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腦內 MAPK 訊息傳遞路徑在「安非它命的厭食作用」及  
「NPY 基因表現」之角色  
研究成果報告(精簡版)

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**Roles of central catecholamine and hypothalamic  
neuropeptide Y genome in the development of tolerance to  
phenylpropanolamine-mediated appetite suppression**

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**Running title:**

D.Y. Kuo et al.

CAT and NPY in phenylpropanolamine tolerance

## **Abstract**

Phenylpropanolamine (PPA) is an appetite suppressant. Repeated treatment with PPA can decrease food intake on initial days, which is followed by a gradual return of normal food intake (tolerant effect) on subsequent days. In an attempt to investigate the underlying mechanisms of PPA tolerance, roles of catecholamine (CAT) and hypothalamic NPY genome were examined. Results revealed that pretreatment with either bupropion, a CAT transporter inhibitor, or  $\alpha$ -methyl-para-tyrosine, a tyrosine hydroxylase inhibitor, could modulate the effect of PPA tolerance. Moreover, results also revealed that the alteration in NPY mRNA level coincided with the change of feeding behavior during PPA treatment and that infusions of NPY antisense oligonucleotide into cerebroventicle could abolish the effect of PPA tolerance. Our current findings suggest that cerebral CAT and hypothalamic NPY genome are involved in the development of tolerance to PPA-induced appetite suppression.

**Keywords:** phenylpropanolamine, NPY, feeding behavior, catecholamine, brain, tolerance.

## Introduction

Phenylpropanolamine (PPA) is an over-the-counter appetite suppressant and may be used in human dieters to improve obesity (Borovicka *et al.*, 2002; Cooper *et al.*, 2005; Schteingart, 1992). PPA-contained chewing gum can be used to reduce the weight gain caused by smoking cessation, however, this strategy is effective only in short-term studies (about 13 weeks) (Klesges *et al.*, 1990; Klesges *et al.*, 1995). In long-term studies, the weight-reducing effect of PPA is attenuated and may eventually lead to a rebound of weight (Borrelli *et al.*, 1999; Cooper *et al.*, 2005) possibly due to a mechanism of PPA tolerance. Furthermore, the rebound of weight can be reduced if they are treated with the drug bupropion (BUP), a dopamine and norepinephrine transporter inhibitor (Hays *et al.*, 2001). Evidence reveal that BUP can exert its antidepressive effect by blocking the action of dopamine transporter in the brain (Argyelan *et al.*, 2005) and that BUP-induced weight loss is primarily due to the effect of drug on catecholamine (CAT)-mediated neurotransmission (Billes & Cowley, 2006). These results revealed that CAT might involve the development of tolerance to PPA-induced anorexia.

In animal studies, evidence reveal that PPA can suppress appetite and promote weight loss (Svec *et al.*, 2003; Winders *et al.*, 1994) due to its pharmacological effect

on CAT receptors, such as  $\alpha$ -adrenoceptor (Moya-Huff & Maher, 1987; Wellman & Davies, 1992) or  $\alpha$ 1-adrenoceptor and dopamine D1 receptor in the brain (Cheng & Kuo, 2003). Moreover, the weight-reducing effect of PPA may reflect a combined effect of this drug on both food intake and brown adipose tissue thermogenesis (Wellman & Sellers, 1986).

In addition to CAT, hypothalamic NPY may also contribute to the anorectic effect of PPA (Kuo, 2003; Kuo *et al.*, 2004). NPY is widely distributed in central nervous system with high concentration found in hypothalamus (Chronwall *et al.*, 1985; De Quidt & Emson, 1986). NPY is a well-known orexigenic neurotransmitter that is postulated to control the energy balance by stimulating feeding behavior and inhibiting thermogenesis, especially under conditions of energy deficiency such as food restriction, intense exercise, obesity and diabetes (Billington *et al.*, 1994; Kalra & Kalra, 2004; Woods *et al.*, 1998).

PPA is a sympathomimetic agent that is structurally and functionally related to amphetamine-like anorectic drugs. Evidence reveals that PPA has a better safety profile than AMPH since a repeated administration of PPA is not subject to the induction of tolerance and abuse (Lee *et al.*, 1989). Until recently, the mechanism for the induction of PPA tolerance is still unknown. Thus, the present study is designed to investigate the mechanism underlying the tolerant effect of PPA-induced anorexia. It

is hypothesized that CAT-ergic pathway and hypothalamic NPY may contribute to the induction of PPA tolerance. We predicted in the current study that a combined administration of BUP/PPA, which presumably could increase CAT neurotransmission, and a combination of  $\alpha$ -methyl-para-tyrosine (AMPT)/PPA, which could decrease CAT neurotransmission, should modify the anorectic response of PPA treatment alone. AMPT is a tyrosine hydroxylase inhibitor that can decrease CAT content in the brain (Yuan *et al.*, 2001). Likewise, a combination of PPA and NPY antisense oligonucleotide, which can interfere with NPY transcript expression, should modulate the anorectic response of PPA treatment alone.

## **Material and Methods**

### ***Animals***

Male Wistar rats (with a weight of 200~300 g, Animal Center of National Cheng Kung University Medical College) were housed individually in transparent plastic cages with stainless steel covering and hardwood bedding (Beta Chip, Northeastern Products Corp., NY, USA). Food (LabDiet, PMI Nutrition International, Brentwood, MO, USA) and tap water were provided *ad libitum*. Animals were maintained at 22  $\pm$

2°C according to a 12 h light: 12 h dark cycle (light on at 6:00 AM) and habituated to frequent handling. Drug administration, food intake and body weight assessment were performed daily at the beginning of dark phase (6:00 PM). This study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

### ***Drug treatments***

To examine the effects of PPA (Sigma-Aldrich, MO, USA) on feeding behavior and body weight, rats were given the PPA (0, 40, 80, or 110 mg/kg; i.p.; n=6-8 each group) daily for 6 days at the beginning of dark phase (at 6:00 PM). Feeding behavior and body weight were examined at 24 h after daily drug treatment. The first injection of PPA was conducted at the end of Day 0 (at 6:00 PM) and the intake data were calculated as the total amount of food during the previous day. The data of body weight change were calculated with respect to the weight of previous day.

