

# The Effect of Neuropeptide Y Knockdown on Hypothalamic Superoxide Dismutase-Associated Appetite Control in Acute Amphetamine-Treated Rats

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It has been reported that reactive oxygen species in the hypothalamus is involved in regulating appetite and that hypothalamic neuropeptide Y (NPY) and cocaine- and amphetamine-regulated transcript (CART) participate in amphetamine (AMPH)-induced appetite suppression. This study examined whether the endogenous anti-oxidative enzyme superoxide dismutase (SOD) was involved in CART-mediated appetite control in AMPH-treated rats. The rats were treated with AMPH for one day, and changes in feeding behavior and expression levels of hypothalamic NPY, CART, and SOD were assessed and compared. The results showed that food intake and NPY decreased during AMPH treatment, whereas CART and SOD increased. Moreover, an intracerebroventricular infusion of NPY antisense oligonucleotide before daily AMPH treatment enhanced the decreases in food intake, body weight, and NPY expression and the increases in CART and SOD expression. The results suggest that hypothalamic SOD participates in regulating NPY- and CART-mediated appetite control during AMPH treatment. These results may advance the knowledge of the molecular mechanism of AMPH-evoked appetite suppression.

**Key words:** superoxide dismutase, cocaine- and amphetamine-regulated transcript, neuropeptide Y, amphetamine, appetite, hypothalamus

## Introduction

The production of reactive oxygen species (ROS) in the hypothalamic neurons during fuel utilization or psychomotor drug treatment plays an essential role in the regulation of appetite. Thus, glucose-utilizing proopiomelanocortin (POMC)

neurons are activated and ROS is accumulated during positive energy balance, whereas fatty acid-utilizing NPY neurons are activated but ROS levels are not increased during negative energy balance<sup>[1,2]</sup>. Moreover, the treatment of AMPH, a psychomotor and anorectic drug, increased oxidative stress in the hypothalamus, which plays a functional role in regulating appetite suppression<sup>[3]</sup>.

The mechanism underlying AMPH-induced appetite suppression is associated with the central release of dopamine<sup>[4,5]</sup>, which decreases NPY expression but increases POMC and CART expression in the hypothalamus during AMPH treatment<sup>[6-8]</sup>. Orexigenic NPY neurons in the hypothalamus may function in a pattern opposite to anorexigenic POMC- and CART-containing

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neurons during the regulation of appetite in rats treated with AMPH or AMPH-like anorectic drug<sup>[3,9]</sup>. The colocalization of CART with both NPY and POMC suggests that CART has a modulatory role in controlling appetite<sup>[10-13]</sup>. Moreover, the CART peptide plays a functional role in the prevention of oxidative stress in the brain<sup>[14]</sup>. Although POMC- and CART-containing neurons may function in a pattern opposite to NPY neurons during AMPH treatment, it is still unknown whether the endogenous antioxidants may also function in the regulation of neuropeptides-mediated appetite control.

AMPH can induce an autooxidation of cytosolic dopamine and thus cause oxidative damage of dopamine terminals<sup>[15]</sup>. In the brain, AMPH treatment increases ROS concentration and anti-oxidative enzymes expression, such as SOD, glutathione peroxidase (GP), catalase, to avoid the oxidative damage<sup>[16,17]</sup>. SOD, including SOD-1 and SOD-2, is known to catalyze the dismutation of superoxide, which results in the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). To prevent damage, H<sub>2</sub>O<sub>2</sub> must be quickly decomposed to water and oxygen by GP and catalase. GP has a great adaptation to avoid the oxidative damage caused by ROS and to maintain the oxygen balance<sup>[18,19]</sup>. Although AMPH increases oxidative stress in the hypothalamus<sup>[7,20,21]</sup>, it is still unclear whether SOD participates in neuropeptides-mediated appetite control during AMPH treatment. As NPY can regulate normal eating<sup>[22]</sup>, energy balance<sup>[23,24]</sup>, and prevention of oxidative stress<sup>[25]</sup>, we hypothesized that NPY knockdown may modulate CART- and SOD- involved appetite control in AMPH-treated rats.

