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Bicuculline Ameliorated Chronic, but not Acute, Stress-Induced Feeding Suppression

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ABSTRACT

This study was conducted to examine if γ-amino Butyric Acid (GABA)-ergic neurotransmission is implicated in the regulation of stress-induced feeding. Rats received $GABA_A$ receptor antagonist bicuculline before each stress session during 10 days of daily restraint stress. The hypothalamic mRNA expressions of corticotropin-releasing hormone and neuropeptide Y were analyzed by *in situ* hybridization and the plasma corticosterone with radioimmunoassay. Bicuculline ameliorated the decrease in food intake by repeated restraints but not by a single restraint. Corticosterone increase responding to acute stress but not to repeated restraints was attenuated by bicuculline. Stress-induced expression of corticotropin-releasing hormone was blunted by bicuculline pre-treatment. Restraint stress did not affect neuropeptide Y expression, regardless of bicuculline pre-treatment. It is concluded that $GABA_A$ receptors may mediate chronic but not acute, stress-induced suppression in food intake, possibly in relation with anorectic action of the hypothalamic corticotropin-releasing hormone and the hypothalamic neuropeptide Y may not be implicated in its regulatory mechanism.

Key words: Corticosterone, food intake, γ-amino butyric acid, stress

INTRODUCTION

Dysfunction of the Hypothalamic-pituitary-adrenal gland (HPA) axis has been reported to be implicated in the pathogenesis of eating disorders (Putignano *et al*., 2001; Gluck *et al*., 2004). Several human studies have demonstrated that acute stress increases not only the frequency and amount of food intake but also the intake of highly palatable food (Oliver *et al*., 2000; Zellner *et al*., 2006). Whereas, some other studies have reported that stress may result in a decreased energy intake in human (Pollard *et al*., 1995; Adam and Epel, 2007). Stress, in fact, can lead to both under and over-eating and little is known about what determines the direction of eating (Adam and Epel, 2007). In contrast to humans, rats and mice consistently lose weight in response to stress and it has been suggested that decreased food intake and weight loss serve as the most reliable marker of stress severity (Armario, 2006). It has been reported that food intake and weight gain were suppressed on the days of stress in adult rats exposed to restraint stress for 3 h daily for three consecutive days (Harris *et al*., 1998; Miragaya and Harris, 2008).

γ-amino butyric acid (GABA)-ergic neurotransmission has been implicated in the regulation of feeding behaviors, i.e., injections of GABA receptor agonists increase or decrease food intake depending on the experimental conditions (Ward *et al*., 2000; Weerts *et al*., 2005; Meena *et al*., 2009). Studies in rodents indicate that GABAergic neurotransmission is highly sensitive to stressful situations, including moderate

early life stress and chronic stress in adulthood (Orchinik *et al*., 2001; Maggio and Segal, 2009). Variety of stressors increases or decreases GABA contents in the brain regions dysfunction of the hypothalamic-pituitary-adrenal gland (HPA) axis has been reported to be implicated in the pathogenesis of eating disorders (Putignano *et al*., 2001; Gluck *et al*., 2004). Several human studies have demonstrated that acute stress increases not only the frequency and amount of food intake but also the intake of highly palatable food (Oliver *et al*., 2000; Zellner *et al*., 2006). Whereas, some other studies have reported that stress may result in a decreased energy intake in human (Pollard *et al*., 1995; Adam and Epel, 2007). Stress, in fact, can lead to both under and over-eating and little is known about what determines the direction of eating (Adam and Epel, 2007). In contrast to humans, rats and mice consistently lose weight in response to stress and it has been suggested that decreased food intake and weight loss serve as the most reliable marker of stress severity (Armario, 2006). It has been reported that food intake and weight gain were suppressed on the days of stress in adult rats exposed to restraint stress for 3 h daily for three consecutive days (Harris *et al*., 1998; Miragaya and Harris, 2008).