To examine the effect of CAT-mediated neurotransmission on PPA-induced feeding behavior, rats were given bupropion (BUP, 20 mg/kg, i.p.; Sigma-Aldrich, MO, USA) at 40 min before the treatment of PPA (0 or 80 mg/kg; n=6-8 each group) once a day for 6 days. It has been reported that BUP at this dose can induce the

anorectic response due to its central effect on CAT-ergic neurotransmission (Klimek *et al.*, 1985; Zarrindast & Hosseini-Nia, 1988). Feeding behavior was examined at 24 h after drug treatment every day.

To examine the effect of endogenous CAT on daily PPA anorexia, the drug AMPT was given prior to the treatment of 80-mg/kg PPA daily for 6 days. AMPT, an inhibitor of tyrosine hydroxylase, was given at the dose of 40 mg/kg (i.p.) repeatedly into rats twice a day at 6 and 2 h prior to PPA administration in order to inhibit CAT synthesis in the brain (Yuan *et al.*, 2001). The treatment of AMPT alone is efficient to decrease cerebral CAT contents measured by HPLC-ECD as indicated in our previous report (Kuo & Cheng, 2002).

To assess the effect of PPA on NPY mRNA level, rats (n=5-6 each group) were treated daily with PPA (0 or 80 mg/kg, i.p.) for 1, 2, 3, 4, 5 and 6 days, respectively, and then were sacrificed. Rats receiving PPA at 40 min before sacrifice were anesthetized with pentobarbital (30 mg/kg, i.p.) and then decapitated. The hypothalamus was removed from the brain immediately and subjected to determinations of mRNA levels, or stored at  $-80^{\circ}\text{C}$  until the day to use.

To determine the effect of NPY antisense on the anorectic response of PPA, rats (n=8-10 for each group) were injected daily with either antisense or missense oligodeoxynucleotide (ODN, 10  $\mu\text{g}/10 \mu\text{l}/\text{day}$ , ICV) at 60 min before daily PPA (80



mg/kg) for 6 days. Moreover, to determine the effect of NPY antisense on hypothalamic NPY mRNA levels during PPA treatment, rats (n=4-6 for each group) were injected with either antisense or missense (10 µg/10 µl/day, ICV) at 60 min before daily PPA (80 mg/kg) for 6 days. Rats receiving drug at 40 min before sacrifice were anesthetized with pentobarbital (30 mg/kg, i.p.) and then were decapitated. The hypothalamus was removed from the brain immediately and subjected to determinations of mRNA levels, or stored at -80°C until the day to use.

### ***RNA extraction***

Hypothalamic NPY mRNA levels in a block of mediobasal hypothalamic tissue were measured as described previously (Morris, 1989). In brief, total RNA was isolated from tissue block using the modified guanidinium thiocyanate-phenol-chloroform method (Chomczynski & 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 sec, incubated at 22°C for 3 min, and then centrifuged at 12,000 ×g for 15 min at 4°C.

After removal of aqueous phase and precipitation with 0.5 ml isopropanol, samples were incubated at 22°C for 10 min and centrifuged at 12,000 ×g for 15 min at 4°C. The gel-like RNA pellets were washed with 75% ethanol by vortexing and centrifugation at 7,500 ×g for 5 min at 4°C. Thereafter, RNA pellets were dried briefly, dissolved in RNase-free water, and stored at -80°C. The content of RNA was determined spectrophotometrically at 260 nm (Hitachi U-3210, Japan).

#### ***Reverse transcription-polymerase chain reaction (RT-PCR)***

Using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim GmbH, Germany), RNA was reversely transcribed into single-stranded cDNA. For each sample, 8 µl of sterile DEPC water containing 2 µg of RNA were added to oligo-p(dT)<sub>15</sub> primer (0.8 µg/µl) followed by a heating at 65°C for 15 min, a cooling at 25°C for 10 min, and then added to a reaction mixture consisting of 10x reaction buffer (100 mM Tris, 500 mM KCl; pH 8.3), deoxynucleotide mix (10 mM each), MgCl<sub>2</sub> (25 mM), RNase inhibitor (40 unit/µl), and AMV reverse transcriptase (25 unit/µl). Reaction mixtures were incubated at 42°C for 2 h and then brought to 95°C for 5 min to terminate the reaction followed by soaking at 16°C. PCR was subsequently carried out by mixing 3µl of cDNA product with mastermix solution

consisting of DEPC water, 10x reaction buffer, MgCl<sub>2</sub> (25 mM), deoxynucleotide mix (10 mM each), P1 and P2 primers (1 µg/µl each), and Taq polymerase (5 unit/µl). GAPDH was used as the internal standard calibrator. PCR reactions for NPY were carried out on a PCR thermocycler (Perkin-Elmer GeneAmp 2400) for 28 cycles with the following steps: 91°C for 1 min (denaturing), 60°C for 1 min (annealing), and 72°C for 30 sec (extension), followed by a final elongation step at 72°C for 7 min, and finally the PCR products were soaked at 16°C.