There are two active CART peptide fragments, CART (55–102) and CART (62–102), that exhibit relatively different activities in various testing paradigms<sup>[26]</sup>. Although CART (55–102) is five-fold more potent than CART (62–102) in the inhibition of food intake<sup>[27]</sup>, these two peptides were examined in this experiment for comparing the possible different activity in AMPH-treated rats.

## Materials and methods

### Animals

Male Wistar rats weighing 200-300 g were obtained from the National Laboratory Animal Center in Taiwan, ROC. The animals were housed individually in cages and maintained at a temperature of 22 ± 2 °C in a room with a 12-h light-dark cycle (light on at 6:00 a.m.). The rats were also habituated to frequent handling. Drugs were administered and food intake was determined every day at the beginning of the dark phase (6:00 p.m.). Water and chow (LabDiet) were freely available throughout the experiment. Food intake data points above 35 g/day were discarded because they indicated food spillage. All of the procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

### Animal treatment

To examine the effect of AMPH (d-amphetamine) on feeding behavior and body weight, rats (N=8 for each group) were injected intraperitoneally (i.p.) with AMPH at doses of 1, 2 or 4 mg/kg. AMPH was injected at the end of a day (i.e. at 6:00 p.m.) and recorded the intake and body weight data after 24-h following AMPH treatment. The data for food intake and body weight change were calculated with respect to the food amount and body weight from the previous day. Rats received AMPH 40 min before being anesthetized (pentobarbital, 35-40 mg/kg; i.p.) and decapitated.

To determine the acute effect of AMPH treatment (2 mg/kg; i.p.) on the changes in hypothalamic NPY, CART, and SOD-1 mRNA levels, rats were injected with the drug once a day. Similarly, to determine the acute effect of AMPH (2 mg/kg; i.p.) on the changes in hypothalamic NPY, CART (55-102), CART (62-102), and SOD-1 expression, rats were injected with the AMPH for 1 day. On the sacrifice day, rats received a treatment of 2 mg/kg AMPH 40 min before being sacrificed to enhance the effects of AMPH. The rats were anesthetized with 35-40 mg/kg pentobarbital and decapitated. Following decapitation, the hypothalamus was removed to determine mRNA levels or protein expression. The rat's hypothalamus

was removed from the brain immediately and subjected to determinations of mRNA levels or protein expression, or stored at  $-80^{\circ}\text{C}$  until its use for analysis.

To assess the effect of pretreatment with NPY antisense oligodeoxynucleotide (ODN) on the anorectic response and on NPY, SOD-1, and CART (52-102) expression in AMPH-treated rats, animals ( $N=8$  per group) were given intracerebroventricularly (i.c.v.) NPY antisense (20  $\mu\text{g}$  in a 10- $\mu\text{l}$  vehicle) 1 h before AMPH (2 mg/kg; i.p.) treatment. Before AMPH treatment, rats were i.c.v. administered a similar dose of NPY antisense daily for 2-3 days until the feeding behavior was slightly reduced. The response is due to the fact that either continuous or repeated i.c.v. injections of antisense may be necessary to maximize behavioral effects and importantly to block the synthesis of a constitutively active gene product<sup>[28,29]</sup>. At 40 min after antisense (missense) and/or AMPH treatment, rats were anesthetized and the hypothalamus of each rat was removed from the brain and its NPY, SOD-1, and CART (52-102) expression levels were determined by Western Blot. The description of the surgery for i.c.v. cannulation and the information of antisense were described in the desired section.

