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Urocortin1-induced anorexia is regulated by activation of the serotonin 2C receptor in the brain

Yumi Harada [a,d], Kiyoshige Takayama [b], Shoki Ro [a,c], Mitsuko Ochiai [a], Masamichi Noguchi [d], Seiichi Iizuka [d], Tomohisa Hattori [d], Koji Yakabi [a,*]

[a] Department of Gastroenterology and Hepatology, Saitama Medical University, Kawagoe, Saitama, Japan
[b] Department of Laboratory Sciences, Gunma University School of Health Sciences, Maebashi, Gunma, Japan
[c] Central Research Laboratories, Teikyo University Chiba Medical Center, Chiba, Japan
[d] Tsumura Research Laboratories, Tsumura & Co., Ibaraki, Japan

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ABSTRACT

This study was conducted to determine the mechanisms by which serotonin (5-hydroxytryptamine, 5-HT) receptors are involved in the suppression of food intake in a rat stress model and to observe the degree of activation in the areas of the brain involved in feeding. In the stress model, male Sprague–Dawley rats (8 weeks old) were given intracerebroventricular injections of urocortin (UCN) 1. To determine the role of the 5-HT2c receptor (5-HT2cR) in the decreased food intake in UCN1-treated rats, specific 5-HT2cR or 5-HT2b receptor (5-HT2bR) antagonists were administered. Food intake was markedly reduced in UCN1-injected rats compared with phosphate buffered saline treated control rats. Intraperitoneal administration of a 5-HT2cR antagonist, but not a 5-HT2bR antagonist, significantly inhibited the decreased food intake. To assess the involvement of neural activation, we tracked the expression of *c-fos* mRNA as a neuronal activation marker. Expression of the *c-fos* mRNA in the arcuate nucleus, ventromedial hypothalamic nucleus (VMH) and rostral ventrolateral medulla (RVLM) in UNC1-injected rats showed significantly higher expression than in the PBS-injected rats. Increased *c-fos* mRNA was also observed in the paraventricular nucleus (PVN), the nucleus of the solitary tract (NTS), and the amygdala (AMG) after injection of UCN1. Increased 5-HT2cR protein expression was also observed in several areas. However, increased coexpression of 5-HT2cR and *c-fos* was observed in the PVN, VMH, NTS, RVLM and AMG. Whereas, pro-opiomelanocortin mRNA expression was not changed. In an UNC1-induced stress model, 5-HT2cR expression and activation was found in brain areas involved in feeding control.

1. Introduction

Stress-induced effects that result in decreased food intake are well known. The decrease of appetite not only leads to a decline in the quality of life of the patients, resulting from associated disorders such as depression or anorexia nervosa, but may also cause nutrition-related problems.

When individuals experience stress, the amygdala (AMG) sends out a signal, induced by an unpleasant feeling, to the paraventricular nuclei (PVN) of the hypothalamus where corticotropin-releasing factor (CRF) neurons are activated, stimulating CRF synthesis. CRF acts through specific binding to the CRF1 and CRF2 receptors to mediate the behavioral and neuroendocrine responses to stress. Activation of the CRF1 receptor (CRF1R) is involved in the acute interruption of feeding, whereas activation of the CRF2 receptor (CRF2R) is involved in long-term responses [3,38]. Among the three types of CRF family peptides (urocortin 1, 2, and 3), urocortin 1 (UCN1) binds both the CRF1R and CRF2R with higher affinities than CRF [33]. In animals and humans, UCN1 actions include elevation of CRF, adrenocorticotropic hormone (ACTH) and stress hormone secretion as well as appetite suppression and gastrointestinal motility [5,20,29,30]. However, the signaling pathways downstream of the CRFR activation, which negatively regulate food intake, are not well understood.

Various physiological functions are mediated by interactions with the 14 different serotonin (5-HT) receptor subtypes. The interaction of the CRF neuron with the 5-HT system has been implicated in the pathophysiology of disease states, such as depression and anxiety disorders. Enhanced 5-HT neurotransmission produces a potent increase in plasma stress hormones [15]. Moreover, it has been reported that the activation of CRF neurons also regulates 5-HT signaling or release [19,25].