### ***Gel electrophoresis***

At the completion of RT-PCR, 8 µl of each PCR product was subsequently separated by flat-bed gel electrophoresis on a 3% agarose gel. Gels stained by ethidium bromide (0.5 µg/ml, Sigma-Aldrich Co., MO, USA) were visualized under UV light, photographed, and then scanned densitometrically. Ratios of NPY and GAPDH mRNA were calculated to determine relative NPY mRNA levels. Contents of NPY mRNA in PPA-treated group were indicated as the percentage of control group. The ratio of NPY/GAPDH mRNA was measured by digital densitometry (KODAK Gel Logic 100 Imaging System, Eastman Kodak Company, NY, USA).

### *Lateral ventricular cannulation*

Stereotaxic surgery (Kopf Model 900, Tujunga, CA, USA) of rats was performed under anesthesia with pentobarbital (30 mg/kg; i.p.). The target of cannulation was close to the junction between the right lateral ventricle and the third ventricle (coordinates: 0.8 mm posterior to Bregma, 1.5 mm from the midline, and 3.5~4.0 mm below the dura) (Paxinos & Watson 1986). A 23-g stainless steel guide cannula was implanted and secured to the skull using stainless-steel screws and dental cement. The accuracy of placement was confirmed by observing a transient and rapid inflow of vehicle in PE tube connected with a 28-g injector cannula. The cannula was then occluded with a 28-g stylet. For intracerebroventricular (ICV) infusion of NPY antisense, the stylet was replaced with a 28-g injector cannula extending 0.5 mm below the tip of guide cannula. Behavioral testing began at 1 week after the surgery. Rats were daily treated with antibiotics (tetracycline) to prevent the infection during the period of experiment. For all experiments, verification of cannula placement was done by the administration of angiotensin II (100 ng/rat; Sigma-Aldrich, USA). Angiotensin II reliably induced water drinking in non-deprived rats when administered into the ventricles (Ritter et al., 1981). Only data from rats drinking more than 10 ml within 30 min were included in this study.

### *ICV administration of NPY antisense*

An 18-mer ODN near the initiation codon encompassing bases 10-27 of the rat NPY mRNA sequence (Genbank access no. 15880) was selected. The antisense ODN (5'-CCCCATTCGTTTGTACC) was inversely complementary to this sequence. Phosphorothioate internucleotide linkages were obtained through a treatment with tetraethylthiuram disulfide, and the resulting phosphorothioate oligodeoxynucleotides (S-ODN) were purified and lyophilized. An 18-mer missense S-ODN (5'-TTATTCCCCCAGTTTGCC) was used as the control. This antisense sequence did not appear to display self-hybridization; therefore it was effective in blocking the message read-through. In addition, daily ICV injection of NPY antisense S-ODN for a week appeared to reduce food intake and body weight as compared with the missense-treated control. Rats were handled and ICV injected with vehicle 4 days prior to the experimental injections to accustom them to the procedure. One hour before the PPA administration (80 mg/kg; i.p.), NPY antisense (10 µg/10µl/day) was administered to 6-8 rats, and the same treatment was repeated for 6 days. An equivalent dose of missense S-ODN was administered to each of the 6-8 additional rats that served as the control. Both antisense and missense S-ODNs were dissolved in

artificial corticospinal fluid (ACSF) containing 140 mM NaCl, 3.35 mM KCl, 1.15 mM MgCl<sub>2</sub>, 1.26 mM CaCl<sub>2</sub>, 1.2 mM Na<sub>2</sub>HPO<sub>4</sub> and 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>; pH 7.4.

### *Statistical analysis*

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

## **Results**

### *The effect of PPA on feeding behavior and body weight*

Changes of daily food intake in rats receiving PPA are shown in upper panel of Figure 1. Using two-way ANOVA to repeatedly measure the effect of PPA treatment for 6 days, a significant dose-dependent [ $F(3,28)=20.2$ ,  $P < 0.05$ ] and time-dependent effect [ $F(6,49)=5.18$ ,  $P < 0.05$ ] were revealed. Moreover, an interaction effect [ $F(18,196)=1.19$ ,  $P < 0.05$ ] was also revealed. Followed by a post-hoc testing, it revealed that a treatment with PPA (80 mg/kg) reduced the feeding from Day 1 to Day

3, and a treatment with PPA (110 mg/kg) reduced the feeding from Day 1 to Day 6 when compared to controls. Moreover, it also revealed that a treatment with PPA (80 mg/kg) produce a significant effect on Day 5 and Day 6 if compared with that on Day 1. These results indicated that daily PPA (80 mg/kg), but not PPA (110 mg/kg), could produce a tolerant effect on food intake during a 6-day period of time. Therefore, a dose of 80 mg/kg was selected to examine the effect of PPA tolerance on the following study.

Similar results can be obtained in body weight changes that are shown in lower panel of Figure 1. Using two-way ANOVA to measure the effect of PPA treatment, it revealed a significant dose-dependent [ $F(3,28)=22.1$ ,  $P<0.05$ ], a time-dependent [ $F(6,49)=6.15$ ,  $P<0.05$ ] and an interaction effect [ $F(18,196)=1.21$ ,  $P<0.05$ ]. These results indicated that daily PPA (80 mg/kg), but not PPA (110 mg/kg), could produce a tolerant effect on body weight change during a 6-day period of time.

#### ***The effect of BUP or AMPT on PPA-induced anorexia***

The effect of bupropion (BUP) pretreatment on PPA-induced anorexia is shown in upper panel of Figure 2. Using two-way ANOVA to repeatedly measure the effect of PPA treatment for 6 days, it revealed significant treatment-dependent [ $F(3,28)=16.2$ ,

$P < 0.05$ ] and time-dependent [ $F(6,49) = 6.58$ ,  $P < 0.05$ ] effects. Followed by a post-hoc testing, it revealed that a treatment with PPA (80 mg/kg) reduced the feeding response from Day 1 to Day 3, while a treatment with BUP/PPA could reduce the feeding response from Day 1 to Day 6. Moreover, comparing the feeding between BUP/PPA- and PPA-treated rats every day, it revealed significant effects on Day 5 and Day 6. These results reveal that BUP can modulate the response of PPA tolerance and suggest that CAT-mediated neurotransmission is involved in the regulation of PPA tolerance.

