

## **5-hydroxy-L-tryptophan Suppressed Food Intake in Rats Despite an Increase in the Arcuate NPY Expression**

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### **ABSTRACT**

This study was conducted to define the underlying mechanism of hypophagia induced by increased central serotonergic action. Rats received 3 daily injections of 5-hydroxy-L-tryptophan (5-HTP), a serotonin precursor, at a dose of 100 mg/kg/10 ml saline at 1 h before lights off. A significant suppression in food intake was observed shortly after the 5-HTP injection and persisted during 3 daily 5-HTP injections. Neuropeptide Y (NPY) expression in the arcuate nucleus increased after 3 days of 5-HTP treatment, as high as in the pair-fed group. Immunoreactivity of phosphorylated extracellular signal-regulated protein kinase (pERK1/2) in the hypothalamic paraventricular nucleus (PVN) was increased markedly by 3 days of 5-HTP treatment, but not by 3 days of pair-fed. mRNA expression levels of serotonin reuptake transporter (5-HTT) was increased in the dorsal raphe nucleus of the 5-HTP treated rats, but not in the pair-fed group. Results suggest that increased pERK1/2 in the PVN of 5-HTP injected rats may be a part of serotonergic anorectic signaling, perhaps blunting the orectic action of NPY; i.e., 5-HTP injected rats showed hypophagia despite of increased NPY expression in the arcuate nucleus.

**Key words:** food intake, hypophagia, hypothalamus, serotonin

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### **INTRODUCTION**

Serotonin, 5-hydroxytryptamine (5-HT), known to play a role in feeding behavior as an anorectic molecule (Curzon, 1990) has been implicated in the

processes of within-meal satiation and post-meal satiety (Halford and Blundell, 2000). The hypothalamus appears to be where 5-HT exerts its anorectic effect in the central control of feeding, perhaps, at least partly, through its interaction with the hypothalamic feeding peptides. It has been reported that 5-HT exhibits a negative correlation with neuropeptide Y (NPY), a potent orexigenic molecule, in the hypothalamus (Dryden et al., 1995; 1996; Jahng et al., 1998b; Currie et al., 2002). We

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have previously reported that the hypothalamic expression of NPY was significantly decreased in anorectic (*anx/anx*) mouse showing drastic activation of the central 5-HT system (Jahng et al., 1998b). Previous studies reported that metergoline, a 5-HT<sub>1</sub>/5-HT<sub>2</sub> receptor antagonist, and 8-hydroxy-2-(di-n-propylamino) tetralin, a 5-HT<sub>1A</sub> receptor agonist, enhances food consumption (Coscina et al., 1994; Currie and Coscina, 1996; Voigt et al., 2002), and that 5-HT<sub>1A</sub> receptor immunoreactivity is observed in the hypothalamic arcuate neurons containing NPY (Collin et al., 2002). Taken together, it is suggested that anorexic effects of the brain 5-HT system may comprise decreased NPYergic activity in the hypothalamus.

NPY dose-dependently increased cellular level of phosphorylated extracellular signal-regulated protein kinase (pERK1/2) in cultured cells (Gur et al., 2002). It has been shown that food deprivation increases neuronal level of pERK1/2 in the hypothalamic paraventricular nucleus (PVN) of mice (Ponsalle et al., 1992) and rat (Ueyama et al., 2004; Lee et al., 2010). Food deprivation increases not only NPY mRNA expression in the arcuate nucleus (Brady et al., 1990; Swart et al., 2002; Kim et al., 2005; Lee et al., 2010) but also release in the PVN (Dube et al., 1992; Yoshihara et al., 1996). Neurons in the PVN are richly supplied by axons of NPY neurons from the arcuate nucleus (Elmqvist et al., 1998; 1999). Thus, if the PVN neurons are second order effectors located downstream of the arcuate nucleus, pERK1/2 could, possibly, be a part of NPY downstream signaling cascade in the PVN during food deprivation.

