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Intracerebral administration of protein kinase A (PKA) or c-AMP response element binding protein (CREB) antisense oligonucleotide can modulate amphetamine-mediated appetite suppression in free moving rats

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ABSTRACT

Although amphetamine (AMPH)-induced appetite suppression has been attributed to its inhibitory action on neuropeptide Y (NPY), an appetite neurotransmitter abundant in the brain, molecular mechanisms underlying this effect are not well known. This manuscript examined the possible role of protein kinase A (PKA) and cAMP response element binding protein (CREB) signaling in this anorectic effect, and the results showed that both PKA and CREB mRNA levels in hypothalamus were increased following AMPH treatment, which were relevant to a reduction of NPY mRNA level. To determine if PKA or CREB was involved in the anorectic response, the intracerebroventricular infusions of antisense oligonucleotide (or missense control) were performed at 60 min before daily AMPH treatment in conscious rats, and results showed that either PKA or CREB knock down could block AMPH-induced anorexia as well as restore NPY mRNA level, indicating the respective involvement of PKA and CREB signaling in the regulation of NPY gene expression. It is suggested that hypothalamic PKA and CREB signaling may involve the central regulation of AMPH-mediated feeding suppression via the modulation of NPY gene expression.

Keywords: Amphetamine, NPY, PKA, CREB, Feeding behavior, Antisense oligonucleotide, Signaling transduction.

INTRODUCTION

Amphetamine (AMPH) is a well-known appetite suppressant. After the approval in the 1940s and 1950s of AMPH and AMPH-like compounds for the treatment of obesity, AMPH is presently served as a prototype for the development of subsequent anorectic drugs, such as phentermine and phenylpropanolamine (11, 38). AMPH derivatives, such as 3,4-methylenedioxymethamphetamine (MDMA, Adam), had emerged over the last two decades as a common recreational psychostimulant or "club drug" due to their hallucinogenic effect with relatively low toxicity (51). In humans, AMPH can be used to treat attention-deficit/hyperactivity disorder (ADHD) due to its psychomotor effects, such as increased attention, restlessness, and feelings of confidence (5, 6). Because of these effects of AMPH, the mechanisms behind the AMPH-induced anorexia, weight loss or psychomotor effect have been investigated extensively.

AMPH is regarded as an indirect dopamine agonist. The anorectic action of AMPH is relevant to the central release of biogenic amines (33, 53) that in turn may lead to an inhibitory action on hypothalamic neuropeptide Y (NPY) (28). Acute treatment with AMPH markedly decreases food intake which was followed by a gradual return of normal food intake with subsequent daily treatment (27, 30, 46).

NPY, an orexigenic neurotransmitter in the brain, appears to play an essential role in the regulation of feeding behavior (7, 61) and is postulated to control the energy balance by stimulating feeding behavior and inhibiting thermogenesis, especially under conditions of energy deficiency such as food restriction, intense exercise, obesity and diabetes (24).

It is unclear whether cAMP-dependent protein kinase (PKA) signaling is required for NPY gene expression during AMPH treatment. PKA signaling can be elicited by various physiological ligands in cells and is critically involved in the regulation of metabolism, cell proliferation and apoptosis (18, 56). In the brain, PKA appears to be implicated in the regulation of behavioral sensitization (12, 57), conditioned locomotion (54) and reward-related feeding (4) behaviors induced by AMPH treatment. Moreover, PKA is involved in the regulation of NPY-induced feeding behavior (25, 48) and several studies demonstrate that NPY gene is regulated by forskolin or cAMP analogs *in vivo* (1, 20) or *in vitro* (35). Thus, one might hypothesize that PKA might involve the regulation of NPY gene expression during AMPH treatment.

PKA plays a major role in the induction of cAMP response element binding protein (CREB), which is a downstream nuclear transcription factor of PKA (19), revealing possible respective roles of PKA and CREB signaling in the regulation of

NPY gene expression. CREB is required for dopamine-dependent gene expression (2, 13); therefore, CRE-mediated gene induction may involve in NPY gene expression in AMPH-treated rats. A primary aim was to investigate if PKA and CREB signaling were involved in the regulation of NPY gene expression in AMPH-treated rats.

In the present study, intracerebroventricular (ICV) administration of PKA or CREB antisense oligodeoxynucleotides (ODN) are used to down regulate PKA or CREB gene expression in freely moving rats. Although the method of antisense knock down has inherent difficulties, antisense ODNs are preferentially taken up by cerebral neurons in rodent animals after central administration (40, 58, 63). Moreover, ICV administration of antisense ODN has been used to interrupt specific gene expression in the brain (9, 17, 41) or in the hypothalamus (22). Therefore, we chose antisense ODNs, which had been previously used to specifically down-regulate PKA (15, 23) or CREB gene expression (8, 26), to examine their effects on AMPH anorexia following central ventricular administration.

MATERIALS AND METHODS

Animals

Male Wistar rats (200~300 g, Animal Center of National Cheng Kung University Medical College) were housed individually in a cage, maintained at 22 ± 2℃

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 (LabDiet, PMI Nutrition International, Brentwood, MO, USA) 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.

To examine the effect of AMPH (Sigma-Aldrich, MO, USA) on feeding behavior, rats (n=6-8 per group) were given the drug intraperitoneally (IP) at a dose of 0, 2 or 4 mg/kg/day for 4 days. The first injection of AMPH was conducted at the end of Day 0 (at 6:00 PM). The intake data were calculated as the total amount of food during the previous day.

To assess the effects of AMPH on NPY, PKA, CREB and CART mRNA levels, rats (n=5-6 each group) were injected daily with AMPH (0 or 2 mg/kg) for 1, 2, 3 or 4 days, and then were sacrificed. To examine the effects of AMPH on NPY, PKA, and CREB protein concentrations, rats (n=5-6 each group) were injected daily with AMPH (0 or 2 mg/kg) for 1, 2, 3 or 4 days, and then were sacrificed. Rats received AMPH 40 min before being anesthetized (pentobarbital, 30 mg/kg, IP) and decapitated. Their hypothalamus was removed from the brain immediately and subjected to determinations of protein or mRNA levels or stored at –80℃ until the

day to use.

RNA Extraction

Hypothalamic NPY, PKA, CREB and CART mRNA levels were measured in a block of mediobasal hypothalamic tissue as described previously (35). In brief, total RNA was isolated from this block using the modified guanidinium thiocyanate-phenol-chloroform method (10). Each hypothalamic block was homogenized in 1 ml of TRIZOL reagent (Life Technologies, Inc., Grand Island, USA) using an Ultrasonic Processor (Vibra Cell, Model CV17; Sonics & Materials Inc., Danbury, Connecticut, USA). After an incubation at 22℃ for 5 min, 0.2 ml of chloroform was added to each sample, shaken vigorously for 15 sec, incubated at 22 ℃ for 3 min, then centrifuged at 12,000 ×g for 15 min at 4℃. After removal of aqueous phase and precipitation with 0.5 ml isopropanol, samples were incubated at 22℃ for 10 min and centrifuged at 12,000 ×g for 15 min at 4℃. The gel-like RNA pellets were washed with 75% ethanol by vortexing and centrifugation at 7,500 ×g for 5 min at 4℃. Thereafter, RNA pellets were dried briefly, dissolved in RNase-free water, and stored at -80 ℃. The content of RNA was determined spectrophotometrically at 260 nm (Hitachi U-3210, Japan).