### RNA extraction

Hypothalamic NPY, SOD, and CART mRNA levels were measured in a block of mediobasal hypothalamic tissue as described previously [30]. In brief, total RNA was isolated from this block using the modified guanidinium thiocyanate-phenol-chloroform method. 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  $22^{\circ}\text{C}$  for 5 min, 0.2 ml of chloroform was added to each sample, shaken vigorously for 15 sec, incubated at  $22^{\circ}\text{C}$  for 3 min, then centrifuged at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . After removal of aqueous phase and precipitation with 0.5 ml isopropanol, samples were incubated at  $22^{\circ}\text{C}$  for 10 min and centrifuged at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The gel-like RNA pellets were washed with 75% ethanol by vortexing and centrifugation at  $7,500 \times g$  for 5 min at  $4^{\circ}\text{C}$ . Thereafter, RNA

pellets were dried briefly, dissolved in RNase-free water, and stored at  $-80^{\circ}\text{C}$ . 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  $\mu\text{l}$  of sterile DEPC (diethyl pyrocarbonate) water containing 2  $\mu\text{g}$  of RNA were added to oligo-p (dT)15 primer (0.8  $\mu\text{g}/\mu\text{l}$ ) followed by a heating at  $65^{\circ}\text{C}$  for 15 min, a cooling at  $25^{\circ}\text{C}$  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),  $\text{MgCl}_2$  (25 mM), RNase inhibitor (40 unit/ $\mu\text{l}$ ), and AMV reverse transcriptase (25 unit/ $\mu\text{l}$ ). Reaction mixtures were incubated at  $42^{\circ}\text{C}$  for 2 h and then brought to  $95^{\circ}\text{C}$  for 5 min to terminate the reaction followed by soaking at  $16^{\circ}\text{C}$ . PCR was subsequently carried out by mixing 3  $\mu\text{l}$  of cDNA product with mastermix solution consisting of DEPC water, 10x reaction buffer,  $\text{MgCl}_2$  (25 mM), deoxynucleotide mix (10 mM each), P1 and P2 primers (1  $\mu\text{g}/\mu\text{l}$  each), and Taq polymerase (5 unit/ $\mu\text{l}$ ). 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:  $91^{\circ}\text{C}$  for 1 min (denaturing),  $60^{\circ}\text{C}$  for 1 min (annealing), and  $72^{\circ}\text{C}$  for 30 sec (extension), followed by a final elongation step at  $72^{\circ}\text{C}$  for 7 min, and finally the PCR products were soaked at  $16^{\circ}\text{C}$ . 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: SOD-1 ( $60^{\circ}\text{C}$ , 25); CART ( $60^{\circ}\text{C}$ , 28 cycles); GAPDH ( $52^{\circ}\text{C}$ , 25 cycles). The sequences of primers used in RT-PCR were shown in Table 1.

### Gel electrophoresis

At the completion of RT-PCR, 8  $\mu\text{l}$  of each PCR product was subsequently separated by flat-bed gel electrophoresis on a 3% agarose gel. Gels stained

**Table 1.** Sequences of primers used in RT-PCR.

Genes	GenBank accession No.	Primer	Sequence 5'→3'	Size of product (base pairs)
<b>NPY</b>	NM_012614	Forward	GGGCTGTGTGGACTGACC	264
		Reverse	GGAAGGGTCTTCAAGCCT	
<b>SOD-1</b>	NM17051	Forward	GAG CAT GGGTTCCATGTCCAT	279
		Reverse	ACTTTCTTCATT TCCACCTTTGCC	
<b>CART</b>	NM_017110.1	Forward	CTCCTGGGCGCCGCCCTGCTGC	252
		Reverse	CATGGGGACTTGGCCGTACTION	
<b>GAPDH</b>	NM_017008	Forward	TCCCTCAAGATTGTCAGCAA	309
		Reverse	AGATCCACAACGGATACATT	

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 glyceraldehydes-3-phosphate dehydrogenase (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 (Hofer, San Francisco, CA, USA). Similar steps were used to determine SOD and CART mRNA levels.

