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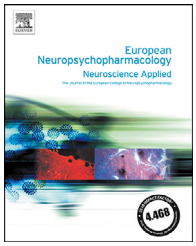

Double immunofluorescent evidence that oxidative stress-associated activation of JNK/AP-1 signaling participates in neuropeptide-mediated appetite control

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Abstract
Amphetamine (AMPH), an appetite suppressant, alters expression levels of neuropeptide Y (NPY) and cocaine- and amphetamine-regulated transcript (CART) in the hypothalamus. This study explored the potential role of cJun-N-terminal kinases (JNK) in appetite control, mediated by reactive oxygen species (ROS) and activator protein-1 (AP-1) in AMPH-treated rats. Rats were given AMPH daily for 4 days. Changes in feeding behavior and expression levels of hypothalamic NPY, CART, cFos, cJun, phosphorylated JNK (pJNK), as well as those of anti-oxidative enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GP) and glutathione S-transferase (GST), were examined and compared. Following AMPH treatment, food intake and NPY expression decreased, whereas the other proteins expression and AP-1/DNA binding activity increased. Both cerebral cJun inhibition and ROS inhibition attenuated AMPH anorexia and modified detected protein, revealing a crucial role for AP-1 and ROS in regulating AMPH-induced appetite control. Moreover, both pJNK/CART and SOD/CART activities detected by double immunofluorescent staining increased in hypothalamic arcuate nucleus in AMPH-treated rats. The results suggested that pJNK/AP-1 signaling and endogenous anti-oxidants participated

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in regulating NPY/CART-mediated appetite control in rats treated with AMPH. These findings advance understanding of the molecular mechanism underlying the role of pJNK/AP-1 and oxidative stress in NPY/CART-mediated appetite suppression in AMPH-treated rats.
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1. Introduction

Reactive oxygen species (ROS) are typically produced in mitochondria as electrons leak which react with oxygen to form superoxide (Sharma et al., 2004). Production of ROS in the hypothalamus during fuel utilization is important in regulating energy homeostasis (Horvath et al., 2009; Jaillard et al., 2009; Thakur et al., 2017). Thus, suppressing the production of ROS in the hypothalamus diminishes proopiomelanocortin (POMC) cell activation and promotes the activity of neuropeptide Y (NPY) neuron and feeding, whereas increasing the production of ROS activates POMC neurons and reduces feeding (Diano et al., 2011). Moreover, glucose-utilizing POMC neurons are fired, which leads to the increases in ROS levels in hypothalamic cells during positive energy balance. By contrast, fatty acid-utilizing NPY neurons are activated, but ROS levels are not increased in these cells during negative energy balance (Leloup et al., 2006; Andrews et al., 2008).

The anti-ROS-associated enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GP), and glutathione S-transferase (GST), are anti-oxidative enzymes that function in the detoxification process and prevention of ROS accumulation (Shao et al., 2008; Rosenfeld et al., 2017). Incomplete reduction of O_2 during respiration in mitochondria produces superoxide anions, which are dismutated by SOD to form H_2O_2. In turn, H_2O_2 is decomposed by catalase to form H_2O and O_2. Both SOD and catalase are thought to play a role in the detoxification process in the brain during anorectic drug treatment (Kuo et al., 2009, 2011). GP prevents ROS accumulation in the brain by breaking down H_2O_2 (Ehrenbrink et al., 2006). GST catalyzes the conjugation of oxidized products with the thiolate anion of glutathione (GSH), the major antioxidant in the brain, to form nontoxic products (Johnson et al., 1993; Sharma et al., 2004). Previous research showed that GST was present a high concentration in the thalamus/hypothalamus (Björk et al., 2006) and that it eliminated metabolites produced by dopamine (DA) oxidation (Baez et al., 1997). There are four main classes of cytosolic GSTs: α, μ, π, and θ. Shao et al. (2008) demonstrated that GSTα was expressed in the brain and that it played an essential role in the function of neuroprotection against lithium-induced oxidative stress. Previous research reported that SOD enzymes, including CuZn-SOD (SOD-1) and Mn-SOD (SOD-2), played essential roles in destroying oxygen-based radicals and that they seemed to play a role in attenuation of DNA binding effect of activator protein-1 (AP-1) in methamphetamine-treated rats (Sheng et al., 1996; Pinto and Moraes, 2014).

Amphetamine (AMPH) is a well-known appetite suppressant. In previous report, we revealed that a non-selective DA D1/D2 receptor antagonist blocked approximately 90% of AMPH-induced anorexia, implicating the central release of DA in regulating AMPH-mediated appetite control (Chen et al., 2001; Hsieh et al., 2014). A previous study showed that AMPH-induced release of DA was injurious to neurons through DA auto-oxidation and the formation of ROS in the brain (Alagarsamy et al., 1997). Increased oxidative stress appeared to influence the expression of orexigenic neuropeptide Y (NPY) gene and anorxigenic cocaine- and amphetamine-related transcript (CART) gene in the hypothalamus (Hsieh et al., 2015). A number of studies suggested that hypothalamic NPY and CART genes may function reciprocally in the regulation of AMPH-induced appetite suppression (Chu et al., 2015; Yu et al., 2018).

The c-Jun N-terminal kinase (JNK) is a member of mitogen-activated protein kinase (Chang and Karin, 2001). Previous studies demonstrated the association of JNK with central regulation of oxidative stress (Turner et al., 1998) and activation of ROS (Torres and Forman, 2003; McCubrey et al., 2006). There are three JNK isoforms (JNK1, JNK2 and JNK3), which are encoded by three different genes (Ip and Davis, 1998). JNK1 and JNK2 are ubiquitously expressed and JNK3 is restricted to the brain, testis, and pancreatic β-cells (Solinas and Karin, 2010). Previous studies demonstrated that JNK activated cJun, a member of the AP-1 transcription factors, and that AP-1 is potently induced in response to cellular stress (Chang and Karin, 2001; Hibi et al., 1993). Previous research also showed that AP-1 and anti-oxidative enzymes played a role in the modulation of NPY gene expression in the brain (Sheng et al., 1996; Rodriguez et al., 1999; Hsieh et al., 2006, 2008). In addition, a recent study reported that phosphorylated JNK (pJNK) activity within specific cellular contexts in specific stage of type-2 diabetes may participate in the modulation of obesity and metabolic stress (Solinas and Becattini, 2016).

The present study investigated whether pJNK/AP-1 signaling and endogenous anti-oxidant in the hypothalamus participated in reciprocal regulation of NPY/CART-mediated appetite control in AMPH-treated rats.

2. Experimental procedures

2.1. Animal treatments

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 normal 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. 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 (8th edition, 2011). All animal surgeries were performed under sodium pentobarbital (35–40 mg kg_1; i.p.) anesthesia. An additional dose of half of the initial dose would be administered if rats showed signs of feeling pain or consciousness.

Every effort was made to minimize suffering in these animals. A total of 255 animals were used in the experiments described here.

2.2. Experimental procedures

There were 5 experiments subdivided in this study.

To investigate the effects of AMPH on feeding behavior and hypothalamic neuropeptide and antioxidants expression, rats ($N = 8$ for each group) were i.p. injected with AMPH at a dose of 1, 2 or 4 mg/kg for 4 days. AMPH was first injected at the end of Day 0 (i.e., at 6:00 p.m.), which is also regarded as the beginning of Day 1. The data for daily food intake were calculated with respect to the food intake amount in the previous day. To determine the effect of AMPH treatment on the changes in hypothalamic NPY, CART (55–102), cFos, cJun, total JNK(tJNK), pJNK, and antioxidants (including SOD-1, SOD-2, GSTα, and GP) expression, rats ($N = 6$) were injected with AMPH (2 mg/kg; i.p.) once a day for 4 day. On the sacrifice day, rats received a treatment of AMPH 40 min before being sacrificed to enhance the effects of AMPH. The rats were anesthetized with pentobarbital and decapitated. Following decapitation, the hypothalamus was removed to determine the desired protein expression. The hypothalamus was removed immediately and subjected to determinations of protein expression, or stored at -80 °C until its use for analysis.

To investigated the effect of AMPH on AP-1/DNA binding activity, rats ($N = 6$) were daily given AMPH (2 mg/kg; i.p.) for 4 days. At 40–50 min after daily AMPH treatment (at 6:00 p.m.), the hypothalamus was removed to determine AP-1/DNA binding activity by a technique of electro-motility shift assay (EMSA).

To explore the effects of JNK inhibitor/AMPH co-administration on changes of feeding behavior, hypothalamic neuropeptides and antioxidants expression, rats were pretreated (intracerebroventricularly, i.c.v.) with JNK inhibitor (SP600125) on the anorectic response and the expression of NPY, CART, cFos, cJun, pJNK and antioxidants in AMPH-treated rats, rats ($N = 8$ per group) were given JNK inhibitor (10 μL in concentration of 1.5 μM; i.c.v.; the infusion rate was 25 μL/h) one hour before AMPH (4 mg/kg; i.p.) treatment once a day for 4 days. The drug SP600125 is a selective JNK inhibitor which is effective in the inhibition of JNK expression (Siddiqui and Reddy, 2010; Gehringer et al., 2015) and can completely inhibit the expression of pJNK and cJun in AMPH-treated rats (Shyu et al., 2004). Moreover, acute i.c.v. injection of SP600125 in the region of hypothalamic ARC can ameliorate the JNK-mediated energy metabolism (Benzleret al., 2013). SP600125 (molecular weight = 220) is poorly soluble in water. Stock solution of 25 μM SP600125 is dissolved in 0.25% dimethyl sulfoxide (DMSO) solution and dilutes with aCSF to the desired concentration. On the sacrifice day, rats received JNK inhibitor and/or AMPH at 40 min prior to being anesthetized and then decapitated immediately to remove the hypothalamus. Following decapitation, the hypothalamus was removed to determine the desired protein expression. The hypothalamus was removed immediately and subjected to determinations of protein expression, or stored at -80 °C until its use for analysis.