Reverse Transcription-polymerase Chain Reaction (RT-PCR)

Using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim GmbH, Germany), RNA was reversely transcribed into single-stranded cDNA. For each sample, 8 μl of sterile DEPC (diethyl pyrocarbonate) water containing 2 μg of RNA were added to oligo-p(dT)15 primer (0.8 μg/ μl) followed by a heating at 65℃ for 15 min, a cooling at 25℃ for 10 min, and then added to a reaction mixture consisting of 10x reaction buffer (100 mM Tris, 500 mM KCl; pH 8.3), deoxynucleotide mix (10 mM each), MgCl2 (25 mM), RNase inhibitor (40 unit/ μl), and AMV reverse transcriptase (25 unit/ μl). Reaction mixtures were incubated at 42℃ for 2 h and then brought to 95℃ for 5 min to terminate the reaction followed by soaking at 16℃. PCR was subsequently carried out by mixing 3 μl of cDNA product with mastermix solution consisting of DEPC water, 10x reaction buffer, MgCl2 (25 mM), deoxynucleotide mix (10 mM each), P1 and P2 primers (1 μg/μl each), and Taq polymerase (5 unit/μl). GAPDH was used as the internal standard calibrator. PCR reactions for NPY were carried out on a PCR thermocycler (Perkin-Elmer GeneAmp 2400) for 28 cycles with the following steps: 91℃ for 1 min (denaturing), 60℃ for 1 min (annealing), and 72℃ for 30 sec (extension), followed by a final elongation step at 72℃ for 7 min, and finally the PCR products were soaked at 16℃. 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: PKA (60℃, 28 cycles); CREB (60℃, 35 cycles); CART (60℃, 28 cycles); GAPDH (52℃, 25 cycles). The sequences of primers used in RT-PCR were shown in Table 1.

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 PKA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or CREB antibodies (Cell Signaling Technology, Beverly, MA) and α-tubulin antibodies (Sigma-Aldrich, St Louis, MO, USA). After incubation with horseradish peroxidase goat anti-rabbit IgG, the color signal was developed by 4-chloro-1-napthol/ 3,3'-diaminobenzidine, 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).

Gel Electrophoresis

At the completion of RT-PCR, 8 μl of each PCR product was subsequently

separated by flat-bed gel electrophoresis on a 3% agarose gel. Gels stained by ethidium bromide (0.5 μg/ml, Sigma-Aldrich Co., MO, USA) 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 AMPH-treated group were indicated as the percentage of control group. The ratio of NPY/GAPDH mRNA was measured by digital densitometry (Hoefer, San Francisco, CA, USA). Similar steps were used to determine the contents of PKA, CREB and CART mRNA.

Lateral Ventricular Cannulation

A stereotaxic surgery (Kopf Model 900, Tujunga, CA, USA) of rat was performed under anesthesia with pentobarbital (30 mg/kg, IP). 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) (43). A 23-g stainless steel guide cannula was implanted and secured to the skull using stainless-steel screws and dental cement. A correct placement was confirmed by observing a transient and rapid inflow of vehicle in PE tube connected with a 28-g injector cannula. The cannula was then occluded with a 28-g stylet. For ICV infusion of PKA or CREB antisense, the stylet was replaced with a 28-g injector

cannula extending 0.5 mm below the tip of guide cannula. Behavioral testing began at 1 week after the surgery. For all experiments verification of cannula placement was done by the administration of angiotensin II (100 ng/rat; Sigma-Aldrich, USA). Angiotensin II reliably induced water drinking in non-deprived rats when administered into the ventricles (44). Only data from rats drinking more than 10 ml within 30 min were included in this study.

ICV Administration of Antisense ODN

To determine the effect of PKA or CREB antisense on the anorectic response of AMPH, rats (n=6-8 for each group) were given with antisense (20 μg in a 10-μl vehicle; ICV) at 1 h before AMPH (4 mg/kg; IP) daily for 4 days. Before AMPH treatment, rats were ICV injected with similar dose of antisense daily for 2-3 days until the response of feeding behavior was slightly reduced. This is due to the fact that either continuous or repeated ICV injections of antisense may be necessary to maximize behavioral effect and especially to block the synthesis of constitutively active gene product (41, 64). The sequences of the CREB antisense ODN and missense ODN were 5'-TGGTCATCTAGTCACCGGTG-3' and 5'-GTCTGCAGTCGATCTACGGT-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 (8, 22, 60). The missense sequence did not show significant matches in the database. The PKA antisense was targeted to the α catalytic subunit of PKA (5'-GCAGTCGCGGCATTGTTG-3') complementary to nucleotides 6-23, which could interrupt PKA gene expression (15). The missense sequence was 5'-CTGCGTGGAGGCATTCGT-3', which did not show significant matches in the database. We used ODNs that were phosphorothioate-modified (S-ODNs) only on the three terminal bases of both the 5' and 3' ends (Proligo Pty Ltd, Singapore), because these S-ODNs had been shown to produce sequence-specific effects without detectable toxicity in brain region (nucleus accumbens) (60) and were regarded as a well-established agent in several vertebrate systems (41). Both antisense and missense S-ODNs were dissolved in artificial corticospinal fluid (ACSF) containing 140 mM NaCl, 3.35 mM KCl, 1.15 mM $MgCl_2$, 1.26 mM $CaCl_2$, 1.2 mM Na_2HPO_4 and 0.3 mM NaH_2PO_4; pH 7.4.

Another control experiment was designed to determine the effect of PKA or CREB antisense S-ODN pretreatment on NPY mRNA level in AMPH-treated rats. Rats (n=6-8 each group) were injected daily with antisense or missense (20 μg in a 10-μl vehicle; ICV) at 1 h before daily AMPH (4 mg/kg; IP) for 2 days (Day 0 and

Day 1). Before AMPH treatment, rats were ICV injected with similar dose of antisense daily for 2-3 days until the response of feeding behavior was slightly reduced. At 40 min after AMPH treatment, the hypothalamus was removed to determine the NPY mRNA content.

To determine the efficiency of PKA or CREB antisense, rats (n=4-6 each group) were injected daily with antisense or missense (20 μg in a 10-μl vehicle; ICV) for 2-3 days until the response of feeding behavior was slightly reduced in antisense group. At 40 min after the last treatment of antisense or missense, the hypothalamus was removed to determine the PKA or CREB mRNA content.

Statistical Analysis

Data were presented as the mean ± SEM. T-test, two-way or one-way ANOVA followed by Dunnett's test was used to detect significances of difference among groups. $P<0.05$ was considered to be statistically significant.

RESULTS

The Effect of AMPH on Feeding Behavior

Changes of daily food intake in rats receiving AMPH were shown in Figure 1.

Statistical analysis (two-way ANOVA) revealed significant dose-dependent [$F(2,20)=9.2$, $P<0.01$] and time-dependent effects [$F(4,35)=3.58$, $P<0.01$], however, the interaction effect failed to achieve significance. It revealed that a treatment with 2 mg/kg AMPH reduced the food intake during Day 1 to Day 3, and a treatment with 4 mg/kg AMPH reduced food intake during Day 1 to Day 4 when compared to controls. This result suggested that daily AMPH (2 mg/kg) produced a marked anorectic response on Day1 and a return to normal intake on the following days, but daily AMPH (4 mg/kg) produced a continuous anorectic response during a 4-day period of time. Therefore, the 2 mg/kg AMPH dose was employed for subsequent measures.

Effects of AMPH on NPY, PKA, CREB and CART mRNA Levels

Results shown in Figure 2 revealed that daily AMPH decreased NPY mRNA level but increased PKA, CREB and CART mRNA levels during a 4-day period. Analysis with one-way ANOVA revealed a decrease of NPY mRNA contents [$F(4,25)=5.7$, $P<0.01$] from Day1 to Day 3, but revealed an increase of PKA mRNA contents [$F(4,25)=7.7$, $P<0.01$] from Day1 to Day 4, an increase of CREB mRNA contents [$F(4,25)=2.6$, $P<0.05$] on Day 2 and an increase of CART mRNA contents [$F(4,25)=3.7$, $P<0.05$] on Day 1 and Day 2 as compared with the control. These results revealed that PKA, CREB and CART genes were activated for 4, 1 and 2 days,

respectively, during AMPH treatment. Moreover, changes in NPY mRNA levels were consistent with changes of feeding behavior, revealing the involvement of NPY gene in AMPH anorexia.