### Lateral ventricular cannulation

Stereotaxic surgery (Kopf Model 900, Tujunga, CA, USA) was performed on each rat under pentobarbital anesthesia (30 mg/kg, i.p.). The target of cannulation was near the junction of the right lateral ventricle and the third ventricle (coordinates: 0.8 mm posterior to the Bregma, 1.5 mm from the midline, and 3.5-4.0 mm below the dura)<sup>[31]</sup>. A 23-g stainless steel guide cannula was implanted and secured to the skull using stainless steel screws and dental cement. The correct placement was confirmed by observing the transient and rapid inflow of the vehicle in polyethylene tubing connected to a 28-g injector cannula. The cannula was then occluded with a 28-g stylet. For the infusion of antisense, the stylet was replaced with a 28-g injector cannula extending 0.5 mm below the tip of the guide cannula. For all experiments, the cannula placement was verified by histochemistry of brain section and by the administration of angiotensin II (100 ng/rat). Angiotensin II reliably induced water drinking in non-deprived rats when administered into the cerebroventricles<sup>[32]</sup>. Only data from rats that drank more than 10 ml of water

in 30 min were included in this study. Behavioral testing of drinking began about 1 week after the cannulation surgery and the restoration of feeding behavior, and then angiotensin II was administered to confirm the cannula placement. It was about two days after the treatment of angiotensin II to confirm the restoration of normal drinking behavior, and then we started the experiment of AMPH treatment.

### Cerebral infusion of NPY antisense

An 18-mer oligonucleotide (ODN) near the initiation codon encompassing bases 10-27 of the rat NPY mRNA sequence (Genbank access no. 15880) was selected. The antisense ODN (5'-CCCCATTCGTTTGTACC) inversely complementary to this sequence. Phosphorothioate internucleotide linkages were obtained through treatment with tetraethylthiuram disulfide, and the resulting phosphorothioate oligonucleotides were purified and lyophilized. An 18-mer missense ODN (5'-TTATTCCCCCAGTTTGCC) was used as the control. As described previously (Gillard et al., 1993), this antisense sequence did not appear to display self-hybridization; therefore it was effective in blocking the message read-through. In addition, one week of daily i.c.v. injection of this antisense appeared to reduce food intake and body weight as compared with the missense-treated control. Rats were handled and i.c.v. injected with vehicle 4 days prior to the experimental injections to accustom them to the procedure. One hour before AMPH (2 mg/kg/day, i.p.) treatment, antisense ODN (10 µg/10µl/day) was administered to 8 rats, and the same treatment was repeated for 7 days. An equivalent dose of missense ODN was

administered to each of the 8 rats that served as the control. Food intake and body weight changes were recorded daily. We used NPY antisense that was phosphorothioate-modified (S-ODNs) only on the three terminal bases of both the 5' and 3' ends, because these S-ODNs can improve hybridization affinity and nuclease resistance and were regarded as a well-established agent in several vertebrate systems<sup>[29]</sup> and rat brain<sup>[33]</sup>. Both antisense and missense S-ODNs were dissolved in artificial cerebrospinal fluid (aCSF) containing 140 mM NaCl, 3.35 mM KCl, 1.15 mM MgCl<sub>2</sub>, 1.26 mM CaCl<sub>2</sub>, 1.2 mM Na<sub>2</sub>HPO<sub>4</sub> and 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>; pH 7.4.

### Western blotting

Hypothalamus tissue extracts were subjected to electrophoresis. Proteins were separated on a 12.5 % polyacrylamide gel, transferred onto a nitrocellulose membrane, and incubated with specific antibodies against NPY, CART (55-102), CART (62-102), SOD-1, and  $\beta$ -actin. After incubation with horseradish peroxidase goat anti-rabbit IgG, the color signal was developed using 4-chloro-1-naphthol/3,3'-diaminobenzidine and 0.9 % (w/v) NaCl in Tris-HCl. The 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).

