Intracerebral administration of protein kinase A or cAMP response element-binding protein antisense oligonucleotide can modulate amphetamine-mediated appetite suppression in free-moving rats

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Hsieh Y-S, Yang S-F, Kuo D-Y. Intracerebral administration of protein kinase A (PKA) or cAMP response element-binding protein antisense oligonucleotide can modulate amphetamine-mediated appetite suppression in free-moving rats. Am J Physiol Endocrinol Metab 292: E123–E131, 2007. First published August 8, 2006; doi:10.1152/ajpendo.00195.2006.—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 study 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 was relevant to a reduction of NPY mRNA level. To determine whether PKA or CREB was involved in the anorectic response, intracerebroventricular infusions of antisense oligonucleotide (or missense control) were performed 60 min before daily AMPH treatment in conscious rats, and results showed that either PKA or CREB knockdown 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.

AMPK 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 has served as a prototype for the development of subsequent anorectic drugs such as phentermine and phenylpropanolamine (11, 38). AMPH derivatives, such as 3,4-methylenedioxyamphetamine (MDMA, Adam), have emerged over the last two decades as common recreational psychostimulants or “club drugs” because of their hallucinogenic effect with relatively low toxicity (51). In humans, AMPH can be used to treat attention-deficit/hyperactivity disorder (ADHD) because of its psychomotor effects, such as increased attention, restlessness, and feelings of confidence (5, 6). Because of these effects of AMPH, the mechanisms behind AMPH-induced anorexia, weight loss, and psychomotor effects 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, 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 behaviors in AMPH treatment. Moreover, PKA is involved in the regulation of AMPH-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 could 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 (CRE)-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, CREB-mediated gene induction may be involved in NPY gene expression in AMPH-treated rats. A primary aim was to investigate whether 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 (ODNs) is used to downregulate PKA or CREB gene expression in freely moving rats. Although the method of antisense knockdown 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).

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Therefore, we chose antisense ODNs, which had previously been used to specifically downregulate 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°C according to a 12:12-h light-dark cycle (lights on at 6:00 AM), and habituated to frequent handling. Drug administration and food intake assessment (LabDiet, PMI Nutrition International, Brentwood, MO) were performed daily at the start 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. All the experimental protocols shown in the present study were approved by the Committee on Animal Research at Chung Shan Medical University.

To examine the effect of AMPH (Sigma-Aldrich) 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⁻¹·day⁻¹ 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 cocaine- and amphetamine-regulated transcript (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 killed. 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 killed. Rats received AMPH 40 min before being anesthetized (pentobarbital sodium, 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°C until the day of 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 guanidium thiocyanate–phenol-chloroform method (10). Each hypothalamic block was homogenized in 1 ml of TRIzol reagent (Life Technologies) using an Ultrasonic Processor (Vibia Cell, model no. CV17; Sonics and Materials, Danbury, CT). After an incubation at 22°C for 5 min, 0.2 ml of chloroform was added to each sample, and samples were 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 of 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 7,500 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).

RT-PCR. With the use of the First-Strand cDNA Synthesis Kit (Boehringer Mannheim), RNA was reverse transcribed into single-stranded cDNA. For each sample, 8 µl of sterile diethyl pyrocarbonate (DEPC)-water containing 2 µg of RNA were added to oligo-(dT)15 primer (0.8 µg/µl) followed by a heating at 65°C for 15 min, a cooling at 25°C for 10 min, and then addition to a reaction mixture consisting of 10× reaction buffer (100 mM Tris, 500 mM KCl; pH 8.3), deoxynucleotide mix (10 mM each), MgCl₂ (25 mM), RNase inhibitor (40 units/µl), and AMV reverse transcriptase (25 units/µl). 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 µl of cDNA product with mastermix solution consisting of DEPC-water, 10× reaction buffer, MgCl₂ (25 mM), deoxynucleotide mix (10 mM each), P1 and P2 primers (1 µg/µl each), and Taq polymerase (5 units/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°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 for changes in two steps (annealing and cycles), described as follows: PKA (60°C, 28 cycles); CREB (60°C, 35 cycles); CART (60°C, 28 cycles); GAPDH (52°C, 25 cycles). The sequences of primers used in RT-PCR are shown in Table 1.