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MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats (200-250 g) were purchased (Orient, Co. Korea) and acclimated to the laboratory condition in a specific-pathogen-free barrier area where the temperature (22±1°C) and humidity (55%) were controlled constantly with a 12/12 h light/dark cycle (lights-on at 07:00 AM). Rats had *ad libitum* access to standard laboratory food (Purina Rodent Chow, Purina Co., Seoul, Korea) and tap water. All rats were habituated to the experimenter at least for 3-5 days before the experiments started. Rats were cared according to the Guideline for Animal Experiments, 2000, edited by the Korean Academy of Medical Sciences which is consistent with the NIH Guidelines for the Care and Use of Laboratory Animals, revised 1996. All animal protocols were approved by the Committee for the Care and Use of Laboratory Animals at Seoul National University.

Experimental protocol and drug treatment: Rats were divided into five groups: No stress control (Veh/NS), single restraint stress (Veh/SRS), single restraint stress with bicuculline pre-treatment (BC/SRS), repeated restraint stress (Veh/RRS) and repeated restraint stress with bicuculline pre-treatment (BC/RRS) (n = 5-8, total 32 rats). Rats were placed in a restraint box for 2 h once (SRS) or once a day repeatedly for 10 days (RRS), during 09:00-11:00 h. In the restraint box, rats were able to move their four limbs but not to change their body orientation. Rats in the NS group were left in their home cage undisturbed. RS rats received an intraperitoneal injection of bicuculline (Sigma-Aldrich Co., MO, USA) at a dose of 0.75 mg kg^{-1} (Ghisleni *et al*., 2008) or aseptic saline 30 min prior to each restraint session. Veh/NS rats received daily injections of aseptic saline omitting restraint stress.

***In situ* hybridization:** One hour after the end of the 1st or 10th restraint session, rats were anesthetized with an overdose of sodium pentobarbital. Once unresponsive, transcardiac perfusion was performed with heparinized isotonic saline (0.9% NaCl and 0.5% $NaNO_2$) followed by ice-cold fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2). Brains were rapidly removed, blocked and post-fixed in the same fixative for 3 h and then transferred into 30% sucrose solution for 24 h for cryoprotection. Coronal sections (40 μm) were cut on a freezing sliding microtome (MICROM Laborgeräte, Walldorf, Germany). Every third sections through the rostral caudal extent of the hypothalamus (between bregma -1.80 and -3.80 mm) (Paxions and Watson, 1986) were collected into 20 mL glass scintillation vials containing ice-cold 2X SSC (0.3 M NaCl and 0.03 M sodium citrate). The SSC was pipetted off and the sections were suspended in 1 mL of prehybridization buffer (50% formamide, 10% dextran sulfate, 2X SSC, 1X Denhardt's solution, 50 mM dithiothreitol and 0.5 mg mL^{-1}

denatured herring sperm DNA), incubated for 2 h at 48°C. *In situ* hybridization was performed with radioactively labeled cDNA probes of CRH (1.0 kb EcoRI restriction fragment) or NPY (0.5 kb EcoRI restriction fragment) (Jahng *et al*., 1998) as we previously described (Choi *et al*., 2003). The tissue sections were then mounted on gelatin-subbed slides, air-dried and apposed to Kodak BioMax film (Eastman Kodak Co., Rochester, NY, USA) at 4°C. Exposure times varied from 12 to 48 h to obtain autoradiographic images within a linear range of optical density after development in Kodak D-19 developer (Eastman Kodak Co.). *In situ* hybridization was carried out on the representative members of each experimental group at the same time under identical conditions, allowing direct comparison of mRNA expression.

Plasma corticosterone assay: Tail bloods were collected at 0, 20, 60, 120 min time points during the first or the 10th restraint session. All blood samples were centrifuged at 2,000 g for 20 min at 4°C and the plasma was transferred into new tubes and stored at -80°C until assayed. Plasma levels of corticosterone were determined by radioimmunoassay using Coat-A-Count kit (DPC Co., Los Angeles, CA, USA). To minimize diurnal variation in the plasma hormone levels, rats were subjected to restraint stress during 900-1200 h.