In order to determine if pERK1/2 is a putative downstream effector of NPY signaling in the hypothalamic PVN, possibly related with increased brain 5-HT level, 5-hydroxy-L-tryptophan (5-HTP), a 5-HT precursor, was administered to rats to increase the brain 5-HT level (Gartside et al., 1992; Yamada et al., 2000; Choi et al., 2003).

## MATERIALS AND METHODS

### *Animals*

Adult male Sprague-Dawley rats (250~300 g, Daehanbiolink Co., Korea) were individually acclimated to the standard laboratory conditions (12 h

light-dark cycle, light on at 9:00 AM) with free access to standard laboratory food (Purina Rodent Chow, Purina Co., Seoul, Korea) and water *ad libitum*. Animals were cared according to The Guideline for Animal Experiments, 2000, edited by The Korean Academy of Medical Sciences, which is consistent with NIH Guideline for the Care and Use of Laboratory Animals, 1996 revised.

### *Drug treatments*

Rats were divided into three treatment groups (n=6/group/time point), such as the control, the 5-hydroxy-L-tryptophan (5-HTP; 100 mg/kg/10 ml, dissolved in sterile physiologic saline; Sigma Co., MO, USA) injected or the pair-fed group. The 5-HTP group received a single intraperitoneal injection of 5-HTP at 1 h before lights off or three daily injections at 9:00 AM every morning. The control group received the same volume of saline instead of 5-HTP at each time point. The pair-fed group was provided with the same amount of food consumed by 5-HTP rats. Rats were sacrificed at 2 or 8 h after the single injection of 5-HTP, or 24 h after the last injection of daily 5-HTP. Total 48 rats (18 control, 18 5-HTP, and 12 pair-fed) were hired for this study.

### *In situ hybridization*

Rats were anesthetized with an overdose of sodium pentobarbital. Once unresponsive, transcardiac perfusion was performed with heparinized isotonic saline containing 0.5% NaNO<sub>2</sub>, then with 4% paraformaldehyde in 0.1 M sodium phosphate buffer. The brains were rapidly dissected, blocked, post-fixed for 3 h, and transferred into 30% sucrose for 24 h for cryoprotection. Forty-micron coronal sections were cut on a freezing sliding microtome. Every other sections through the rostral-caudal extent of the hypothalamus (between bregma -1.80 mm and -3.80 mm; Paxinos and Watson, 1986) and the raphe nucleus (between bregma -7.64 mm and -8.80 mm; Paxinos and Watson, 1986) were collected into 20 ml glass scintillation vials containing ice-cold 2×SSC (0.3 M NaCl, 0.03 M Na Citrate) for *in situ* hybridization. The SSC was pipetted off, and sections were suspended in 1 ml of prehybridization buffer (50% formamide, 10% dextran sulfate, 2×SSC, 1×Denhardt's solution, 50 mM

DTT, and 0.5 mg/ml denatured herring sperm DNA), incubated for 2 h at 48°C. *In situ* hybridization was performed with radioactively labeled cDNA probes of NPY (Jahng et al., 1998b; for the arcuate sections) or serotonin reuptake transporter (5-HTT, Jahng et al., 1998a; for the raphe sections) 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., 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. *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.

#### **Immunohistochemistry**

Free-floating tissue sections were washed twice for 15 min in 0.1 M sodium phosphate buffered saline (PBS), and then permeabilized in 0.2% Triton, 1% bovine serum albumin (BSA) in PBS for 30 min. After washing twice in PBS-BSA, sections were incubated overnight with anti-rabbit phospho-p44/42 MAPK (Thr202/Tyr204) antibody (1 : 300 dilution, Cell signaling, Beverly, MA, USA). Sections were washed in PBS-BSA twice and incubated for 1 h with biotinylated anti-rabbit-goat antibody (Vector Laboratories, CA, USA); bound secondary antibody was then amplified with commercial ABC kit (Vectastain Elite Kit, Vector Laboratories, CA, USA). Antibody complexes were visualized by a 5 min 0.05% diaminobenzadine reaction. Immunostained sections were mounted onto gelatin-coated slides, air dried overnight, consequently dehydrated through a graded ethanol to xylene, and then cover-slipped with Permount.