Western blotting. Protein samples extracted from hypothalamic 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 β-tubulin antibodies (Sigma-Aldrich, St Louis, MO). 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% (wt/vol) NaCl in Tris·HCl (Sigma Chemical). Relative photographic density was quantified by scanning the photographic negative film on a Gel Documentation and Analysis System (AlphaImager 2000; Alpha Innotech, San Leandro, CA).

Gel electrophoresis. At the completion of RT-PCR, 8 µl of each PCR product were subsequently separated by flat-bed gel electrophoresis on a 3% agarose gel. Gels stained by ethidium bromide (0.5 µg/ml, Sigma-Aldrich) 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 the AMPH-treated group were indicated as the percentage of control group. The ratio of NPY to GAPDH mRNA was measured by digital densitometry (Hoefer, San Leandro, CA).

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’→3’</th>
<th>Size of Product, base pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>Sense</td>
<td>GGGCTGTGTGCGACCTGACCC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GGAAGGGTCTTCAAGGCCTT</td>
</tr>
<tr>
<td>PKA</td>
<td>Sense</td>
<td>AGATGGAATGCGATCGCCAGCG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GCGAGGATTTGTCATGACTGAC</td>
</tr>
<tr>
<td>CREB</td>
<td>Sense</td>
<td>GAAAAGGATGAGGAGGGTTCCTTA</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GGGCTAACAGTAGTGGTGGAGGATGCA</td>
</tr>
<tr>
<td>CART</td>
<td>Sense</td>
<td>CTCTCTGGGGCGCCGCTGTCGC</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CATGGGAGATCTGCGGATCTTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
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</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AAGAGCACAACGAGAGTATTT</td>
</tr>
</tbody>
</table>

NPY, neuropeptide Y; PKA, protein kinase A; CREB, CAMP response element-binding protein; CART, cocaine- and amphetamine-regulated transcript.
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) of rat was performed under anesthesia with pentobarbital sodium (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 ivc infusion of PKA or CREB antisense, the stylet was replaced with a 28-g injector cannula extending 0.5 mm below the tip of the guide cannula. Behavioral testing began at 1 wk after the surgery. For all experiments, verification of cannula placement was done by the administration of angiotensin II (100 ng/rat, Sigma-Aldrich). Angiotensin II reliably induced water drinking in nondeprived rats when administered into the ventricles (44). Only data from rats drinking >10 ml within 30 min were included in this study.

**Ivc 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 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 a 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 effects and especially to block the synthesis of a constitutively active gene product (41, 64). The sequences of the CREB antisense ODN and missense ODN were 5′-TGTTGCTATCTATGCACCGGTG-3′ and 5′-GTTGTGAGGTCTTCCCGTGT-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. We used ODNs that were phosphorothioate modified (S-ODNs) only on the three terminal bases of both the 5′- and 3′-ends (Proligo Pty), 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 cortico-spinal fluid (ACSF) 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 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 (days 0 and 1). Before AMPH treatment, rats were icv injected with a 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 are presented as means ± SE. A t-test or two- 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**

**Effect of AMPH on feeding behavior.** Changes of daily food intake in rats receiving AMPH are shown in Fig. 1. Statistical analysis (2-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 days 1–3, and a treatment with 4 mg/kg AMPH reduced food intake during days 1–4 compared with controls. This result suggested that daily AMPH (2 mg/kg) produced a marked anorectic response on day 1 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 Fig. 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 day 1 to day 3 but revealed an increase of PKA mRNA contents [F(4,25) = 7.7, P < 0.01] from day 1 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 days 1 and 2 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, PKA, and CREB protein levels.** As described in our previous report (27), AMPH could decrease hypothalamic NPY contents that were markedly de-
creased on day 1 with a gradual return to normal level on the following days during a 4-day period in AMPH-treated rats. 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 Fig. 3 revealed that daily AMPH increased PKA and CREB protein contents during a 4-day period. With the use of \( ^{\text{35}}\text{H}9251\)-tubulin as the internal standard, the ratio of PKA (or CREB) over \( ^{\text{35}}\text{H}9251\)-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 day 1 to day 4 and an increase of CREB contents \( [F(4,25) = 2.1, P < 0.05\] on day 2 compared with the control. These results revealed that PKA and CREB were activated for 4 days and 1 day, respectively, during AMPH treatment.