Effects of AMPH on NPY, PK and CREB Protein Levels

AMPH could decrease hypothalamic NPY contents that were markedly decreased on Day 1 with a gradual return to normal level on the followings during a 4-day period in AMPH-treated rats as described in our previous report (27). This change of NPY contents following AMPH treatment was in a manner consistent with the alteration of NPY mRNA levels shown in the present study. Results shown in Figure 3 revealed that daily AMPH increased PKA and CREB protein contents during a 4-day period. Using α-tubulin as the internal standard, the ratio of PKA (or CREB) over α-tubulin in each group was calculated and compared. Analysis with one-way ANOVA revealed an increase of PKA contents [$F(4,25)=6..5$, $P<0.01$] from Day1 to Day 4 and an increase of CREB contents [$F(4,25)=2.1$, $P<0.05$] on Day 2 as compared with the control. These results revealed that PKA and CREB were activated for 4 and 1 days, respectively, during AMPH treatment.

The Effect of PKA or CREB Antisense on AMPH Anorexia

As shown in upper panel of Figure 4, PKA antisense could partially block the anorectic response of AMPH, indicating the involvement of PKA gene in AMPH-treated rats. Repeatedly measuring the responses from Day 1 to Day 4 revealed a significant treatment effect [$F(3,28)=4.6$, $p<0.01$] and time effect [$F(4,35)=3.9$, $p<0.01$]. Comparing the intake between antisense/AMPH-treated and AMPH-treated rats every day revealed significant effects on Day 1, Day 3 and Day 4 ($P<0.05$). Furthermore, significant effects on Day 1, Day 2 and Day 4 were found between antisense/AMPH-treated and missense-treated (control) rats. These results indicate that PKA knock down could modify the feeding response of AMPH.

As shown in lower panel of Figure 4, CREB antisense could block the anorectic response of AMPH, indicating the involvement of CREB gene in AMPH-treated rats. Repeatedly measuring the responses from Day 1 to Day 4 revealed a significant treatment effect [$F(3,26)=5.8$, $p<0.01$] and time effect [$F(4,33)=4.1$, $p<0.01$]. Comparing the food intake between antisense/AMPH-treated and AMPH-treated groups in every day, significant effects were seen in all four days. These results indicate that CREB knock down could block the anorectic response of AMPH.

No statistical significance was obtained in 2 mg/kg AMPH-treated rats receiving missense/CSF (vehicle) injection (shown in Fig. 3) or not (shown in Fig. 1) (t-test), indicating the noninterference of missense treatment and vehicle on AMPH's action.

Similar comparisons could be observed in our previous report (30).

The Effect of PKA or CREB Antisense on NPY mRNA Levels

Results shown in Figure 5 revealed that PKA or CREB antisense reversed partially or completely the decreased level of NPY mRNA in AMPH-treated rats. 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 NPY mRNA content was decreased in both AMPH and PKA antisense/AMPH groups [$F(3,18)=3.19$, $P<0.05$], but was not changed in CREB antisense/AMPH group as compared with the control group. Moreover, significant effects were observed in both PKA antisense/AMPH and CREB antisense/AMPH groups when compared with the AMPH group. Statistical analysis revealed that the ratio of NPY/GAPDH mRNA was about 47 ± 5 % in AMPH group, about 75 ± 6 % in PKA antisense/AMPH group and about 91 ± 5 % in CREB antisense/AMPH group as compared with the control group. Moreover, it appears that the effect of CREB antisense/AMPH (or PKA antisense/AMPH) on NPY mRNA level is similar from Day 1 to Day 4. These results suggest that PKA and CREB signaling are involved in the regulation of NPY gene expression during a 4-day repeated AMPH treatment.

Effects of Antisense S-ODN on PKA or CREB mRNA Level

Results shown in Figure 6 revealed that ICV injection of PKA or CREB antisense S-ODN in rats resulted in a significant decrease of PKA or CREB mRNA level. Using GAPDH as the internal standard, the ratio of PKA or CREB mRNA over GAPDH mRNA in each group was calculated and compared. Results from statistical analysis revealed that PKA or CREB mRNA content was decreased in antisense-treated rats as compared with the missense-treated group (t-test, $P<0.05$). These results revealed that ICV injection of PKA or CREB antisense was effective to reduce the hypothalamic PKA or CREB mRNA level in rats. Moreover, CREB and PKA antisenses used in this study are sequence specific since they didn't interfere with each other.

DISCUSSION

NPY has been reported to involve the anorectic action of AMPH. However, molecular mechanisms underlying this action are not well known. In this study, we found that the alteration in NPY mRNA content following daily AMPH treatment was consistent with the change of feeding behavior, confirming the involvement of NPY gene in AMPH anorexia and suggesting that AMPH tolerance was related to the

restoration of NPY gene expression.

In addition to NPY gene, PKA and CREB genes were also involved in AMPH anorexia. However, instead of being inhibited, PKA and CREB genes were activated following AMPH treatment. On the first day of dosing, a decrease in NPY mRNA level was accompanied with an increase in PKA mRNA level, implying the participation of PKA signaling in the inhibition of NPY gene expression. This finding was supported by a previous report showing that cAMP agonist or PKA activator administered into hypothalamus could decrease NPY-induced feeding behavior for up to 4 h (48). Moreover, a study also indicated that leptin, a potent adipocyte hormone acting on hypothalamus to reduce appetite, could inhibit fasting-stimulated NPY gene expression through the modulation of PKA/CRE signaling in hypothalamus (50). However, in contrast to our present findings, several *in vitro* and *in vivo* studies had shown that NPY gene was activated by forskolin or cAMP analogs (1,35) and that PKA inhibitors could attenuate the stimulatory effect of feeding induced by peptides (ghrelin or orexin) in NPY neurons (25). Mechanisms underlying this contradictory effect of PKA on NPY gene expression are unknown. To clarify this contradiction, we examined the effect of PKA or CREB antisense on NPY gene expression in AMPH-treated rats, and found that either PKA or CREB knock down in the brain could block the anorectic response of AMPH with a restoration of NPY gene

expression. This result supported that activations of PKA and CREB signaling were involved in the inhibition of NPY gene expression in AMPH-treated rats.

Similar result could be observed in a previous report (8) although the injected site and the method for detecting CREB were different. Using a similar sequence of CREB antisense S-ODN injected directly into the perifornical hypothalamic area, Chance et al found that both NPY-stimulated feeding and ad lib feeding were reduced. Both reductions were accompanied with a decrease in CREB protein (vs. a decrease in CREB mRNA level in the present study). These results revealed that CREB protein was involved in NPY-mediated feeding and that perifornical area might be one of the sites on which antisense exerted its effect.

As the induction of either PKA or CREB signaling normally served to activate gene transcription, including NPY gene (49), it was possible that AMPH might activate both signals in a distinct population of hypothalamic neurons, such as CART (cocaine- and amphetamine-regulated transcript)-producing neurons, and in turn inhibit NPY neurons. CART is a potent appetite-suppressing peptide closely associated with the action of NPY (31). A recent investigation indicating that a CRE site in the area of CART proximal promoter was involved in cAMP/PKA/CREB signaling in neuron-like cells (14). The present data shown in Figure 2 revealed that CART mRNA levels were elevated following AMPH treatment and were expressed in

a manner opposite to the change of NPY mRNA level during a 4-day AMPH treatment. This result indicated that the increased CART following AMPH treatment might play an inhibitory role on NPY gene expression and might support our hypothesis to some extent. The possible involvement of CART needs to be investigated further. The activation of PKA/CREB signaling during AMPH treatment is leptin-independent since plasma leptin level is not changed during AMPH treatment as described in our previous report (29).

Changes in PKA mRNA levels were markedly increased on Day 2 in AMPH-treated rats, which were not parallel with changes in NPY mRNA levels that showed the lowest level on Day 1, implying that the inhibition of NPY gene on Day 1 might not be completely modulated by PKA signaling. A recent report by our laboratory showed that protein kinase C-α (PKC-α) signaling could modulate AMPH anorexia in a manner similar to PKA signaling (21). AMPH could increase the oxidative stress in the brain (16, 62); therefore, co-activation of PKA and PKC signaling in AMPH-treated rats might play an anti-oxidative role (39, 55) in modulating NPY gene expression. The current data (Figure 4) showing that NPY mRNA content can be reversed by CREB or PKA antisense to a different level (about 91% and 75% of the control, respectively) in AMPH-treated rats may be due to the convergence of PKA and PKC signaling on CREB pathway and NPY gene

expression.