#### **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). Tissue levels of NPY, 5-HTT mRNA, or pERK were determined by quantifying the mean relative optical density of pixels with densities of at least 2 S.D. above the mean density

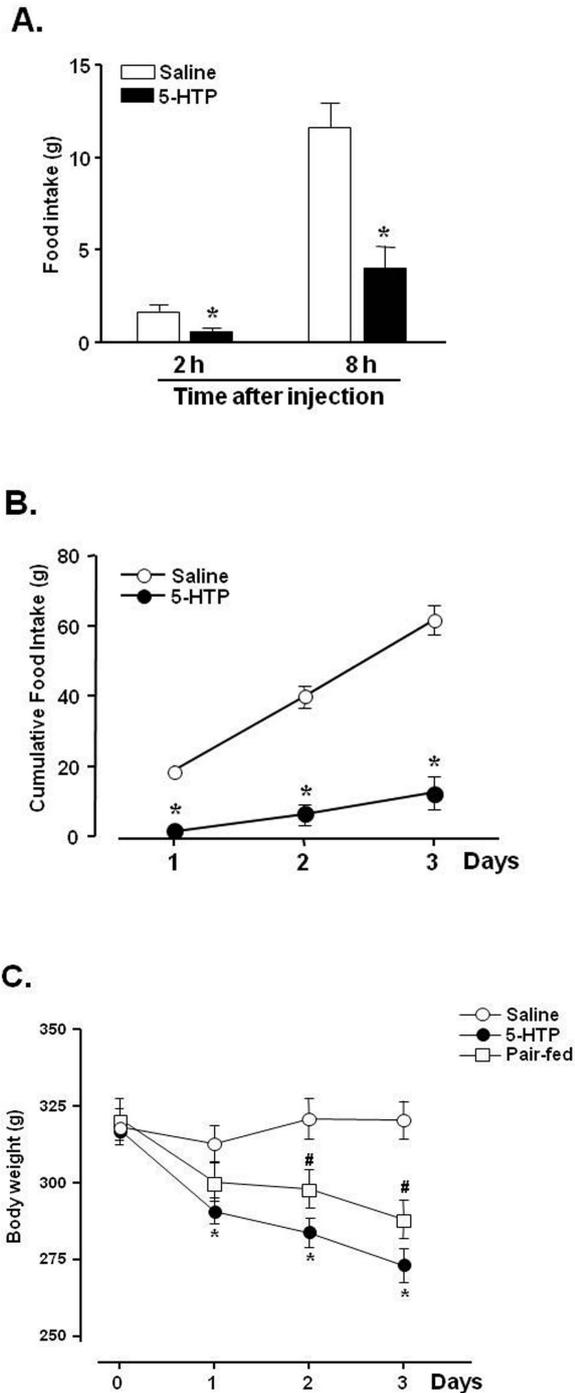
of the image background (mRNA or protein pixels). For each section, the mean background value was subtracted from the mean pixel value. The pixel values were averaged across three sections from each individual rat and then the average value of each rat averaged across all rats in each experimental group. All the data was analyzed by one way analysis of variance (ANOVA) and preplanned comparisons with the control were performed by *post-hoc* Fisher's PLSD test or unpaired t-test using StatView software (Abacus, Berkeley, CA). Significance was set at  $p < 0.05$ , and all values were presented as means  $\pm$  SEM.

