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期末報告

下視丘之CART(55-102)神經胜肽、活性氧族群和deltaFosB轉錄因子、及血液瘦身素和飢餓素在厭食劑作用之角色

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中 文 摘 要 ： 安非他命引發之厭食與DNA上Glucocorticoid response element受reactive oxygen species (ROS抑制有關

中文關鍵詞 ： 氧化壓力,糖皮質素反應元素,腦

英 文 摘 要 ： Amphetamine (AMPH)-induced appetite suppression is associated with changes in hypothalamic reactive oxygen species (ROS), antioxidants, neuropeptides, and plasma glucocorticoid. This study explored whether ROS and glucocorticoid response element (GRE), which is the promoter site of corticotropinreleasing hormone (CRH) gene, participated in neuropeptides-mediated appetite control. Rats were treated daily with AMPH for four days, and changes in food intake, plasma glucocorticoid and expression
levels of hypothalamic neuropeptide Y (NPY), proopiomelanocortin (POMC), superoxide dismutase (SOD), CRH, and glucocorticoid receptor (GR) were examined and compared. Results showed that food intake decreased and NPY gene down-regulated, while POMC, SOD, and CRH gene up-regulated during AMPH treatment. GR and GRE-DNA bindings were disrupted on Day 1 and Day 2 when glucocorticoid levels were still high. Pretreatment with GR inhibitor or ROS scavenger modulated mRNA levels in NPY,POMC, SOD and CRH in AMPH-treated rats. We suggest that disruptions of negative GRE (nGRE) on Day 1 and Day 2 are associated with an increase in oxidative stress during the regulation of NPY/POMCmediated
appetite control in AMPH-treated rats. These results advance the understanding of molecular mechanism in regulating AMPH-mediated appetite suppression

英文關鍵詞 ： Oxidative stress,Glucocorticoid response element,NPY, POMC, Brain

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Role of oxidative stress in disrupting the function of negative glucocorticoid response element in daily amphetamine-treated rats

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ABSTRACT

Amphetamine (AMPH)-induced appetite suppression is associated with changes in hypothalamic reactive oxygen species (ROS), antioxidants, neuropeptides, and plasma glucocorticoid. This study explored whether ROS and glucocorticoid response element (GRE), which is the promoter site of corticotropin-releasing hormone (CRH) gene, participated in neuropeptides-mediated appetite control. Rats were treated daily with AMPH for four days, and changes in food intake, plasma glucocorticoid and expression levels of hypothalamic neuropeptide Y (NPY), proopiomelanocortin (POMC), superoxide dismutase (SOD), CRH, and glucocorticoid receptor (GR) were examined and compared. Results showed that food intake decreased and NPY gene down-regulated, while POMC, SOD, and CRH gene up-regulated during AMPH treatment. GR and GRE-DNA bindings were disrupted on Day 1 and Day 2 when glucocorticoid levels were still high. Pretreatment with GR inhibitor or ROS scavenger modulated mRNA levels in NPY, POMC, SOD and CRH in AMPH-treated rats. We suggest that disruptions of negative GRE (nGRE) on Day 1 and Day 2 are associated with an increase in oxidative stress during the regulation of NPY/POMC-mediated appetite control in AMPH-treated rats. These results advance the understanding of molecular mechanism in regulating AMPH-mediated appetite suppression.

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1. Introduction

The hypothalamus is the major site to integrate appetite-controlled neurotransmitter and peripheral hormone in the control of energy metabolism. Several hunger and satiety mediators in the hypothalamus regulate appetite by controlling the activities of orexigenic neuropeptide Y (NPY)- and anorexigenic proopiomelanocortin (POMC)-containing neurons. Moreover, peripheral hormones, such as insulin, ghrelin and leptin, primarily bind and activate their cognate receptors directly in the hypothalamic arcuate nucleus or in the dorsal vagal complex in the medulla which communicates with the hypothalamus (Kim et al., 2014).

Amphetamine (AMPH) is a well-known appetite suppressant (Fleckenstein et al., 2007; Kuo et al., 2012; Chu et al., 2014); it acts by the central release of dopamine, which in turn down regulates NPY gene as well as up regulates POMC and melanocortin receptor 4 (MC4R) gene in the hypothalamus (Gillard et al., 1993; Chen et al., 2001; Hsieh et al., 2013, 2014). MC4R is a member of POMC system. AMPH can induce auto-oxidation of cytosolic dopamine and thus cause oxidative damage of dopamine terminals (Cadet et al., 2007), which is associated with increases in reactive oxygen species (ROS) (Kuo et al., 2011, 2015) and anti-oxidative enzyme, such as superoxide dismutase (SOD) and glutathione peroxidase in the hypothalamus (Frey et al., 2006; Tata and Yamamoto, 2007; Hsieh et al., 2015). Thus, AMPH can be regarded as a stressor (Schaefer et al., 2010; Grace et al., 2012). Moreover, AMPH can activate the hypothalamic–pituitary–adrenal (HPA) axis to increase plasma glucocorticoids (Schaefer et al., 2010), which may exert feedback inhibition on hypothalamus to decrease CRH release (Ostrander et al., 2006; Papadimitriou and Priftis, 2009).

The sequence of events leading to HPA activation by additive drugs appears to start within the brain, suggesting that central activation is not due to peripheral stimulation (Armario, 2010). Glucocorticoids released during stress have a wide impact on the brain through binding with glucocorticoid receptors (GR), a cytoplasmic protein (de Kloet et al., 2008). Glucocorticoid/GR complex can move to the nucleus and then interact with the glucocorticoid response element (GRE) located at the promoter site of the

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corticotrophin-releasing hormone (CRH) gene (Stahn et al., 2007; Lee et al., 2013). Previous evidence revealed that, in acute stress induced by additive drugs, an increase in CRH provides feedback inhibition by a mechanism of negative GRE (nGRE)-mediated control to inhibit CRH gene expression (Kyrou et al., 2006). However, in chronic stress and impaired GR conditions, both have been proposed to lead to the dysregulation of HPA axis activity (Simms et al., 2012).

In regard to the appetite control, acute stress exerts an anorexigenic effect through the stimulation of POMC neurons by increasing CRH gene expression and decreasing NPY secretion (Chrousos, 2000; Cadet et al., 2014). By contrast, chronic stress is associated with chronic activation of the HPA axis and prolonged glucocorticoid secretion, which exert orexigenic effects caused by the inhibition of CRH and the stimulation of NPY expression (Chrousos, 2000; Kyrou et al., 2006; Sobrino Crespo et al., 2014). It is unclear whether the chronically elevated levels of glucocorticoids during daily AMPH treatment can modulate nGRE-involved and NPY/POMC-mediated appetite control. We hypothesize that prolonged glucocorticoid secretion during daily AMPH treatment might disrupt nGRE-involved feedback inhibition due to an increase of oxidative stress in the hypothalamus in AMPH-treated rats.

