased NPY mRNA levels but increased CREB, PKA, and SOD-2 mRNA levels during a 4-day period. Analysis with one-way ANOVA revealed a decrease in NPY mRNA content [$F(4,24) = 6.02$, $p < 0.05$] from day 1 to 3, but revealed an increase in CREB mRNA content [$F(4,24) = 5.15$, $p < 0.05$] from day 1 to 2, an increase in PKA mRNA content [$F(4,24) = 4.32$, $p < 0.05$] from day 1 to 2, an increase in SOD-2 mRNA content [$F(4,24) = 4.92$, $p < 0.05$] from day 1 to 4, and an increase in CART mRNA contents [$F(4,24) = 5.69$, $p < 0.05$] from day 1 to 4 when compared with the control. Statistical analysis revealed that ratios of NPY/GAPDH, CREB/GAPDH, PKA/GAPDH, SOD-2/GAPDH, and CART/GAPDH mRNA were about $65 \pm 6\%$, $805 \pm 25\%$, $245 \pm 35\%$, $292 \pm 40\%$, and $345 \pm 35\%$, respectively, in PPA-treated group when compared with the control group. These results revealed that NPY gene was inhibited for 3 days but CREB, PKA, SOD-2, and CART genes were activated for 2, 2, 4, and 4 days, respectively, during a 4-day period of PPA treatment. Moreover, changes in NPY mRNA levels were consistent with changes of feeding behavior, revealing the involvement of NPY gene in PPA anorexia.

The effect of PPA on CREB DNA binding activity

Results shown in Fig. 3 revealed that PPA could increase CREB DNA binding activity in the hypothalamus. Analysis with one-way ANOVA revealed an increase in CREB from day 1 to 3 [$F(4,24) = 2.89$, $p < 0.05$] when compared with the control. This result revealed that CREB DNA binding activity was increased during the first 3 days of PPA treatment.

The effect of CREB antisense on PPA anorexia

As shown in upper panel of Fig. 4, CREB antisense could partially block the anorectic response of PPA (115 mg/kg), indicating the involvement of CREB gene in PPA-treated rats. Statistical analysis with two-way ANOVA revealed a significant treatment effect [$F(3,28) = 5.82$, $p < 0.05$] and time effect [$F(4,35) = 4.1$, $p < 0.05$]. Comparing the intake between antisense/PPA-treated and PPA-treated rats every day revealed significant effects from day 1 to 4 ($p < 0.05$). These results indicate that CREB knockdown could modify the feeding response of PPA.