The effect of AMPT pretreatment on PPA-induced anorexia is shown in lower panel of Figure 2. Using two-way ANOVA to repeatedly measure the effect of PPA treatment for 6 days, a significant treatment-dependent effect [ $F(3,27) = 9.5$ ,  $P < 0.05$ ] was revealed. Comparing with the control group, it showed that a treatment with 80 mg/kg PPA could reduce the feeding response during Day 1 to Day 3, while pretreatment with AMPT could attenuate this response. The present results indicated that AMPT could alter the response of PPA tolerance, revealing that PPA tolerance was relevant to the endogenous CAT.

### ***The effect of PPA treatment on hypothalamic NPY mRNA level***

Results shown in Figure 3 reveal the effect of daily PPA (80 mg/kg) on the



decrease of NPY mRNA level during a 6-day period of time. Analysis with one-way ANOVA revealed a decrease of NPY mRNA contents [ $F(6,35)=5.7$ ,  $P<0.05$ ] from Day 1 to Day 3 and a restoration of NPY mRNA content on Day 5 and Day 6 in PPA-treated rats as compared with the control. This result revealed that changes in NPY mRNA levels were consistent with changes of feeding behavior, revealing the involvement of NPY genome in both PPA anorexia and PPA tolerance.

#### ***The effect of NPY antisense on PPA-induced anorexia***

As shown in upper panel of Figure 4, NPY antisense alone can decrease the feeding behavior revealing the effectiveness of this drug **as described in our previous report (Kuo *et al.*, 2001)**. Moreover, NPY antisense can enhance the anorectic response of PPA and block the tolerant response of PPA, indicating the involvement of NPY in PPA tolerance. Repeated measurement for the responses from Day 1 to Day 6 revealed a significant treatment effect [ $F(3,28)=4.9$ ,  $P<0.05$ ] and time effect [ $F(6,49)=5.1$ ,  $P<0.05$ ]. **Followed by a post hoc Dunnett's test, it revealed** that the intake between antisense/PPA-treated and PPA-treated rats every day revealed significant effects on Day 5 and Day 6. Furthermore, significant effects from Day 1 to Day 3 were found between missense/PPA-treated and control groups, while those

from Day 1 to Day 6 were found between antisense/PPA-treated and control groups. These results indicated that NPY knock down could modify the tolerant effect induced by PPA. No statistical significance was obtained in 80 mg/kg PPA-treated rats receiving missense/CSF (vehicle) injection (shown in Fig. 4) or not (shown in Fig. 1) (t-test), indicating the noninterference of missense treatment and vehicle on PPA's action.

Similar results can be obtained in body weight changes that are shown in lower panel of Figure 4. Using two-way ANOVA to repeatedly measure the effect from Day 1 to Day 6, it revealed a significant dose-dependent effect [ $F(3,28)=4.1$ ,  $P<0.05$ ] and a time-dependent effect [ $F(6,48)=5.51$ ,  $P<0.05$ ]. These results indicated that daily PPA (80 mg/kg), but not PPA (110 mg/kg), could produce a tolerant effect on body weight change during a 6-day period of time.

#### ***The effect of co-application of NPY antisense and PPA on NPY mRNA levels***

Results shown in Figure 5 revealed that NPY antisense alone or together with PPA both could decrease hypothalamic NPY mRNA level during 6-day period. Using GAPDH as the internal standard, the ratio of NPY/GAPDH mRNA in each group was calculated and compared. A one-way ANOVA revealed that both NPY antisense and

NPY antisense/PPA groups have a significant effect on the decrease of NPY mRNA content as compared with the control (missense) group [F(7,54)=2.19, P<0.05]. Statistical analysis revealed that the ratio of NPY/GAPDH mRNA was about  $85 \pm 5$  % in NPY antisense alone group and about  $66 \pm 6$  % in NPY antisense/PPA group as compared with the control group. Moreover, it appears that the co-administration of NPY antisense and PPA can abolish the reversion of NPY mRNA to normal level during 6-day period. These results suggest that NPY genome is involved in regulating the development of tolerance to PPA anorexia.

## **Discussion**

Via an animal model of rats, current results showed that a moderate dose of PPA (80 mg/kg) could decrease food intake and weight change on the initial 3 days and a tolerant effect being observed during the following days. However, when rats were treated with a higher dose (110 mg/kg) of PPA, this tolerant response disappeared. These results revealed that CAT-ergic transmission might play a functional role in the control of PPA tolerance. To clarify this possibility, we took a further step to examine whether pretreatments with other CAT-ergic drugs could modify the tolerant effect of PPA. Results revealed that pretreatment with either BUP or AMPT, which can

increase or decrease the effect of CAT-ergic transmission, respectively, could modulate the tolerant response of PPA. These findings suggest that cerebral CAT is involved in the regulation of PPA tolerance.

In addition to CAT, we also found that the alteration of NPY mRNA level was consonant with the change of feeding behavior following PPA treatment, indicating that NPY might involve the regulation of PPA tolerance. To clarify this possibility, we further examined the functional role of NPY during PPA treatment. In the presence of NPY antisense, food intakes were decreased continuously with no evidence of tolerance and NPY mRNA levels were also decreased continuously throughout the entire 6-day period in PPA-treated rats. This result was consistent with the previous finding that injection of NPY antisense into brain could prevent the tolerant effect induced by dopaminergic drugs (Kuo, 2003). This result suggests that hypothalamic NPY genome is involved in the regulation of PPA tolerance.