2. Materials and methods

2.1. Animals treatments

Male Wistar rats weighing 200–300 g were obtained from the National Laboratory Animal Center (Taipei, Taiwan), which is certificated by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The animals were individually housed in cages, were maintained at a temperature of 22 ± 2 °C in a room with a 12-h light-dark cycle (the light was turned on at 6:00 a.m. and turned off at 6:00 p.m.), and were habituated to frequent handling. Drugs were administered and food intake was determined daily at the beginning of the dark phase. Water and chow (LabDiet) were freely available to the rats throughout the experiment. Daily food intake amounted above 40 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. This study was approved by the Chung-Shan Medical University Experimental Animal Center (permit number 1489). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Experimental procedures

2.2.1. Protocol 1: effects of AMPH on food intake and hypothalamic gene expression

To examine the effect of AMPH (*d*-amphetamine) on feeding behavior, rats (n = 8 for each group) were injected intraperitoneally (i.p.) with the AMPH at a dose of 1, 2 or 4 mg/kg daily for four days. AMPH was first injected at the end of Day 0 (i.e., at the beginning of Day 1), and the amounts of daily food intake were calculated by accounting the difference of food amount between the previous day and the present day.

To determine the effect of daily AMPH (2 mg/kg, i.p.) on changes in hypothalamic NPY, POMC, MC4R, CRH, and SOD-1 gene expression, the rats were injected with the drug once a day for 1, 2, 3, or 4 days, depending on the rat group. On the sacrifice day, rats received a treatment of 2 mg/kg AMPH 40–50 min before being sacrificed to enhance the effects of the drug. Previous evidence reveals significant effects of AMPH on both central dopamine-associated locomotion (Kuczenski and Segal, 1989) and reductions in hypothalamic NPY expression (Kuo et al., 2001) are seen 40–50 min following treatment. The rats were anesthetized with 35–40 mg/kg pentobarbital and were then decapitated. Following decapitation, the hypothalamus was removed to determine the expression of protein or the levels of mRNA.

2.2.2. Protocol 2: effect of GR antagonist pretreatment on AMPH-induced responses

To investigate the effect of pretreatment with GR antagonist on AMPH-induced anorexia and hypothalamic NPY, POMC, CRH and SOD-1 mRNA levels, rats were treated daily with RU-486 (20 mg/kg; i.p.) 60 min before AMPH (4 mg/kg; i.p.) treatment. RU-486 (Mifepristone) is a highly selective GR antagonists (Simms et al., 2012), which can decrease AMPH-induced behavioral response via HPA stress axis (Aynara et al., 2010; Dustin et al., 2011) and may have a role in the treatment of a number of neuropsychiatric disorders (Peter and Allan, 2006). RU486 was suspended in 1% Tween-80 and 25% *b*-cyclodextrin in saline and stirred for 2 h before systemic injections (i.p.). For a desired concentration, RU486 was first dissolved in DMSO and then diluted with phosphate-buffered saline (PBS).

2.2.3. Protocol 3: effects of AMPH on GR expression and GR-GRE binding activity

To examine the effect of AMPH on GR expression and GR-GRE binding activity, rats were given AMPH (2 mg/kg; i.p.; N = 6 per group) daily for four consecutive days. At 40–50 min after daily AMPH treatment, the rats were sacrificed and the hypothalamus was removed to determine the GR expression and GRE/DNA binding activity by an electromobility shift assay (EMSA).

2.2.4. Protocol 4: effect of ROS scavenger pretreatment on AMPH-induced responses

To examine the effect of pretreatment with ROS scavenger on food intake and hypothalamic NPY, POMC, CRH, and SOD-1 expression in AMPH-treated rats, rats (n = 6–8 for each group) were infused daily with glutathione ethyl-ester (GSH-EE) (20 μl in concentration of 1 μmole/l; i.c.v.; the infusion rate was 4 μl/min) 40 min before AMPH treatment (4 mg/kg; i.p.) for 4 days. GSH-EE is a potent ROS scavenger that is particularly effective in restoring the mitochondrial glutathione redox state to a reduced state (GSH) (Benani et al., 2007). At 40–50 min after AMPH treatment, rats were anesthetized and the hypothalamus of each rat was removed from the brain and its mRNA levels were determined by qPCR. GSH-EE was dissolved in artificial corticospinal fluid (aCSF).

2.2.5. Protocol 5: to compare the difference of feeding in a 24-h AMPH treatment

To compare the differences in feeding behavior induced by a single treatment of AMPH (4 mg/kg; i.p.; N = 8 per group) between normal and AMPH-treated rats, the amounts of food intake were measured every 6 h over a 24-h period after drug treatment. There were four separate period of time to be recorded, i.e. 0–6 h, 6–12 h, 12–18 h, and 18–24 h, in the measure of 24-h feeding behavior.

2.2.6. Protocol 6: the effect of drug treatment on plasma corticosterone concentrations

To investigate the change of plasma corticosterone concentrations during daily AMPH (2 mg/kg, i.p.) injection, rats' blood samples were collected from Day 1 to Day 4 at 40–50 min after AMPH treatment. Tested groups included (1) AMPH-treated group, (2) GR inhibitor/AMPH co-administration group, and (3) ROS scavenger/AMPH co-administration group.

2.3. 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) (Paxinos and Watson, 1986). A 23-g stainless steel guide cannula was implanted and secured to the skull using stainless steel screws and dental cement. The 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. Behavioral testing began 1 week after the surgery. For all experiments, the cannula placement was verified by histochemistry or by the administration of angiotensin II (100 ng/rat). Angiotensin II reliably induced water drinking in non-deprived rats when administered into the ventricles (Ritter et al., 1981). Only data from rats that drank more than 10 ml of water in 30 min were included in this study.

2.4. Cerebral infusion of ROS scavenger

The rats (n = 6–8 for each group) were infused daily with GSH-EE (20 μl in concentration of 1 μmole/l; i.c.v.; the infusion rate was 4 μl/min) 40 min before AMPH treatment (2 mg/kg; i.p.) for 4 days. GSH-EE is a potent ROS scavenger that is particularly effective in restoring the mitochondrial glutathione redox state to a reduced state (GSH) (Benani et al., 2007). GSH-EE 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.

2.5. RNA extraction

Hypothalamic NPY, POMC, CRH, SOD-1, and GR mRNA were extracted in a block of mediobasal hypothalamic tissue as described previously (Magni and Barnea, 1992). In brief, total RNA was isolated from this block using the modified guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Each hypothalamic block was homogenized in 1 ml of TRIZOL reagent (Life Technologies, Inc., Grand Island, USA) using an Ultrasonic Processor (Vibra Cell, Model CV17; Sonics & Materials Inc., Danbury, Connecticut, USA). After an incubation at 22 °C for 5 min, 0.2 ml of chloroform was added to each sample, shaken vigorously for 15 s, incubated at 22 °C for 3 min, and then centrifuged at 12,000g for 15 min at 4 °C. After removal of aqueous phase and precipitation with 0.5 ml isopropanol, samples were incubated at 22 °C for 10 min and centrifuged at 12,000g for 15 min at 4 °C. The gel-like RNA pellets were washed with 75% ethanol by vortexing and centrifugation at 7500g 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, Japan). RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A260/A280 between 1.9 and 2.1. In the present study, the A260/A280 ratio of our RNA sample was between 1.9 and 2.1.

2.6. Quantitative real-time polymerase chain reaction (qPCR)

The total RNA was isolated from the hypothalamus using Trizol (Life Technologies, Grand Island, NY, USA) as described above. The qPCR analysis was performed using a Taqman one-step PCR Master Mix (Applied Biosystems, CA, USA). In briefly, 100 ng of cDNA was added per 25 μl reactions with each NPY, POMC, GR, CRH, SOD-1, or GAPDH primer and TaqMan probes. The qPCR assays were performed in triplicate on a StepOnePlus sequence detection system. The oligonucleotide sequences of TaqMan probes and primers were used according to the manufacturer's instructions. The threshold was set above the non-template control background and within the linear phase of the target gene amplification to determine the cycle number at which the transcript was detected.