## **RESULTS**

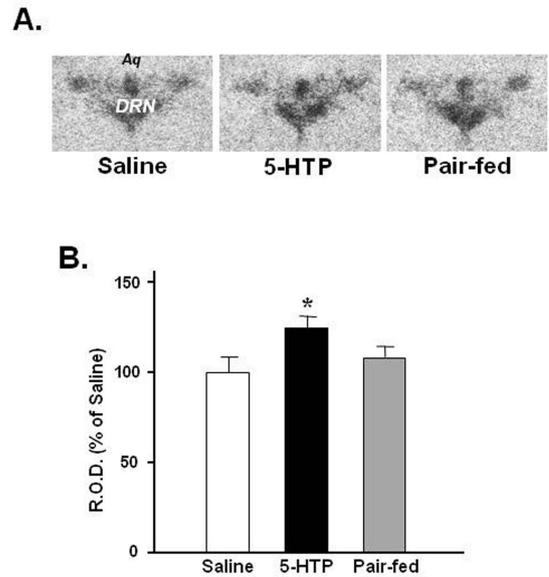
Rats received an intraperitoneal injection of 5-HTP (100 mg/kg/10 ml saline) or saline at 1 h before lights off, and the spontaneous feeding with *ad libitum* access to food (standard rodent chow) and water for 2 or 8 h were measured. Food intake of 5-HTP injected rats decreased significantly ( $p < 0.05$ ) compared with the saline injected controls at both time points (Fig. 1A). Cumulative food intake was suppressed persistently ( $p < 0.05$  vs. saline on each day) during 3 daily administrations of 5-HTP (Fig. 1B). Weight losses in 5-HTP rats compared with saline controls appeared to be more severe than in its pair-fed group (Fig. 1C).

mRNA expression levels of 5-HT reuptake transporter 5-HTT in the dorsal raphe nucleus, where most of 5-HT neurons in the brain are located, were examined after 3 days of 5-HTP treatment when not only the 5-HTP group but also the pair-fed group showed significant reductions in body weight compared to their saline controls. 5-HTT mRNA levels increased in the 5-HTP treated group ( $p < 0.05$ ), but not in its pair-fed group, as compared with the free-fed saline group (Fig. 2), suggesting an increased 5-HTergic activity in the brain by 5-HTP injections, but not by pair-feeding.

A single injection of 5-HTP appeared not to acutely affect NPY mRNA expression levels in the arcuate nucleus; i.e., the arcuate NPY mRNA level did not differ from the saline group at 2 or 8 h after a single injection (Fig. 3). Three days of 5-HTP treatment significantly increased NPY expression levels in the arcuate nucleus ( $p < 0.05$  vs. saline),



**Fig. 1.** Food intake and body weight gain during 3 daily 5-HTP injections. (A) Food intake after a single 5-HTP injection. (B) Cumulative food intake during three daily 5-HTP injections. (C) Body weight changes during the injection period. Intraperitoneal injections of 5-HTP were given at a dose of 100 mg/kg/10 ml saline daily at 1 h before the lights off. Control group received the same volume of injections omitting 5-HTP. Pair-fed rats (Pair-fed) received the same amount of food that the 5-HTP rats consumed daily. 5-HTP; 5-hydroxy-L-tryptophan, \* $p < 0.05$ ; 5-HTP vs. Saline at each time point, # $p < 0.05$ ; Pair-fed vs. Saline at each time point.



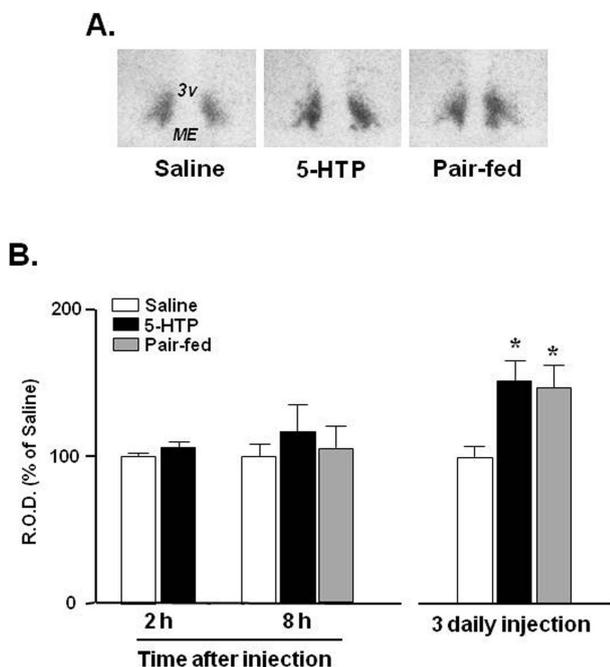
**Fig. 2.** 5-HTT mRNA *in situ* hybridization after three daily 5-HTP or pair-fed, Representative autoradiographies (A) and quantitative analyses of 5-HTT *in situ* signals on the films (B), Aq: aqueduct, DRN: dorsal raphe nucleus, \* $p < 0.05$  vs. saline control.