Changes in NPY mRNA levels during the period of AMPH tolerance were accompanied with a gradual decrease in PKA mRNA levels (although their levels were still elevated), implying a disinhibitory effect of PKA on NPY gene expression. Possibly, this disinhibitory effect of PKA might be relevant to a gradual decrease in dopamine released from presynaptic nerve terminals during a repeated treatment of AMPH (30). The physiological state of AMPH-treated rats on Day 1 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.

A transient increase in CREB mRNA level (about 6 fold) on Day 2 was accompanied with an increase in PKA mRNA level (about 6 fold) on the same day, implying a consistent role for PKA and 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 (32). Thus, PKA was indispensable for both CREB phosphorylation and CRE-mediated gene expression in NPY neurons in fasted rats (50). 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 (49), our results suggested that CREB gene was markedly activated to modulate NPY gene expression after 2 days of daily AMPH treatment.

There are inconsistencies in the present data if compared the alteration of CERB mRNA levels with the change of NPY mRNA levels. The reason is unknown but may be relevant to the CREB antisense which functions in the area outside the hypothalamus. Some catecholaminergic neurons originated in the brain stem and projected to the hypothalamus contain NPY and are the main action site of AMPH to suppress appetite (33, 45). Evidence revealed that CRE-mediated transcription in several brain regions including brain stem may influence the development of neuronal plasticity associated with AMPH-induced behavioral actions (42, 47). Thus, CREB genes in hypothalamus, brain stem, or other areas in the brain, may be blocked down together by CREB antisense and thus leads to a significant effect on NPY gene expression in AMPH-treated rats. This possibility might explain why ICV infusion of CREB antisense could significantly interrupt the response of AMPH anorexia and restore the effect of NPY gene expression, although CREB mRNA levels in hypothalamus were low (except Day 2) during AMPH treatment.

The present data provided a molecular basis for the anorectic effect of AMPH and implied that manipulations at the level of PKA or CREB might allow the development of therapeutic agents to improve the undesirable properties of AMPH. This message is of relevance in understanding the cAMP regulation of the NPY neurons and physiological feeding and as a base for developing AMPH-related or

cAMP-related substances as potential therapeutic drugs to treat hyperphagia. Moreover, it seems possible that targeting one particular protein kinase or its down-stream molecules could avoid many undesirable side effects of drug abuse. In recent years, approaches modulating protein kinase inhibitors or activators have been applied in the improvement of nervous diseases (3); therefore, our results may be helpful for the therapeutic research of AMPH-like anti-obesity drugs.

Future work will identify the cells in the hypothalamus wherein PKA and CREB are activated. Thus, the effect of PKA, PKC or CREB antisense pretreatment on AMPH-induced anorexia will be investigated by injecting the antisense directly into arcuate or paraventricular nucleus. Moreover, the effect of PI3K or STAT3 antisense on AMPH-induced anorexia will also be investigated since they are involved in the regulation of NPY gene expression (37).

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REFERENCES

1. **Akabayashi A, Zaia CT, Gabriel SM, Silva I, Cheung WK and Leibowitz SF.** Intracerebroventricular injection of dibutyryl cyclic adenosine 3',5'-monophosphate increases hypothalamic levels of neuropeptide Y. *Brain Res* 660: 323-328, 1994.
2. **Andersson M, Konradi C and Cenci MA.** cAMP response element-binding protein is required for dopamine-dependent gene expression in the intact but not the dopamine-denervated striatum. *J Neurosci* 21: 9930–9943, 2001.
3. **Battaini F.** Protein kinase C isoforms as therapeutic targets in nervous system disease states. *Pharmacol Res* 44: 353-361, 2001.
4. **Beninger RJ and Gerdjikov T.** The role of signaling molecules in reward-related incentive learning. *Neurotox Res* 6: 91-104, 2004.
5. **Biederman J, Spencer TJ, Wilens TE, Weisler RH, Read SC and Tulloch SJ.** Long-term safety and effectiveness of mixed amphetamine salts extended release in adults with ADHD. *CNS Spectr* 10: 16-25, 2005.
6. **Biel JH and Bopp BA.** Amphetamine: Structure-activity relationship. In *Handbook of psychopharmacology.* Iversen LL, Iversen SD and Snyder SH eds. pp 1-39, Plenum Press, New York, 1978.
7. **Billington CJ, Briggs JE, Harker S, Grace M and Levine AS.** Neuropeptide Y

in hypothalamic paraventricular nucleus: a center coordinating energy metabolism. *Am J Physiol* 266: R1765-R1770, 1994.

8. **Chance WT, Sheriff S and Balasubramaniam A.** Antagonism of NPY-induced feeding by pretreatment with cyclic AMP response element binding protein antisense oligonucleotide. *Neuropeptides* 34:167-72, 2000.
9. **Chiasson BJ, Hooper ML, Murphy PR and Robertson HA.** Antisense oligonucleotide eliminates in vivo expression of c-fos in mammalian brain. *Eur J Pharmacol* 227: 451-453, 1992.
10. **Chomczynski P and Sacchi N.** Single-step method of RNA isolation by acid quanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159, 1987.
11. **Colman E.** Anorectics on trial: a half century of federal regulation of prescription appetite suppressants. *Ann Intern Med* 143: 380-385, 2005.
12. **Crawford CA, Choi FY, Kohutek JL, Yoshida ST and McDougall SA.** Changes in PKA activity and Gs alpha and Golf alpha levels after amphetamine- and cocaine-induced behavioral sensitization. *Synapse* 51: 241-248, 2004.
13. **Das S, Grunert M, Williams L and Vincent SR.** NMDA and D1 receptors regulate the phosphorylation of CREB and the induction of c-fos in striatal neurons in primary culture. *Synapse* 25: 227-233, 1997.

14. **Dominguez G and Kuhar MJ.** Transcriptional regulation of the CART promoter in CATH.a cells. *Brain Res Mol Brain Res* 126: 22-29, 2004.

15. **Fiala A, Muller U and Menzel R.** Reversible downregulation of protein kinase A during olfactory learning using antisense technique impairs long-term memory formation in the honeybee, Apis mellifera. *J Neurosci* 19: 10125-10134, 1999.

16. **Fukami G. Hashimoto K, Koike K, Okamura N, Shimizu E and Iyo M.** Effect of antioxidant N-acetyl-L-cysteine on behavioral changes and neurotoxicity in rats after administration of methamphetamine. *Brain Res* 1016: 90-95, 2004.

17. **Ghosh MK and Cohen JS.** Oligodeoxynucleotides as antisense inhibitors of gene expression. *Prog Nucleic Acid Res Mol Biol* 42: 79-126, 1992.

18. **Griffioen G and Thevelein JM.** Molecular mechanisms controlling the localization of protein kinase A. *Curr Genet* 41: 199–207, 2002.

19. **Habener JF, Miller CP and Vallejo M.** cAMP-dependent regulation of gene transcription by cAMP response element-binding protein and cAMP response element modulator. *Vitam Horm* 51: 1-57, 1995.

20. **Higuchi H, Yang HYT and Sabol SL.** Rat neuropeptide Y precursor gene expression: mRNA structure, tissue distribution, and regulation by glucocorticoids, cyclic AMP, and phorbol ester. *J Biol Chem* 263: 6288-6295, 1988.

21. **Hsieh YH, Yang SF, Chiou HL and Kuo DY.** Transcriptional involvement of

protein kinase C-alpha isozyme in amphetamine-mediated appetite suppression. *Eur J Neurosci* 22: 715-723, 2005.

22. **Hulsey MG, Pless CM, Whit, BD and Martin RJ.** ICV administration of anti-NPY antisense oligonucleotide: effects on feeding behavior, body weight, peptide content and peptide release. *Regul Peptides* 59: 207-214, 1995.