2.7. Western blotting

The extracted hypothalamus tissues were subjected to electrophoresis. Hypothalamic proteins were separated on a 12.5% polyacrylamide gel, transferred onto a nitrocellulose membrane, and incubated with specific antibodies against NPY, MC4R, GR, 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).

2.8. GR and GRE-DNA binding assay

Binding of GR and GRE-DNA in nuclear extracts was assessed by EMSA with double-stranded deoxyoligonucleotides carrying regulatory elements of GRE sequence (5′-GATCA GAACA CAGT GTTCTCTA-3′) were used, which was labeled on the 3′ end with biotin (Roy et al., 2002). 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 × 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).

2.9. Blood collection and enzyme-linked immunosorbent assay (ELISA)

To investigate the change of plasma corticosterone concentrations during daily AMPH injection, rats' blood samples were collected from Day 1 to Day 4 at 40–50 min after AMPH treatment. Corticosterone is a major type of glucocorticoid in rodent animals. Blood were centrifuged at 3000 rpm for 15 min. Plasma was removed and frozen at −20 °C for subsequent biochemical determinations. Plasma samples were analyzed by using ELISA kits to determine corticosterone concentration. Plasma corticosterone concentrations were analyzed using the indirect immunoperoxidase method as described previously (Sternberger and Sternberger, 1986) on a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at the wavelength of 405 nm.

2.10. Drugs, chemicals, and reagents

Chow (LabDiet) was purchased from PMI Nutrition International (Brentwood, MO, USA). d-AMPH sodium salt, RU-486 (Mifepristone), angiotensin II, pentobarbital sodium salt, GSH-EE and Tris–HCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Corticosterone ELISA kit was from Enzo Life Sciences (Farmingdale, NY, USA). Antibodies against NPY and β-actin were purchased from

Santa Cruz Biotechnology (Santa Cruz, CA, USA). The qPCR analysis was performed using a TaqMan one-step PCR Master Mix (Applied Biosystems, USA). The TRIZOL reagent, which was purchased from Life Technologies, Inc. (Grand Island, NY, USA), was used for tissue homogenization. The drugs *d*-AMPH and pentobarbital were dissolved in water, and angiotensin II and GSH-EE were dissolved in aCSF.

2.11. 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

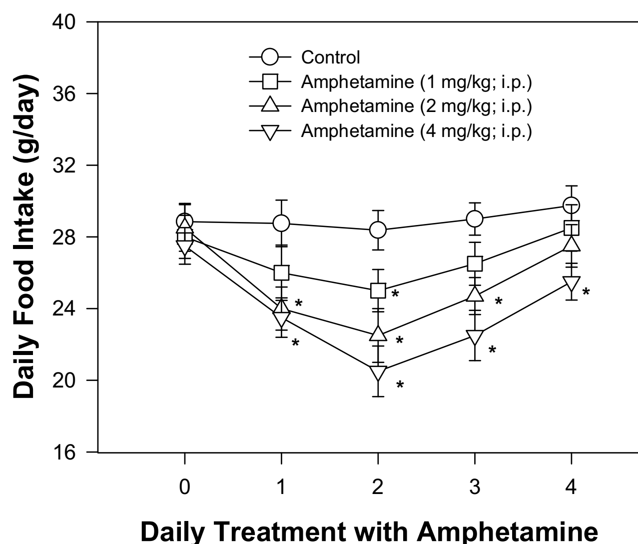
3.1. Effects of AMPH on feeding behavior

Changes of feeding behavior in rats receiving AMPH (1, 2, 4 mg/kg; i.p.) treatment were shown in the 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 significant dose-dependent [$F(3,28) = 11.25$, $p < 0.05$] and time-dependent effects [$F(4,35) = 7.21$, $p < 0.05$]. 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 rats treated with 2 mg/kg AMPH could develop a tolerance to AMPH on Day 4. However, with a dose of 4 mg/kg AMPH, it produced a continuous anorectic response during AMPH treatment. The effect of AMPH treatment on the changes of body weight revealed that changes occurred in a manner consistent with the alteration of daily food intake during AMPH treatment (Hsieh et al., 2015).

Therefore, AMPH at dose of 2 mg/kg was employed for (1) the measure of mRNA/protein levels in AMPH administration and (2) behavioral study in GR inhibitor/AMPH co-administration, because there was a restoration of food intake back to normal (control) level on Day 4. However, AMPH at dose of 4 mg/kg was used for the behavioral study in ROS inhibitor/AMPH co-administration, because AMPH (4 mg/kg) could exert a greater and continuous effect on anorexia from Day 1 to Day 4 and was thus more suitable than AMPH (2 mg/kg) for the examination of blocking effect of ROS inhibitor.

3.2. Effects of GR inhibitor on AMPH-induced anorexia

Results shown in the lower panel of Fig. 1 indicated that pretreatment with GR inhibitor before AMPH (2 mg/kg/day, i.p.) modified anorectic response, indicating the involvement of GR in regulating AMPH-induced anorexia. Using statistical analysis with two-way ANOVA followed by Dunnett's test ($p < 0.05$) to measure the effect of AMPH, results revealed significant treatment-dependent [$F(3,28) = 8.15$, $p < 0.05$] and time-dependent effects [$F(4,35) = 7.22$, $p < 0.05$]. GR inhibitor enhanced anorectic response on Day 3 and Day 4 compared to that in AMPH-treated rats. No significance was obtained in saline-treated rats receiving GR inhibitor alone or not, indicating the noninterference of GR inhibitor alone on food intake.



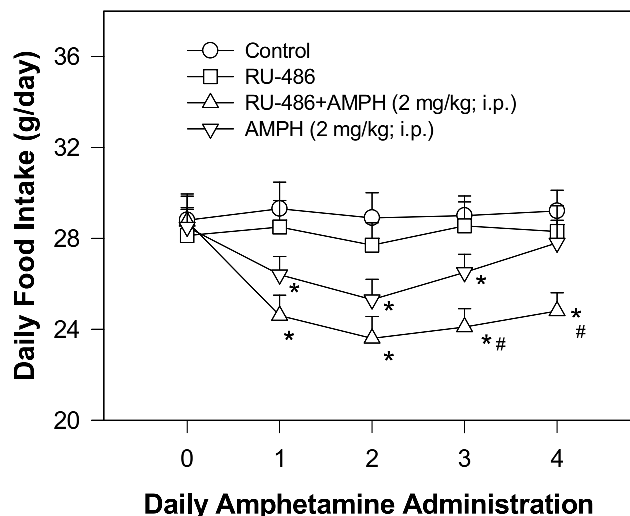
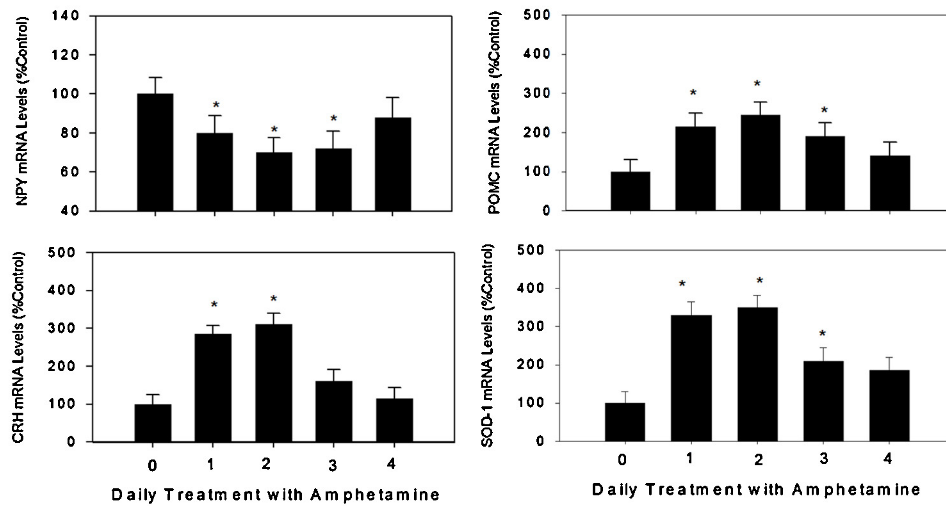