and this increase was also observed by 3 days of pair-fed (Fig. 3B), suggesting that the increased NPY expression is a consequence of chronic weight loss.

Rats were sacrificed 24 h after the last injection of 3 daily 5-HTP and the tissue sections of the paraventricular nucleus (PVN), where NPYergic fibers are richly innervated from the arcuate nucleus, were processed for pERK immunohistochemistry (Fig. 4A). pERK levels in the PVN markedly increased ( $p < 0.05$ ) in 5-HTP rats, but not in pair-fed rats, compared to their saline controls (Fig. 4B).

## DISCUSSION

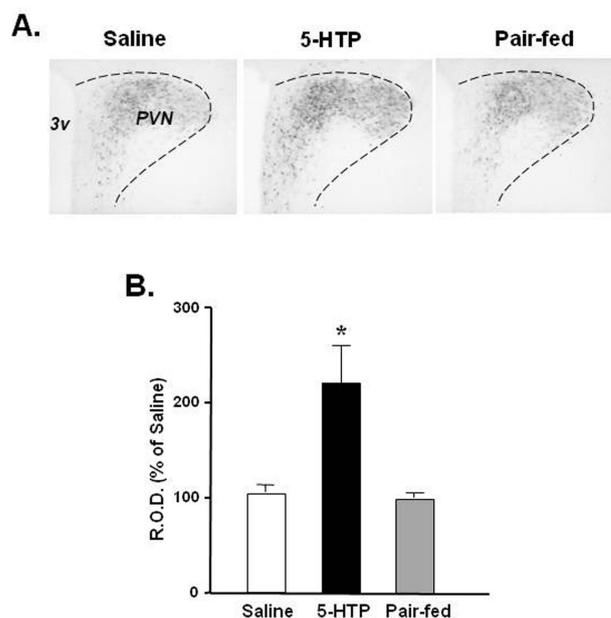
We have demonstrated that 5-HTP injections persistently suppresses food intake and induces weight loss, in accordance with our previous report suggesting that anorexia by 5-HTP injections is due to increased 5-HTergic activities in the brain regions (Choi et al., 2003). Weight losses in 5-HTP injected rats appeared to be bigger than in its pair-fed group, consistently with our previous report (Choi et al., 2003), suggesting an additional anorectic effect of the 5-HTP injection, other than decreasing energy intake. Previous studies have reported that



**Fig. 3.** Representative autoradiography of NPY *in situ* hybridization in the hypothalamic arcuate nucleus of rats received 3 daily 5-HTP injections or pair-fed (A), and relative optical density of the *in situ* signals on the autoradiographic films (B). 3v: third ventricle, ME: median eminence, \* $p < 0.05$  vs. saline control.

5-HT may increase the resting energy expenditure (Leibowitz and Alexander, 1998; Walsh et al., 1999). That is, not only decreased energy intake but also increased energy expenditure may contribute to the weight loss by systemic 5-HTP.