Effect of PKA or CREB antisense on AMPH anorexia. As shown in Fig. 4, top, 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 increase on day 1 with a gradual return to normal level on the following days during a 4-day period in AMPH-treated rats. 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 Fig. 3 revealed that daily AMPH increased PKA and CREB protein contents during a 4-day period. With the use of \( ^{\text{35}}\text{H}9251\)-tubulin as the internal standard, the ratio of PKA (or CREB) over \( ^{\text{35}}\text{H}9251\)-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 day 1 to day 4 and an increase of CREB contents \( [F(4,25) = 2.1, P < 0.05\] on day 2 compared with the control. These results revealed that PKA and CREB were activated for 4 days and 1 day, respectively, during AMPH treatment.

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Effect of PKA or CREB antisense on AMPH (4 mg/kg, ip)-induced feeding suppression over a 4-day period. Daily antisense or missense treated (vehicle) was administered 1 h before daily AMPH treatment. Bars are means ± SE; n = 6–8 per group. *P < 0.05 vs. control (missense treated) groups of each treatment day. 

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 treated (vehicle) was administered 1 h before daily AMPH treatment. Bars are means ± SE; n = 6–8 per group. *P < 0.05 vs. control (missense treated) groups of each treatment day. 

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Fig. 5. Effects of PKA or CREB antisense on NPY mRNA levels. Results shown in Fig. 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 to 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 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 to GAPDH mRNA was ~47 ± 5% in the AMPH group, ~75 ± 6% in the PKA antisense/AMPH group, and ~91 ± 5% in the CREB antisense/AMPH group compared with the control group. Moreover, it appears that the effect of CREB antisense/AMPH (or PKA antisense/AMPH) on the 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.

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 content was decreased in antisense-treated rats 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 did not interfere with each other.
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 knockdown 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 results were 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. (8) found that both NPY-stimulated feeding and ad libitum 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.

Because 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-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 indicated 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 Fig. 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 Cα (PKCα) 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, coactivation 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 (Fig. 4) showing that NPY mRNA content can be reversed by CREB or PKA antisense to a different level (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

![Fig. 5. A, top: effects of pretreatment with PKA antisense on NPY mRNA level in AMPH-treated rats. Daily antisense or missense was administered 1 h before daily AMPH (4 mg/kg) treatment over a 4-day period. A, bottom: relative NPY mRNA values in rats treated with AMPH or PKA antisense/AMPH compared with missense-treated group. B, top: effects of pretreatment with CREB antisense on NPY mRNA level in AMPH-treated rats. B, bottom: relative NPY mRNA values in rats treated with AMPH or CREB antisense/AMPH compared with missense-treated group. Content of NPY mRNA in drug-treated rats was indicated as the percentage of control. Bars are means ± SE; n = 4–6 per group. *P < 0.05 vs. the control group. #P < 0.05 vs. the AMPH-treated group.](image-url)
mRNA levels (although their levels were still elevated), implying a disinhibitory effect of PKA on NPY gene expression. 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 a state of negative energy balance, resulting in the induction of NPY gene expression on subsequent days.

A transient increase in CREB mRNA level (~6-fold) on day 2 was accompanied with an increase in PKA mRNA level (~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 when comparing the alteration of CREB 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, thus leading 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 provide a molecular basis for the anorectic effect of AMPH and imply 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 downstream 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

![Fig. 6. Effects of icv injection of PKA or CREB antisense on hypothalamic PKA mRNA (A) or CREB mRNA (B) level. Top: results of RT-PCR analyzing mRNA levels of PKA or CREB in stained ethidium bromide gels. Bottom: 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 are means ± SE; n = 4–6 each group. *P < 0.05 vs. control.](image-url)
improvement of nervous diseases (3); therefore, our results may be helpful for the therapeutic research of AMPH-like anti-obesity drugs.

Future work can 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 could be investigated by injecting the antisense directly into arcuate or paraventricular nucleus. Moreover, the effect of phosphorylidyinositol 3-OH-kinase or signal transducer and activator of transcription-3 antisense on AMPH-induced anorexia could also be investigated, since they are involved in the regulation of NPY gene expression (37).

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REFERENCES

NPY, PKA, CREB, and Amphetamine Anorexia