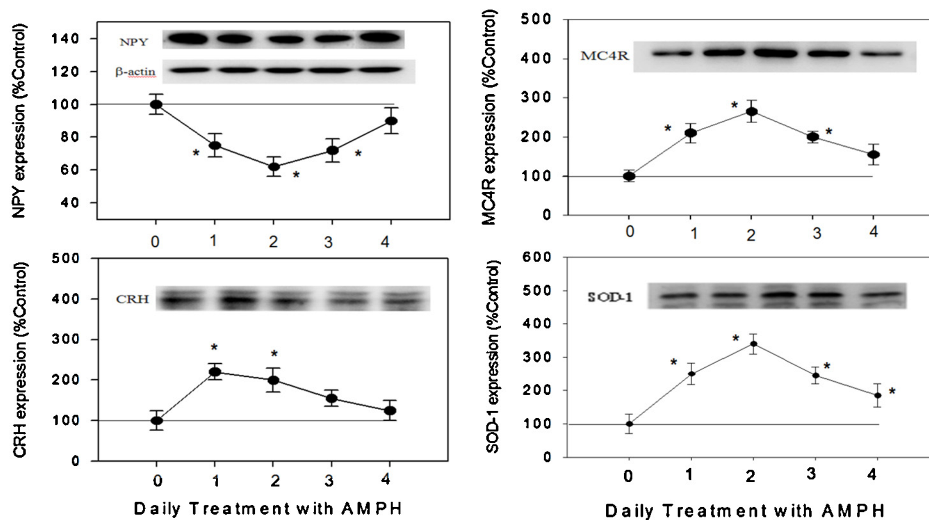
Fig. 1. Upper panel: the effect of daily AMPH treatment on food intake over a 4-day period. The first injection of AMPH (1, 2 and 4 mg/kg; i.p.) was conducted at the end of Day 0. Lower panel: the effect of RU-43044 pretreatment on AMPH-induced food intake. The RU-486 could enhance the decrease of AMPH-induced anorexia. Each point represents the mean ± SEM of 8 rats. Bars were mean ± SEM. $N = 8$ each group. *$P < 0.05$ vs. control. #$P < 0.05$ vs. AMPH-treated groups of each treatment day.

3.3. Effects of AMPH on NPY, POMC, CRH, and SOD-1 gene expression

Results shown in the upper panel of Fig. 2 revealed that daily AMPH injections decreased NPY mRNA levels but increased POMC, CRH and SOD-1 mRNA levels. Using GAPDH as the internal standard, the ratio of each mRNA over GAPDH mRNA was calculated and compared. Analysis with one-way ANOVA revealed a significant decreases in NPY mRNA levels [$F(4,25) = 8.87$, $p < 0.05$] compared to the controls. Significant increases were observed in POMC mRNA levels [$F(4,25) = 10.21$, $p < 0.05$], CRH mRNA levels [$F(4,25) = 9.64$, $p < 0.05$] and SOD-1 mRNA levels [$F(4,25) = 11.68$, $p < 0.05$] compared to the controls.

Results shown in the middle panel of Fig. 2 were similar to that in the upper panel of Fig. 2, indicating the changes in protein expression were similar to that in mRNA expression. It revealed that daily AMPH decrease NPY but increased MC4R, CRH and SOD-





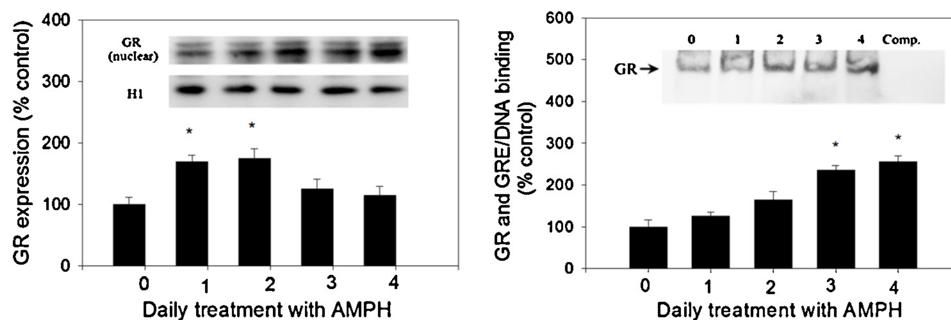


Fig. 2. Effects of daily AMPH on hypothalamic mRNA levels (upper panel), on hypothalamic protein expression (middle panel), and on GR and GRE-DNA binding ability (lower panel). Upper panel: results showed relative densitometric values of mRNA expression levels using the analysis of qPCR. Middle panel: results showed relative densitometric values using Western Blot in AMPH (2 mg/kg; i.p.)-treated groups. Lower panel: (left side) results showed the values using Western blots in GR expression in AMPH-treated rats. Right side: results showed the values using EMSA assay. Contents of GR and GRE-DNA binding ability were indicated as the percentage of the control group. Lane 6 represented nuclear extracts incubated with unlabeled oligonucleotide (competitive control) to confirm the specificity of binding. Bars are the means ± SEM. N = 6–8 each group. $^{*}p < 0.05$ vs. control. Comp: competitive control. GR: glucocorticoid receptor.

1 expression. Using β-actin as the internal standard, the ratio of NPY, MC4R, CRH, and SOD-1 over β-actin in each group was calculated and compared. Analysis with one-way ANOVA revealed a significant decreases in NPY expression [$F(4,25) = 9.16$, $p < 0.05$] compared to the controls. Significant increases were observed in MC4R [$F(4,25) = 11.23$, $p < 0.05$], CRH [$F(4,25) = 10.66$, $p < 0.05$], and SOD-1 [$F(4,25) = 9.98$, $p < 0.05$] compared to the controls.