In this study, 5-HTT mRNA expression in the dorsal raphe nucleus was increased by 3 days of 5-HTP treatment, but not by 3 days of pair-fed (food restriction). Previous studies have reported that long-term food restriction decreases the brain density (Huether, 1999) and expression levels of 5-HTT (Haider and Haleem, 2000; Jahng et al., 2007), and that the depletion of brain 5-HT with chronic para-chlorophenylalanine treatment decreases 5-HTT mRNA expression in the raphe nucleus (Linnet et al., 1995; Yu et al., 1995). Also, we have reported that increased 5-HTT mRNA expression in the raphe nucleus by 3 daily 5-HTP injections is accompanied with increased 5-HT levels in the brain regions (Choi et al., 2003). Thus, it is concluded that increased 5-HTT mRNA expression in the dorsal raphe of 5-HTP treated rats may not be a consequence of reduced food intake and weight loss, and it reveals an increased 5-HTergic activity,



**Fig. 4.** pERK immunoreactivities (-ir) in the hypothalamic paraventricular nucleus after 3 daily 5-HTP or pair-fed. Representative photos of pERK1/2 immunohistochemistry (A) and the quantitative analyses of pERK1/2-ir (B), 3v: third ventricle, PVN: paraventricular nucleus, \* $p < 0.05$  vs. saline control.

likely due to increased 5-HT levels in the brain regions.

Previous studies have reported that 5-HT exhibits a negative correlation with NPY in the hypothalamus (Dryden et al., 1995; 1996; Jahng et al., 1998b; Currie et al., 2002), suggesting that anorexic effects of the brain 5-HT system may comprise decreased NPYergic activity in the hypothalamus. However, in this study, NPY mRNA expression in the arcuate nucleus markedly increased after 3 daily injections of 5-HTP when the hypothalamic 5-HT level increased (Choi et al., 2003). Although a single injection of 5-HTP instantly increased the hypothalamic 5-HT levels (Choi et al., 2003), the arcuate NPY expression was not acutely affected by a single 5-HTP injection in this study, suggesting that increased NPY expression in the arcuate nucleus with 3 daily injections of 5-HTP is not a direct effect of increased 5-HT neurotransmission in the hypothalamus. Meanwhile, the arcuate NPY expression was increased in the pair-fed (food restriction) group as much as in the 3 daily 5-HTP group. A negative energy balance, such as food restriction or food deprivation, increases NPY mRNA expression in the hypothalamic arcuate

nucleus (Ponsalle et al., 1992; Bi et al., 2003; Makimura et al., 2003; Jahng et al., 2005). Thus, it is concluded that the increased NPY expression in the arcuate nucleus of 5-HTP treated rats is a consequence of decreased food intake, a negative energy balance, rather than of increased 5-HTergic activity in the hypothalamus. The hypothalamic NPY, a potent orexic peptide, stimulates feeding (Stanley and Leibowitz, 1985; Kalra et al., 1999; Schwartz et al., 2000), and increased NPY expression in the arcuate nucleus correlates with obese phenotype; i.e., increased food intake and weight gain (Sancora et al., 1990; Kowalski et al., 1999). However, food intake of 5-HTP injected rats was persistently suppressed despite of the increased NPY expression in the arcuate nucleus, suggesting that a tentative orectic action by increased hypothalamic NPY is inhibited in the 5-HTP injected rats. Also, it should be noticed that a single injection of 5-HTP instantly suppressed food intake (Choi et al., 2003), but not acutely affected the arcuate NPY expression in this study. Thus, it is hypothesized that the anorectic action by increased 5-HTergic activity in the hypothalamus is not mediated by decreased NPY expression in the arcuate nucleus, but it may comprise a blunted orectic signaling of NPY in the paraventricular nucleus where the axons of the arcuate NPY neurons are richly supplied (Elmqvist et al., 1998; 1999; Schwartz et al., 2000).