### Drugs, Chemicals, and Reagents

Chow (LabDiet) was purchased from PMI Nutrition International (Brentwood, MO, USA). AMPH, angiotensin II, and Tris-HCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against NPY, SOD-1 and  $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CART (55-102) and CART (62-102) antibodies were from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Semi-quantitative real-time PCR analysis was performed using a Taqman one-step PCR Master Mix (Applied Biosystems, USA). TRIZOL reagent (Life Technologies, Inc., Grand Island, USA) was used in tissue homogenization. Antisense and DNA primer were synthesized by Proligo Pty Ltd

(Singapore).

### Statistical analysis

Data are presented as the means  $\pm$  SEM. A t-test or one-way ANOVA followed by Dunnett's test was used to detect significant differences between the groups. Statistical significance was set at  $p < 0.05$ .

## Results

### The effect of AMPH on feeding behavior and body weight

Changes of 24-h feeding behavior and body weight in rats receiving AMPH were shown in our previous report<sup>[51]</sup>. Using statistical analysis with one-way ANOVA followed by Dunnett's test ( $p < 0.05$ ) to measure the effect of AMPH on food intake, it revealed a significant dose-dependent effect [ $F(4,35)=4.11$ ]. Statistical results revealed that 2 and 4 mg/kg AMPH reduced the food intake compared to the control. Using statistical analysis with one-way ANOVA followed by Dunnett's test ( $p < 0.05$ ) to measure the effect of AMPH on body weight, it revealed a significant dose-dependent effect [ $F(4,35)=3.85$ ]. Statistical results revealed that 2 and 4 mg/kg AMPH reduced the 24-h body weight changes compared to the control. These results revealed that the decreases of 24-h body weight in AMPH-treated rats were expressed in a pattern similar to the decreases of 24-h food intake.

### The effect of AMPH on NPY, SOD-1, and CART mRNA levels

Results shown in Figure 1 revealed that AMPH treatment decreased the expression of NPY but increased the expression of SOD-1 and CART mRNA levels during a 24-h period. Using GAPDH as the internal standard, the ratio of NPY, SOD-1, and CART mRNA over GAPDH in each group was calculated and compared. Statistical analysis [t-test,  $p < 0.05$ ] revealed a decrease in NPY mRNA level, but revealed increases in SOD-1 and CART mRNA levels compared to the control. These results revealed that SOD-1 and CART mRNA levels were increased but NPY mRNA level was decreased during 24-h period of AMPH treatment.

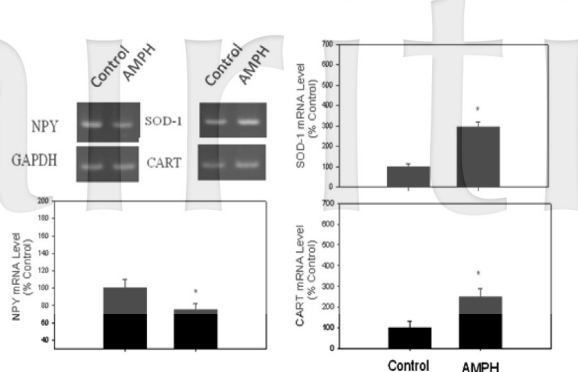


Fig. 1. The effect of AMPH (2 mg/kg; i.p.) on the changes of hypothalamic NPY, SOD-1, and CART mRNA levels over a 24-h period. Results showed the relative densitometric value for the mRNA levels. Contents of mRNA in AMPH-treated groups were indicated as the percentage of the control group. Inserted photo showed the images of NPY, SOD-1, and CART mRNA. Bars were mean  $\pm$  SEM. N=6 each group. \* $p$ <0.05 vs. control. AMPH: amphetamine; NPY: neuropeptide Y; SOD: superoxide dismutase; CART: cocaine and amphetamine regulated transcript.

### The effect of AMPH on NPY, SOD-1, and CART expression

Results shown in Figure 2 revealed that AMPH treatment decreased NPY but increased SOD-1 and CART (55-102) expression compared to the control. However, CART (62-102) expression remained unchanged during AMPH treatment. Using  $\beta$ -actin as the internal standard, the ratio of NPY, SOD-1, CART (55-102), and CART (62-102) over  $\beta$ -actin in each group was calculated and compared. Statistical analysis [t-test,  $p$ <0.05] revealed a decrease in NPY, but increases in SOD-1, and CART (55-102) expression compared to the control.