23. **Itoi K, Horiba N, Tozawa F, Sakai Y, Sakai K, Abe K, Demura H and Suda T.** Major role of 3',5'-cyclic adenosine monophosphate-dependent protein kinase A pathway in corticotropin-releasing factor gene expression in the rat hypothalamus in vivo. *Endocrinology* 137: 2389-2396, 1996.

24. **Kalra SP and Kalra PS.** NPY and cohorts in regulating appetite, obesity and metabolic syndrome: beneficial effects of gene therapy. *Neuropeptides* 38: 201-211, 2004.

25. **Kohno D, Gao HZ, Muroya S, Kikuyama S and Yada T.** Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca^{2+} signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* 52: 948-956, 2003.

26. **Konradi C, Cole RL, Heckers S and Hyman SE.** Amphetamine regulates gene expression in rat striatum via transcription factor CREB. *J Neurosci* 14: 5623–5634, 1994.

27. **Kuo DY.** Involvement of hypothalamic neuropeptide Y in regulating the amphetamine-induced appetite suppression in streptozotocin diabetic rats. *Regul Peptides* 127: 19-26, 2005.

28. **Kuo DY.** Further evidence for the mediation of both subtypes of dopamine D1/D2 receptors and cerebral neuropeptide Y (NPY) in amphetamine-induced appetite suppression. *Behav Brain Res* 147:149-155, 2003.

29. **Kuo DY and Cheng JT.** Role of cerebral dopamine but not plasma insulin, leptin and glucocorticoid in the development of tolerance to the anorectic effect of amphetamine. *Neurosci Res* 44: 63-69, 2002.

30. **Kuo DY, Hsu CT and Cheng JT.** Role of hypothalamic neuropeptide Y (NPY) in the change of feeding behavior induced by repeated treatment of amphetamine. *Life Sci* 70: 243-251, 2001.

31. **Lambert PD, Couceyro PR, McGirr KM, Dall Vechia SE, Smith Y and Kuhar MJ.** CART peptides in the central control of feeding and interactions with neuropeptide Y. *Synapse* 29: 293-298, 1998.

32. **Larhammar D, Ericsson A and Persson H.** Structure and expression of the rat neuropeptide Y gene. *Proc Natl Acad Sci USA* 84: 2068-2072, 1987.

33. **Leibowitz SF.** Catecholaminergic mechanisms of the lateral hypothalamus: their role in the mediation of amphetamine anorexia. *Brain Res* 98: 529-545, 1975b.

34. **Lowry OH, Rosebrough NJ, Farr AL and Randall RJ.** Protein measurement with the folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.

35. **Magni P and Barnea A.** Forskolin and phorbol ester stimulation of neuropeptide Y (NPY) production and secretion by aggregating fetal brain cells in culture: evidence for regulation of NPY biosynthesis at transcriptional and posttranscriptional levels. *Endocrinology* 130: 976-984, 1992.

36. **Morris BJ.** Neuronal localization of neuropeptide Y gene expression in rat brain. *J Comp Neurol* 290: 358-368, 1989.

37. **Morrison CD, Morton GJ, Niswender KD, Gelling RW and Schwartz MW.** Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling. *Am J Physiol Endocrinol Metab* 289:E1051-1057, 2005.

38. **Nichols DE.** Medicinal chemistry and structure-activity relationships. In *Amphetamine and its analogs: psychopharmacology, toxicology, and abuse.* Cho AK and Segal DS eds, pp.3-41, Academic Press, San Diego, 1994.

39. **Numazawa S, Ishikawa M, Yoshida A, Tanaka S and Yoshida T.** Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. *Am J Physiol Cell Physiol* 285: C334-342, 2003.

40. **Ogawa S, Brown HE, Okano HJ and Pfaff DW.** Cellular uptake of

intracerebrally administered oligodeoxynucleotides in mouse brain. *Regul Peptides* 59:143-149, 1995.

41. **Ogawa S and Pfaff DW.** Current status of antisense DNA methods in behavioral studies. *Chem Senses* 23: 249-255, 1998.

42. **Olson VG, Zabetian CP, Bolanos CA, Edwards S, Barrot M, Eisch AJ, Hughes T, Self DW, Neve RL and Nestler EJ.** Regulation of drug reward by cAMP response element-binding protein: evidence for two functionally distinct subregions of the ventral tegmental area. *J Neurosci* 25:5553-5562, 2005.

43. **Paxinos G and Watson C.** The rat brain in stereotaxic coordinates. (2nd Ed.) Academic Press, Sydney, Australia, 1986.

44. **Ritter RC, Slusser PG. and Stone S.** Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. *Science* 213: 451–452, 1981.

45. **Sawchenko PE, Swanson LW, Grzanna R, Howe PR, Bloom SR and Polak JM.** Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nucleus of the hypothalamus. *J Comp Neurol* 241:138-153, 1985.

46. **Seiden LS, Sabol KE and Ricaurte GA.** Amphetamine: effects on catecholamine systems and behavior. *Annu Rev Pharmacol Toxicol* 23: 639-677, 1993.

47. **Shaw-Lutchman TZ, Impey S, Storm D and Nestler EJ.** Regulation of

CRE-mediated transcription in mouse brain by amphetamine. *Synapse* 48:10-7, 2003.

48. **Sheriff S, Chance WT, Iqbal S, Rizvi TA, Xiao C, Kasckow JW and Balasubramaniam A.** Hypothalamic administration of cAMP agonist/PKA activator inhibits both schedule feeding and NPY-induced feeding in rats. *Peptides* 24: 245-254, 2003.

49. **Sheriff S, Chance WT, Fischer JE and Balasubramaniam A.** Neuropeptide Y treatment and food deprivation increase cyclic AMP response element-binding in rat hypothalamus. *Mol Pharmacol* 51: 597-604, 1997.

50. **Shimizu-Albergine M, Ippolito DL and Beavo JA.** Downregulation of fasting-induced cAMP response element-mediated gene induction by leptin in neuropeptide Y neurons of the arcuate nucleus. *J Neurosci* 21: 1238-1246, 2001.

51. **Skrinska VA and Gock SB.** Measurement of 3,4-MDMA and related amines in diagnostic and forensic laboratories. *Clin Lab Sci* 18: 119-123, 2005.

52. **Sternberger LA and Sternberger NH.** The unlabeled antibody method: comparison of peroxidase-antiperoxidase with avidin-biotin complex by a new method of quantification. *J Histochem Cytochem* 34: 509-605, 1986.

53. **Sulzer D, Chen TK, Lau YY, Kristensen H, Rayport S and Ewing A.** Amphetamine redistributes dopamine from synaptic vesicles to the cytosol and

promotes reverse transport. *J Neurosci* 15: 4102-4108, 1995.

54. **Sutton MA, McGibney K and Beninger RJ.** Conditioned locomotion in rats following amphetamine infusion into the nucleus accumbens: blockade by coincident inhibition of protein kinase A. *Behav Pharmacol* 11: 365-376, 2000.

55. **Taminato A, Bagattini R, Gorjao R, Chen G, Kuspa A and Souza GM.** Role for YakA, cAMP, and protein kinase A in regulation of stress responses of Dictyostelium discoideum cells. *Mol Biol Cell* 13: 2266-2275, 2002.

56. **Tasken K, Skalhegg BS, Tasken KA, Solber R, Knutsen HK, Levy FO, Sandberg M, Orstavik S, Larsen T, Johansen AK, Vang T, Schrader HP, Reinton NT, Torgersen KM, Hansson V and Jahnsen T.** Structure, function, and regulation of human cAMP-dependent protein kinases. *Adv Second Messenger Phosphoprotein Res* 31: 191–204, 1997.

57. **Tolliver BK, Ho LB, Fox LM and Berger SP.** Necessary role for ventral tegmental area adenylate cyclase and protein kinase A in induction of behavioral sensitization to intraventral tegmental area amphetamine. *J Pharmacol Exp Ther* 289: 38-47, 1999.

58. **Wahlestedt C.** Antisense oligonucleotide strategies in neuropharmacology. *Trends Pharmacol Sci* 15: 42-46, 1994.

