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

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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].

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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.