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Role of Neuropeptide Y and Proopiomelanocortin in Fluoxetine-Induced Anorexia

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Fluoxetine is an anorexic agent known to reduce food intake and weight gain. However, the molecular mechanism by which fluoxetine induces anorexia has not been well-established. We examined mRNA expression levels of neuropeptide Y (NPY) and proopiomelanocortin (POMC) in the brain regions of rats using RT-PCR and *in situ* hybridization techniques after 2 weeks of administering fluoxetine daily. Fluoxetine persistently suppressed food intake and weight gain during the experimental period. The pair-fed group confirmed that the reduction in body weight in the fluoxetine treated rats resulted primarily from decreased food intake. RT-PCR analyses showed that mRNA expression levels of both NPY and POMC were markedly reduced by fluoxetine treatment in all parts of the brain examined, including the hypothalamus. POMC mRNA *in situ* signals were significantly decreased, NPY levels tended to increase in the arcuate nucleus (ARC) of fluoxetine treated rats (compared to the vehicle controls). In the pair-fed group, NPY mRNA levels did not change, but the POMC levels decreased (compared with the vehicle controls). These results reveal that the chronic administration of fluoxetine decreases expression levels in both NPY and POMC in the brain, and suggests that fluoxetine-induced anorexia may not be mediated by changes in the ARC expression of either NPY or POMC. It is possible that a fluoxetine raised level of 5-HT play an inhibitory role in the orectic action caused by a reduced expression of ARC POMC (α-MSH).

Key words: Fluoxetine, Neuropeptide Y, Proopiomelanocortin, Hypothalamus, Rats, Anorexia

INTRODUCTION

The energy balance depends on food intake, while energy storage in fat and energy expenditure is important for the maintenance of body weight. The regulation of this energy homeostasis requires: (i) a mechanism for sensing energy level storage in body fat and relaying the information to control sites in the hypothalamus; and (ii) the integration of information in the hypothalamus which in turn, determines the energy balance through controlling food intake and energy expenditure (Bernardis *et al.*, 1996; Rang *et al.*, 2003). In the central regulation of the energy balance, two groups of neurons in both paraventricular (PVN) and arcuate (ARC) nuclei of hypothalamus are mainly involved. In one group, neuropeptide Y (NPY) is a powerful feeding stimulant (Wolf, 1997). The other contains the protein proopiomelanocortin (POMC), which is an anorexigenic factor, releasing α-melanocyte-stimulating hormones (α-MSH) (Yaswen *et al.*, 1999). In addition, many other orexigenic factors (that stimulate appetite) and anorexigenic factors (that reduce appetite) can be involved in regulating food intake and energy expenditure. Orexigenic factors include agouti related protein (AGRP), co-localized with NPY in the arcuate nucleus (Ollmann *et al.*, 1997) and orexin (Sakurai *et al.*, 1998). Anorexigenic factors include serotonin (5-HT) (Pollock *et al.*, 1981) and cocaine- and amphetamine-regulated transcript (CART) (Kristensen *et al.*, 1998).

5-HT is an anorexigenic neurotransmitter to modulate the energy balance. This is found in neurons projecting from the raphe nucleus to the hypothalamus, especially in the PVN and ARC (Leibowitz *et al.*, 1990). According to the monoamine theory, depression results from functionally

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deficient monoaminergic transmissions in the CNS, inhibiting monoamine uptake and ameliorating moods. Fluoxetine is currently the most prescribed antidepressant among drugs that inhibit 5-HT uptake. This selective 5-HT reuptake inhibitor (SSRI) caused anorexia. Fluoxetine induced an anorexigenic activity acting on the hypothalamic 5-HT systems in the control of carbohydrate intake in a circadian-related manner (Weiss *et al.*, 1991). The anorexigenic activity of fluoxetine was examined in mice, rats and guinea pigs (Anelli *et al.*, 1992). In obese Zucker rats (a well-characterized animal obesity model), the chronic administration of fluoxetine generated a decrease in NPY in the PVN not in the arcuate. This suggests that NPY could be involved in the anorexigenic effect of fluoxetine in the animal obesity model (Gutierrez *et al.*, 2002). However, the anorexigenic mechanism of fluoxetine in normal rats has not yet been investigated carefully. Thus, understanding the anorexigenic mechanism of the inhibition of 5-HT reuptake is necessary for elucidating the pathophysiological mechanism and pharmacological therapeutics of obesity.

This study was designed to investigate whether fluoxetine exerts anorexigenic effects in normal rats (as seen in obese Zucker rats), and examine the possible involvement of NPY and α-MSH in the anorexigenic mechanism of fluoxetine. To more fully understand this experimental hypothesis, changes in body weight and food intake were measured in normal rats after being treated with fluoxetine. Striking alterations of the expression levels of mRNAs encoded with NPY and POMC by fluoxetine were observed in 12 different parts of rat brains, including the hypothalamus and pituitary glands.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from Hallym Experimental Animal Laboratories (Hwaseong, South Korea). Upon arrival, the animals were housed two per cage in a temperature-controlled room (22 ± 2°C) with adjustable humidity (50 ± 5%) and lighting (12 h dark-light cycle, lights on 7:00 a.m.). Food (standard rat chow diet) and water were available *ad libitum* at all times. All procedures were conducted in accordance with the Guide for the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by Chungnam National University Animal Care and Use Committee.