59. **Whitesell L, Geselowitz D, Chavany C, Fahmy B, Walbridge S, Alger JR and**

Neckers LM. Stability, clearance, and disposition of intraventricularly administered oligodeoxynucleotides: implications for therapeutic application within the central nervous system. *Proc Natl Acad Sci USA* 90: 4665-4669, 1993.

60. **Widnell KL, Self DW, Lane SB, Russell DS, Vaidya V, Miserendino MJD, Rubin CS, Duman RS and Nestler EJ.** Regulation of CREB expression: in vivo evidence for a functional role in morphine action in the nucleus accumbens. *J Pharmacol Exp Ther* 276: 306-315, 1996a.

61. **Woods SC, Figlewicz DP, Madden L, Porte D Jr, Sipols AJ and Seeley RJ.** NPY and food intake: discrepancies in the model. *Regul Peptides* 75: 403-408, 1998.

62. **Yamamoto BK and Zhu W.** The effects of methamphetamine on the production of free radicals and oxidative stress. *J Pharmacol Exp Ther* 287: 107-114, 1998.

63. **Yee F, Ericson H, Reis DJ and Wahlestedt C.** Cellular uptake of intracerebroventricularly administered biotin- or digoxigenin-labeled antisense oligodeoxynucleotides in the rat. *Cell Mol Neurobiol* 14: 475-486, 1994.

64. **Zhang M and Creese I.** Antisense oligodeoxynucleotide reduces brain dopamine D2 receptors: behavioral correlates. *Neurosci Lett* 161: 223-226, 1993.

FIGURE LEGEND

Fig. 1. Effects of repeated treatments of amphetamine (AMPH) on daily food intake over a 4-day period. Various doses of AMPH (0, 2 or 4 mg/kg, IP) were administered to rats once a day (at 6:00 PM of each day) for 4 days. The first injection of AMPH was conducted at the end of Day 0. Each point represents the mean ± SEM of 6-8 rats. *P < 0.05 vs. control group of each treatment day.

Fig. 2. Effects of repeated treatments of AMPH (2 mg/kg) on hypothalamic NPY, PKA, CREB and CART mRNA levels over a 4-day period. Upper panel: the RT-PCR results of NPY, PKA, CREB, CART and GAPDH mRNA levels. Lower panel: relative densitometric values for RT-PCR products of hypothalamic NPY, PKA, CREB and CART mRNA between AMPH- and control groups. Content of each mRNA in AMPH-treated group was indicated as the percentage of control. Bars are mean ± SEM. N=5-6 per group. *P<0.05 vs. control.

Fig. 3. Effects of repeated treatments of AMPH (2 mg/kg) on hypothalamic PKA and CREB protein contents over a 4-day period. Upper panel: the results of Western

Blotting analyzing the contents of PKA and CREB. Lower panel: relative densitometric values for Western Blotting of PKA and CREB in saline- and AMPH-treated groups. Contents of PKA and CREB in AMPH-treated groups were indicated as the percentage of the control group. Bars were mean ± SEM. N=4-6 each group. *P<0.05 vs. control.

Fig. 4. Effects of the pretreatment of PKA or CREB antisense on AMPH (4 mg/kg, IP)-induced feeding suppression over a 4-day period. Daily antisense or missense (20 μg/10μl/day, ICV) was administered one hour before daily AMPH treatment. * P<0.05 vs. the control (missense-treated) groups of each treatment day. # P<0.05 vs. the AMPH-treated groups of each treatment day. Bars are mean ± SEM. N=6-8 per group.

Fig. 5. (A) Upper panel: effects of pretreatment with PKA antisense on NPY mRNA level in AMPH-treated rats. Daily antisense or missense was administered one hour before daily AMPH (4 mg/kg) treatment over a 4-day period. Lower panel: relative NPY mRNA values in rats treated with AMPH or PKA antisense/AMPH as compared to that of the missense-treated group. (B) Upper panel: effects of pretreatment with CREB antisense on NPY mRNA level in AMPH-treated rats. Lower panel: relative

NPY mRNA values in rats treated with AMPH or CREB antisense/AMPH as compared to that of the missense-treated group. Content of NPY mRNA in drugs-treated rats was indicated as the percentage of control. Bars are mean ± SEM. N=4-6 per group. * $P<0.05$ vs. the control group, $^{\#}$ $P<0.05$ vs. the AMPH-treated group.

Fig. 6. Effects of ICV injection of PKA or CREB antisense on hypothalamic (A) PKA mRNA or (B) CREB mRNA levels. Upper panel: the results of RT-PCR analyzing mRNA levels of PKA or CREB in stained ethidium bromide gels. Lower panel: relative densitometric values for RT-PCR products of PKA or CREB mRNA in missense- and antisense-treated groups. Content of PKA or CREB mRNA in antisense-treated group was indicated as the percentage of the missense-treated group. Bars were mean ± SEM. N=4-6 each group. *$P<0.05$ vs. control.

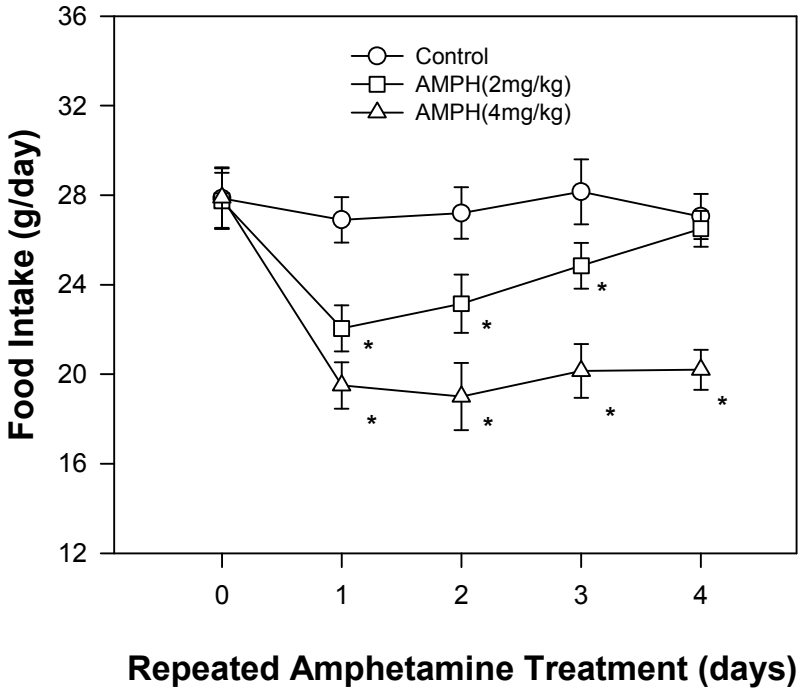

Control
AMPH(2mg/kg)
AMPH(4mg/kg)
Food Intake (g/day)
36
32
28
24
20
16
12
0
1
2
3
4
Repeated Amphetamine Treatment (days)
*