To investigate the effects of ROS scavenger/AMPH co-administration on changes of feeding behavior, hypothalamic neuropeptides (NPY, CART, cFos, cJun, pJNK) and antioxidants, rats ($N = 8$ for each group) were daily infused with N-acetyl-L-cysteine (NAC) (5 μL in concentration of 10 μg; i.c.v.; the infusion rate was 5 μL/min) (Koppen et al., 2014) 40 min before AMPH treatment (4 mg/kg; i.p.) for 4 days. NAC is an antioxidant which can increase levels of glutathione and is widely used in the research of ROS. NAC results from its free radical scavenging property and was used as an antioxidant through increasing intracellular GSH levels and through its thiol-disulfide exchange activity (Zafarullah et al., 2003; Sun, 2010). NAC was 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.

To examine the effect of AMPH treatment on immunofluorescent activities of (1) pJNK/CART and (2) SOD/CART in hypothalamic arcuate nucleus (ARC), rats were given AMPH (4 mg/kg; $N = 5$) once a day for 2 days. Forty minutes after the second day of AMPH treatment, rats were under ether anaesthesia and killed by transaortic perfusion with a fixative solution, as described in the desired section below.

2.3. AP-1/DNA binding assay

Polymerase chain reaction (PCR) amplifications of the AP-1 response elements on the collagen promoter region were performed using the following primers: AP-1, 5′-CCT AAG GCA TAG AGC AAT GAC-3′ (sense) and 5′-GGT GAG AAA CAT GAC TAG GTG-3′ (antisense). Electromobility shift assay (EMSA) was carried out using the Lightshift kit. Briefly, 10 μg of nuclear protein was preincubated with 10 mM Tris, 50 mM KC1, 1 mM DTT, 5 mM $MgCl_2$, 2 μg poly (dI $\times$ dC) and 2 pmol of oligonucleotide probe for 20 min at room temperature. Specific binding was confirmed by using a 200-fold excess of unlabeled probes as specific competitor. Protein-DNA complexes were separated by a 6% nondenaturing acrylamide gel electrophoresis. Complexes were transferred to positively charged nylon membranes and UV-crosslinked in a crosslinker. Gel shifts were visualized with a streptavidin-horseradish peroxidase followed by chemiluminescent detection (Chen et al., 2006).

2.4. 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). 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. 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 (Day 0).

2.5. 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), cFos, cJun, AP-1, SOD-1, GSTα, GP, and β-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).

2.6. Immunofluorescent staining

The rats were killed under ether anesthesia by transaortic perfusion with 50 ml heparinized saline, and then treated with 250 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer; pH 7.4). Brains were removed and immersed immediately in the fixative for 90 min, washed in 0.1 M phosphate buffer saline (PBS), and then placed in 0.1 M PBS containing 30% sucrose at 4 °C for 48 h. Brains were prepared as 7 μm coronal sections containing the third ventricle of hypothalamus using Leica CM3050 S cryostat (Buffalo Grove, IL, USA) at −20 °C. Sections were fixed with pre-cooling acetone for 10 min, and then washed with phosphate buffered saline (PBS) for 3 times. Immersed the slides into antigen retrieval buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) and boiled for 10 min. Slides were rinsed for 3 times in PBS. Sections were covered with the blocking reagent (Innovex, Richmond, CA, USA) for 2 h at room temperature. Slides were washed using tris buffered saline with tween 20 (TBST) for 3 times. In the double staining procedure, sections were incubated at 4 °C for 24 h with both anti-CART (1:100 dilution) and anti-pJNK (1:50 dilution) or both anti-CART and anti-SOD1 (1:200) primary antibodies. In the next day, all sections were incubated at 37 °C for 1 h with corresponding secondary antibodies (fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:50) for anti-pJNK and anti-SOD1 whereas rhodamine-conjugated goat anti-rabbit IgG (1:50) for anti-CART) plus 4′,6-diamidino-2-phenylindole (DAPI, 0.5 μg/ml) in the dark. Slides were rinsed with TBST for a total 3 washes. Tissues were then covered with mounting media (Vector Laboratories, Burlingame, CA, USA) and sealed with cover slips. Images were visualized by upright fluorescent microscope (ZEISS AXioskop2, Carl Zeiss Microscopy, Thornwood, NY, USA) or observed under confocal microscope (ZEISS LSM 510 META). The digitally results of fluorescence intensities were quantified by ImageJ software (National Institute of Health, Bethesda, MD, USA). In the procedure, Global calibration, Global scale and Threshold were setting to remove the background and select the area for quantification. Colocalization results of yellow color were analyzed by using RGB method that converts pixel in brightness values.

2.7. Drugs, chemicals, and reagents

Chow (LabDiet) was purchased from PMI Nutrition International (Brentwood, MO, USA). AMPH, angiotensin II, NAC, and Tris-HCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against NPY, cFos, SOD-1, SOD-2, JNK, pJNK, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against GST-α was purchased from Oxford Biomedical Research (Oxford, MI, USA), those against AP-1 and c-Jun antibodies were from Cell Signaling Technology, (Beverly, MA, USA), and those against GP was from Gibco BRL, Life Technologies, Inc., (Rockville, MD, USA). CART (55–102) antibody was from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). SOD-1 antibody used in immunofluorescent staining was purchased from Novus, Inc., (Littleton, CO, USA). TRIZOL reagent (Life Technologies, Inc., Grand Island, USA) was used in tissue homogenization. EMSA was carried out using the Lightshift kit (Promega Life Science).

2.8. Statistical analysis

Data are presented as the means ± SEM. A two-way or one-way ANOVA followed by Dunnett's test was used to detect significant differences between the groups. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of AMPH on feeding and hypothalamic protein levels

The change of feeding behavior in rats receiving AMPH (1, 2, 4 mg/kg; i.p.) treatment were shown in the left upper panel of Fig. 1. Using statistical analysis with two-way ANOVA followed by Dunnett's test ($p<0.05$), to measure the effect of AMPH, results revealed a significant dose-dependent [$F(3,28)=11.16$, $p<0.05$] and time-dependent effect [$F(4,35)=6.27$, $p<0.05$], but it was not conclusive of the interaction (Dose × Time) effect. Statistical results revealed that AMPH (1 mg/kg) reduced the food intake on Day 2, AMPH (2 mg/kg) reduced the food intake from Day 1 to Day 3, and that AMPH (4 mg/kg) reduced food intake from Day 1 to Day 4 compared to the controls. Moreover, the effect of 2 mg/kg AMPH on Day 4 was significant compared to that on Day 2, revealing that 2 mg/kg AMPH could induce gradually the tolerant effect to AMPH. However, with a dose of 4 mg/kg AMPH, it produced a continuous anorectic response during a 4-day period of drug treatment.

Therefore, AMPH (2 mg/kg) was employed for the experiments in hypothalamic neuropeptides and antioxidants expression (Figs. 1 and 2) and AP-1/DNA binding activity (Fig. 3) because there was a reversion of food intake (tolerant effect of AMPH) during a 4-day period of AMPH treatment. However, AMPH (4 mg/kg) was used for the experiments in JNK inhibitor/AMPH co-administration (Fig. 4), ROS inhibitor/AMPH co-administration (Fig. 5), and double immunofluorescent staining (Figs. 6 and 7) because it could exert a greater anorectic effect and neuropeptides expression, and was thus more suitable to study the inhibitory effect of AMPH.

3.2. Effects of AMPH on NPY, CART, cFos, cJun, and pJNK/tJNK expression

Results shown in Fig. 1 revealed that daily AMPH treatment decreases NPY but increased CART (55–102), cFos, cJun, and pJNK/tJNK expression during AMPH treatment. Using β-actin as the internal standard, the ratio of detected proteins over β-actin in each group was calculated and compared. Analysis with one-way ANOVA revealed a decrease in NPY from Day1 to Day 3 [$F(4,25)=5.56$, $p<0.05$], but revealed the increases in CART (55–102) expression from Day 1 to Day 4 [$F(4,25)=6.68$, $p<0.05$], cFos expression from Day 1 to Day 3 [$F(4,25)=3.61$, $p<0.05$], cJun expression from Day 1 to Day 3 [$F(4,25)=4.13$, $p<0.05$], and pJNK/tJNK expression from Day 1 to Day 4 [$F(4,25)=5.42$, $p<0.05$].

Results shown in Fig. 2 revealed a significant effect on antioxidants expression, which included SOD-1 expression from Day 1 to Day 3 [$F(4,25)=4.22$, $p<0.05$], SOD-2 expression from Day 1 to Day 4 [$F(4,25)=6.32$, $p<0.05$], GP expression from Day 1 to Day 3 [$F(4,25)=3.32$, $p<0.05$], and GSTα expression from Day 1 to Day 3 [$F(4,25)=4.45$, $p<0.05$] compared to the control. These results revealed that CART (55–102), cFos, cJun, and antioxidants expression were increased with maximum response on Day 2 during AMPH treatment, which were expressed just opposite to the expression of NPY with the biggest reduction on Day 2.