3.4. Effects of AMPH on GR expression and on GR-GRE binding activity

Results shown in the lower panel of Fig. 2 revealed that daily AMPH treatment increased GR expression during AMPH treatment. Using histone 1 as the internal standard, the ratio of GR over in each group was calculated and compared. Analysis with one-way ANOVA revealed increases in GR [$F(4,25) = 4.56$] on Day1 and Day 2 compared to the control. Results shown in the lower panel of Fig. 2 revealed that daily AMPH could increase hypothalamic GR-GRE binding activity. Analysis with one-way ANOVA revealed that GR and GRE/DNA binding activity was increased on Day 3 and Day 4 [$F(4,25) = 5.22$, $P < 0.05$], which was not consistent with the pattern in GR expression during AMPH treatment. This result revealed that GR and GRE/DNA binding might be disrupted on Day 1 and Day 2 during AMPH-treatment.

3.5. Effects of pretreatment with GR inhibitor on hypothalamic mRNA levels

Results shown in Fig. 3 revealed that pretreatment with GR inhibitor enhanced the decrease in NPY mRNA levels and the increases in POMC, CRH, and SOD-1 mRNA levels compared to the control groups. Using statistical analysis with two-way ANOVA followed by Dunnett's test ($p < 0.05$) to measure the effect of AMPH, results revealed significant treatment-dependent [$F(3,28) = 10.25$, $p < 0.05$] and time-dependent effects [$F(4,35) = 9.56$, $p < 0.05$]. GR inhibitor enhanced the decrease in NPY mRNA levels on Day 3 and Day 4 if compared between GR/AMPH- and AMPH-treated group. By contrast, GR inhibitor enhanced the increases in POMC mRNA levels, CRH mRNA levels, and SOD-1 mRNA levels on Day 3 and Day 4 if compared between GR/AMPH- and AMPH-treated group.

3.6. The effect of the pretreatment with GSH-EE on AMPH-induced food intake

Results shown in the upper panel of Fig. 4 reveal that pretreatment with GSH-EE before daily AMPH (4 mg/kg) could attenuate an AMPH-induced anorectic response. Statistical analysis with two-way ANOVA revealed significant treatment-dependent [$F(3,28) = 15.23$, $p < 0.05$] and time-dependent effects [$F(4,35) = 8.79$, $p < 0.05$]. Moreover, it revealed that co-administration with GSH-EE/AMPH attenuated the decreases of food intake from Day1 to Day 4 compared to the AMPH-treated group. The food intake shown in vehicle-treated rats was similar to that in saline-treated rats. Moreover, the expression of feeding in GSH-EE-treated rats remained unchanged compared to that in vehicle-treated rats, revealing the noninterference of vehicle in this study.

3.7. The effect of pretreatment with GSH-EE on hypothalamic mRNA levels

Results shown in the lower panel of Fig. 4 indicate the statistical result of qPCR. By one-way ANOVA, results revealed that NPY mRNA level decreased in AMPH-treated rats and GSH-EE/AMPH-treated rats, but showed no change in GSH-EE-treated rats compared to the control group [$F(6,35) = 5.25$, $p < 0.05$]. By contrast, POMC mRNA level increased in AMPH-treated group and GSH-EE/AMPH-treated groups, but showed no change in GSH-EE-treated group compared to the control group [$F(6,35) = 6.65$, $p < 0.05$]. Similarly, CRH mRNA level increased in AMPH-treated rats and GSH-EE/AMPH-treated rats, but showed no change in GSH-EE-treated rats compared to the control group [$F(6,35) = 5.11$, $p < 0.05$]. SOD-1 mRNA level increased in AMPH-treated rats and GSH-EE/AMPH-treated rats, but showed no change in GSH-EE-treated rats compared to the control group [$F(6,35) = 6.32$, $p < 0.05$]. Taken together, results revealed that a pretreatment with GSH-EE in daily AMPH-treated rats resulted in partial restoration of NPY, POMC, CRH, and SOD-1 mRNA levels back to normal compared to the AMPH-treated group. These results revealed that GSH-EE pretreatment could modify the AMPH-induced changes in NPY, POMC, CRH and SOD-1 mRNA levels, indicating the involvement of ROS (or SOD-1) in regulating NPY/POMC-mediated appetite control in AMPH-treated rats.

3.8. Effects of daily AMPH on plasma concentration of corticosterone

Results shown in Table 1 revealed that plasma corticosterone increased during AMPH administration, GR/AMPH co-administration, or ROS scavenger/AMPH co-administration. Analysis with one-way ANOVA revealed a significant increase in AMPH administration group [$F(4,35) = 9.61$, $p < 0.05$], GR/AMPH co-administration group [$F(4,35) = 11.56$, $p < 0.05$] or ROS scavenger/AMPH co-administration group [$F(4,35) = 10.88$, $p < 0.05$] from Day 1 to Day 4 compared to control group. This result indicated the increases of plasma corticosterone in AMPH-treated, GR/AMPH-cotreated, or ROS scavenger/AMPH-cotreated rats compared to the controls.

3.9. Time courses for the change of food intake after a single injection of AMPH

Results shown in Supplementary Fig. 1 reveal that: (1) basic patterns of feeding response are that they eat more in the dark period (0–12 h) but less in the light period after a single AMPH (4 mg/kg; i.p.) treatment; and (2) a single AMPH treatment leads to a decrease of food intake only at the first 0–6 h time interval compared to normal rats (t-test, $P < 0.05$). There are no changes during the other time intervals (6–12, 12–18, and 18–24) after drug treatment in both rats. These results revealed that the AMPH-induced anorexia was mainly occurred at the initial 6-h period compared to the normal rats.

4. Discussion

Our results showed that (1) NPY decreased but POMC, CRH, SOD, and GR increased during AMPH treatment, implying the involvement of SOD and GR in the control of NPY/POMC-mediated appetite suppression; (2) the effect of GR inhibition (using RU-43044) enhanced the decrease in NPY and the increases in POMC, CRH, and SOD, indicating the involvement of GR signaling; (3) the reduction of oxidative stress (using an ROS scavenger) attenuated the decrease in NPY and the increases in POMC, SOD, and CRH, revealing the involvement of oxidative stress in regulating NPY/POMC-mediated appetite control; and (4) despite the chronic elevation of plasma glucocorticoid from Day 1 to Day 4, CRH levels significantly increased but GR-GRE binding slightly increased on Day 1 and Day 2. By contrast, on Day 3 and Day 4, GR-GRE binding significantly increased but CRH levels gradually return to normal, revealing a disruption of nGRE-mediated feedback inhibition on Day 1 and Day 2. Taken together, the results suggest that the increases in oxidative stress and CRH on Day 1 and Day 2 are