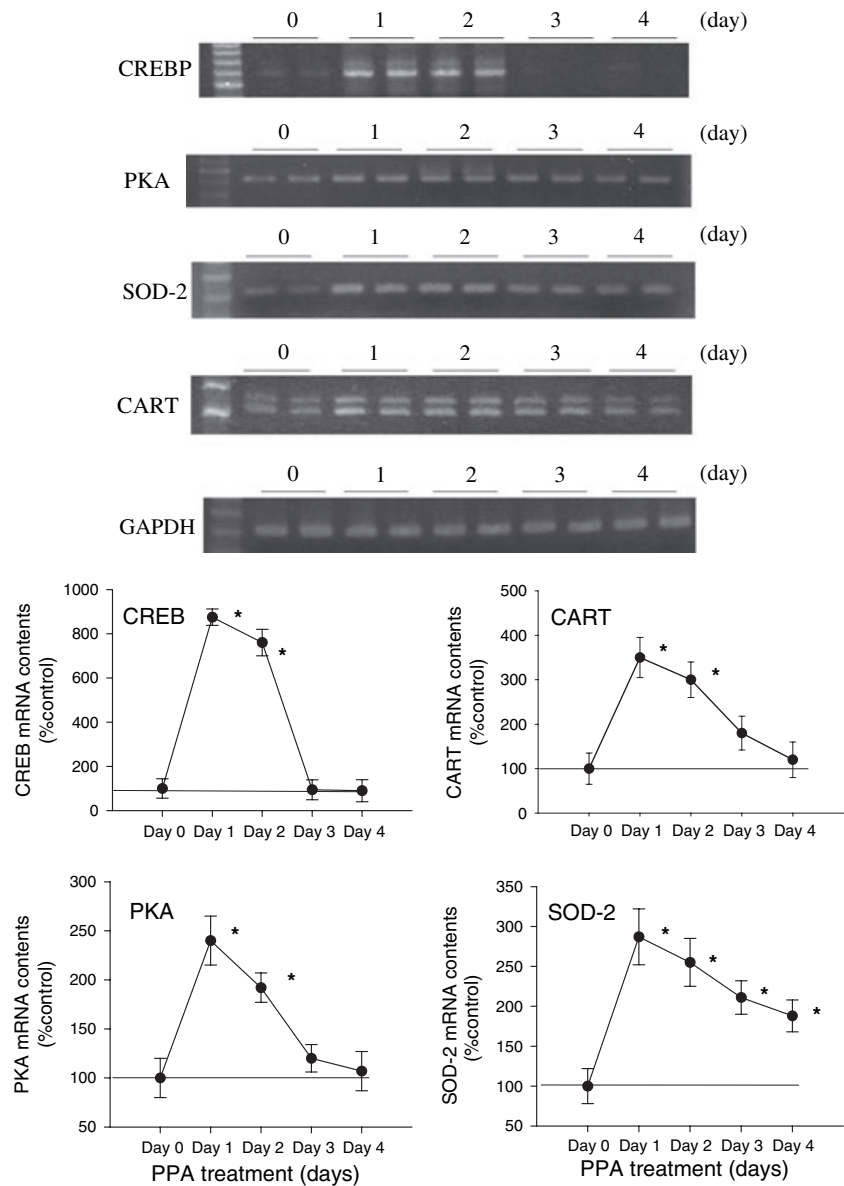


Fig. 2 The effect of daily treatment of PPA (75 mg/kg) on hypothalamic CREB, PKA, SOD-2, CART, and GAPDH mRNA levels over a 4-day period. Upper: the RT-PCR results of CREB, PKA, SOD-2, CART, and GAPDH mRNA levels. Lower: relative densitometric values for RT-PCR products of CREB, PKA, SOD-2, and CART mRNA between PPA and control groups. Content of each mRNA in PPA-treated group was indicated as the percentage of control. Bars are mean \pm SEM. $n = 5-6$ per group. * $p < 0.05$ versus control.

No statistical significance was obtained in 115 mg/kg PPA-treated rats receiving missense/CSF (vehicle) injection (shown in Fig. 4) or not (shown in Fig. 1) (t -test), indicating the non-interference of missense treatment and vehicle on PPAs action.

The effect of antisense pre-treatment on NPY and SOD-2 mRNA levels

Results shown in Fig. 5 revealed that coadministration of CREB antisense and PPA could decrease hypothalamic NPY mRNA level during 4-day period. Using GAPDH as the internal standard, the ratio of NPY/GAPDH mRNA in each group was calculated and compared. A one-way ANOVA revealed that CREB antisense/PPA coadministration has a significant effect [$F(5,29) = 3.65, p < 0.05$] on the decrease

of NPY mRNA content when compared with the control (missense) group. Statistical analysis revealed that the ratio of NPY/GAPDH mRNA was about $51 \pm 5\%$ in PPA alone group and about $88 \pm 6\%$ in CREB antisense/PPA group when compared with the control group. These results suggest that CREB is involved in the regulation of NPY gene expression in PPA-treated rats.