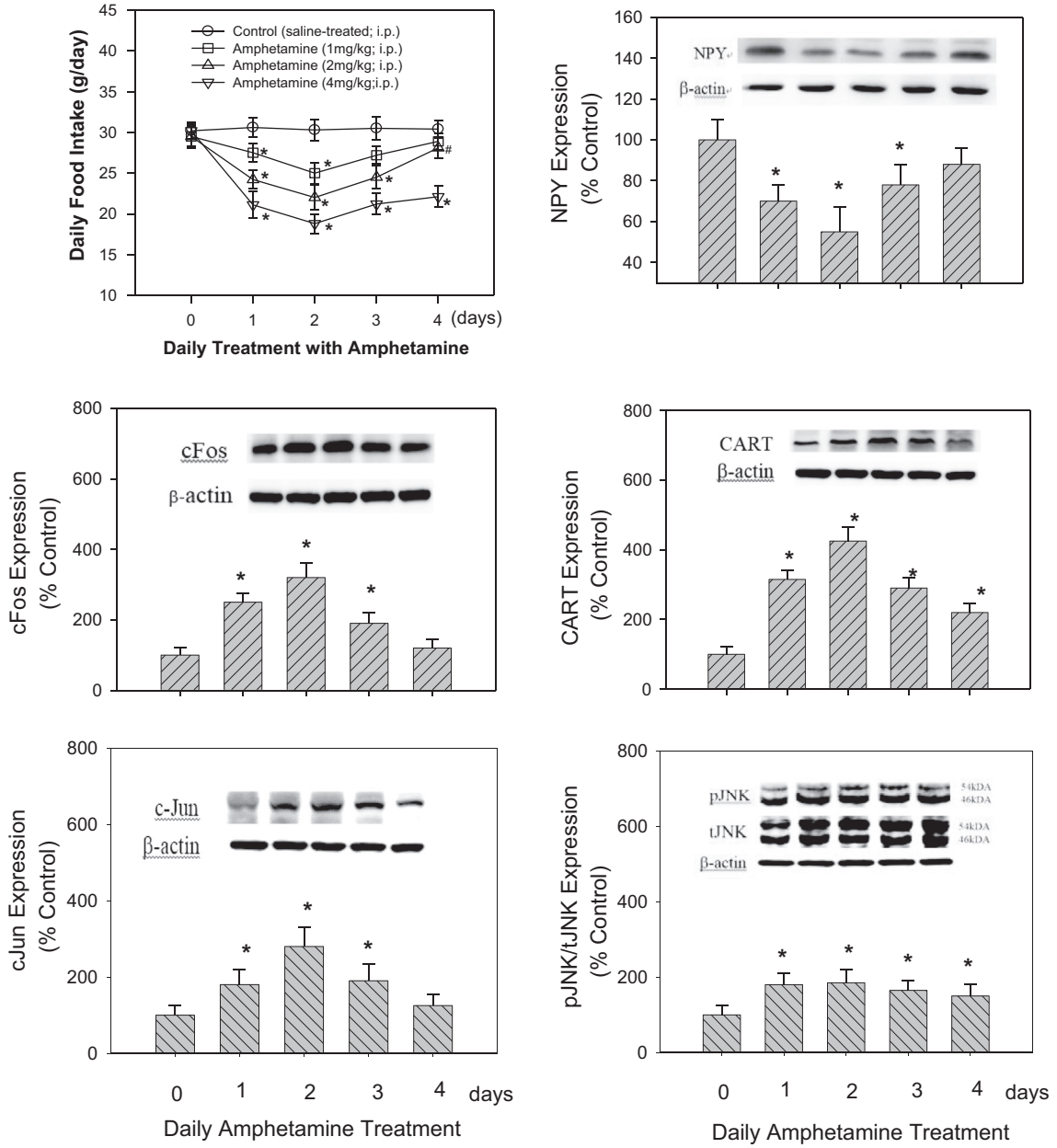


Fig. 1 The effect of daily AMPH treatment on food intake (linear figure) and expression levels of hypothalamic protein (columned figures) over a 4-day period. *Linear figure in upper left*: the first injection of AMPH (1, 2 and 4 mg/kg; i.p.) was conducted at the end of Day 0. Each point in figures represents the mean ± SEM ($N = 8$ each group). $^{*}P < 0.05$ vs. the control group. $^{\#}P < 0.05$ vs. the AMPH (2 mg/kg)-treated group on Day 2. ***Columned figures (others):*** effects of AMPH (2 mg/kg; i.p.) on expression levels of NPY, CART, cFos, cJun, pJNK, and β-actin in the hypothalamus. Results of column showed the relative densitometric value of Western Blots which were inserted with each figure. Bars were mean ± SEM. $N = 6$ each group. $^{*}P<0.05$ vs. control.

3.3. The effect of AMPH on AP-1 and DNA binding activity

As shown in Fig. 3, daily treatment with AMPH for four days could increase hypothalamic AP-1/DNA binding activity. Analysis with one-way ANOVA revealed that AP-1/DNA binding activity was increased during the first 3 days of AMPH treatment [$F(4,25)=3.88$, $P<0.05$] compared to controls. Moreover, the expressive pattern of AP-1/DNA binding activity was similar to that in pJNK expression during a 4-day period of AMPH treatment.

3.4. The effect of co-administration with JNK inhibitor/AMPH on food intake and hypothalamic protein expression levels

Results shown in the left upper panel of Fig. 4 revealed that a pretreatment of JNK inhibitor (SP600125) in AMPH-treated rats could modify the anorectic response of AMPH, indicating the involvement of JNK signaling in AMPH anorexia. Using two-way ANOVA to analyze the effect of JNK inhibitor pretreatment on AMPH anorexia, it revealed significant dose-dependent [$F(3,28)=8.42$, $p<0.05$] and time-

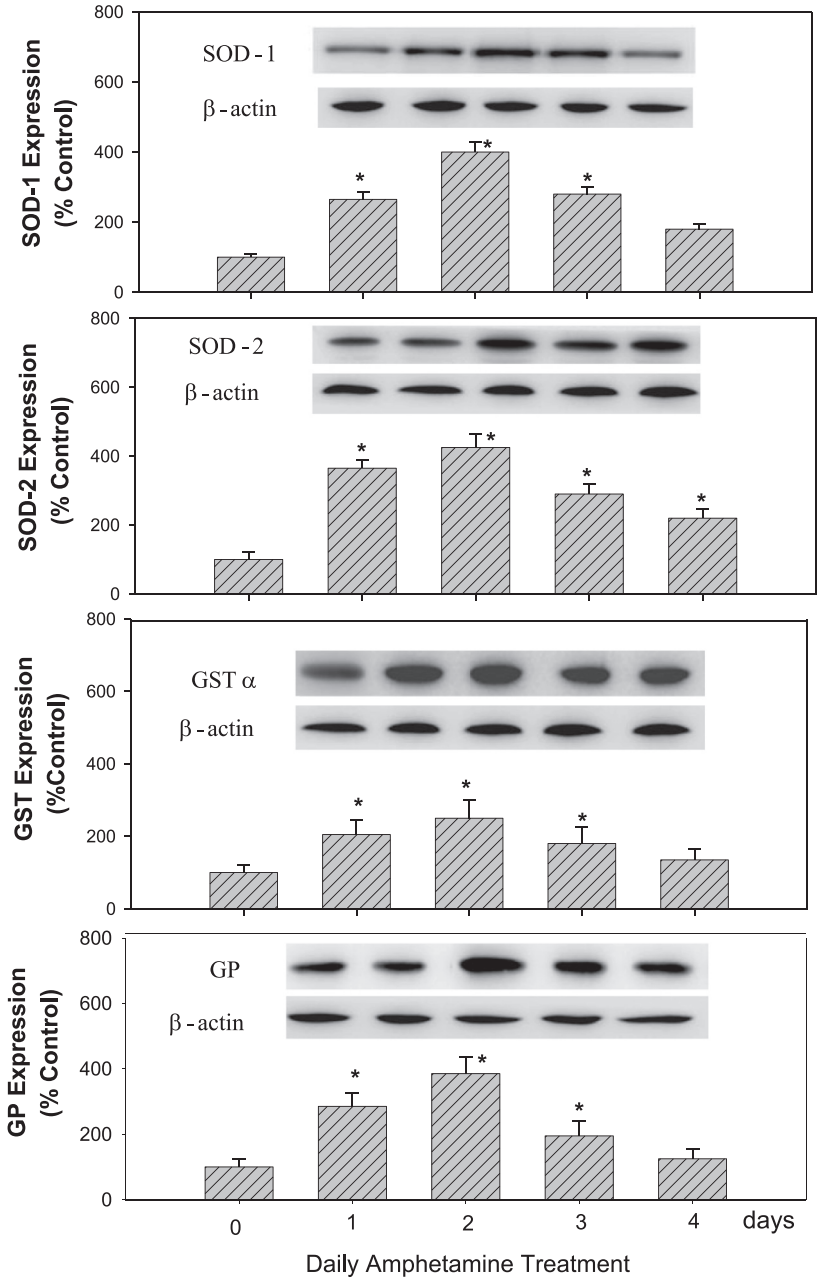


Fig. 2 The effect of daily AMPH (2 mg/kg; i.p.) on expression levels of anti-oxidants (SOD-1, SOD-2, GP, and GSTα) and β-actin in the hypothalamus over a 4-day period. Results of column showed the relative densitometric value of Western Blots which were inserted with each figure. Bars were mean ± SEM. $N = 6$ each group. $^{*}P<0.05$ vs. control.