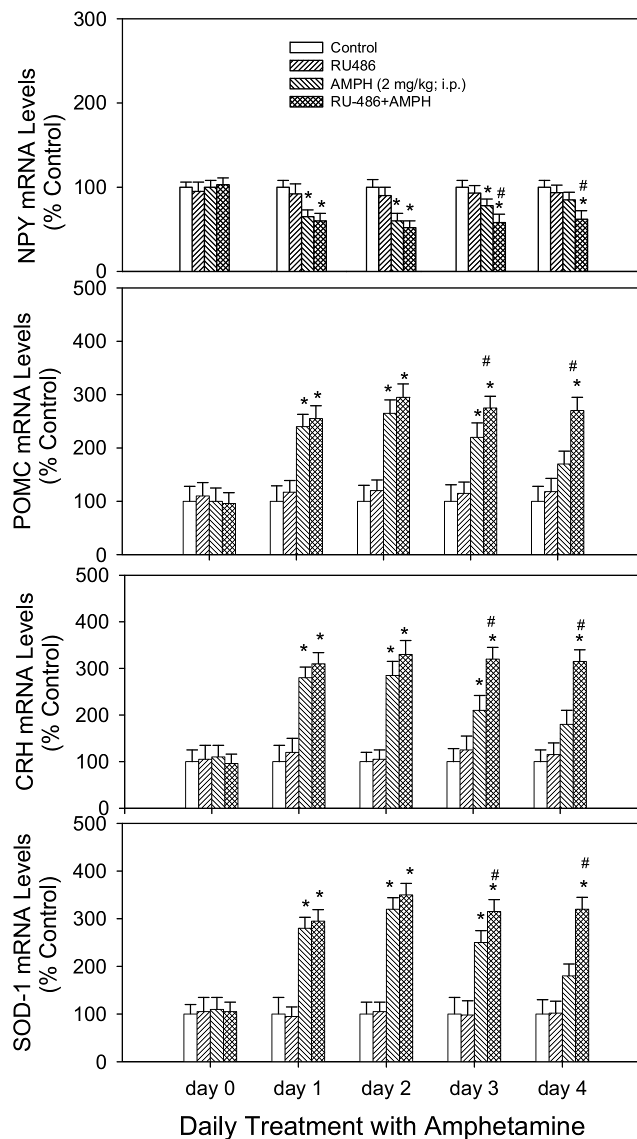


Fig. 3. The effect of pretreatment with RU-486 on hypothalamic NPY, POMC, SOD-1, and CRH mRNA levels in AMPH (2 mg/kg)-treated rats. The results showed relative densitometric values of hypothalamic mRNA levels using the analysis of qPCR. Contents of mRNA levels were indicated as the percentage of the vehicle-treated (control) group. Bars are the means ± SEM. $N = 6$ each group. *$p < 0.05$ vs. control. #$P < 0.05$ vs. AMPH-treated groups. RU-486 was dissolved in vehicle as described in the Section 2.

Table 1
Effects of pretreatment with GR inhibitor or ROS scavenger prior to daily AMPH on plasma corticosterone concentrations over 4-day period.

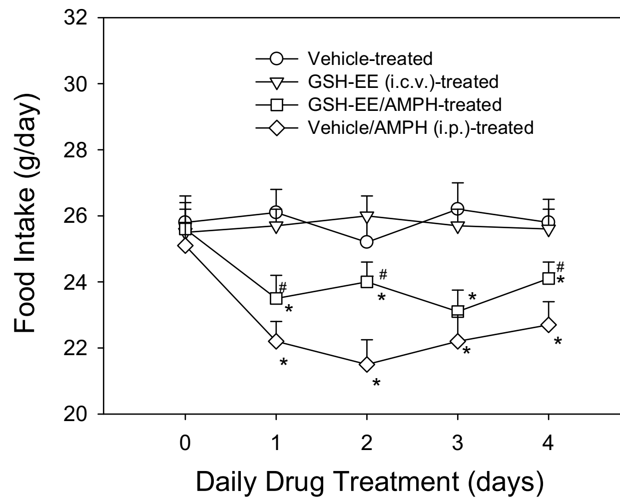
	Plasma corticosterone concentration (ng/ml)				
	Day 0 (control)	Day 1	Day 2	Day 3	Day 4
AMPH-treated group	62.93 ± 10.86	163.91 ± 14.65[a]	183.31 ± 27.76[a]	149.87 ± 23.19[a]	198.89 ± 36.08[a]
GR inhibitor/AMPH co-administration group	71.78 ± 11.88	170.72 ± 11.67[a]	193.52 ± 17.53[a]	168.75 ± 18.16[a]	199.54 ± 22.56[a]
ROS scavenger/AMPH co-administration group	60.22 ± 10.86	156.61 ± 12.35[a]	172.31 ± 17.65[a]	150.65 ± 13.37[a]	177.66 ± 16.85[a]

Plasma corticosterone levels were determined by ELISA methods from Day 0 to Day 4 during repeated amphetamine treatment and were expressed as mean ± SEM of eight rats for each group.

responsible for the disruption of nGRE-mediated feedback inhibition in the regulation of NPY/POMC-mediated appetite control in AMPH-treated rats.

Our results showed that the response of daily food intake during AMPH treatment was expressed in a pattern similar to that of NPY, with a maximum reduction on Day 2; however, it was expressed in a pattern reciprocal to that of POMC, with the greatest increase on Day 2. These results confirmed that NPY and POMC may function antagonistically in the regulation of appetite suppression in rats treated with AMPH as described in our previous reports (Hsieh et al., 2013, 2014). Moreover, our results also confirmed that the NPY/POMC pathway participated in the regulation of AMPH anorexia (on Day 1 and Day 2) and the development of AMPH tolerance (from Day 2 to Day 4) (Kuo et al., 2009). In the present study, the up-regulation of GR, CRH, and SOD-1 was concomitant with the up-regulation of POMC but against the down-regulation of NPY during AMPH treatment, implying that GR, CRH, and oxidative stress

(A) The effect of pretreatment with ROS scavenger on AMPH-induced anorexia



(B) The effect of pretreatment with ROS scavenger on hypothalamic mRNA levels

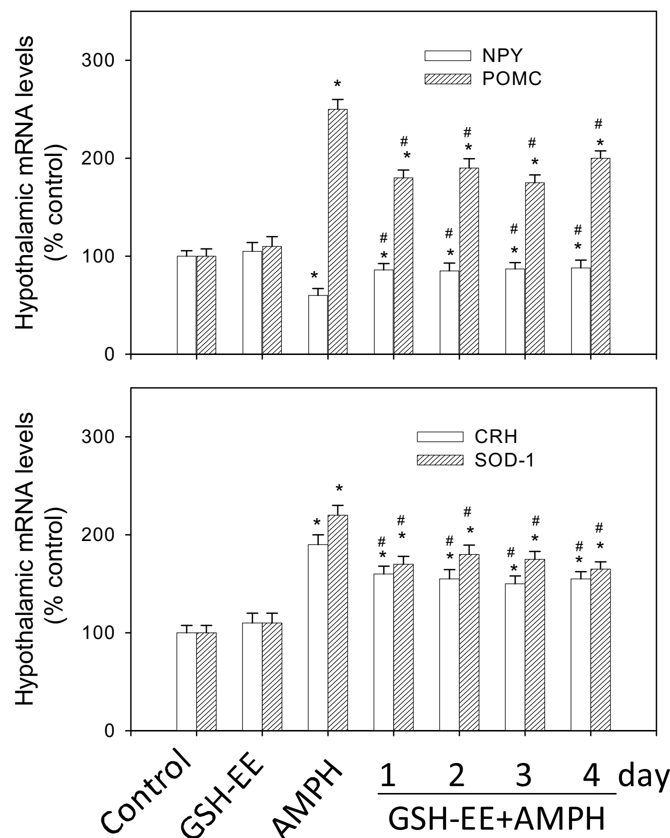


Fig. 4. Upper panel: the effect of daily pretreatment with ROS scavenger (GSH-EE; i.c.v.) on AMPH-induced feeding behavior. AMPH at the dose of 4 mg/kg was i.p. administered to rats once a time for 4 days. Lower panel: results of qPCR showing the GSH-EE/AMPH-induced changes of hypothalamic NPY, POMC, CRH, and SOD-1 mRNA levels. GSH-EE was i.c.v. administered at 40 min before AMPH treatment. $^{*}P < 0.05$ vs. the control (vehicle-treated) groups. $n = 8$ per group. $^{\#}P < 0.05$ vs. the vehicle/AMPH-treated group. GSH-EE: glutathione ethyl-ester, which is dissolved in aCSF solution.

together might be activated in POMC- but not NPY-containing neurons.