Quantitative and statistical analysis: Images on the autoradiographic films were digitized with a Zeiss Stemi-2000 stereoscope attached to a Dage-MTI CCD 72 camera and MCID image analysis system (MCID Imaging Research Inc., Ontario, Canada). Messenger RNA (mRNA) expression level was determined by quantifying the mean relative optical density of pixels with densities of at least 2 s.d. above the mean density of the image background (mRNA pixels). For each section, the mean background value was subtracted from the mean mRNA pixel value. The mRNA pixel values were averaged across six sections from each individual rat and the average mRNA values of each rat were then averaged across all the rats within each experimental group. The average mRNA values of each experimental group were then converted to relative values to their control groups.

Statistical analysis: Data was analyzed by one-way, two-way or repeated measures analysis of variance and preplanned comparisons with the controls performed by post hoc Fisher's Protected Least Significant Difference LSD) test using StatView software (Abacus, Berkeley, CA, USA). Values are presented by Means±SEM For all comparisons, the level of significance was set at $p \leq 0.05$.

RESULTS

Body weight gain of rats was significantly suppressed by repeated restraint stress (Fig. 1a). Repeated measures ANOVA revealed significant difference between no stress controls

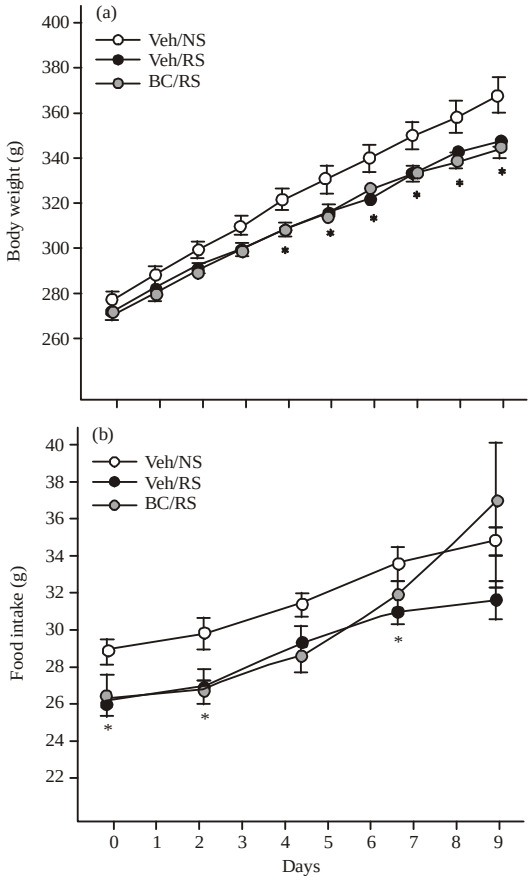

Fig. 1(a-b): Body weight gain (a) Food intake (b) During the experimental period. Rats received 2 h of restraint stress daily for 10 days with an intraperitoneal injection of bicuculline (0.75 mg kg^{-1}) or aseptic saline 30 min prior to each restraint session. No stress control rats received daily injections of aseptic saline omitting restraint stress. Veh/NS: Vehicle injection and no stress, Veh/RS: Vehicle injection and restraint stress, BC/RS: Bicuculline injection and restraint stress, *p<0.05L: Veh/NS vs. Veh/RS, Data are presented as Means±SEM

(Veh/NS) and stressed (Veh/RS) rats [F(1, 9) = 6.472, p<0.0001]. Bicuculline prior to each restraint session did not rescue the stress-induced weight loss. Daily food intake of Veh/RS rats was decreased significantly (p<0.05) on day 1, 3 and 7 as compared with Veh/NS rats (Fig. 1b). Daily food intake of BC/RS rats did not differ from Veh/RS rats until day 7. On day 9, food intake of BC/RS rats tended to be increased relative to Veh/RS rats but statistical significance was not found between the groups (Fig. 1b). Total amount of food consumed during the whole experimental period was significantly reduced (p<0.05) in Veh/RS group (259.39±2.38 g) but not in BC/RS group (268.97±7.11 g), compared to Veh/NS group (284.10±5.65 g).