dependent effects [F(4,35)=5.36, $p<0.05$], but it was not conclusive of the interaction effect. Moreover, results shown in Fig. 4 also revealed that JNK inhibitor/AMPH co-administration could change the expression levels of NPY, CART (55–102), cFos, cJun, pJNK, and antioxidant expression compared to the control group during a 4-day period of drug treatment. Using β-actin as the internal standard, the ratios of detected proteins over β-actin in each group were calculated and compared. Using one-way ANOVA followed by Dunnett's test ($P<0.05$), it revealed that significant decrease in NPY [F(6,35)=7.78, $P<0.05$] and increases in CART (55–102) [F(6,35)=3.21, $P<0.05$], cFos [F(6,35)=2.77, $P<0.05$], cJun [F(6,35)=2.98, $P<0.05$], cJNK [F(6,35)=3.08, $P<0.05$], SOD-1 [F(6,35)=5.12, $P<0.05$], SOD-2 [F(6,35)=4.38, $P<0.05$], GP [F(6,35)=3.85, $P<0.05$], and GSTα [F(6,35)=5.12, $P<0.05$] could be observed in pJNK inhibitor/AMPH co-administrated groups compared to the control group. The feeding behavior shown in vehicle-treated rats was similar to that in saline-treated rats (Fig. 1). Moreover, the anorectic response in vehicle/AMPH-treated rats (Fig. 4) was not significantly changed compared to that in AMPH-treated rats (Fig. 1). These results revealed the noninterference of vehicle treatment in this study. Moreover, a treatment with pJNK inhibitor alone

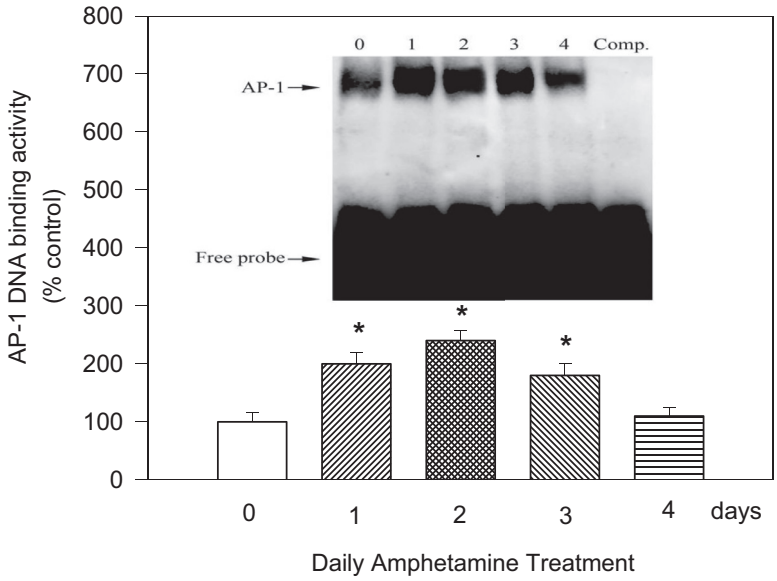


Fig. 3 The result of EMSA analyzing AP-1/DNA binding ability following daily AMPH treatment over a 4-day period. Columned figure indicated relative densitometric values for EMSA assay. Contents of AP-1/DNA binding ability were indicated as the percentage of the control group. *Inserted image:* nuclear extracts in the hypothalamus were analyzed by EMSA assay with biotin labeled AP-1 specific oligonucleotide as described in Experimental procedures. Lane 6 represented nuclear extracts incubated with unlabeled oligonucleotide (competitive control) to confirm the specificity of binding. Bars were mean ± SEM. $N = 6$ each group. $^{*}P<0.05$ vs. control. Comp: competitive control.

could change pJNK expression but didn't change the expression levels of other protein, indicating the specific effect of pJNK inhibitor on pJNK expression.

3.5. The effect of the pretreatment with NAC on AMPH-induced food intake and hypothalamic protein levels

NAC is a drug used to detect the effect of ROS scavenger on AMPH-treated feeding behavior. Results shown in the upper panel of Fig. 5 revealed that daily pretreatment with ROS scavenger (NAC) could modify AMPH-induced anorectic response during a 4-day period. Statistical analysis with two-way ANOVA revealed significant dose-dependent [$F(3,28)=10.32$, $p<0.05$] and time-dependent effects [$F(4,35)=7.58$, $p<0.05$]. Moreover, it revealed that co-administration with NAC/AMPH attenuated the decreases of food intake from Day1 to Day 4 compared to that in AMPH-treated group. The food intake in vehicle-treated rats was similar to that in saline-treated rats. Moreover, the food intake in NAC alone-treated rats remained unchanged compared to that in vehicle-treated rats, revealing the noninterference of vehicle in this study. Results shown in the lower panel of Fig. 5 revealed that NAC/AMPH-treated co-administration resulted in partial restoration of detected proteins and antioxidants expression toward normal level during the first day (24 h) of testing. By one-way ANOVA followed by Dunnett's test ($P<0.05$), it revealed that a significant decrease in NPY [$F(3,20)=8.78$, $P<0.05$] but increases in CART (55–102) [$F(3,20)=6.12$, $P<0.05$], cFos [$F(3,20)=4.38$, $P<0.05$], cJun [$F(3,20)=3.77$, $P<0.05$], cJNK [$F(3,20)=4.16$, $P<0.05$], SOD-1 [$F(3,20)=6.76$, $P<0.05$], SOD-2 [$F(3,20)=4.18$, $P<0.05$], GP [$F(3,20)=3.52$, $P<0.05$], and GSTα [$F(3,20)=5.17$, $P<0.05$] in NAC/AMPH co-administrated groups compared to the control group. NAC treatment alone can slightly but not significantly decrease cJNK expression and didn't affect the expression levels of other hypothalamic proteins compared to the control group during a 24-h of drug treatment.

3.6. The effect of AMPH treatment on hypothalamic pJNK, SOD-1, and cart (55-102) immunofluorescent expression

Fig. 6 showed the results of immunofluorescent staining, which indicated the significant influences of AMPH on the fluorescent intensity of pJNK, CART (55–102), and SOD-1 in hypothalamic ARC area. The expression of pJNK, CART (55–102) and SOD-1 positive cells appeared to be increased approximately 205%, 170%, and 180%, respectively, in rats receiving 4 mg/kg AMPH compared to the control groups (t-test, $P<0.05$). In Fig. 7, results of double immunofluorescent staining, which indicated the co-localization of two detected protein, revealed that merged pJNK/CART and merged SOD-1/CART immunofluorescences (yellow color) were increased about 208% and 135%, respectively, in AMPH-treated group (t-test, $P<0.05$).

4. Discussion

The primary findings in the present study were as follows: (1) In AMPH-treated rats, feeding behavior and NPY decreased, whereas CART (55–102), JNK, anti-oxidative

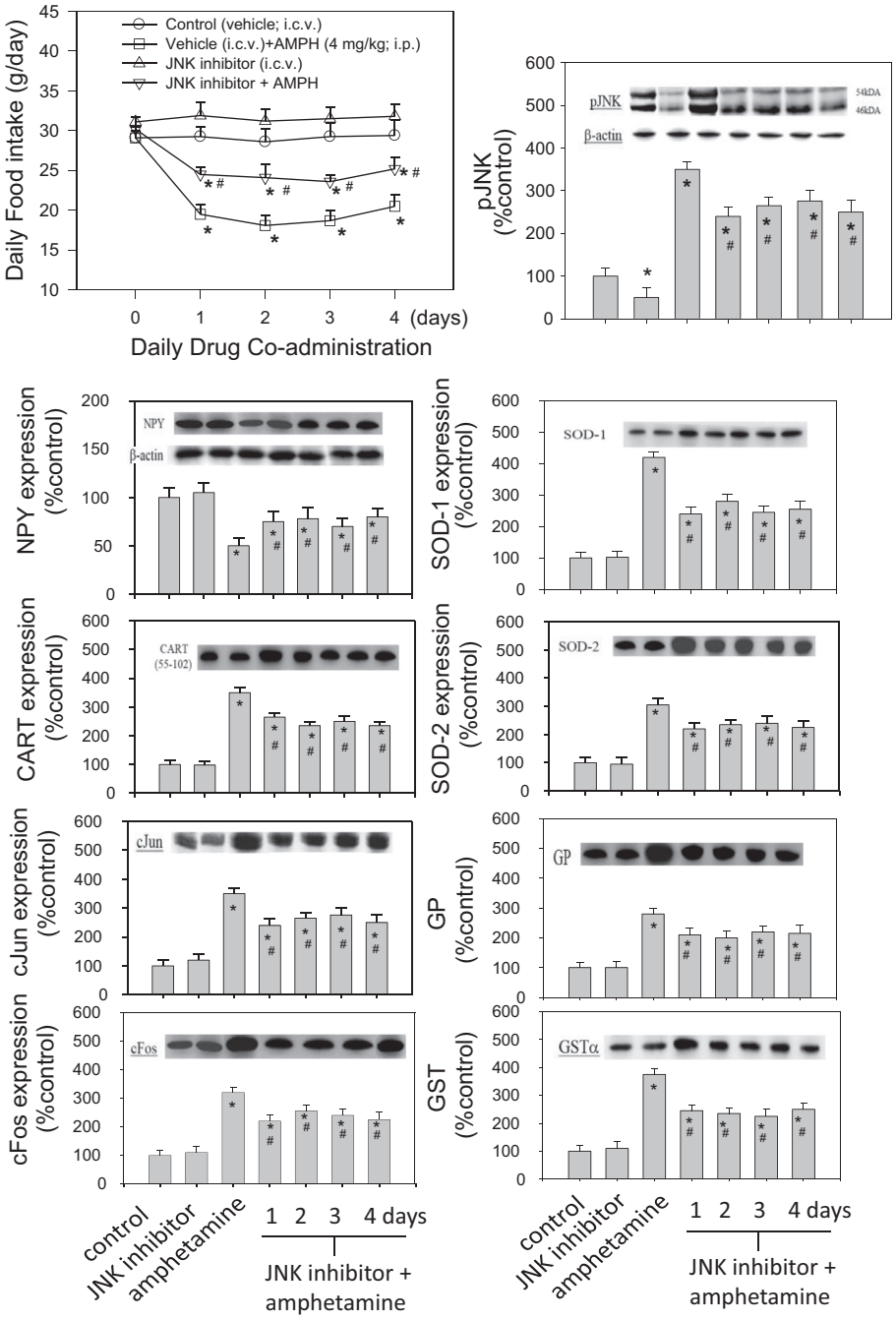
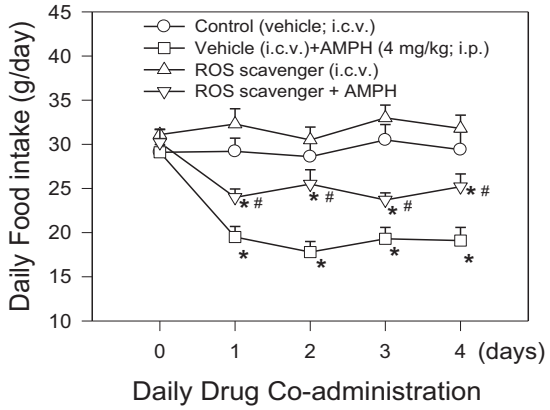