### Effects of antisense/AMPH co-administration on feeding behavior

A pretreatment with NPY antisense could modulate the anorectic response of AMPH during 24-h period, indicating the involvement of NPY in AMPH anorexia as described in our previous report<sup>[51]</sup>. Using one-way ANOVA to analyze the effect of NPY antisense on AMPH anorexia, it revealed significant treatment-dependent effect

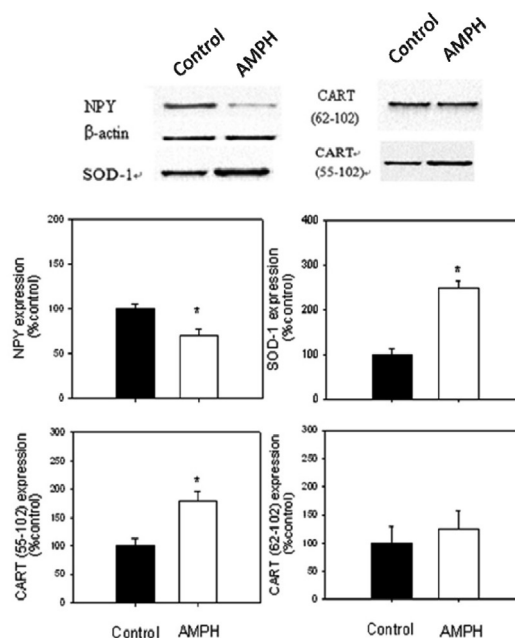


Fig. 2. The effect of AMPH (2 mg/kg; i.p.) on expression levels of hypothalamic NPY, CART (55-102), CART (62-102), SOD-1, and  $\beta$ -actin over a 24-h period. Upper panel: the results of Western Blot. Lower panel: relative densitometric values for Western Blot in control and 2 mg/kg AMPH-treated groups. Contents of protein in AMPH-treated groups were indicated as the percentage of the control group. Bars are the means  $\pm$  SEM. N=8 each group. \*  $p$ <0.05 vs. control. AMPH: amphetamine; NPY: neuropeptide Y; SOD: superoxide dismutase; CART: cocaine and amphetamine regulated transcript.

[F(3,28)=4.41]. Significant difference was observed in antisense-treated, AMPH-treated, and antisense/AMPH-treated groups compared to missense-treated (control) groups. Moreover, it revealed significant difference to compare the food intake between antisense/AMPH- and AMPH-treated groups. Similarly, a pretreatment with NPY antisense could modulate the body weight change during 24-h period in AMPH-treated rats shown in our previous report<sup>[51]</sup>. Taken together, the present results indicated that NPY knockdown could enhance the decreases in food intake and body weight in AMPH-treated rats.

The feeding behavior and body weight change

shown in missense-treated rats was similar to that in saline-treated rats during a 24-h period. Moreover, the anorectic response and body weight change in missense/AMPH-treated rats was not significantly changed compared to that in AMPH-treated rats. These results revealed the noninterference of missense treatment in this study.

### The effect of NPY antisense on NPY, SOD-1, and CART expression

As shown in Figure 3, NPY antisense by itself could reduce NPY but showed no significant effect on CART (55-102) and SOD-1 expression compared to the control (missense-treated) group, revealing a specific effect of NPY antisense on the decrease of NPY expression. Using  $\beta$ -actin as the internal standard, the ratio of NPY, SOD-1, or CART (55-102) over  $\beta$ -actin in each group was calculated and compared. By one-way ANOVA followed by Dunnett's test ( $p < 0.05$ ), it revealed

that NPY decreased by approximately 48% in antisense-treated, 55% in AMPH-treated, and 50% in antisense/AMPH-treated rats compared to the control group [ $F(3,28)=4.56$ ]. By contrast, CART (55-102) expression increased by approximately 200% in both AMPH-treated and antisense/AMPH-treated groups compared to the control group [ $F(3,28)=4.05$ ]. Similarly, SOD-1 increased by approximately 200-230% in AMPH-treated and antisense/AMPH-treated rats compared to the control group [ $F(3,28)=3.68$ ]. Taken together, results revealed that a pretreatment with NPY antisense in AMPH-treated group resulted in reduction of NPY, but increases of CART (55-102) and SOD-1, compared to the AMPH-treated group.