* Corresponding author at: Department of Gastroenterology and Hepatology, Saitama Medical University, 1981, Kamoda, Kawagoe, Saitama 350-8550, Japan. Tel.: +81 49 228 3741; fax: +81 49 225 6649.
E-mail address: kjyakabi@saitama-med.ac.jp (K. Yakabi).

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The 5-HT2c receptor (5-HT2cR) is a prominent serotonin receptor that is distributed throughout the central nervous system. Activation of 5-HT2cR induces a decrease in food intake and increases anxiety [6,9]. Because 5-HT2cR is expressed on CRF neurons and is involved in the stimulating effect of ACTH and corticosterone secretion [12] it has been suggested that interactions exist between the 5-HT2cR and the CRFR. 5-HT2cRs are found in neurons of the hypothalamic arcuate nucleus (ARC) known to express pro-opiomelanocortin (POMC), a peptide that acts to suppress appetite, and the 5-HT2cRs play an important role in regulating the activity of the neurons. POMC neurons are believed to decrease food intake by releasing α-melanocortin-stimulating hormone (α-MSH), which activates the melanocortin-4 receptors. α-MSH is secreted from the axon terminals of POMC neurons that project to the PVN. 5-HT2cRs are also found in the AMG [10] and may be involved in anxiety and fear responses. However, few studies have focused on the underlying mechanism by which 5-HT2cRs may contribute to appetite loss, followed by CRF1R and CRF2R stimulation.

Here, we present a study that represents a first step toward elucidating the role of brain 5-HT2cRs on suppression of feeding behavior followed by activation of CRFRs. We specifically aimed to determine the mechanisms by which 5-HT2cRs can regulate the food intake of rats intracerebroventricularly (i.c.v.) injected with UCN1 and to observe the amount of activation in areas of the brain known to be involved in food intake. We histologically analyzed *c-fos* expression in relation to 5-HT2cRs as indicators of brain activity.

2. Materials and methods

2.1. Experimental animals

Seven-week-old, male Sprague-Dawley rats weighing 210–230 g were purchased from Japan SLC, Inc. (Shizuoka, Japan). The UCN1-injected stress model was induced as reported previously [36]. In brief, rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) and placed on a stereotaxic frame. A stainless steel guide cannula (AG-8; Eicom, Kyoto, Japan) was implanted into the right lateral ventricle (coordinates: 0.8 mm posterior and 1.4 mm right lateral from the bregma, and 3.4 mm ventral from the skull surface) using a rat brain atlas [23]. Rats were allowed to recover for at least five days before starting the experiment. All animals were housed in polycarbonate cages (width: 265 mm, depth: 427 mm, and height: 204 mm) that were maintained at room temperature (23 ± 2 °C), relative humidity of 55% ± 10%, and a 12-h light: 12-h dark cycle (lights were kept on from 07:00 to 19:00 h daily). Free access to water and standard laboratory food were provided. Intraperioneal and i.c.v. injection into rats was performed by skilled experimenters. Standard laboratory food was removed 16 h before the experiment.

All experimental procedures were performed according to the "Guidelines for the Care and Use of Laboratory Animals" and approved by the Laboratory Animal Committee of Tsumura & Co.

2.2. Drugs and reagents

Rat UCN1 was purchased from the Peptide Institute (Osaka, Japan). SB242084 hydrochloride (SB242084), the 5-HT2cR antagonist, and SB215505 hydrochloride (SB215505), the 5-HT2bR antagonist, were purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA). All other reagents used for analysis were of the highest purity commercially available.

2.3. Effects of 5-HT2 receptor antagonists on food intake in UCN1-injected rats

To test the 5-HT2cR antagonistic effects on food intake in UCN1-injected rats, SB242084 and SB215505 were administered because 5-HT2b/2cR antagonism has been reported to increase food intake in several stress models and in healthy rodents [31,38]. UCN1 was dissolved in phosphate buffered saline (PBS), and SB242084 or SB215505 were dissolved in saline. The doses used in this study were based on previous reports. It has been demonstrated that when these agents are administered i.p. to rats, they transit to the brain and act as antagonists for their target receptors [10,14]. SB242084 or SB215505 at a dose of 3 mg/kg was immediately injected (0 h) after either UCN1 or saline injection. Food intake was measured at 1, 2, 4 and 6 h. Control rats were administered PBS and then saline rather than the test compounds. A preweighed amount of chow was distributed in each cage. Cumulative food intake was calculated at 1, 2, 4 and 6 h after injection.