Fig. 4 *Upper panel:* the effect of daily JNK inhibitor (SP600125) pretreatment on AMPH-induced feeding behavior over a 4-day period. JNK inhibitor (1.5 μM; i.c.v.; 25 mL) was administered 60 min before receiving 4 mg/kg AMPH once a day for 4 days. * $p<0.05$ vs. the vehicle-treated group of each treatment day. # $p<0.05$ vs. the AMPH-treated groups of each treatment day. Bars are mean ± SEM. $N=8$ per group. *Lower panel:* the effect of JNK inhibitor/AMPH co-administration on expression levels of NPY, CART, cFos, cJun, pJNK, and antioxidants over a 4-day period. * $p<0.05$ vs. the control (vehicle) groups of each treatment day. # $p<0.05$ vs. the AMPH-treated groups of each treatment day. Bars are the means ± SEM. $N=6$ per group.

enzymes, and AP-1/DNA binding activity increased; (2) JNK inhibition modified feeding behavior and expression levels of CART (55–102), cFos/cJun, and anti-oxidative enzymes; (3) central inhibition of ROS production modified the expression levels of NPY, CART (55–102), cFos/cJun, and JNK; and (4) the results of double immunofluorescent staining showed that both pJNK/CART and SOD/CART immunofluorescent particles increased in hypothalamic ARC. These results suggested that both the activation of pJNK/AP-1 signaling and the production of antioxidants in the hypothalamus participated in the regulation of NPY/CART-mediated appetite control in AMPH-treated rats.

Our previous results (Kuo et al., 2011) showed that both NPY reduction and CART (55–102) elevation on Day 1 and Day

(A) Feeding behavior during ROS scavenger/AMPH co-administration



(B) Hypothalamic protein expression levels during drug co-administration

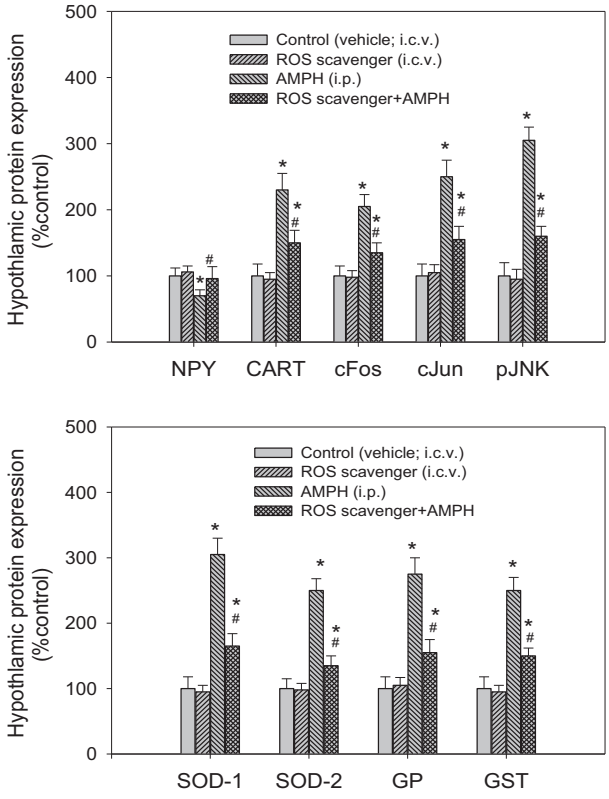


Fig. 5 *Upper panel*:results of Western blots showing the effect of NAC (a ROS scavenger) pretreatment on AMPH-induced anorexia during a 4-day period of drug treatment. *Middle and Lower panel*:results of Western blots showing the NAC/AMPH-induced changes of hypothalamic protein and the relative densitometric values for these protein during the first day (24 h) of AMPH (4 mg/kg; i.p.) treatment. Contents of NPY, CART (55–102), cFos, cJun, pJNK, antioxidants (including SOD-1, SOD-2, GP, and GSTα) were indicated as the percentage of controls. $^{*}P<0.05$ vs. the control (vehicle-treated, i.c.v.) group. $^{\#}P<0.05$ vs. the vehicle/AMPH-treated groups of each treatment day. Bars are mean $\pm$ SEM. $N=6$–8 per group. NAC: N-acetyl-L-cysteine. Vehicle: aCSF solution.

2 participated in the anorectic response of AMPH (anorectic effect). This anorectic effect was associated with satiety or a positive energy balance. In contrast, the expression levels of NPY and CART (55–102) were reversed back to normal levels from Day 2 to Day 4 (tolerant effect). This tolerant effect was associated with hunger or a negative energy balance. These results implied that CART neurons might function in a manner reciprocal to that of NPY neurons during the regulation of AMPH-evoked appetite suppression.

To examine the functional role of JNK in the regulation of NPY/CART-mediated appetite control, rats were pretreated with a JNK inhibitor prior to AMPH treatment. JNK