*In vitro* study demonstrated that NPY dose-dependently increases cellular level of pERK1/2 in cultured cells (Gur et al., 2002). *In vivo* studies have shown that food deprivation increases not only NPY release (Dube et al., 1992; Yoshihara et al., 1996) but also the neuronal level of pERK1/2 in the hypothalamic PVN (Ponsalle et al., 1992; Ueyama et al., 2004; Lee et al., 2010). These studies suggest that NPY signaling cascade in the PVN neurons may involve the activation of ERK1/2, and this idea seemed to be further supported by the present result demonstrating that pERK1/2 level is increased in the PVN when NPY expression increased in the arcuate nucleus following 3 daily injections of 5-HTP. However, in the pair-fed group, the PVN-pERK1/2 level did not increase despite a significant increase of NPY expression in the arcuate nucleus. We have previously shown that

intracerebroventricular injection of NPY, although it stimulated feeding, did not increase pERK1/2 level in the PVN (Lee et al., 2010). Thus, it is suggested that pERK1/2 may not be a downstream signaling of increased NPYergic activity in the PVN of 5-HTP injected rats. Previous studies have supported the idea that increased 5-HTergic activity may be implicated in the pERK1/2 increase in the PVN of 5-HTP injected rats. That is, activation of the 5-HT<sub>1A</sub> receptor resulted in ERK activation in cell lines (Millan et al., 2001) and injections with 5-HT<sub>1A</sub> agonists increased pERK1/2 level in the rat PVN (Sullivan et al., 2005; Crane et al., 2007), suggesting that 5-HT<sub>1A</sub> receptors may stimulate pERK1/2 levels in the PVN. Neurons expressing 5-HT<sub>1A</sub> receptors are observed in the PVN (Marvin et al., 2010). Currie and Coscina (1996) demonstrated that intra-PVN injections of metergoline, a 5-HT<sub>1/5-HT2</sub> receptor antagonist, antagonize the hypophagia following 5-HT injections into the PVN. We have observed that ketanserin, a 5-HT<sub>2A/2C</sub> antagonist, although it abolished the hypophagia by 5-HTP injections, did not block the pERK1/2 increase in the PVN (unpublished data). A 5-HT<sub>2A/2C</sub> agonist DOI attenuated NPY-induced feeding and this was antagonized by ketanserin (Currie et al., 2002). Thus, it is concluded that increased pERK1/2 level in the PVN of 5-HTP injected rats may be a part of 5-HTergic signaling mediated by 5-HT<sub>1</sub> receptors, but not by 5-HT<sub>2</sub> receptors, possibly blunting the orectic NPYergic signal in the PVN.

It is speculated that 5-HT-induced hypophagia is predominantly mediated by central 5-HT pathways, although the mechanism has not been fully defined. Heisler and colleagues (2006) have suggested that 5-HT-induced hypophagia is mediated by downstream activation of melanocortin 4 receptor (Mc4r). They hypothesized that 5-HT action at the arcuate nucleus would lead to a decrease in the release of the endogenous melanocortin receptor (MCR) antagonist AgRP and an increase in the release of the endogenous MCR agonist  $\alpha$ -MSH at downstream MCR-expressing target sites. Mc4rs are expressed in the hypothalamic PVN (Kishi et al., 2003), where the axons of AgRP and  $\alpha$ -MSH neurons are richly innervated from the arcuated nucleus (Elmqvist et al., 1998; 1999). Previous

study has suggested that pERK1/2 is a downstream effector of the central Mc4rs mediating hypophagia (Sutton et al., 2005). Thus, one can prospect that the increased pERK1/2 level in the PVN of 5-HTP injected rats may be a consequence of Mc4rs activation by increased release of its agonist  $\alpha$ -MSH in the PVN. However, 5-HTP injections did not increase the expression of proopiomelanocortin (POMC), functional precursor of  $\alpha$ -MSH, in the arcuate nucleus (our unpublished observation). We have previously reported that the arcuate POMC expression is decreased by chronic treatment with selective 5-HT reuptake inhibitor fluoxetine known to increase the brain 5-HT level (Myung et al., 2005). Thus, it is concluded that the increased pERK1/2 in the PVN of 5-HTP injected rats is less likely due to an activation of melanocortin pathways.

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