### Discussion

The current results revealed that the hypothalamic NPY gene was down-regulated, whereas SOD-1 and CART genes were up-regulated, during the control of AMPH-induced decreases of food intake and body weight. Moreover, NPY knockdown in the brain modulated the decreases in food intake, body weight, and hypothalamic NPY expression in the AMPH-treated rats, but modulated the increases in hypothalamic CART and SOD-1 expression, during a 24-h testing period of AMPH treatment. These results suggest that hypothalamic SOD-1 participate in NPY/CART-mediated appetite and body weight controls in AMPH-treated rats.

The present results showed that hypothalamic NPY decreased but CART (55-102) increased during AMPH treatment. These results confirm our previous finding that CART-containing neurons are functionally reciprocal to that of NPY-containing neurons during the regulation of AMPH-evoked appetite suppression<sup>[34]</sup>. Numerous separate studies have revealed the relationships among NPY-, POMC-, and CART-containing neurons. These studies revealed that (1) there are functional interactions between CART and NPY in the hypothalamus, with neural varicosities containing NPY forming dense pericellular baskets around CART-immunoreactive cell bodies<sup>[35]</sup>, (2) NPY can inhibit POMC neurons via the release

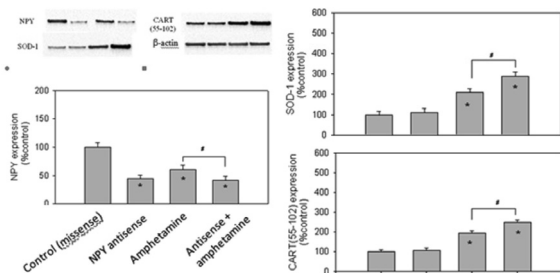


Fig. 3. The effect of NPY antisense (or missense) pretreatment on AMPH-induced changes of NPY, CART (55-102), SOD-1, and  $\beta$ -actin expression over a 24-h period. Missense or antisense treatment (20  $\mu$ g/10  $\mu$ l/day, i.c.v.) was administered one hour before 4 mg/kg AMPH treatment. Results showed that NPY antisense could enhance NPY reduction and partially reverse CART (55-102) and SOD-1 expression back to normal in AMPH-treated rats. \*  $p < 0.05$  vs. the control (missense) groups. #  $p < 0.05$  comparing between AMPH- and antisense/AMPH-treated groups. Bars are the means  $\pm$  SEM. N=8 per group. AMPH: amphetamine; NPY: neuropeptide Y; SOD: superoxide dismutase; CART: cocaine and amphetamine regulated transcript.

of  $\gamma$ -aminobutyric acid<sup>[36]</sup> or via unidirectional inhibitory input from NPY to POMC neurons<sup>[37,38]</sup>; (3) food deprivation can suppress the expression of hypothalamic CART and POMC but increase that of NPY<sup>[39]</sup>; and (4) glucocorticoids are required for meal-induced changes in NPY, CART, and POMC mRNA expression in the hypothalamic arcuate nucleus<sup>[40]</sup>. Moreover, recent evidence revealed that NPY- and POMC-containing neurons might action in an antagonistic manner in the regulation of drug-induced appetite suppression in AMPH-treated rats<sup>[9]</sup>. In the present study, the results revealed that NPY decreased, but CART increased, during the regulation of food intake and body weight in the AMPH-treated rats. Thus, it is possible that both POMC- and CART- containing neurons may function together in a manner opposite to that of NPY-containing neurons during the control of AMPH-induced appetite suppression.