Fig. 1

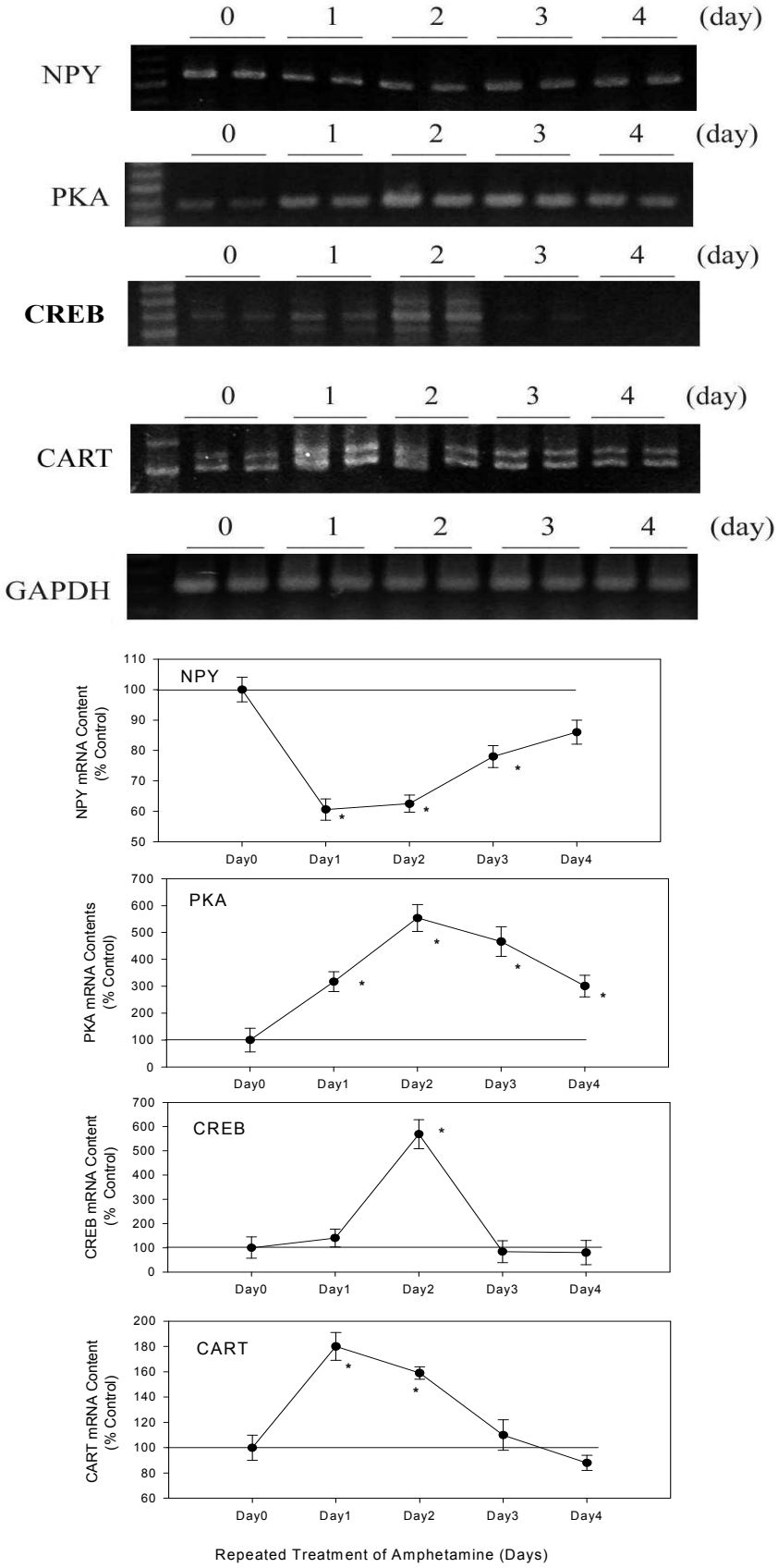

0 1 2 3 4 (day)
NPY
0 1 2 3 4 (day)
PKA
0 1 2 3 4 (day)
CREB
0 1 2 3 4 (day)
CART
0 1 2 3 4 (day)
GAPDH
NPY
NPY mRNA Content (% Control)
PKA
PKA mRNA Contents (% Control)
CREB
CREB mRNA Content (% Control)
CART
CART mRNA Content (% Control)
Day0 Day1 Day2 Day3 Day4
Repeated Treatment of Amphetamine (Days)

Fig. 2

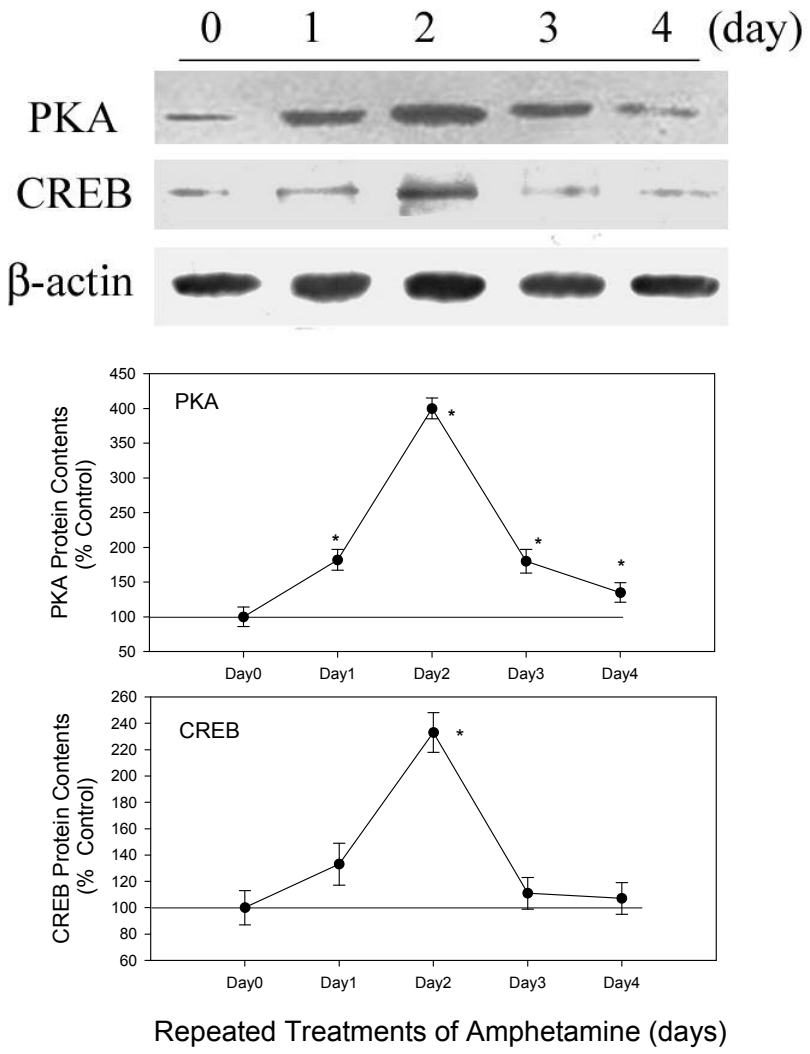

0 1 2 3 4 (day)
PKA
CREB
β-actin
PKA
PKA Protein Contents (% Control)
450
400
350
300
250
200
150
100
50
Day0 Day1 Day2 Day3 Day4
CREB
CREB Protein Contents (% Control)
260
240
220
200
180
160
140
120
100
80
60
Day0 Day1 Day2 Day3 Day4
Repeated Treatments of Amphetamine (days)

Fig. 3.

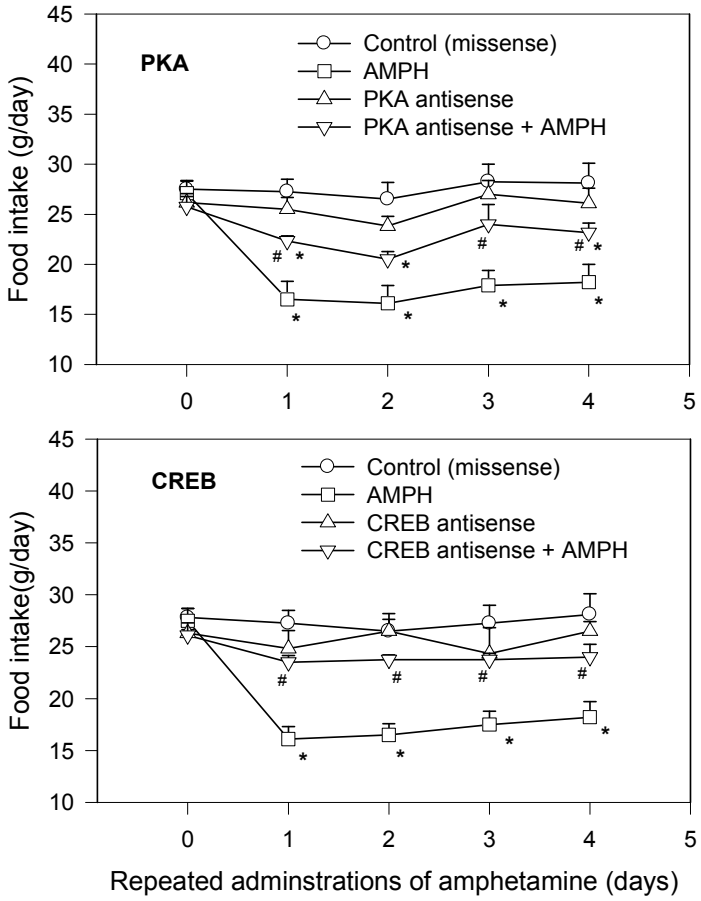

PKA
Control (missense)
AMPH
PKA antisense
PKA antisense + AMPH
Food intake (g/day)
CREB
Control (missense)
AMPH
CREB antisense
CREB antisense + AMPH
Food intake(g/day)
Repeated adminstrations of amphetamine (days)