The NPY-ergic pathway in hypothalamus can be stimulated under conditions of negative energy balance, such as food restriction, diabetes, intense exercise and obesity (Bi *et al.*, 2003; Lewis *et al.*, 1993; Jeanrenaud *et al.*, 2001). In the present study, the metabolic state of reduced food intake and body weight gain observed on Day 1, Day 2 and Day 3 during PPA treatment was similar to that of negative energy balance, which can stimulate the activity of NPY-ergic pathway, and in turn the

increase of NPY mRNA level might serve as a homeostatic regulator in maintaining energy balance by stimulating feeding, which could eventually reverse the food intake back to the normal level.

Some evidence show that CAT-ergic pathway can regulate the level of NPY in several brain regions (Gillard *et al.*, 1993; Leibowitz & Rossakis, 1979) and that PPA (or some psychotomimetic drugs) can alter the level of NPY (Hsieh *et al.*, 2004; Yoshihiro *et al.*, 1996). Thus, PPA-induced tolerance toward the CAT-ergic effect might prevent the NPY mRNA level from decreasing during repeated PPA treatment, and in turn the normal level of NPY led to the normalization of feeding behavior. Based on these findings, our results suggest that PPA tolerance may be implicated in the decreased inhibitory action of PPA on NPY-ergic neurons.

It appears that co-administration of NPY antisense and PPA can enhance the decrease of food intake during a 6-day period, which may be due to the additive decrease of NPY level, although the underlying mechanism is unknown. The hypothalamic NPY level was declined partly due to PPA's dopaminergic action and partly due to NPY antisense's action. Actually, NPY antisense will effect on the biosynthesis of NPY genome only, without a direct influence on the action of PPA. However, combined administration of PPA and NPY antisense may enhance the inhibition of food intake. Thus, co-application of PPA and NPY antisense may be

superior to the application of PPA alone in an attempt to reduce the body weight gain and to slow down the effect of PPA tolerance.

Although amphetamine-induced hypophagia is intact in NPY-deficient mice (Cannon et al., 2004), it has been hypothesized that NPY participates in the hypophagic response to amphetamine (Gillard et al. 1993; Kuo 2003; Kuo et al. 2001). The reason for this effect of amphetamine is unknown. It is possible that transgenic animals may produce an altered brain state in which animals cannot respond selectively to internal homeostatic and motivational drives. For example, NPY-deficient mice are more sensitive to the locomotor effects of amphetamine and quickly entered stereotypy (Cannon et al., 2004). Dopamine  $\beta$ -hydroxylase knockout mice were hypersensitive to the behavioral response of amphetamine or cocaine because they have alterations in dopamine signaling (Schank et al., 2006). Moreover, NPY-overexpressing mice have a normal feeding behavior but are more sensitive to amphetamine-induced anorectic response than wild-type mice as described in our previous report (Kuo et al., 2002).

The mechanisms underlying the therapeutic efficacy of PPA or BUP as a smoking cessation agent are still unknown. Most studies have been focused on the presynaptic action of drug (Cooper *et al.*, 2005; Rauhut *et al.*, 2003) and therefore little is known about the postsynaptic mechanisms such as the role of NPY. NPY has

pleiotropic activities ranging from the control of obesity to anxiolysis and cardiovascular function (Zukowska *et al.*, 2003). Hence, it is possible that the therapeutic efficacy of PPA or BUP as a smoking cessation agent may be relevant to the anxiolytic effect of NPY in amygdala, a site which is abundant of NPY. Evidence reveal that NPY in amygdala is implicated in pathophysiology of certain mood disorders, including anxiety and depression (Kokare *et al.*, 2005; Sorensen *et al.*, 2004) and that CAT in amygdala can modulate some behavior or activate the HPA axis in response to acute stress (Kroner *et al.*, 2005; Ma & Morilak. 2005).

Some case-control studies in humans indicated that the use of PPA in diet may aid the risk of hemorrhagic stroke (Delorio, 2004; Mersfelder, 2001). It is not know whether cerebral NPY is involved in the induction of this stroke. NPY is known to participate in central mechanisms of blood pressure control (Coelho *et al.*, 2004; Morris *et al.*, 2004; Velkoska *et al.*, 2005). Released by sympathetic activity, NPY is a major mediator of stress, responsible for prolonged vasoconstriction via Y1 receptors (Zukowska *et al.*, 2003). Recently, some reports indicated that NPY is involved in the regulation of stress-induced cerebral ischemia in rodents (Arsenijevic *et al.*, 2006; Li *et al.*, 2005). An unpublished data by our laboratory showed that rats treated with higher doses of PPA (>160 mg/kg) would induce a lethal effect which might be relevant to the oxidative stress or the incident of stroke. Perhaps, co-administration of

BUP/PPA or NPY antisense/PPA is a safer and better therapeutic method than the administration of PPA alone in the improvement of human obesity. It is because co-administration of each drug may lower the risk of PPA and slow down the speed of PPA tolerance, and therefore may prevent the incident of stroke caused by PPA. The possible role of NPY in PPA-induced stroke needs to be investigated further.



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## Figure legends

**Figure 1.** The effect of repeated administrations of phenylpropanolamine (PPA) on daily food intake (upper panel) and body weight change (lower panel) over a 6-day period. Various doses of PPA (0, 40, 80 or 110 mg/kg, IP) were administered to rats once a day (at 6:00 PM of each day) for 6 days. The first injection of PPA was conducted at the end of Day 0. Each point represents the mean  $\pm$  SEM of 6-8 rats. \*P < 0.05 vs. the control group of each treatment day. #P < 0.05 vs. the Day-1 group.