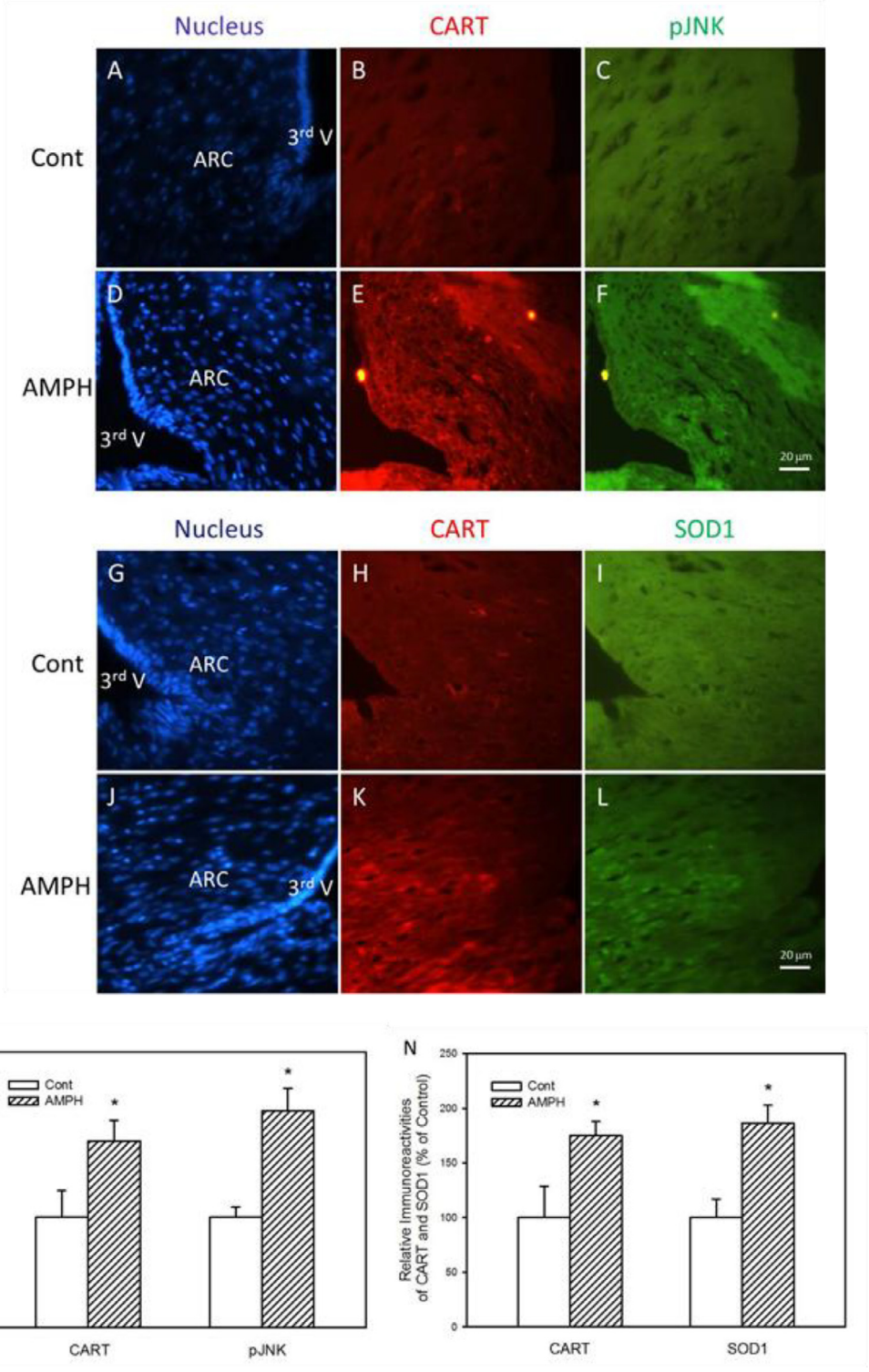


Fig. 6 Results of immunofluorescent staining showing CART (55–102), pJNK and SOD-1 immuno-reactivities in the hypothalamic ARC regions in rats receiving saline or AMPH (4 mg/kg; i.p.) treatment. Frontal sections through the level of median eminence were obtained. *Upper panel (from A to L)*:photo images A–C and G–I show sections in control group, while photo images D–F and J–L show sections in AMPH-treated group. Blue color represented nucleus, red color stood for CART (55–102) and green color showed pJNK or SOD-1. *Lower panel (M and N)*:results showed relative densitometric values of fluorescence intensity for CART (55–102) and pJNK (M) as well as CART and SOD-1 (N) in ARC regions compared to the control group in AMPH-treated rat ($N = 5$). Each value represents mean ± SEM. $^{*}p < 0.05$ vs. control. Cont: control. Scale bar: 20 μm (400X). 3rd V: the third ventricle. SOD-1 is the only antioxidants detected by immunofluorescent staining in this experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

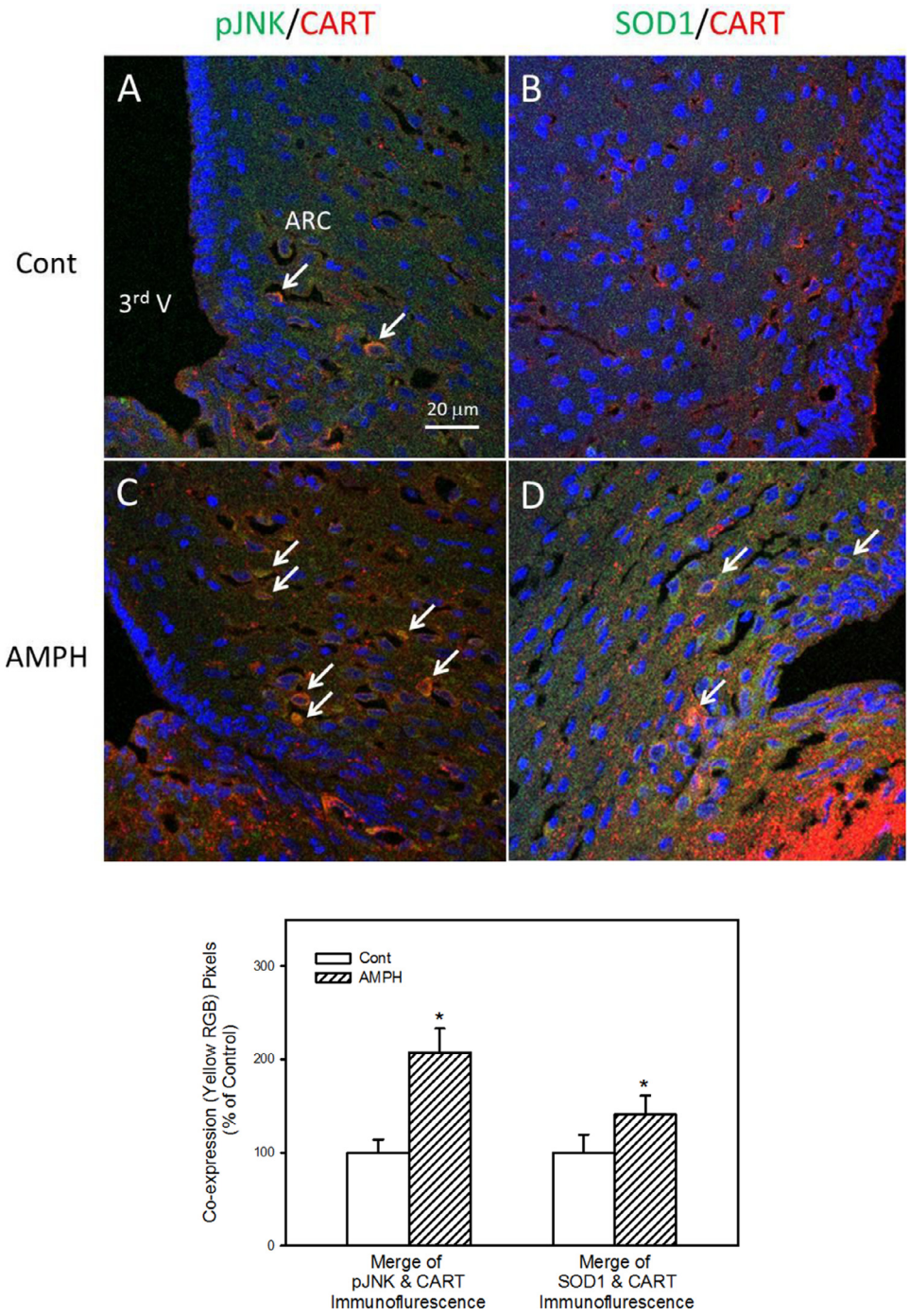


Fig. 7 Results of double immunofluorescent staining showing the co-localizations of either pJNK/CART or SOD-1/CART immunofluorescences in the ARC area in AMPH-treated rats. Under the analysis of confocal microscope, co-localization images were seen by merging of pJNK with CART (A) and (C) or SOD-1 with CART (B) and (D) immunofluorescent images, giving yellow color in the merged image panel (E). Quantification of co-localization images (shown in yellow color) by using RGB method that converts pixel in brightness values. Each value represents mean ± SEM. $N = 5$ per group. $^{*}P<0.05$ vs. control. $\#P<0.05$ between the two groups. Blue: nucleus. Scale bar: 20 μm. 3rd V: the third ventricle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inhibition modified NPY/CART, cFos/cJun, and antioxidants expression in rats co-administered with JNK inhibitor/AMPH as compared with AMPH-treated rats. This result provided evidence that JNK/AP-1 signaling in the hypothalamus participated in the regulation of antioxidants expression and NPY/CART-mediated appetite control in AMPH-treated rats. Similarly, pretreatment with NAC, a ROS scavenger, modified NPY/CART, cFos/cJun, and JNK expression in NAC/AMPH-treated rats as compared with AMPH-treated rats. These results reveal that activations

of JNK/AP-1 signaling and endogenous antioxidants in the hypothalamus may play a functional role in regulating NPY/CART-mediated appetite control in AMPH-treated rats.

Current results revealed that NPY/CART-mediated appetite suppression in AMPH-treated rats might be associated with the activation of anti-oxidants located in CART-containing neurons in the hypothalamus. This is because SOD-1, SOD-2, GP and GSTα expression increased and were expressed in a pattern similar to that of CART (55-102) in AMPH-treated rats. This finding was in accordance with a previous report indicating that forced swimming-induced stress elicited region-specific changes in CART expression in areas of the brain involved in stress regulation (Balkan et al., 2012). Our findings suggested that the activation of anti-oxidative enzymes in CART-containing neurons may possibly play a functional role in the prevention of oxidative damage in the brain. In addition, as NPY expression level is associated with the prevention of oxidative stress in the brain (dos Santos et al., 2013), JNK inhibition observed in the present study may thus reverse NPY levels and decrease anti-oxidants and CART (55–102) during AMPH treatment.

Furthermore, in the present study, the inhibition of ROS production in the brain modulated NPY, CART and GSTα expression levels and modified AMPH-mediated appetite suppression. This result is in accordance with a previous finding indicating that central administration of ROS alone reduced feeding and increased the activity of POMC neurons (Diano et al., 2011). Previous studies reported that AMPH treatment induced DA oxidation, which produced metabolites in the brain, and that these were eliminated by increased activity of GST (Baez et al., 1997). Research also showed that GST conjugated GSH, the major antioxidant in the brain, with a variety of oxidized products to form non-toxic products (Sharma et al., 2004). Thus, an increase in GSTα during AMPH treatment may have neuroprotective effects against oxidative stress in the brain.

In addition, in the present study, the activities of SOD/GP and CART increased in AMPH-treated rats, and their expression levels were similar, with a maximum response observed on Day 2, pointing to a close relationship between SOD/GP and CART-containing neurons. This result was consistent with that of a previous report, which indicated that feeding rodents with a palatable-diet decreased the activities of anti-oxidants, including SOD, catalase, and GP, in the brain as compared with those of rodents fed a normal-diet. Previous studies also provided evidence that endogenous antioxidants, including SOD and GP, were involved in the control of NPY/CART-mediated feeding behavior induced by phenylpropanolamine, an AMPH-like anorectic drug (Kuo et al., 2011, 2012).