In the analysis of SOD-2 mRNA level, using one-way ANOVA followed by Dunnett's test ($p < 0.05$), it revealed that SOD-2 mRNA contents were increased in PPA-treated rats when compared with the control (missense-treated) group. However, SOD-2 mRNA contents were decreased in CREB antisense/PPA-treated group when compared with the PPA-treated group [$F(5,30) = 4.75, p < 0.05$]. Statistical analysis revealed that the ratio of SOD-2/GAPDH mRNA was about

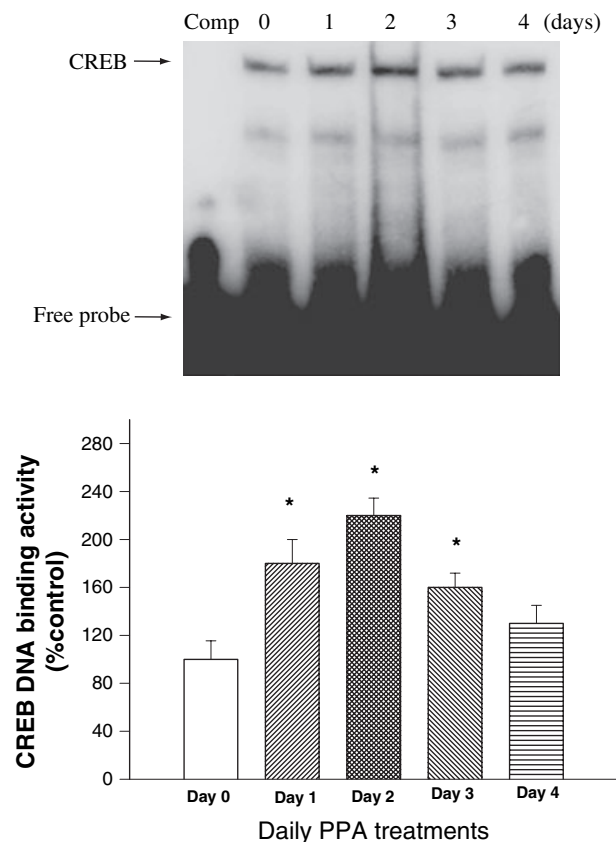


Fig. 3 Effects of daily PPA (75 mg/kg) treatment on CREB DNA binding activity over a 4-day period. Nuclear extracts in hypothalamus were analyzed by EMSA assay with biotin labeled CREB specific oligonucleotide as described in Experimental procedures. Lane 6 represented nuclear extracts incubated with unlabeled oligonucleotide (competitive control) to confirm the specificity of binding. Upper panel: the result of EMSA analyzing CREB DNA binding activity. Lower panel: relative densitometric values for EMSA assay. Contents of CREB binding activity were indicated as the percentage of the control group. Bars were mean \pm SEM. $n = 4-6$ each group. * $p < 0.05$ versus control. Comp: competitive control.

$302 \pm 30\%$ in PPA group and $185 \pm 20\%$ in CREB antisense/PPA group when compared with the control group. These results revealed that CREB signaling was involved in the modulation of SOD-2 gene expression in PPA-treated rats.

Time courses for the change of feeding after a single injection of PPA

Results shown in Fig. 6 reveal that (i) basic patterns of feeding response are similar in normal and PPA-tolerant rats. They eat more in the dark period (i.e. 0–12 h after a single treatment of PPA) but less in the light period and (ii) a single treatment of PPA leads to a decrease in food intake only at the first 0–6 h time interval in normal rats only (t -test, $p < 0.05$). There are no changes during the other time

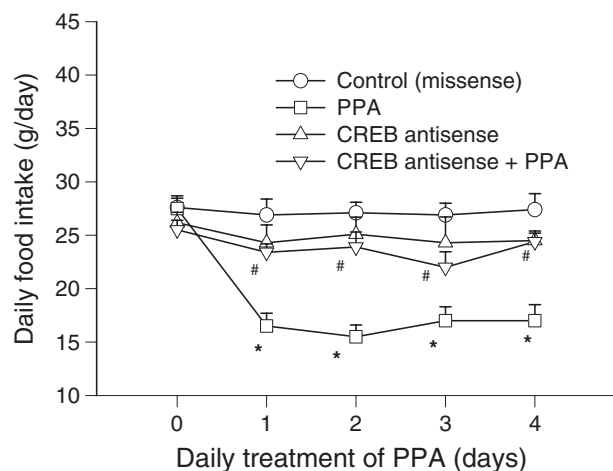


Fig. 4 The effect of daily treatment of CREB antisense oligodeoxynucleotide on PPA-induced food intake over a 4-day period. Daily CREB antisense or missense (20 μ g/10 μ L/day, i.c.v.; $n = 6-8$ each group) was administered into brain 60 min before daily PPA (115 mg/kg, i.p.) treatment every day for 4 days. * $p < 0.05$ versus missense (control) group. # $p < 0.05$ versus the PPA-treated group. PPA: missense + PPA.