**Figure 2.** Upper: The effect of bupropion (BUP) pretreatment on PPA-induced daily food intake. BUP (0 or 20 mg/kg, IP) was administered to rat at 40 min before PPA (80 mg/kg) treatment daily for 6 days. Lower: The effect of  $\alpha$ -methyl-para-tyrosine (AMPT) pretreatment on PPA-induced daily food intake. AMPT (0, or 40 mg/kg, IP) was administered to rat twice a day at 6 h and 2 h before PPA (80 mg/kg) treatment daily for 6 days. The first injection of PPA was conducted at the end of Day 0. Each point represents the mean  $\pm$  SEM of 6-8 rats. \*P < 0.05 vs. the control group of each treatment day. #P < 0.05 vs. the BUP/PPA-treated group of each treatment day. B.i.d: twice a day.

**Figure 3.** The effect of repeated administrations of PPA (80 mg/kg) on hypothalamic NPY mRNA levels over a 6-day period. Upper panel: the RT-PCR results of hypothalamic NPY and GAPDH mRNA levels. Lower panel: relative densitometric values for RT-PCR products of hypothalamic NPY mRNA between PPA and control groups. Content of each mRNA in PPA-treated group was indicated as the percentage of control. Bars are mean  $\pm$  SEM. N=5-6 per group. \*P<0.05 vs. control.

**Figure 4.** The effect of NPY antisense oligodeoxynucleotide on PPA-induced daily food intake (upper panel) and body weight change (lower panel). Antisense or missense (10  $\mu$ g/10  $\mu$ l/day, i.c.v.; n=6-8 each group) was administered into brain 60 min before PPA (80 mg/kg, i.p.) treatment every day for 6 days. \*P<0.05 vs. the control (missense) group of each treatment day. #P < 0.05 vs. the antisense/PPA-treated group of each treatment day.

**Figure 5.** The effect of NPY antisense and PPA co-administration on hypothalamic NPY mRNA levels during a 6-day period. Antisense or missense (10  $\mu$ g/10  $\mu$ l/day, i.c.v.; n=6-8 each group) was administered daily into brain 60 min before daily PPA (80 mg/kg, i.p.) treatment for 6 days. \*P<0.05 vs. the missense group of each treatment day.