To investigate the possible involvement of GR and CRH, we used a GR antagonist (RU-486) to inhibit the action of GR. Previous reports have shown that a GR blockade in the brain can modify AMPH-induced dopamine release and motor activity (Morme'de et al., 1994; Ago et al., 2009; Sébastien et al., 2014). Our results showed that GR inhibition could gradually enhance the decreases

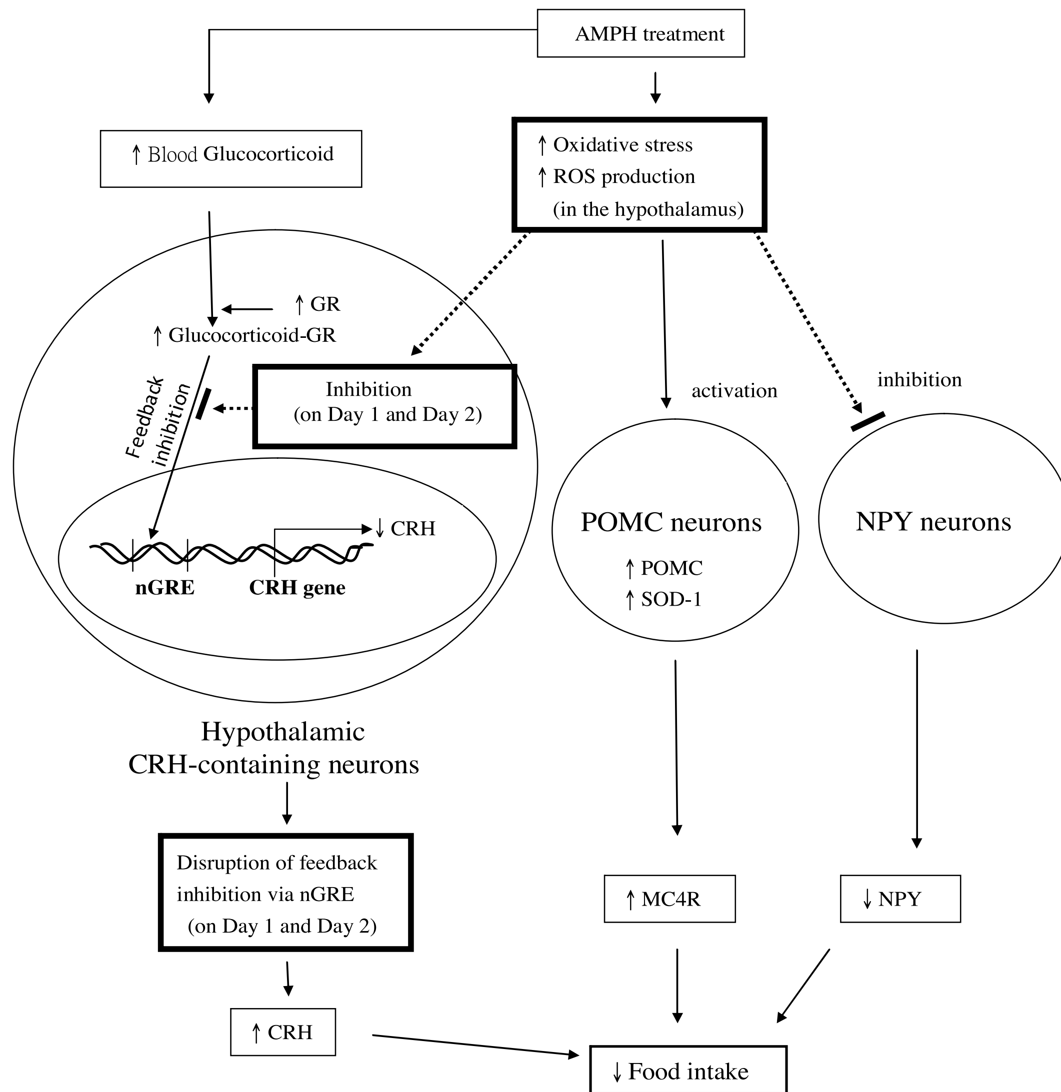


Fig. 5. Schematic illustration showed the flow of ROS-nGRE-CRH signaling in regulating AMPH-evoked decrease of food intake. AMPH treatment activates ROS production in the hypothalamus, which may change hypothalamic CRH, POMC and NPY expression, and finally decrease food intake. The increased ROS inhibited the binding of glucocoticoid-GR and nGRE-DNA, which disrupted the feedback inhibition of glucocorticoid-GR, and thus increased CRH level and decreased food intake. The decrease of NPY and the increase of MC4R during AMPH treatment may also contribute to the reduction of food intake. The scheme can be supported by histochemical evidence that AMPH could decrease NPY immunoreactivity in rat hypothalamic paraventriculum as described in our previous report (Hsieh et al., 2005).

in food intake and NPY expression, but enhanced the increases in POMC and CRH expression from Day 1 to Day 4, compared to the AMPH-treated groups. These results indicated that GR participated in the regulation of CRH release and NPY/POMC-mediated appetite control. Based on these findings, we suggest that high levels of glucocorticoid and GR during AMPH treatment failed to provide feedback to inhibit the release of CRH on Day 1 and Day 2; i.e., nGRE-mediated inhibition of CRH release was disrupted on Day 1 and Day 2. The reason for this might be related to the interference of GR and GRE binding because GR-GRE binding ability remained constant on Day 1 and Day 2 compared to that on Day 3 and Day 4 while GR expression remained high from Day 1 to Day 4. Consistent with the present findings, previous reports have shown that alcohol exposure can disrupt the normal feedback mechanisms of glucocorticoids by interfering with the binding between GR and GRE-DNA, leading to increases in CRH level, stress and anxiety (Roy et al., 2002; Przybycien-Szymanska et al., 2011). Moreover, the actions of glucocorticoid normally are tightly regulated to ensure that the body can respond quickly to stressful events by activation of HPA axis and return to a normal state just as rapidly (Mary and Stephens, 2012). The present study revealed that disruption of the nGRE-mediated mechanism on Day 1 and Day 2 might be associated with the increase in hypothalamic ROS because SOD-1 expression increased on Day 1 and Day 2 during AMPH treatment.

To investigate the possible role of ROS in the disruption of nGRE-mediated appetite control, we injected (i.c.v.) an ROS scavenger in AMPH-treated rats. Results showed that decreased hypothalamic ROS could modify feeding behavior and partially reverse the expression levels in NPY, POMC, and CRH back to normal compared to the AMPH-treated groups. Thus, it is possible that the increases in GR and CRH expression on Day 1 and Day 2 were associated with the increases in oxidative stress when dopamine-induced stress and glucocorticoid (stress hormone) were still high in AMPH-treated rats. The increase in the ROS level disrupted the nGRE-mediated inhibition of CRH release on Day 1 and Day 2, while the gradual return of CRH, POMC, and NPY expression to normal on Day 3 and Day 4 was associated with a successful decrease in ROS and restoration of nGRE-mediated inhibition when the concentration of corticosterone was still high. Finally, the restoration of NPY/POMC-mediated appetite control could be observed on Day 3 and Day 4.