Taken together, current results revealed that the increases in SOD-1, SOD-2, GSTα and GP on Day 1 and Day 2 played functional roles in decreasing ROS production and regulating NPY/CART-mediated appetite suppression. In contrast, the return of SOD, GSTα and GP to normal levels from Day 2 to Day 4 indicated successful inhibition of oxidative stress on Day 2 and gradual restoration of NPY/CART-mediated appetite control. It is suggested that activations of ROS and increased expression of endogenous antioxidants during AMPH treatment may be involved in the regulation of NPY/CART-mediated appetite control.

In our previous reports, using single immunohistochemcal staining, we found that both decreased NPY expression and increased SOD expression in the hypothalamus participated in AMPH-mediated appetite suppression. In the present study, using double immunofluorescent staining, we further demonstrated that both JNK/CART and SOD/CART immunofluorescent activities (Fig. 7, yellow color) increased in hypothalamic ARC region in AMPH-treated rats. This result revealed that JNK, SOD, and CART were co-activated and involved in NPY/CART-mediated appetite suppression in AMPH-treated rats.

The present results revealed that i.c.v. administration of NAC, a ROS inhibitor, prior to AMPH treatment modified the hypothalamic expression of JNK, NPY, anti-oxidants, and CART (55–102). Previous evidence revealed that the effect of low-dose repeated methamphetamine exposure on the GSH-centered antioxidant system and oxidative stress response in rat brain were regional specific. Brain regions studied include thalamus, frontal cortex, striatum, cerebellum and hippocampus. In accordance with our previous reports, which revealed that pretreatment with another ROS scavenger (glutathione ethyl-ester) modulated hypothalamic GST and SOD expression (Kuo et al., 2011, and in turn altered NPY/CART expression and AMPH-induced appetite suppression.

Despite intensive efforts to develop JNK inhibitors, researchers have failed to identify a compound that inhibits JNK1 and JNK2 with selectivity against JNK3 (Siddiqui and Reddy, 2010; Gehringer et al., 2015). Therefore, it is reasonable to propose that the activated JNK isoform in the hypothalamus during AMPH treatment may be JNK3. In support of this idea, previous research demonstrated that (1) JNK1/2 promote feeding efficiency and adiposity in rodents, whereas JNK3 reduced food intake and adiposity (Solinas and Becattini, 2016), and (2) JNK1/2 were ubiquitously expressed, but JNK3 expression was restricted to the brain, testis, and pancreatic β-cells (Solinas, and Karin, 2010).

A recent report raised safety concerns about anti-obesity drugs and suggested that routine preclinical toxicology studies should precede all studies of anti-obesity drugs (dos Santos et al., 2013). The results of the present study provide preclinical toxicology evidence that hypothalamic anti-oxidants play a functional role in the anorectic effect of AMPH. Although the molecular mechanisms of AMPH appeared to be clear, AMPH may not prove to be an ideal anti-obesity drug due to its neurotoxic effects. The risks of AMPH are possibly related to the increase of ROS in injured tissues. The finding of the present result that inhibition of ROS production modulated the behavioral response of AMPH pointed to the possible modulation of the ROS-sensitive pathway under physiological conditions. The present findings may enhance understanding of anorectic or anti-obesity drugs and aid further development of such drugs.

In summary, the results of the present study suggested that both JNK/AP-1 signaling and endogenous antioxidants in the hypothalamus participated in the regulation of NPY/CART-mediated appetite control in AMPH-treated rats (Fig. 8).

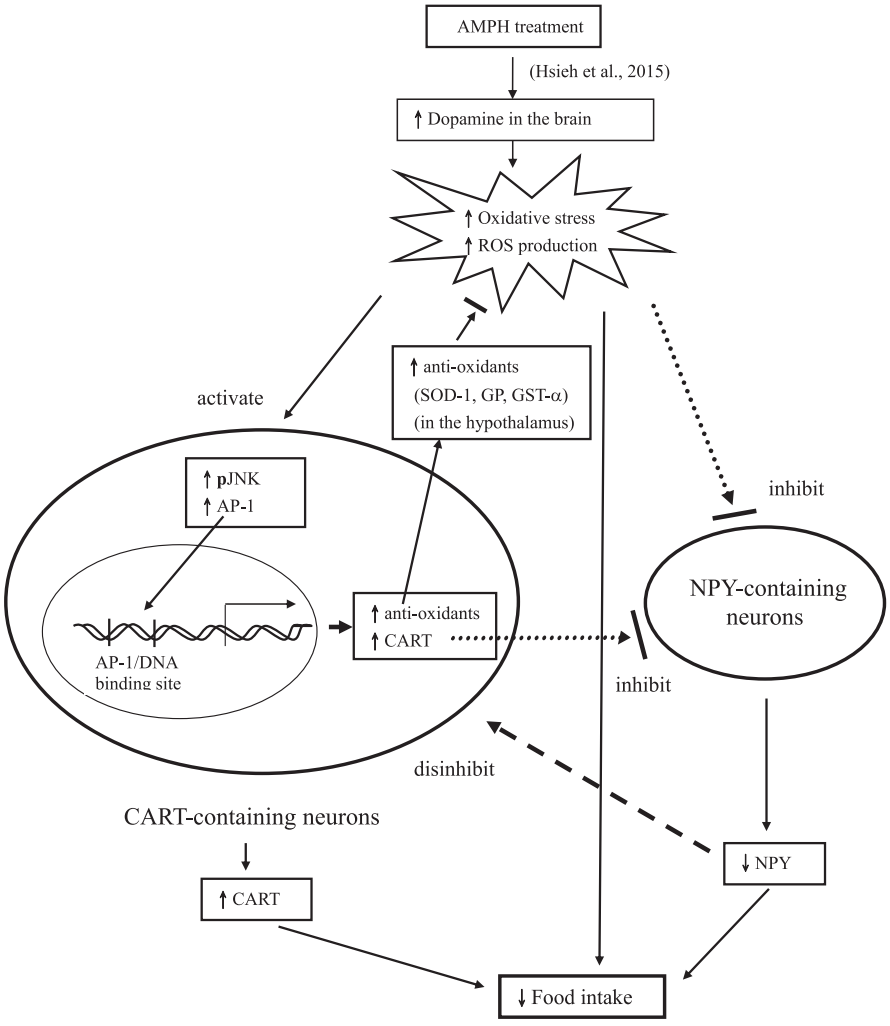


Fig. 8 Schematic diagram showing the putative mechanisms behind AMPH-induced decrease of food intake. AMPH treatment activates dopamine release in the brain, which may in turn increase the oxidative stress, activate hypothalamic CART expression, inhibit hypothalamic NPY expression, and finally decrease food intake. The activation of CART-containing neurons, which is partly due to the disinhibition of NPY-containing neurons, may result in the increased expression of pJNK, AP-1, CART, and anti-oxidants (including SOD-1, SOD-2, GP, and GSTα). Finally, the decrease of NPY and the increase of CART and ROS may contribute to the reduction of food intake. The *solid black arrow* indicates the activation, the *dotted arrows* indicate the inhibition, and the *dashed arrow* represents the disinhibition (or activation). The *upward and downward arrows* in the squares indicate the responses of increase and decrease.

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Contributors authors

Dr. DY Kuo designed the study. Authors SC Chu, YS Hsieh, PN Chen, and CH Yu undertook the analysis of the data. Author DY Kuo wrote the first draft. All authors contributed to and have approved the final manuscript.

Declaration of Competing Interest

There are no conflicts of interest for this manuscript.

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References

Alagarsamy, S., Phillips, M., Pappas, T., Johnson, K.M., 1997. Dopamine neurotoxicity in cortical neurons. Drug Alcohol Depend. 48, 105–111.

Andrews, Z.B., Liu, Z.W., Walllingford, N., Erion, D.M., Borok, E., Friedman, J.M., et al., 2008. UCP2 mediates ghrelin's action on NPY/AGRP neurons by lowering free radicals. Nature 454, 846–851.

Baez, S., Segura-Aguilar, J., Widersten, M., Johansson, A.S., Mannervik, B., 1997. Glutathione transferases catalyse the detoxication of oxidized metabolites (o-quinones) of catecholamines and may serve as an antioxidant system preventing degenerative cellular processes. Biochem. J. 324, 25–28.

Balkan, B., Keser, A., Gozen, O., Koylu, E.O., Dagci, T., Kuhar, M.J., Pogun, S., 2012. Forced swim stress elicits region-specific changes in CART expression in the stress axis and stress regulatory brain areas. Brain Res. 1432, 56–65.

Benzler, J.1., Ganjam, G.K., Legler, K., Stöhr, S., Krüger, M., Steger, J., Tups, A., 2013. Acute inhibition of central c-Jun N-terminal kinase restores hypothalamic insulin signalling and alleviates glucose intolerance in diabetic mice. J. Neuroendocrinol. 25, 446–454.

Björk, K., Saarikoski, S.T., Arlinde, C., Kovanen, L., Osei-Hyiaman, D., Ubaldi, M., et al., 2006. Glutathione-S-transferase expression in the brain: possible role in ethanol preference and longevity. FASEB J. 20, 1826–1835.