Fig. 4

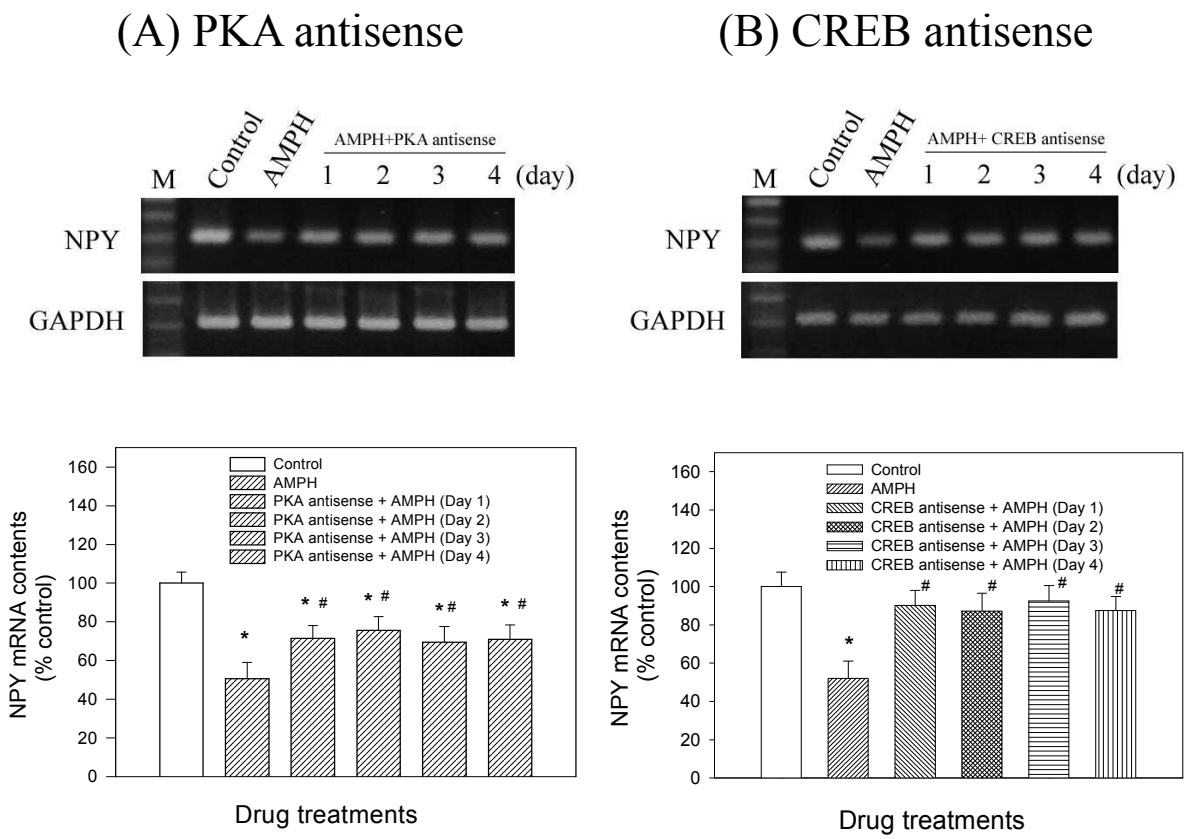
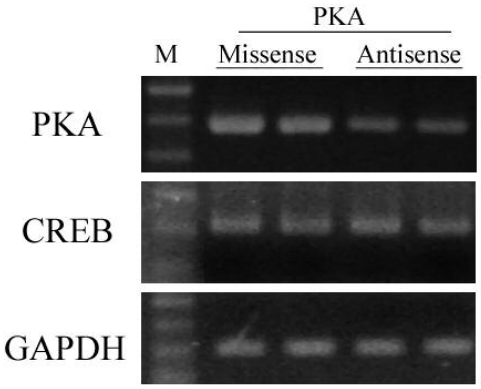
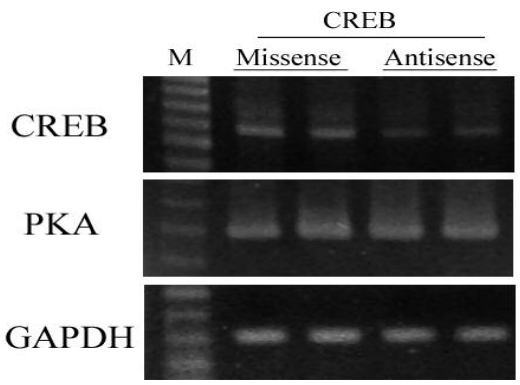

(A) PKA antisense
(B) CREB antisense
M
Control
AMPH
AMPH+PKA antisense
AMPH+ CREB antisense
1 2 3 4 (day)
NPY
GAPDH
NPY mRNA contents (% control)
Control
AMPH
PKA antisense + AMPH (Day 1)
PKA antisense + AMPH (Day 2)
PKA antisense + AMPH (Day 3)
PKA antisense + AMPH (Day 4)
CREB antisense + AMPH (Day 1)
CREB antisense + AMPH (Day 2)
CREB antisense + AMPH (Day 3)
CREB antisense + AMPH (Day 4)
Drug treatments

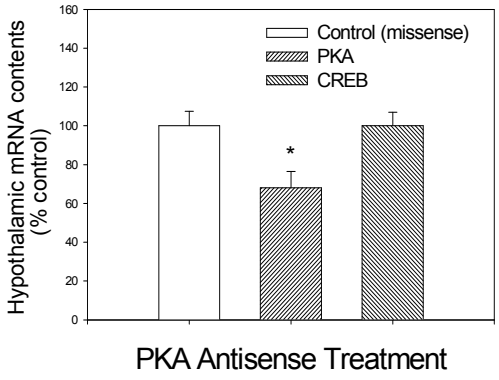
Fig. 5

(A) PKA antisense



(B) CREB antisense





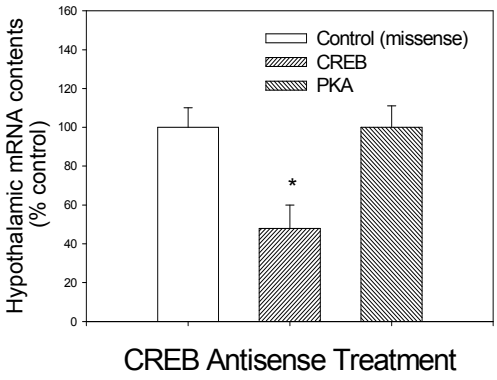


Fig. 6

Table 1: The sequences of primers used in the experiment of RT-PCR

	Primer	Sequence 5'→3'	Size of product (base pairs)
NPY	Sense	GGGCTGTGTGGACTGACC	264
	Antisense	GGAAGGGTCTTCAAGCCT	
PKA	Sense	AGAGTGAATCGGACTCGGACG	383
	Antisense	GCCACGGTTTGCATACTGACC	
CREB	Sense	GAAAGCAGTGACTGAGGAGCTTGTA	616
	Antisense	GGGCTAAGCAGTTGGTGGTGCAGGATGCA	
CART	Sense	CTCCTGGGCGCCGCCCTGCTGC	252
	Antisense	CATGGGGACTTGGCCGTACTTC	213
GAPDH	Sense	TCCCTCAAGATTGTCAGCAA	309
	Antisense	AGATCCACAACGGATACATT	