2.4. Determination of 5-HT2cR protein expression and in situ hybridization for c-fos

Rats were divided into two groups ($n = 4$), anesthetized with sodium pentobarbital (50 mg/kg, i.p.) at 1 h after i.c.v. injection of UCN1 (UCN1 was dissolved in PBS, 300 pmol in 10 μL) or PBS, and then transcardially perfused with saline followed by tissue fixative (Genostaff Co. Ltd., Tokyo, Japan). Following perfusion, the rat brains were dissected. Fixed whole brains were cut along the coronal plane, embedded in paraffin, and cut into 6μm sections. Paraffin-embedded tissue blocks and sections of rat brain were used for immunohistochemistry (IHC) to detect 5-HT2cR or for *in situ* hybridization to detect *c-fos* or, according to procedures described previously by Noguchi et al. [22]. The cDNA template used for *c-fos* was a 458-base-pair (bp) fragment corresponding to bp 884–1341 of *fos* cDNA (GenBank accession no. NM_022197.2). Sense and antisense cRNA probes for *c-fos* mRNA were synthesized using a DIG RNA Labeling Kit (Roche, Switzerland) according to the manufacturer's protocol. For IHC, the 5-HT2cR Antibody (Novus Biologicals, Colorado, USA: NBP1-01015) and Biotinylated Goat anti-Rabbit Immunoglobulin (Dako E0432) were used. Each positive cell was counted in bilateral brain sections and averaged.

2.5. Real-time reverse transcription polymerase chain reaction (RT-PCR) mRNA expression assay

Rats were divided into two groups ($n = 8$) and euthanized 1 h after i.c.v. injection of PBS or UCN1 (300 pmol in 10 μL) before the hypothalamus was removed.

Total RNA was extracted from the isolated hypothalamus using an RNeasy® Lipid Tissue Mini Kit (Qiagen, CA, USA) according to the manufacturer's protocol. Quantitative gene expression analysis was performed using RT-PCR according to a previously described procedure [22]. TaqMan Gene Expression Master Mix (Applied Biosystems) and Assays-on-Demand POMC Gene Expression probes (Rn00595020-m1) were used for subsequent PCR reactions according to the manufacturer's protocol. All RT-PCR reactions were performed in triplicate.

2.6. Statistical analysis

All values are represented as the mean ± standard error of the mean. Statistical significance was evaluated by Student's *t*-test or Aspin–Welch *t*-test. For all tests, $p < 0.05$ was considered statistically significant.

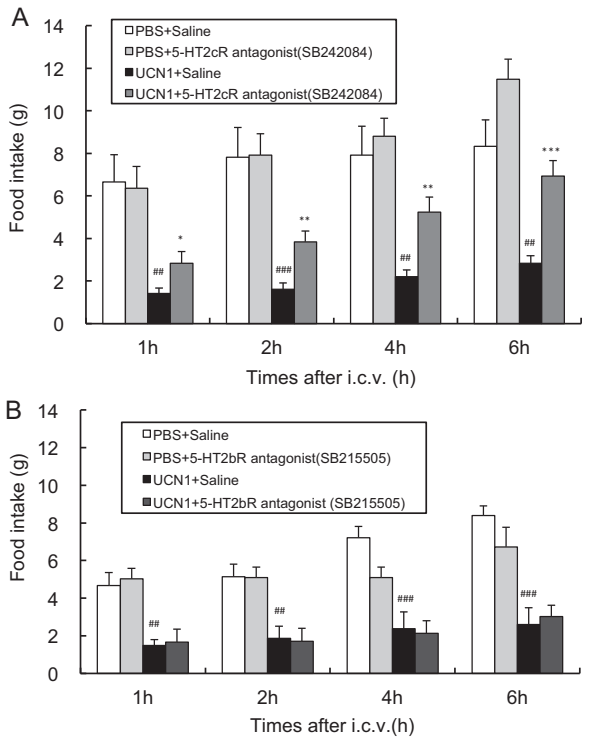

Fig. 1. Effects of SB242084 and SB215505 on cumulative food intake in UCN1-injected rats. SB242084 (A) or SB215505 (B) at a dose of 3 mg/kg was i.p. injected 0 h after PBS or UCN1 injection, and food intake was measured at 1, 2, 4, and 6 h. Normal control rats were administered PBS plus saline, instead of a test compound. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. PBS + saline-treated rats by Student's *t*-test or Aspin–Welch *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. UCN1 + saline-treated rats by Student's *t*-test or Aspin–Welch *t*-test. $n = 6–8$, Mean ± SEM.