Chang, L., Karin, M., 2001. Mammalian MAP kinase signalling cascades. Nature 410, 37e40.

Chen, T.Y., Duh, S.L., Huang, C.C., Lin, T.B., Kuo, D.Y., 2001. Evidence for the involvement of dopamine D1 and D2 receptors in mediating the decrease of food intake during repeated treatment with amphetamine. J. Biomed. Sci. 8, 462–466.

Chen, P.N., Chu, S.C., Chiou, H.L., Kuo, W.H., Chiang, C.L., Hsieh, Y.S., 2006. Mulberry anthocyanins, cyanidin 3-rutinoside and cyaniding 3-glucoside, exhibited an inhibitory effect on the migration and invasion of a human lung cancer cell line. Cancer Lett. 235, 248–259.

Chu, S.C., Chen, P.N., Ho, Y.J., Yu, C.H., Hsieh, Y.S., Kuo, D.Y., 2015. Both neuropeptide Y knockdown and Y1 receptor inhibition modulate CART-mediated appetite control. Horm Behav. 67, 38–47.

Diano, S., Liu, Z.W., Jeong, J.K., Dietrich, M.O., Ruan, H.B., Kim, E., et al., 2011. Peroxisome proliferation-associated control of reactive oxygen species sets melanocortin tone and feeding in dietinduced obesity. Nat. Med. 17, 1121–1127.

dos Santos, V.V., Santos, D.B., Lach, G., Rodrigues, A.L., Farina, M., De Lima, T.C., Prediger, R.D., 2013. Neuropeptide Y (NPY) prevents depressive-like behavior, spatial memory deficits and oxidative stress following amyloid-β (Aβ(1-40)) administration in mice. Behav. Brain Res. 244, 107–115.

Ehrenbrink, G., Hakenhaar, F.S., Salomon, T.B., Petrucci, A.P., Sandri, M.R., Benfato, M.S., 2006. Antioxidant enzymes activities and protein damage in rat brain of both sexes. Exp. Gerontol. 41, 368–371.

Gehringer, M., Muth, F., Koch, P., Laufer, S.A., 2015. c-Jun N-terminal kinase inhibitors: a patent review (2010e2014). Exp. Opin. Ther. Patents 25, 849e872.

Hibi, M., Lin, A., Smeal, T., Minden, A., Karin, M., 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. 7, 2135e2148.

Horvath, T.L., Andrews, Z.B., Diano, S., 2009. Fuel utilization by hypothalamic neurons: roles for ROS. Trends Endocrinol. Metab. 20, 78–87.

Hsieh, Y.S., Yang, S.F., Chiou, H.L., Kuo, D.Y., 2006. Activations of c-FOS/c-JUN signaling are involved in the modulation of hypothalamic superoxide dismutase (SOD) and neuropeptide y (NPY) gene expression in amphetamine-mediated appetite suppression. Toxicol. Appl. Pharm. 212, 99–109.

Hsieh, Y.S., Yang, S.F., Chu, S.C., Kuo, D.Y., 2008. Interrupting activator protein-1 signaling in conscious rats can modify neuropeptide Y gene expression and feeding behavior of phenylpropanolamine. J. Neurochem. 104, 50–61.

Hsieh, Y.S., Chen, P.N., Yu, C.H., Kuo, D.Y., 2014. Central dopamine action modulates neuropeptide-controlled appetite via the hypothalamic PI3K/NFκB-dependent mechanism. Genes Brain Behav. 13, 784–793.

Hsieh, Y.S., Chen, P.N., Yu, C.H., Chen, C.H., Tsai, T.T., Kuo, D.Y., 2015. Involvement of oxidative stress in the regulation of NPY/CART-mediated appetite control in amphetamine-treated rats. Neuro Toxicol. 48, 131–141.

Jaillard, T., Roger, M., Galinier, A., Guillou, P., Benani, A., Leloup, C., et al., 2009. Hypothalamic reactive oxygen species are required for insulin-induced food intake inhibition: an NADPH oxidasedependent mechanism. Diabetes 58, 1544–1549.

Leloup, C., Magnan, C., Benani, A., Bonnet, E., Alquier, T., Offer, G., et al., 2006. Mitochondrial reactive oxygen species are required for hypothalamic glucose sensing. Diabetes 55, 2084–2090.

Ip, Y.T., Davis, R.J., 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)-from inflammation to development. Curr. Opin. Cell Biol. 10, 205–219.

Johnson, J.A., el Barbary, A., Kornguth, S.E., Brugge, J.F., Siegel, F.L., 1993. Glutathione S-transferase isoenzymes in rat brain neurons and glia. J. Neurosci. 13, 2013–2023.

Koppen, A.1., van Riel, A., de Vries, I., Meulenbelt, J., 2014. Recommendations for the paracetamol treatment nomogram and side effects of N-acetylcysteine. Neth. J. Med. 72, 251–257.

Kuo, D.Y., Yang, S.F., Chu, S.C., Chen, C.H., Hsieh, Y.S., 2009. Amphetamine-evoked changes of oxidative stress and neuropeptide Y gene expression in hypothalamus: regulation by the protein kinase C-delta signaling. Chem.-Biol. Int. 180, 193–201.

Kuo, D.Y., Chen, P.N., Yang, S.F., Chu, S.C., Chen, C.H., Kuo, M.S., Yu, C.H., Hsieh, Y.S., 2011. Role of reactive oxygen species-related enzymes in neuropeptide y and proopiomelanocortin-mediated appetite control: a study using atypical protein kinase c knockdown. Antioxid. Redox Signal. 15, 2147–2159.

Kuo, D.Y., Chen, P.N., Chu, S.C., Hsieh, Y.S., 2012. Knocking down the transcript of NF-KAPPAB modulates the reciprocal regulation of endogenous antioxidants and feeding behavior in phenylpropanolamine-treated rats. Arch. Toxicol. 86, 453–463.

McCubrey, J.A., Lahair, M.M., Franklin, R.A., 2006. Reactive oxygen species-induced activation of the MAP kinase signaling pathways. Antioxid. Redox Signal. 8 (9–10), 1775–1789.

Pinto, M., Moraes, C.T., 2014. Mitochondrial genome changes and neurodegenerative diseases. Biochim. Biophys. Acta 1842 (8), 1198–1207.

Rodriguez, J.J., Montaron, M.F., Aurousseau, C., Le Moal, M., Abrous, D.N., 1999. Effects of amphetamine and cocaine treatment on c-Fos, Jun-b, and Krox-24 expression in rats with intrastriatal dopaminergic grafts. Exp. Neurol. 159, 139–152.

Rosenfeld, M.A.1., Vasilyeva, A.D.1., Yurina, L.V.1., Bychkova, A.V.1, 2017. Oxidation of proteins: is it a programmed process? Free Radic. Res. 28, 1–25.

Shao, L., Cui, J., Young, L.T., Wang, J.F., 2008. The effect of mood stabilizer lithium on expression and activity of glutathione s-transferase isoenzymes. Neuroscience 151, 518–524.

Sharma, R., Yang, Y., Sharma, A., Awasthi, S., Awasthi, Y.C., 2004. Antioxidant role of glutathione S-transferases: protection against oxidant toxicity and regulation of stress-mediated apoptosis. Antioxid. Redox Signal. 6, 289–300.

Sheng, P., Wang, X.B., Ladenheim, B., Epstein, C., Cadet, J.L., 1996. AP-1 DNA-binding activation by methamphetamine involves oxidative stress. Synapse 24, 213–217.

Shyu, K.G.1., Wang, B.W., Yang, Y.H., Tsai, S.C., Lin, S., Lee, C.C., 2004. Amphetamine activates connexin43 gene expression in cultured neonatal rat cardiomyocytes through JNK and AP-1 pathway. Cardiovasc. Res. 63, 98–108.

Siddiqui, M.A., Reddy, P.A., 2010. Small molecule JNK (c-Jun N-terminal kinase) inhibitors. J. Med. Chem. 53, 3005e3012.

Solinas, G., Karin, M., 2010. JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction. FASEB J. 24, 2596e2611.

Solinas, G.1., Becattini, B.1, 2016. JNK at the crossroad of obesity, insulin resistance, and cell stress response. Mol. Metab. 6, 174–184.

Sun, S.Y., 2010. N-acetylcysteine, reactive oxygen species and beyond. Cancer Biol. Ther. 9, 109–110.

Thakur, P., Kumar, A., Kumar, A., 2017. Targeting oxidative stress through antioxidants in diabetes mellitus. J. Drug Target 29, 1–41.

Torres, M., Forman, H.J., 2003. Redox signaling and the MAP kinase pathways. Biofactors 17, 287–296.

Turner, N.A., Xia, F., Azhar, G., Zhang, X., Liu, L., Wei, J.Y., 1998. Oxidative stress induces DNA fragmentation and caspase activation via the c-Jun NH_2-terminal kinase pathway in H9c2 cardiac muscle cells. J. Mol. Cell Cardiol. 30, 1789–1801.

Yu, C.H., Hsieh, Y.S., Chen, P.N., Chen, J.R., Kuo, D.Y., 2018. Knockdown of the transcript of extracellular signal-regulated kinase in the brain modulated hypothalamic neuropeptide-mediated appetite control in amphetamine-treated rats. Br. J. Pharmacol. 175, 726–739.

Zafarullah, M., Li, W.Q., Sylvester, J., Ahmad, M., 2003. Molecular mechanisms of N-acetylcysteine actions. Cell Mol. Life Sci. 60, 6–20.