To examine further the possible role of NPY and CART in the reciprocal regulation of NPY-mediated appetite control, the following experiment was performed. Using a cerebral infusion with an NPY antisense oligonucleotide, which aims to inhibit NPY mRNA expression, we found that NPY knockdown enhanced the decrease in NPY expression and the increase in CART (55-102) expression in the AMPH-treated rats. Together with our previous findings indicating that NPY knockdown can modulate POMC expression in AMPH-treated rats<sup>[9]</sup>, our results show that NPY knockdown can modulate both POMC and CART expression. The modulation might be due to a unidirectional inhibitory input from NPY to both POMC and CART neurons<sup>[37]</sup>. Thus, we suggest that both POMC- and CART-containing neurons participate in the reciprocal regulation of NPY-containing neuron during AMPH treatment.

In addition to CART, the present results showed that SOD-1 expression increased during the AMPH treatment and that NPY knockdown modulated SOD-1 expression. This result implies that SOD-1 and CART together might participate in an anti-oxidative stress mechanism via the modulation of NPY expression during AMPH treatment. As POMC and SOD function to prevent AMPH-induced neurotoxicity<sup>[3,20]</sup>, it is possible that POMC,

CART, and SOD-1 together might play a protective role to prevent neuronal damage caused by AMPH, thereby helping to normalize feeding behavior. Moreover, this protective effect might be mediated by the modulation of NPY expression because (1) NPY knockdown can modulate POMC, CART, and SOD expression, and (2) NPY was reported to be associated with modulating oxidative stress<sup>[25]</sup>. Current concepts of the molecular mechanisms underlying the long-lasting neurodegenerative effects of AMPH center on the formation of oxidative stress<sup>[25,41,42]</sup>. Thus, it is rational that increases in CART, POMC, and SOD expression during AMPH treatment might involve an anti-oxidative stress mechanism that favors the normalization of feeding and NPY gene expression.

In addition to the prevention of AMPH-induced neurotoxicity, hypothalamic SOD by itself might be associated with the regulation of AMPH-induced feeding. Evidence reveals that activation of ROS in the hypothalamus during fuel utilization is important in regulating energy homeostasis<sup>[1]</sup>; therefore, suppression of ROS in the hypothalamus diminishes the activity of POMC-containing neuron but promotes the activity of NPY-containing neuron<sup>[43]</sup>. SOD is a ROS-related enzyme which may function in a mechanism of anti-oxidative stress and in the control of appetite suppression during AMPH treatment. It is because the inhibition of ROS production (via i.c.v. infusion of ROS scavenger) in the brain could modulate feeding and the expression of SOD during anorectic drug treatment as described in our previous report<sup>[3]</sup>.

The present results showed that CART (55-102), but not CART (62-102), is activated during AMPH treatment, revealing a functional effect of CART (55-102) on AMPH-induced appetite suppression. Previous evidence revealed that that CART (55-102) is more potent than CART (62-102) in decreasing food intake and controlling body weight<sup>[44-46]</sup>. Although CART (62-102) was not increased following acute treatment with AMPH (once a day for one day) in the present study, it was increased at the third and fourth days following repeated treatment with AMPH (once a day for four days) (data not shown). Thus, CART (62-102)



might operate via a different mechanism in appetite control during repeated AMPH treatment.

Clinical drugs targeting the expression of hypothalamic neuropeptides, including NPY, CART, and POMC, have been suggested as therapeutic agents to combat obesity<sup>[47]</sup>. Based on previous reports indicating that CART is co-localized with hypothalamic POMC neurons<sup>[48]</sup> and that food restriction reduces POMC and CART levels in the hypothalamus in lean animals<sup>[49]</sup>, CART and POMC are currently regarded as endogenous satiety neuropeptides<sup>[50]</sup>.

In conclusion, the present results suggest that hypothalamic SOD-1 participate in the regulation of NPY/CART-mediated appetite control in AMPH-treated rats.

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