intervals in normal rats and during all time intervals in tolerant rats after drug treatment. This result revealed that inductions of PPA anorexia and PPA tolerance were occurred at the initial 0–6 h time interval after a drug treatment.

Effects of PPA on NPY, CREB, and SOD-2 protein contents

The effect of PPA on the change of NPY content had been shown in our previous report (Hsieh *et al.* 2004). NPY was measured by radioimmunoassay and results indicated that the decrease in NPY content during PPA treatment was coincided with the reduction of NPY mRNA level. Results shown in Fig. 7 reveal that daily PPA increase CREB and SOD-2 protein contents during a 4-day period. Using β -actin as the internal standard, the ratio of CREB (or SOD-2) over β -actin in each group was calculated and compared. Analysis with one-way ANOVA revealed increases of CREB contents [$F(4,25) = 2.1$, $p < 0.05$] and SOD-2 contents [$F(4,25) = 5.8$, $p < 0.05$] from day 1 to 3 when compared with the control. These results revealed that CREB and SOD-2 were activated for 3 days during PPA treatment.

Effects of CREB antisense alone on CREB protein and mRNA levels

Results shown in upper panel of Fig. 8 revealed that i.c.v. injection of CREB antisense in rats resulted in a significant decrease of CREB mRNA level. Using GAPDH as the internal standard, the ratio of CREB mRNA over GAPDH mRNA in each group was calculated and compared. Statistical data revealed that CREB mRNA content was decreased in antisense-treated rats when compared with the missense-treated group (t -test, $p < 0.05$). These results revealed that

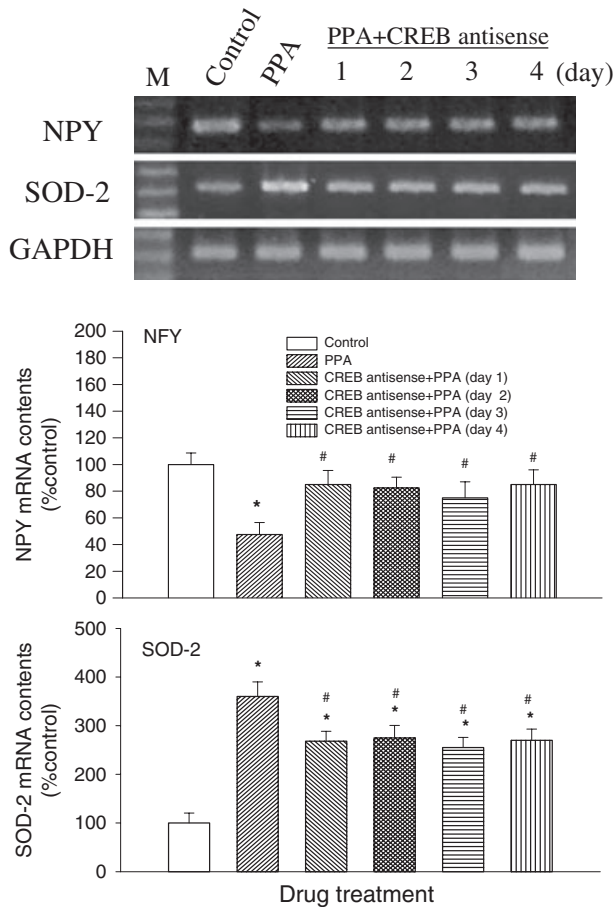


Fig. 5 Effects of daily treatment of CREB antisense on PPA-induced NPY and SOD-2 mRNA levels over a 4-day period. Daily CREB antisense or missense (20 µg/10 µL/day, i.c.v.; $n = 5-6$ each group) was administered into brain 60 min before daily PPA (115 mg/kg, i.p.) treatment for 4 days. * $p < 0.05$ versus control group. # $p < 0.05$ versus the PPA-treated group. Control: missense-treated; PPA: missense/PPA-treated.

i.c.v. injection of CREB antisense was effective to reduce the hypothalamic CREB mRNA level in conscious rats.