NPY mRNA levels in the arcuate nucleus (ARC) tended to be decreased after 2 h of single restraint stress with near significance ($p = 0.058$, Veh/NS vs. Veh/RS) which was not influenced by bicuculline pre-treatment (Fig. 2). Two hours of restraint stress with or without bicuculline pre-treatment did not alter NPY mRNA levels in the Dorso-Medial Hypothalamus (DMH). NPY expression levels neither in the ARC nor in the DMH were changed after the 10 sessions of daily restraint stress (Fig. 2).

Tail bloods were collected at 0, 20, 60 and 120 min time points during the restraint session and used for plasma corticosterone assay (Fig. 3). The plasma corticosterone level was significantly increased at 20 and 60 min time points ($p<0.05$ vs. 0 time point in Veh/RS) after the onset of the 1st restraint session (Fig. 3a). However, in bicuculline pre-treated rats (BC/RS), a significant increase in the plasma corticosterone levels was observed only at 20 min time point ($p<0.05$ vs. 0 time point). A two-way ANOVA with a

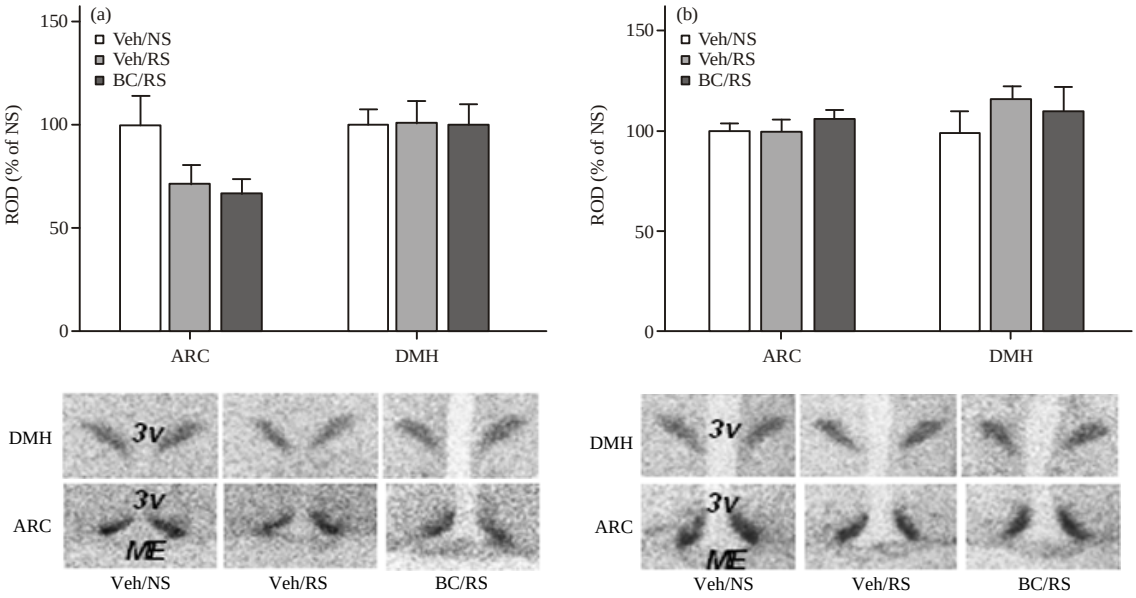

Fig. 2(a-b): Autoradiography and quantitative analysis of NPY mRNA *in situ* hybridization in the arcuate nucleus (ARC) and dorso-medial hypothalamus (DMH). Rats were transcardially perfused at 1 h after the end of the (a) 1st (single restraint) or the (b) 10th (repeated restraints) restraint session. Veh/NS: Vehicle injection and no stress, Veh/RS: Vehicle injection and restraint stress, BC/RS: Bicuculline injection and restraint stress, ROD: Relative optical density, 3v: 3rd ventricle, ME: Median eminence, Data are presented as Means±SEM