3. Results

3.1. The effects of SB242084 and SB215505 on reduced food intake in UCN1-treated rats

Specific 5-HT2cR or 5-HT2bR antagonists were administered to determine the role of 5-HT2cR on decreased feeding behavior in UCN1-treated rats. Food intake after UCN1 injection was markedly reduced (64–79%) compared with PBS-treated controls (Fig. 1). SB242084 administration (i.p., 3 mg/kg), a 5-HT2cR antagonist, significantly inhibited the decreased food intake from 1 to 6 h after UCN1 injection, but it failed to change the food intake in control saline-treated rats (Fig. 1A). However, SB215505, a 5-HT2bR antagonist, did not change the food intake during the experimental time course in either UCN1-treated or PBS-injected control rats (Fig. 1B).

3.2. Changes in 5-HT2cR protein expression and in situ hybridization for c-fos

Using *c-fos* mRNA expression as a marker, the involvement of neural activation in the brain containing the dorsal vagal complex (DVC) following i.c.v. injection of UCN1 was examined (Fig. 2). Expression in the ARC, ventromedial hypothalamic nucleus (VMH), dorsal motor nucleus of the vagus (DMV), lateral hypothalamic nucleus (LH), area postrema (AP), and rostral ventrolateral medulla (RVLM) was very low; however, expression in the ARC, VMH, and RVLM in UNC1-injected rats was significantly higher than in the PBS-injected rats. Increased *c-fos* mRNA expression was observed in the PVN, the nucleus of the solitary tract (NTS), and the AMG after i.c.v. injection of UCN1 (Fig. 2A).

5-HT2cR protein expression in PBS-injected rats was markedly increased in the PVN, RVLM and AMG. Increased 5-HT2cR protein expression induced by UCN1 was also observed in several areas, excluding the PVN, RVLM and AMG (Fig. 2B). Coexpression of *c-fos* mRNA and 5-HT2cR protein in the PBS-injected rats was weak in the ARC, VMH, DMV, LH, AP and NTS (Fig. 2C). However, increased coexpression after UCN1 injection was observed in the PVN (Fig. 2C–E), VMH, NTS (Fig. 2C, F and G), RVLM and AMG (Fig. 2C, H and I), but not in the ARC, AP, LH and DMV (Fig. 2C).

3.3. Changes in POMC mRNA expression

Expression of mRNA in POMC neurons in the ARC and PVN (Fig. 3A), or NTS (Fig. 3B) was similar in both the PBS and UCN1-induced groups.

4. Discussion

In this study, 5-HT2cR activation was observed in hypothalamic PVN, VMH, RVLM and AMG and medulla oblongata NTS after UCN1-injection. Furthermore, the administration of a selective 5-HT2cR antagonist prevented the UCN1-induced food intake inhibition. These findings suggest that 5-HT2cR activation is correlated with suppressed food intake, followed by the activation of CRFRs.

We have previously shown that in rats stressed by the intracerebroventricular (i.c.v.) administration of UCN1, a CRF2R antagonist, astressin 2B, but not a CRF1R antagonist, suppressed acute inhibition of food intake [36]. In addition, in this study, the selective 5-HT2cR, but not the 5-HT2bR antagonist, significantly inhibited the decrease in food intake during the same time period. 5-HT2cR and 5-HT2bR are expressed throughout the mammalian brain [17]. 5-HT2bR is also expressed in the stomach, small intestine, kidney and heart [4,16,18,34]. According to these findings, the central and peripheral 5-HT2bRs may not be involved in the control of food intake in UCN1-induced anorexia. Some reports have indicated that 5-HT2cR activation may inhibit the central or peripheral signaling of ghrelin, a peptide that acts to enhance appetite [8,28,37]. Therefore, the 5-HT2cR antagonist may have suppressed inhibition of ghrelin signaling. Together with these findings, the present results suggest that the activation of 5-HT2cR may contribute to the suppression of food intake via CRF2R stimulation after UCN1 injection.