Results shown in lower panel of Fig. 8 revealed that i.c.v. injection of CREB antisense in rats resulted in a significant decrease of CREB protein. Using α -tubulin as the internal standard, the ratio of CREB over α -tubulin in each group was calculated and compared. Statistical analysis by t -test ($p < 0.05$) revealed that CREB content was decreased in antisense-treated rats when compared with the control (missense-treated) group. This result revealed that i.c.v. injection of CREB antisense was effective to reduce the hypothalamic CREB protein in rats.

Discussion

Recently, the mechanism for appetite-suppressing effect of PPA has been attributed to its inhibitory effect on hypothalamic

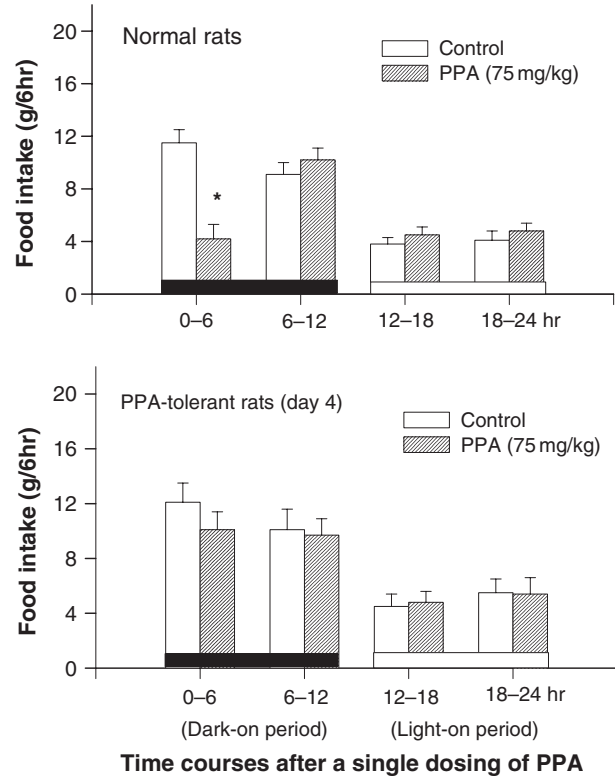


Fig. 6 Comparisons for the time course of a 24-h feeding behavior between normal (upper panel) and tolerant rats (lower panel) after a single dosing of PPA (75 mg/kg; i.p.). A 24-h food intake was divided into four intervals (0-6, 6-12, 12-18, and 18-24 h). Rats were given with PPA or saline at 6:00 PM (i.e. at the beginning of dark-on period) on each testing day. Comparisons were made using t -test. *Indicated $p < 0.05$ compared with the control group of each time intervals.

amic NPY neurons. However, molecular mechanisms underlying this effect are yet to be elucidated. In this study, we found that CREB was activated during PPA treatment and that CREB knockdown could block the decreasing effect of PPA on NPY mRNA level. These results suggest that CREB signaling is involved in the regulation of NPY gene expression.