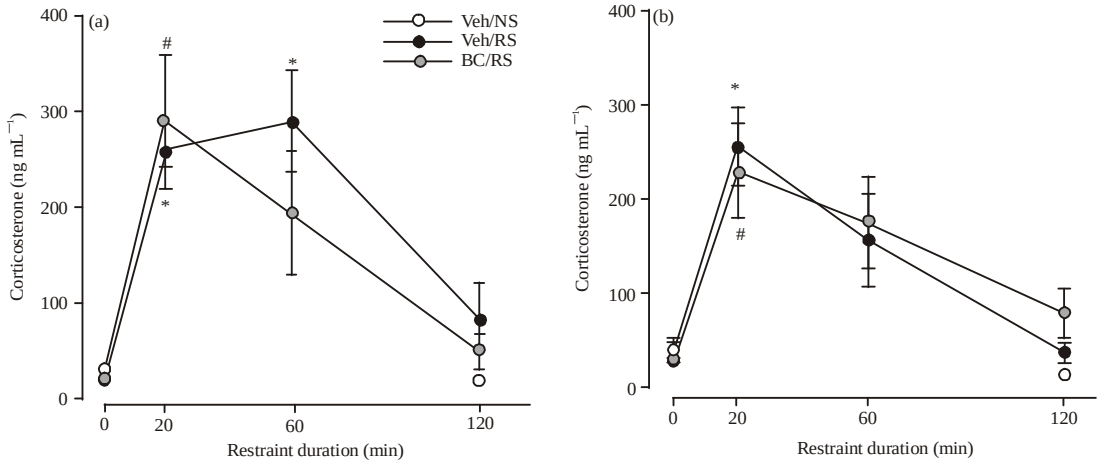

Fig. 3(a-b): Plasma corticosterone levels. Tail bloods were collected at 0, 20, 60 and 120 min time points during the (a) 1st or the (b) 10th Restraint session. Veh/NS: Vehicle injection and no stress, Veh/RS: Vehicle injection and restraint stress, BC/RS: Bicuculline injection and restraint stress, *$p<0.05$ vs. 0 time point in Veh/RS, #$p<0.05$ vs. 0 time point in BC/RS, Data are presented as Means±SEM