Serotonergic neurons in the raphe nuclei send widespread projections throughout the brain, where 5-HT exerts its effects through a variety of 5-HT receptors. 5-HT2cRs are widely distributed in the choroid plexus, frontal cortex, hippocampus, hypothalamus, ventral tegmental area and AMG [10,35]. In this study, we also observed overexpression of 5-HT2cR proteins in the PVN, RVLM and AMG in control rats treated with PBS. One hour after UCN1 administration, the protein expression of 5-HT2cR in these areas did not increase further. In other words, in these particular areas, 5-HT2cRs may be primarily involved in the regulation of other physiological responses rather than feeding responses followed by the activation of CRFRs.

Slight 5HT2cR protein expression was observed in the following areas in the control rats: ARC, VMH, LH, AP, NTS and DMV. Interestingly, in these same areas, the protein expression increased immediately after UCN1 injection. These findings suggest that the increase in 5-HT2cR protein expression may induce stress-related symptoms, such as anorexia. However, in the ARC, LH, AP and DMV, there were no differences in the coexpression of the 5-HT2cR protein and *c-fos* mRNA between UCN1-injected and control rats. These results suggest that even after the increase in 5-HT2cR protein expression following UCN1 injection, activation did not occur

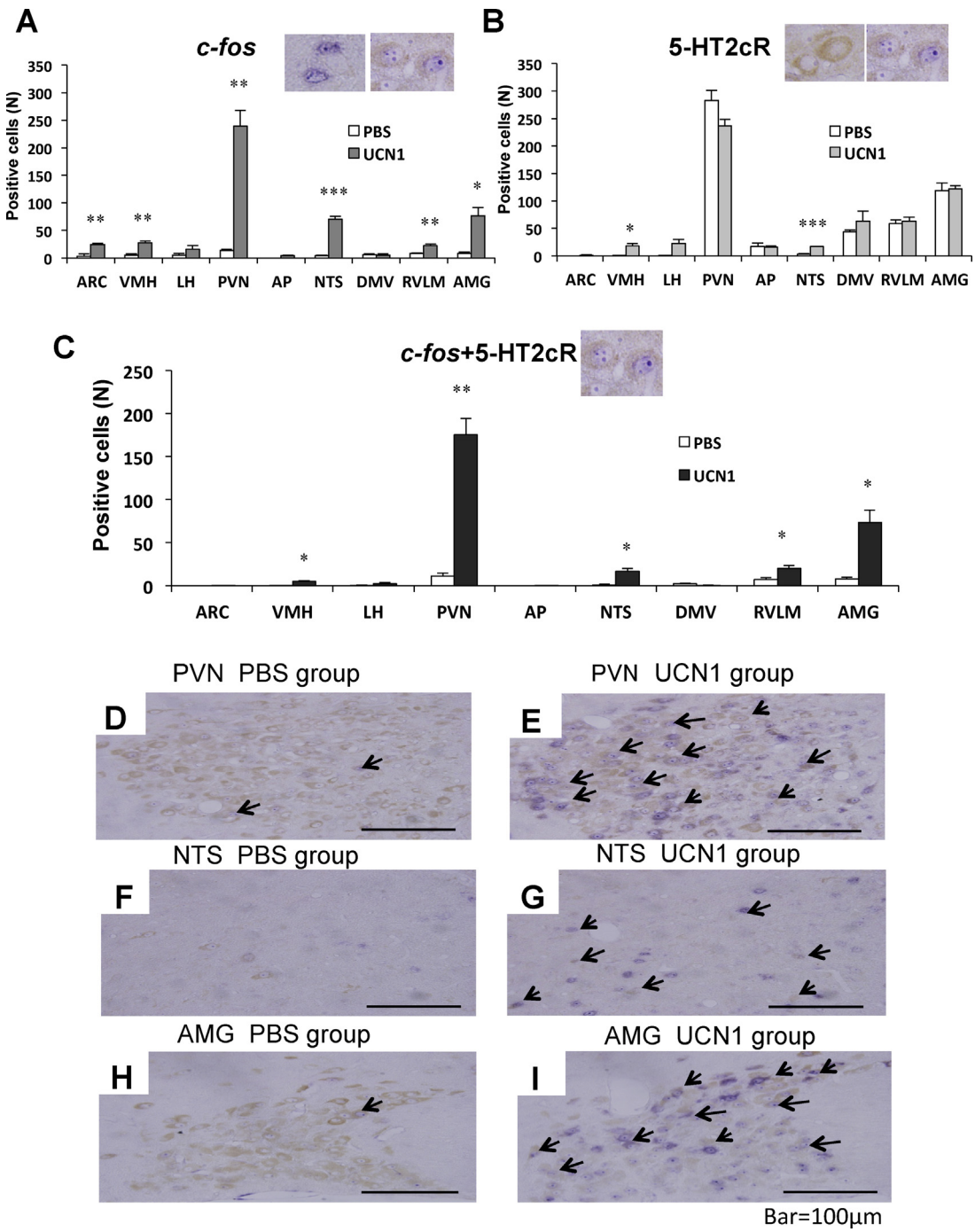

Fig. 2. Changes in *c-fos* mRNA and 5-HT2cR protein and coexpression of 5-HT2cR protein and *c-fos* mRNA in UCN1-injected rats. Brains were collected 1 h after UCN1 injection. (A) Changes in *c-fos* mRNA were assessed by *in situ* hybridization. (B) Changes in 5-HT2cR protein expression were measured using immunohistochemistry. (C) Changes in coexpression of 5-HT2cR protein and *c-fos* mRNA were monitored. The number of *c-fos*-positive cells and the 5-HT2cR protein-positive cells were counted in one representative section from each rat. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. PBS + saline-treated rats by Student's *t*-test or Aspin–Welch *t*-test. ARC, arcuate nucleus; VMH, ventromedial hypothalamic nucleus; LH, lateral hypothalamic nucleus; PVN, paraventricular nucleus; AP, area postrema; NTS, nucleus solitary tract; DMV, dorsal motor nucleus of the vagus; RVLM, rostral ventrolateral medulla; AMG, amygdala. $n = 4$, Mean ± SEM. (D–I) Coexpression of *c-fos*, assessed by *in situ* hybridization, and 5-HT2cR protein, assessed using immunohistochemistry, in the PVN, NTS, and AMG of the PBS- and UCN1-injected rats. (D) PBS PVN, (E) UCN1 PVN, (F) PBS NTS, (G) UCN1 NTS, (H) PBS AMG, and (I) UCN1 AMG. Blue cells: Expression of *c-fos* mRNA. Brown cells: Expression of 5-HT2cR protein. ←: Coexpression of *c-fos* and 5-HT2cR protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

during the first hour or that the peak activation had already passed. Further investigation is required to determine the activation of 5-HT2cR at various time intervals. In the LH, AP and DMV, there was a slight increase in 5-HT2cR protein or c-fos mRNA between the control and UCN1-treated rats. Though it is reasonable to assume that the 5-HT2cRs in these regions play important roles in controlling physiological responses associated with anxiety and feeding, the role of 5-HT2cR in the LH, AP and DMV is still poorly understood.

Different afferent pathways convey stress inputs to the hypothalamic PVN. After UCN1 injection, a marked activation of 5-HT2cRs was observed in the hypothalamic PVN. Because 5-HT2cRs are expressed on CRF neurons [12] and CRF2Rs are also located in the