This finding was supported by previous reports showing that cAMP agonist administered into hypothalamus could decrease NPY-induced feeding behavior (Sheriff *et al.* 2003) and that CREB signaling was involved in the modulation of NPY gene expression (Sheriff *et al.* 1997). However, some *in vitro* and *in vivo* studies had shown that NPY gene was activated by phorbol ester or cAMP analog (Magni and Barnea 1992; Akabayashi *et al.* 1994). Mechanisms underlying this contradictory effect of CREB on NPY gene expression are unknown. To clarify this contradiction, infusion of CREB antisense into brain was administered to PPA-treated rat, and results showed that CREB knockdown in the brain could interrupt the anorectic response of PPA

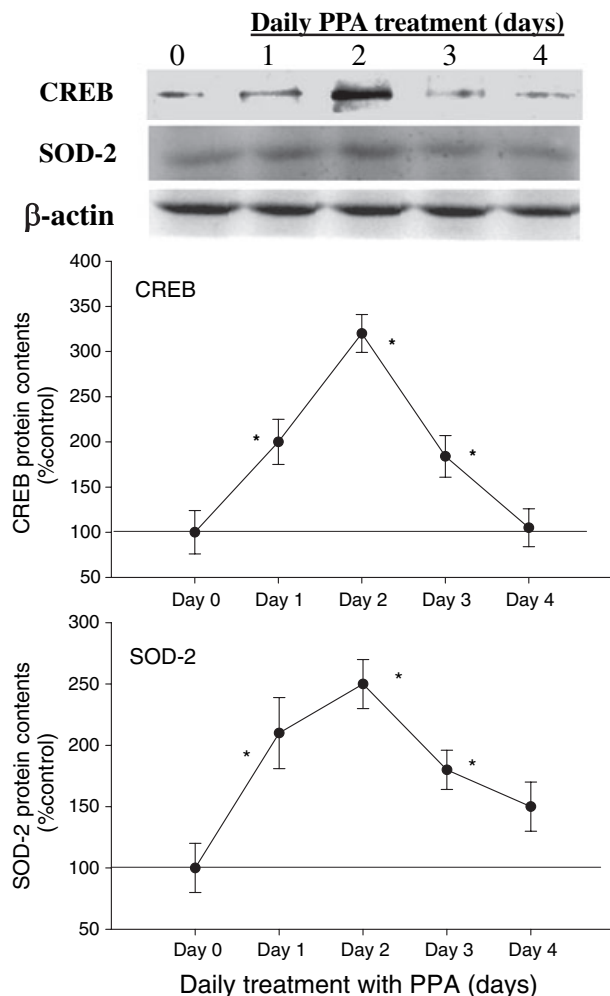


Fig. 7 Effects of daily treatment of PPA (75 mg/kg) on hypothalamic CREB and SOD-2 protein contents over a 4-day period. Upper panel: the results of western Blot analyzing the contents of CREB and SOD-2. Lower panel: relative densitometric values for western Blotting of CREB and SOD-2 in saline- and PPA-treated groups. Contents of CREB and SOD-2 in PPA-treated groups were indicated as the percentage of the control group. Bars were mean \pm SEM. $n = 4-6$ each group. * $p < 0.05$ versus control.

with a restoration of NPY gene expression. This result supported our hypothesis that activation of CREB signaling was involved in the inhibition of NPY gene expression in PPA-treated rats.

Although the injected site and the method for detecting CREB were different, the present findings were consistent with the result shown in a previous report (Chance *et al.* 2000). Using a similar sequence of CREB antisense phosphorothioate-ODN injected directly into the hypothalamic perifornical area, it revealed that both NPY-stimulated feeding and *ad libitum* feeding were reduced and that both reductions were accompanied with a decrease in CREB protein. These results indicated that CREB signaling was involved in NPY-mediated feeding behavior and that

perifornical area might be one of the sites where antisense exerted its effect.

An increase in CREB mRNA level on day 2 was accompanied with a significant decrease in NPY mRNA level on the same day, implying a consistent role for CREB signaling in the regulation of NPY gene. Indeed, a site resembling CRE had been shown to exist on the 5'-flanking region of the rat NPY gene (Larhammar *et al.* 1987). Thus, CREB phosphorylation and CRE-mediated gene expression were indispensable for NPY gene expression in fasted rats (Shimizu-Albergine *et al.* 2001). Consistent with a previous report indicating that a 48-h fasting in rats could increase CRE binding activity and NPY gene expression in hypothalamic nuclear extracts (Sheriff *et al.* 1997), our results suggested that CREB gene was markedly activated to decrease NPY gene expression after 1-2 days of daily PPA treatment.