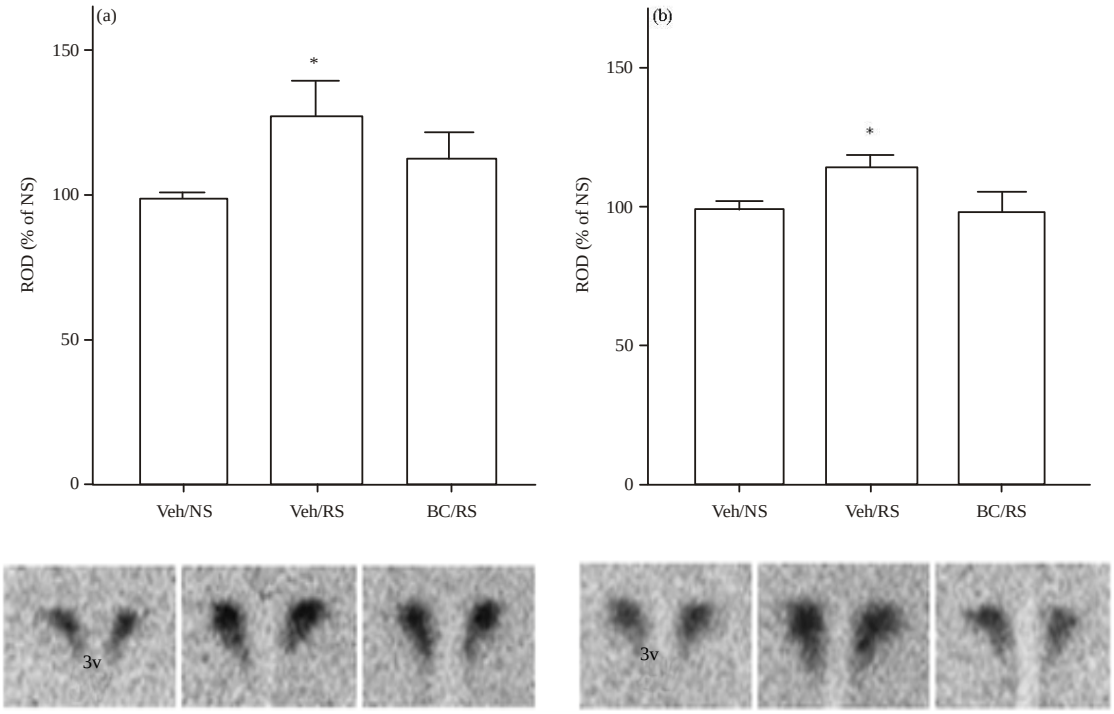

Fig. 4(a-b): Autoradiography and quantitative analysis of CRH mRNA *in situ* hybridization in the hypothalamic paraventricular nucleus. Rats were sacrificed at 1 h after the end of the (a) 1st (single restraint) or the (b) 10th (repeated restraints) restraint session. Veh/NS: Vehicle injection and no stress, Veh/RS: Vehicle injection and restraint stress, BC/RS: Bicuculline injection and restraint stress, ROD: Relative optical density, 3v: 3rd ventricle, *p<0.05 vs. Veh/NS, Data are presented as Means±SEM

split-plot design showed a main effect of time [$F_{(1,35)}$=25.591, p<0.0001] but no interaction between drug and time. During the 10th restraint session, a significant increase in the plasma corticosterone levels was observed only at 20 min time point (p<0.05 vs. 0 time point) both in Veh/RS and BC/RS rats (Fig. 3b). A two-way ANOVA with a split-plot design showed a main effect of time [$F_{(1,40)}$=14.820, p<0.0001] but no interaction between drug and time. Further analysis of the restraint-induced corticosterone increase during the 1st and the 10th restraint session with two-way ANOVA with a split-plot design revealed significant main effects of stress [$F_{(1,40)}$=5.931, p<0.05] and time [$F_{(1,40)}$=26.321, p<0.0001].

CRH mRNA levels in the hypothalamic PVN were examined with *in situ* hybridization after a single or 10 sessions of restraint stress (Fig. 4). Two hours of restraint stress significantly increased the PVN-CRH mRNA levels (p<0.05 vs. Veh/NS) and this increase was blunted by bicuculline pre-treatment (Fig. 4a). CRH mRNA expression in the PVN was significantly increased after the 10th restraint session (p<0.05 vs. Veh/NS) and the restraint-induced increases of the PVN-CRH was blunted by bicuculline pre-treatments (Fig. 4b).

DISCUSSION

Many studies have reported that GABAergic neurotransmission is involved in the regulation of feeding behaviors and the HPA axis activities (Cullinan and Wolfe, 2000; Ward *et al*., 2000; Herman *et al*., 2003; Kovacs *et al*., 2004), suggesting an important role of GABAergic system in the regulation of stress-induced eating. It has been reported that stress activates GABAergic neurons which project to the hypothalamic paraventricular nucleus (Cullinan *et al*., 1995) and GABA agonists have been shown to increase stress-induced corticosterone levels (Borycz *et al*., 1992; Sarkar *et al*., 2011). Stress-induced increase of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD65) mRNA was observed in the anterior hypothalamic nucleus (Bowers *et al*., 1998). Also, restraint or immobilization stress increased the GABA contents in the nucleus accumbens and hypothalamus (Yoneda *et al*., 1983; Noh *et al*., 2012), the brain regions deeply implicated in feeding behaviors and the HPA axis activity. It was reported that acute restraint stress causes a depolarizing shift in the reversal potential of $GABA_A$ receptors, resulting to convert GABA synapses to excitatory (Sarkar *et al*., 2011). In this

study, repeated treatment with bicuculline, a potent $GABA_A$ receptor antagonist, ameliorated the stress-induced suppression of food intake, although it did not rescue the stress-induced weight loss. Together, it is suggested that the activation of GABAergic neurons projecting to the hypothalamus which is mediated by $GABA_A$ receptor may be implicated in the stress-induced suppression of food intake but not of body weight gain.