These results suggest that the increase in oxidative stress on Day 1 and Day 2 participates in the disruption of nGRE-mediated inhibition of CRH release, while the improvement in preventing the increase in oxidative stress, which was reversed to normal on Day 3 and Day 4, is associated with the restoration of the nGRE-mediated mechanism in AMPH-treated rats.

In addition to the effect of oxidative stress on the disruption of nGRE, another transcription factor binding site, c-AMP response element (CRE), which is also located at the CRH promoter site, plays a role against to nGRE during AMPH treatment. CRE and nGRE sites can antagonistically regulate CRH gene expression because nGRE can inhibit but CRE can stimulate CRH release (Przybycien-Szymanska et al., 2011). We previously showed that CRE binding protein (CREB) increased and is expressed in a pattern similar to that of CRH and POMC, with the biggest response on Day 2, during a 4-day period of AMPH treatment (Hsieh et al., 2007). Thus, these results could explain why the decrease in GR–GRE binding ability on Day 1 and Day 2 (due to an increase in CREB–CRE binding) could increase CRH expression during AMPH treatment.

From the time course of a 24-h feeding behavior, the present data indicated that an AMPH-induced anorectic effect was short-lived (0–6 h). Thus, we ruled out the possibility that the change in the NPY level was secondary to reduced feeding rather than the direct action of AMPH on hypothalamic NPY neurons (Kuo, 2002). Consistent with this result, recent evidence has shown that the sequence of events leading to HPA activation by additive drugs appears to start within the brain, and the activation is not due to a secondary effect from peripheral alterations (Armario, 2010). Previous reports have shown that the maximum release of ACTH is usually observed 5–10 min after the start of exposure to stressors and that maximum corticosterone levels are achieved after only 20–30 min (Armario, 2010). In addition, the half-life of plasma corticosterone is only about 60 min during the acute stressful response (Vachon and Moreau, 2001), but it can remain at a high level for more than one week during chronic stressful conditions in rodent animals daily treated with stressors (Gong et al., 2015). Thus, following AMPH treatment on Day 1 and Day 2 (i.e. the period of AMPH-induced anorexia), the anorectic response was associated with the activation of the HPA axis, resulting in the increases in plasma glucocorticoid levels and hypothalamic GR and CRH expression within about 40–50 min. Based on this information, we conclude that the disruption of nGRE-mediated CRH release and the elevation in POMC, MC4R, CRH, and SOD expression in the hypothalamus should be associated with a decrease in NPY and direct action of AMPH but not be due to the secondary response of feeding behavior. These findings further support our viewpoint that the mechanism of nGRE feedback inhibition was interrupted during the initial two days of daily AMPH treatment.

Recent findings suggested that the studies on routine preclinical toxicology should precede strategies in the development of anti-obesity drugs and their safety concerns (dos Santos et al., 2013). Our present results and previous findings (Kuo et al., 2011, 2015) provide the preclinical toxicology evidence that hypothalamic ROS and anti-oxidants play functional roles in the disruption of nGRE-DNA and anorectic effect of AMPH. Although the molecular mechanisms are more evident from the present study, AMPH may not prove to be an ideal anti-obesity drug due to the neurotoxic effects of this compound (Yamamoto et al., 2010). The risks of AMPH are possibly related to the increase of oxidative stress in injured tissues. The present result showing the decrease of ROS production can modulate the behavioral response of AMPH implies the possible modulation of the ROS-sensitive pathway under certain physiological conditions.

In conclusion, the present results suggest that the disruption of nGRE-mediated feedback inhibition on Day 1 and Day 2 is associated with an increase in oxidative stress in the hypothalamus during the regulation of NPY/POMC-mediated appetite control in AMPH-treated rats (Fig. 5).

Conflict of interest

The authors declare no conflicts of interest in this study.

Role of the funding source

The funding source was not involved in study design or in the decision to submit the article for publication.

Contributors Authors

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2016.04.025.

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科技部補助計畫衍生研發成果推廣資料表

日期:2016/06/24

<table>
<tr><td rowspan="3">科技部補助計畫</td><td>計畫名稱: 下視丘之CART(55-102)神經胜肽、活性氧族群和deltaFosB轉錄因子、及血液瘦身素和飢餓素在厭食劑作用之角色</td></tr>
<tr><td>計畫主持人: 郭東益</td></tr>
<tr><td>計畫編號: 104-2320-B-040-014- 學門領域: 生理</td></tr>
<tr><td colspan="2">無研發成果推廣資料</td></tr>
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104年度專題研究計畫成果彙整表

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<tr><td colspan="4">計畫主持人：郭東益</td><td colspan="3">計畫編號：104-2320-B-040-014-</td></tr>
<tr><td colspan="7">計畫名稱：下視丘之CART(55-102)神經胜肽、活性氧族群和deltaFosB轉錄因子、及血液瘦身素和飢餓素在厭食劑作用之角色</td></tr>
<tr><td colspan="4">成果項目</td><td>量化</td><td>單位</td><td>質化
（說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）</td></tr>
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<tr><td colspan="2">研討會論文</td><td>1</td><td>第三十一屆生物醫學聯合學術年會
安非他命引發之游離氧壓力在DNA糖皮質反應要素之角色
Role of oxidative stress in disrupting the function of negative DNA-glucocorticoid response element in amphetamine-treated rats
郭東益1,余青翰1, 謝易修2,陳霈霓2,廖娟妙1</td></tr>
<tr><td colspan="2">專書</td><td>0</td><td>本</td><td></td></tr>
<tr><td colspan="2">專書論文</td><td>0</td><td>章</td><td></td></tr>
<tr><td colspan="2">技術報告</td><td>0</td><td>篇</td><td></td></tr>
<tr><td colspan="2">其他</td><td>0</td><td>篇</td><td></td></tr>
<tr><td rowspan="9">智慧財產權及成果</td><td rowspan="3">專利權</td><td>發明專利 申請中</td><td>0</td><td rowspan="9">件</td><td></td></tr>
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		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
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		收入			0	千元	
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		碩士生			0		
		博士生			0		
		博士後研究員			0		
		專任助理			0		
	非本國籍	大專生			0		
		碩士生			0		
		博士生			0		
		博士後研究員			0		
		專任助理			0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）							

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
      □實驗失敗
      □因故實驗中斷
      □其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：■已發表 □未發表之文稿 □撰寫中 □無
   專利：□已獲得 □申請中 ■無
   技轉：□已技轉 □洽談中 ■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   Highlights

   1.Daily amphetamine (AMPH) increased plasma corticosterone levels at initial 60 min.
   2.Disruptions of nGRE (negative glucocorticoid response element) are associated with an increase in oxidative stress in AMPH-treated rats.
   3.These results advance the understanding of the role of nGRE in regulating NPY/POMC-mediated appetite suppression.

4. 主要發現
   本研究具有政策應用參考價值：□否 ■是，建議提供機關藥廠,醫院或學術機構
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：□否 ■是
   說明：（以150字為限）
   Disruptions of nGRE during amphetamine treatment are associated with an increase in oxidative stress in AMPH-treated rats.