Instead of being inhibited, CREB gene was activated following PPA treatment. This result suggests that CREB signaling may participate in the inhibition of NPY gene expression during PPA treatment. As the induction of CREB signaling normally served to activate gene transcription, including NPY gene (Sheriff *et al.* 1997), it was possible that PPA might activate CREB signaling in a distinct population of hypothalamic neurons, such as anorexigenic CART-producing neurons, and in turn inhibit NPY neurons. CART is a potent appetite-suppressing peptide closely associated with the action of NPY (Lambert *et al.* 1998). Evidence revealed that CART expression appeared to be regulated via CREB-mediated signaling in rat brain (Jones and Kuhar 2006) and that a CRE site in the area of CART proximal promoter was involved in cAMP/PKA/CREB signaling in neuron-like cells (Dominguez and Kuhar 2004). The present findings revealed that CART gene was activated following PPA treatment and that the alteration of CART mRNA levels during a 4-day period of PPA treatment is consistent with that of CREB mRNA levels. This result supports our view that PPA anorexia may be through CART. Moreover, our results shown that, except AMPH and cocaine, the drug PPA which is an AMPH-like anorectic drug can also activate the expression of CART gene.

Comparing the changes between CREB and PKA mRNA levels in PPA-treated rats, it appears that PKA and CREB signaling may play a consistent role in the regulation of NPY gene expression. Indeed, CREB is one of the downstream nuclear transcription factors of PKA; therefore, it is rational that PKA and CREB signaling are co-activated during PPA treatment. However, the increase in CREB mRNA content on day 1 was about eightfold but that of PKA mRNA content on the same day was only about 2.5-fold. This difference might be because of the possibility that other signaling, such as protein kinase C and c-jun, might play a convergent role to regulate CREB gene expression. It has been suggested that several intracellular signaling, including PKA, protein kinase

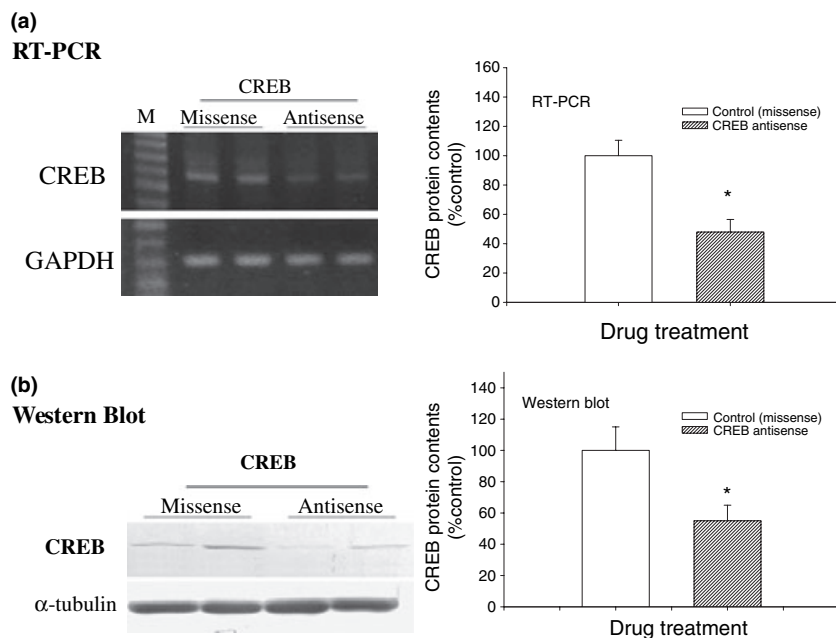


Fig. 8 Effects of CREB antisense or missense treatment on hypothalamic (a) CREB mRNA and (b) CREB protein levels. (a): Left panel: the results of RT-PCR analyzing mRNA levels of CREB in stained ethidium bromide gels. Right panel: relative densitometric values for RT-PCR products of CREB mRNA in missense- and antisense-treated groups. Content of CREB mRNA in antisense-treated group was indicated as the percentage of control (missense-treated). (b): Left

panel: the results of western blotting analyzing the contents of CREB protein. Right panel: relative densitometric values for western blotting of CREB protein in missense- and antisense-treated groups. Content of CREB protein in antisense-treated group was indicated as the percentage of control (missense-treated). Bars were mean \pm SEM. $n = 4-6$ each group. * $p < 0.05$ versus control.