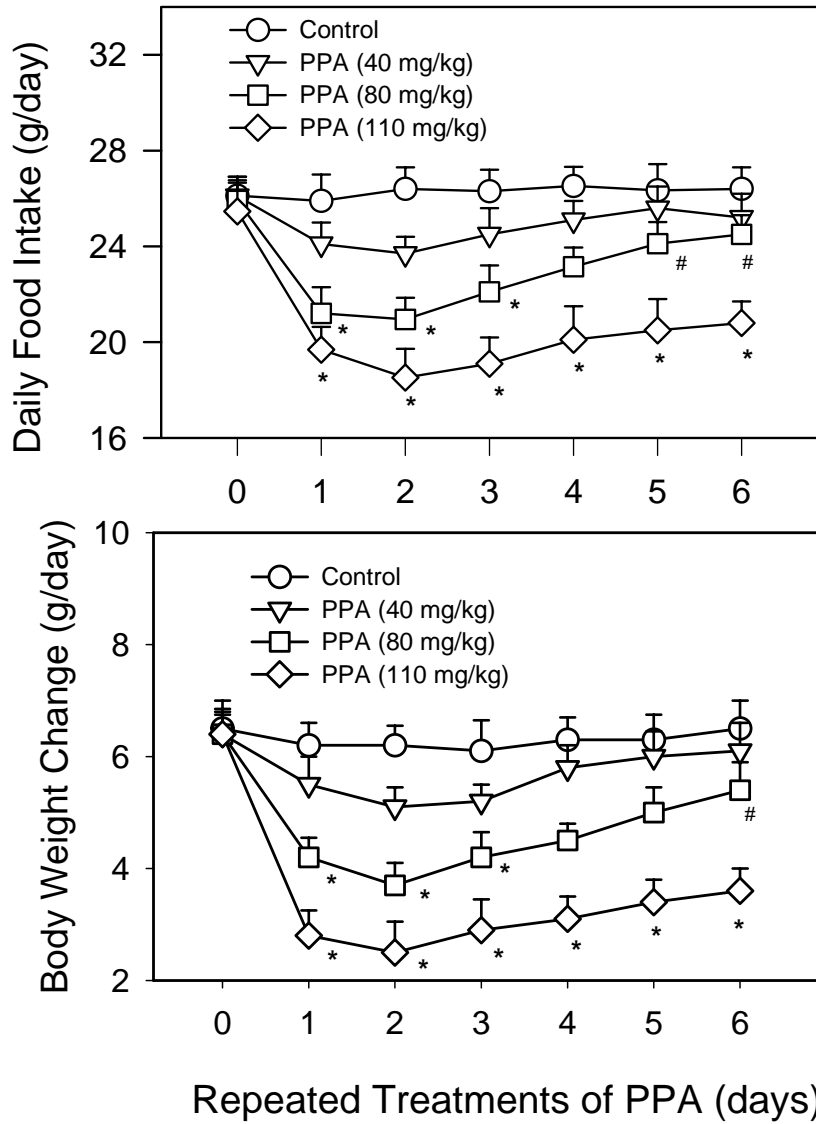


Fig.1

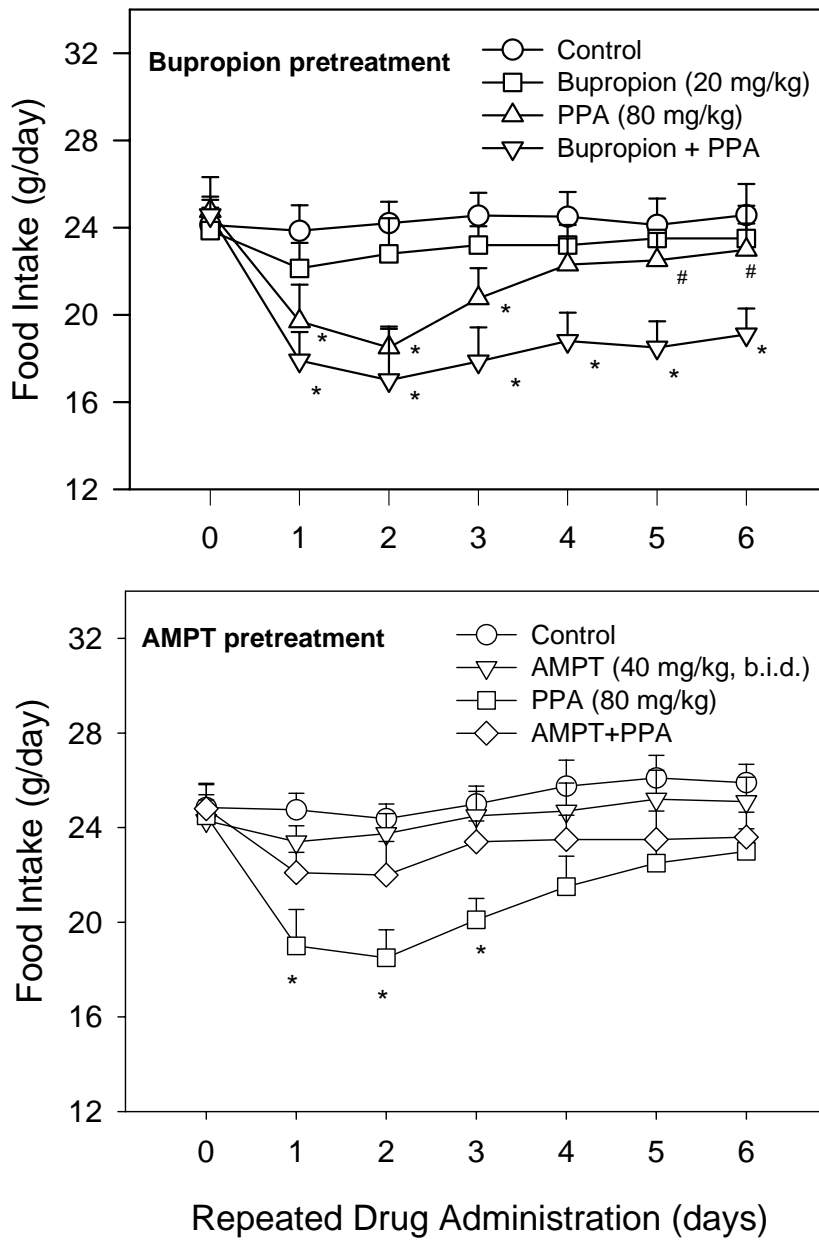


Fig. 2

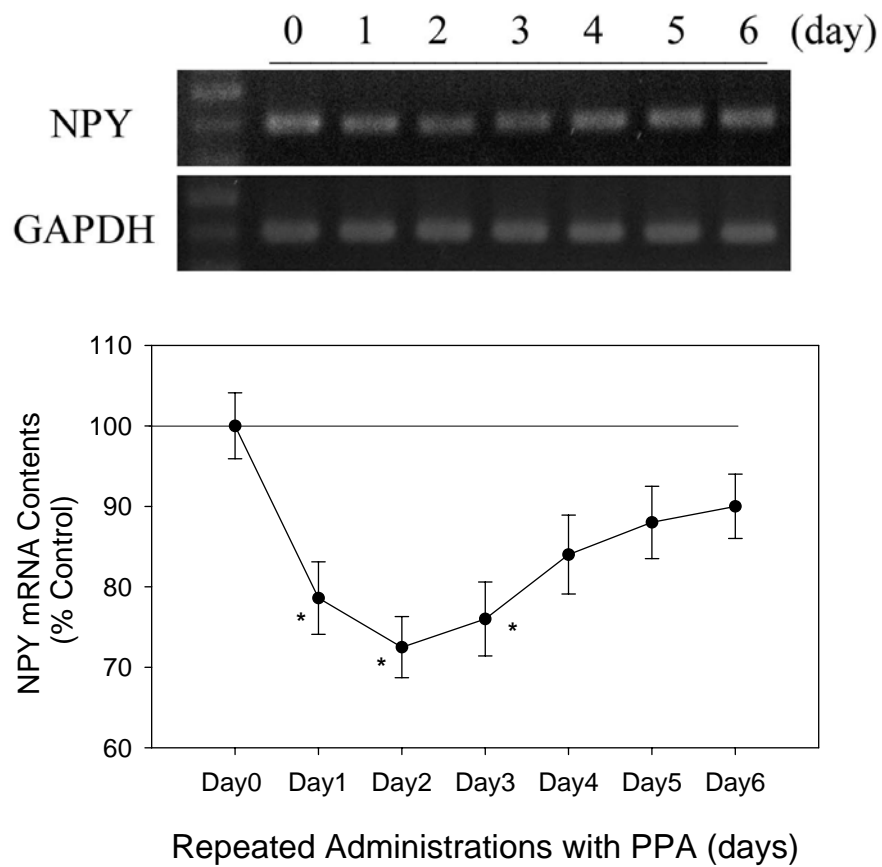