Studies have reported that GABAergic neurotransmission is implicated in the regulation of the HPA axis responses to stressors (Herman *et al*., 2003; Kovacs *et al*., 2004; Briones-Aranda *et al*., 2005). The majority of the local synaptic inputs to the hypothalamic PVN neurons are GABAergic (Decavel and van den Pol, 1990; Herman *et al*., 2003) and a subunit of the $GABA_A$ receptors distributed within the rat PVN have been confirmed to be expressed in the neurons containing CRH, a major stress hormone released by the HPA axis activation (Cullinan, 2000). It was reported that bicuculline reduces acute corticosterone responses to restraint stress (Jones *et al*., 2011). In this study, bicuculline pre-treatment blunted corticosterone response to single restraint stress but not to repeated restraints, suggesting that the hypothalamic $GABA_A$ receptors is involved in the corticosterone response, if any, to acute restraint stress but not to repeated restraints. Increased plasma corticosterone appears to be related with stress-induced suppression in food intake (Armario, 2006; Ortolani *et al*., 2011) and systemic injection of glucocorticoids has been reported to suppress food intake and weight gain (De Vos *et al*., 1995; Jahng *et al*., 2008; Liu *et al*., 2011). In this study, stress-induced suppression in food intake persisted throughout the whole experimental period, however, the basal corticosterone level was not increased following repeated restraint stress. Furthermore, bicuculline pre-treatment attenuated corticosterone response to single restraint but not to repeated restraints, whilst its effect on food intake was observed only after repeated restraints but not single restraint. It is concluded that $GABA_A$-mediated corticosterone response to acute stress may not affect food intake and the plasma corticosterone may not be involved in the long-term effect of bicuculline on stress-induced anorexia.

Stress hormones such as CRH, ACTH and corticosterone secreted by the HPA axis activation have been related in regulations of the feeding mechanism and rate of energy expenditure (Oliver *et al*., 2000; Putignano *et al*., 2001; Gluck *et al*., 2004; Zellner *et al*., 2006). CRH potently inhibits food intake through type 2 CRH receptors in the ventromedial hypothalamus and stimulates energy expenditure via the sympathetic nervous system (Richard *et al*., 2000; Makino *et al*., 2003). Pre-treatment with CRH antagonists reversed stress-induced anorexia (Krahn *et al*., 1986; Smagin *et al*., 1999). In this study, CRH expression in the hypothalamic PVN was significantly increased either by a single or by repeated restraints and these increases were blunted by a $GABA_A$ receptor antagonist bicuculline pre-treatment. It should be noticed that bicuculline rescued anorexia by repeated restraints but not by acute restraint, in this study. Thus, it is suggested that $GABA_A$ receptor may mediate CRH-induced anorexia, if any, by repeated restraints but not one by acute restraint.

NPY which is the most potent orexigenic neuropeptides acting in the hypothalamic arcuate nucleus (ARC), stimulates the food intake (Schwartz *et al*., 2000). NPY mRNA expression in the ARC is increased during negative energy state (Brady *et al*., 1990; Kim *et al*., 2005; Ryu *et al*., 2008; Yoo *et el*., 2011) and NPY expression in the dorso-medial hypothalamus has been implicated in energy expenditure (Yang *et al*., 2009; Bi *et al*., 2012). GABA and NPY are co-expressed in the same neurons of several brain regions, including the hypothalamus (Horvath *et al*., 1997; Oberto *et al*., 2001). These reports together suggest that the hypothalamic NPY expression may be implicated in stress-induced anorexia, possibly in relation with GABAergic action. However, neither the single nor the repeated restraints affected the hypothalamic NPY expressions in this study, regardless of bicuculline pre-treatment. Consistently, acute or repeated restraints reduced NPY expression in the brain area excluding the hypothalamus (Thorsell *et al*., 1998, 1999) and acute or subchronic diazepam, a typical anxiolytic acting on the $GABA_A$ receptor, did not affect the hypothalamic NPY immunoreactivity (Krysiak *et al*., 1999). Thus, it is concluded that the hypothalamic NPY expression may not play a role in the underlying mechanism of stress-induced anorexia, regardless of $GABA_A$ mediation.

CONCLUSION

$GABA_A$ receptors may mediate chronic but not acute, stress-induced suppression in food intake, possibly in relation with anorectic action of the hypothalamic CRH.

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