C, and c-jun signaling, may be activated together in rats treated repeatedly with moderate dose of AMPH. These co-activated signaling could effect together on CREB for the purpose to regulate SOD gene expression, which may reduce the oxidative stress in the brain and thus favored the restoration of NPY gene expression (Hsieh *et al.* 2005, 2006). PPA was reported to involve the oxidative stress-related brain disturbances following a chronic treatment of drug (Levin 2005). Moreover, an acute treatment of PPA was employed to improve the stress-related urinary incontinence because of the action of drug on CNS (Scott *et al.* 2002). The present findings revealed that SOD-2 mRNA levels were increased during moderate dose (75 mg/kg) of PPA treatment and their alterations were coincided with those of PKA and CREB mRNA levels. Moreover, CREB knockdown in PPA-treated rats could reduce SOD-2 mRNA level. This result suggests that PKA/CREB signaling may participate in the activation of SOD-2 gene, which favors the correction of NPY gene expression during moderate dose of PPA treatment.

The activation of PKA/CREB signaling may be a critical pathway for AMPH-like anorectic drugs to induce appetite-suppressing effect. Except the current finding that PKA/CREB is activated in PPA treatment, several evidence reveal that (i) the intracellular cAMP is increased following PPA

treatment in minces of rat heart (Hull *et al.* 1993), (ii) PKA/CREB signaling is activated following AMPH treatment (Hsieh *et al.* 2007), (iii) intracellular cAMP is accumulated following ephedrine treatment in rat leukemia cell (Saito *et al.* 2004) or human adipose tissue (Diepvens *et al.* 2007), and (iv) daily oral administration of phentermine to rats can change the activity of adenylate cyclase-cAMP system in renal and hepatic tissues (Kacew *et al.* 1977). PPA, ephedrine, and phentermine are AMPH-like anorectic drugs and are classified as sympathomimetic agents that may exert their effect via the activation of monoaminergic system in the brain (Alexander *et al.* 2005; Nelson and Gehlert 2006). Except sympathomimetic agents, it has been suggested that psychostimulant and opiate drugs, such as AMPH and cocaine, may target at similar CREB gene to induce behavioral responses (Brenhouse *et al.* 2007; Hsieh *et al.* 2007). It was then rational to speculate that induction of PKA signaling by AMPH-like anorectic drugs should be viewed as a group of concerted events that occurred against a complex background of intra- and intercellular signal pathway.

The physiological state during the first 2 days in PPA-treated rats was similar to that of fasting, which is in a state of negative energy balance, resulting in the induction of NPY gene expression on subsequent days. Restoration of NPY mRNA level on day 4 during PPA treatment was accompa-

nied with a gradual decrease in CREB mRNA level, implying a disinhibitory effect of CREB on NPY gene expression. Possibly, this disinhibitory effect of CREB might be relevant to a gradual decrease in catecholamine released from pre-synaptic nerve terminals during a repeated treatment of PPA (Cheng and Kuo 2003).

Although evidence revealed that substantial food deprivation might lead to increased NPY gene expression (Richard 1995), we ruled out the possibility that the change in 24-h NPY level following PPA treatment was simply secondary to reduced feeding, rather than the rapid action of PPA on hypothalamic NPY. It is because PPA-induced anorexia following PPA treatment was occurred only at 0–6 h time interval. We also ruled out the possibility that the change of NPY level was due to a disturbance of neuroendocrine in tolerant rats as they showed no change in feeding behavior compared with that in normal animals.

In summary, the present data provide a molecular message to understand the role of central CREB in the regulation of hypothalamic NPY gene expression *in vivo* in PPA-treated rats.

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