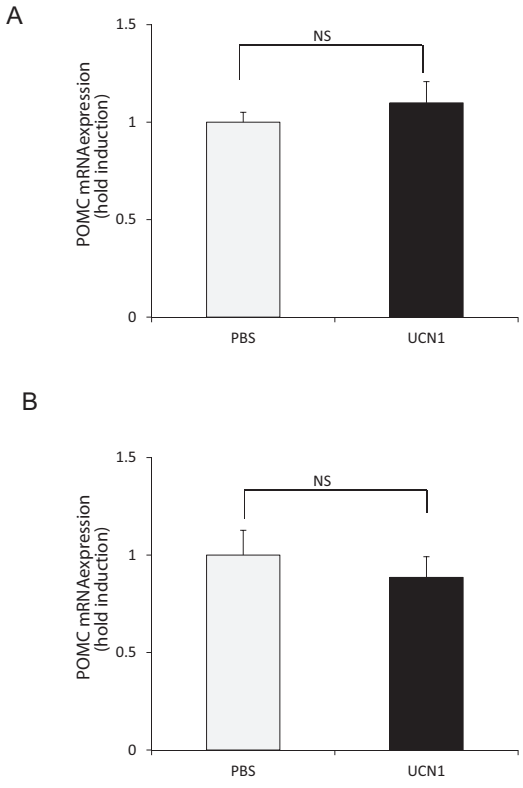

Fig. 3. (A) Effect on POMC mRNA expression in the hypothalamus containing ARC and PVN by real time RT-PCR 1 h after UCN1-injection. (B) Effect on POMC mRNA expression in the medulla oblongata containing NTS by real time RT-PCR 1 h after UCN1-injection. Effect of UCN1 was evaluated by Student's *t*-test. $n = 8$, Mean ± SEM.

PVN, i.c.v. administration of UCN1 is likely to have activated the CRF2Rs, leading to 5-HT2cR activation. In addition, activation and overexpression of 5-HT2cRs occurred simultaneously in the VMH and AMG. The AMG is rich in CRF neurons [26]. There have been some reports that anxiety behavior can appear after infusion of CRF into the AMG or in 5-HT2cR overexpressing mice [13,27]. Furthermore, *Htr2c* knockout ($Htr2c^{KO}$) mice displayed anxiolytic effects in mice exposed to novel environments and showed decreased neuronal activities, specifically in the AMG [13]. These findings imply that the activation of 5-HT2cRs, which is induced by stimulating CRFRs in the AMG, may be directly involved in inducing anxiety, rather than in responses associated with feeding. VMH serves as the primary satiety center in the hypothalamus; however, the role of 5-HT2cRs in the VMH is unclear. As CRFRs are found in the VMH [32], there is most likely an interaction between CRFRs and 5-HT2cRs. Although novelty stress did not enhance the 5-HT biosynthesis, it suppressed 5-HT turnover [21,24]. Therefore, in these areas, 5-HT2cR may be activated.

The UCN1-injected rat demonstrated significantly higher *c-fos* mRNA and 5-HT2cR protein coexpression in the NTS and RVLM compared with the control rats. The NTS play important roles in relaying peripheral stress information to the PVN [2]. Furthermore, *c-fos* mRNA expression was also significantly increased in these areas. Previous findings suggest that peripheral stress information, such as decreased appetite, reduced gastric emptying, and stomach discomfort due to accelerated gastric acid secretion, is transported to the NTS via the vagal afferent pathway within 1 h following UCN1 injection. Asarian et al. [1] showed that the peripheral administration of cholecystokinin (and not Glucagon-like peptide-1) to $5\text{-}Htr2c^{KO}$ mice failed to reduce food intake, which they explained by demonstrating that in $5\text{-}Htr2c^{KO}$ mice, *c-fos* protein expression did not appear in the NTS. These results indicate that activation of 5-HT2cRs in the NTS may be indispensable for transmitting peripheral satiation signals to the PVN.

The RVLM is involved in cardiovascular regulation, such as blood pressure control. Sympathetic pathways related to cardiovascular regulation are relayed by the RVLM premotor neurons acting on the peripheral heart and blood vessels. In rodent studies, mCPP, a 5-HT2cR agonist, induced activation of 5-HT2cR, which led to tachycardia and increased blood pressure [7]. This finding suggests that 5-HT2cR activation may be involved when these conditions are caused by stress.

POMC neurons in the ARC suppress feeding in response to peripheral negative signals via the afferent vagus nerve [11]. Here, we observed no significant difference in the hypothalamic POMC mRNA expression between the UCN1-injected and control rats at 1 h after UCN1 injection. In addition, no significant 5-HT2cR activity was observed, even after food intake decreased considerably in rats treated with UCN1. These results suggest that ARC POMC neurons play a relatively minor role in this period and receive little or no peripheral input.

The effects of 5-HT2cR agonists and UCN1 microinjection on food intake must be further investigated to clarify the involvement of brain regions and 5-HT2cRs.

5. Conclusion

In an UNC1-induced stress model, acute phase feeding responses are mediated by the activation of 5-HT2cR in the brain. 5-HT2cR activation induced by UCN1 injection occurs not only in brain regions associated with food intake but also in areas that control physiological functions.

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