Fig.3

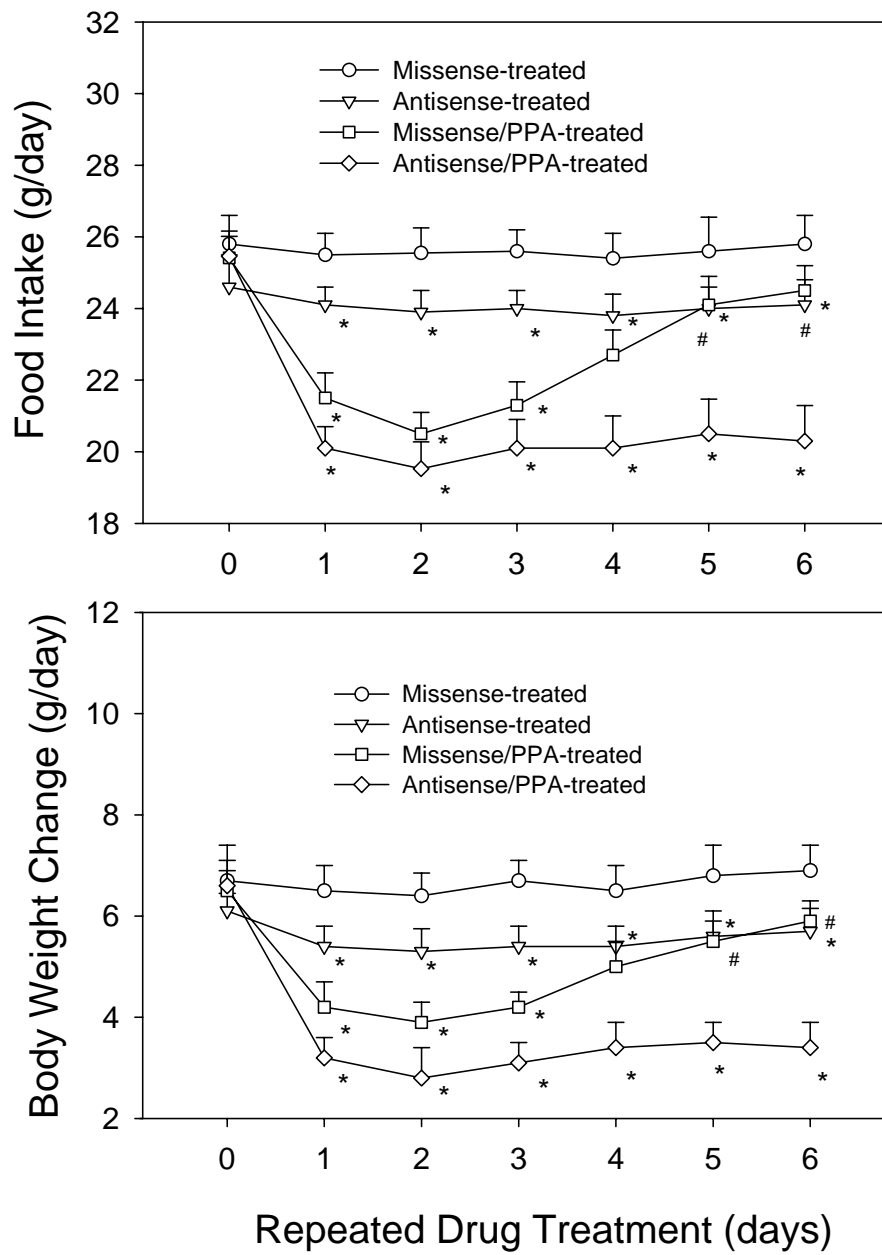


Fig. 4.



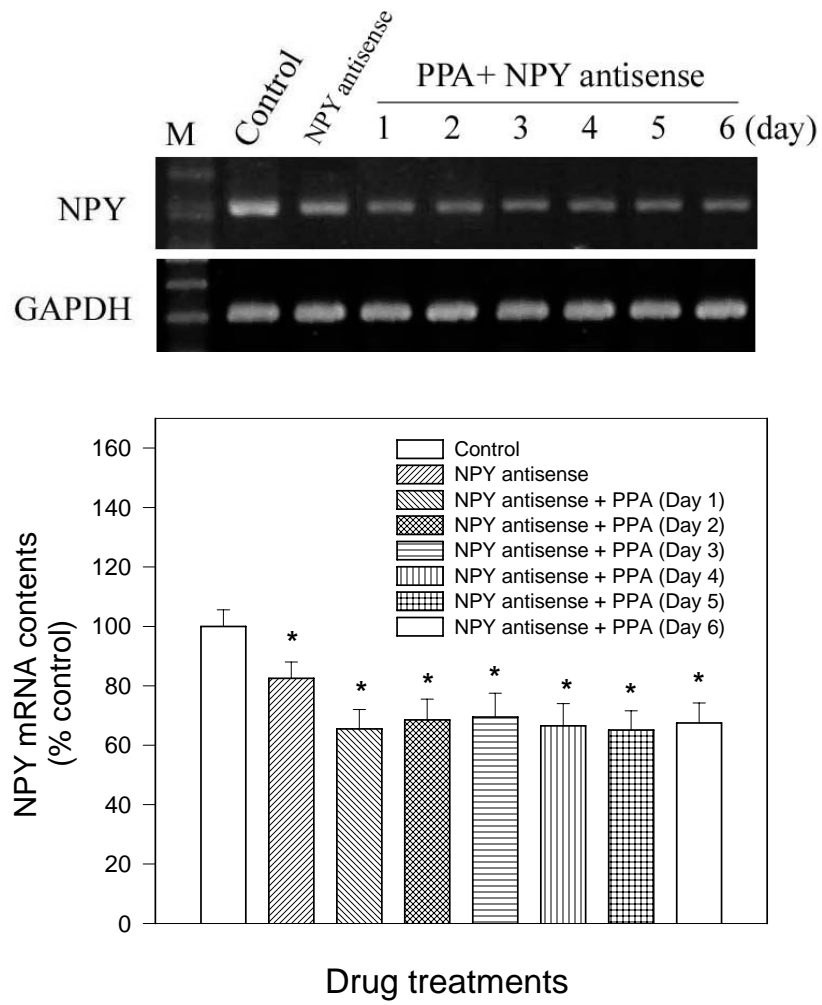


Fig. 5.