Drug treatment

After 7 days of acclimatizing to these conditions, the rats (290 ± 5 g) were randomly divided into two groups - controlled and experimental. Body weight and food intake were measured daily. Rats received an intraperitoneal injection of fluoxetine (10 mg/kg) or the same volume of aseptic physiologic saline daily for 2 weeks (n=12 in each group). Rats in the pair-fed group (n=6) were supplied with the same amount of food consumed by the fluoxetine group on each previous day during the entire experimental period. Fluoxetine hydrochloride was provided by Lilly Korea (Seoul, South Korea).

Brain dissection

On the 15th day (24 h after the last treatment), rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and decapitated. Their complete brains were exposed and anatomically divided into 12 different areas: frontal cortex, cerebral cortex, striatum, hippocampus, thalamus, brain stem, cerebellum, hypothalamus, septum, amygdala, preoptic area, and pituitary gland. Each tissue sample was pooled from 2-5 rats and processed for determination of mRNA levels.

RNA isolation

Total RNAs were isolated from each brain tissue. Equal amounts of tissue (30 mg) were processed under identical conditions using a QIAGEN RNeasy kit in accordance with the manufacturer's protocol.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

cDNAs were obtained using oligo pd$(T)_{12-18}$ primer and SuperScript™ II RNase H-Reverse Transcriptase (Invitrogen) according with the manufacturer's protocol. Each of the forward and the reverse primers (2 pmol/25 μL reaction), 1 unit of *Taq* DNA polymerase (Bioneer) and 1 μL of first strand reaction mix (taken without purification), were used during each amplification. All reactions were assembled using the same master mix containing all the reaction components. Amplification conditions were 1 cycle of 5 min at 95°C, and 30 cycles of 1.5 min at 95°C, 1.5 min at 57°C and 1.5 min at 72°C followed by 5 min at 72°C. The described conditions and the number of amplification cycles used resulted in a linear amplification of the 852 b.p. of β-actin cDNAs identical to the initial control experiments using rat brain derived mRNA (data not shown). Amplification was performed using primers: (i) forward 5'-GTAACCAACTGGGACGAT-3' and reverse 5'-CTTGCTGATCCACATCTG-3' for rat β-actin; (ii) forward 5'-AAGCTCATTCCTCGCAGA-3' and reverse 5'-GGGGGGAAACTAGGAAAA-3' for rat NPY; and (iii) forward 5'-TGCCGAGATTCTGCTACA-3' and reverse 5'-ATGGCGTTCTTGAAGAGC-3' for rat POMC. The same cDNA samples were used for analyzing the expression of the NPY and POMC mRNAs.

Following the amplification, equal quantities of the amplified products were analyzed by electrophoresis in

1% agarose gels containing 0.5 μg/mL ethidium bromide. All the obtained cDNA fragments were subsequently gel-purified and cloned. Individual clones obtained from each of the cloned 408 b.p. cDNA bands for NPY and 676 b.p. for POMC were sequenced to confirm the identity of the amplified products. The amount of cDNAs obtained from each tissue was quantified by direct measurements using a "ChemiDoc XRS" digital imaging system and 'MultiAnalist' software from Bio-Rad laboratories, Inc..

In situ hybridization

Rats were transcardially perfused, first with heparinized isotonic saline containing 0.5% $NaNO_2$, then with 4% paraformaldehyde in a 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. Coronal sections 40 microns long were cut on a freezing sliding microtome. Every third section through the rostral-caudal extent of the hypothalamus (between bregma – 1.40 mm and – 3.80 mm), was collected into 20 mL glass scintillation vials containing ice-cold 2×SSC (0.3 M NaCl, 0.03 M Na Citrate) for NPY and the adjacent sections for POMC *in situ* hybridization. The coordinates were based on Paxions and Watson (Paxions *et al.*, 1986). 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). It was then incubated for 2 h at 48°C. *In situ* hybridization was performed with cDNA probes of NPY or POMC (Baker *et al.*, 1996) as previously described (Jahng *et al.*, 1998). The tissue sections were then mounted on gelatin-subbed slides, air-dried, and at 4°C, apposed to Kodak BioMax film (Eastman Kodak Co., NY, U.S.A.). Exposure times varied from 12 to 48 h to obtain autoradiographic images within a linear range of optical density after development in a 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 for direct comparison of mRNA expression.

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 expression levels 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 pixels). For each section, the mean background value was subtracted from the mean mRNA pixel value. The mRNA pixel values were averaged across 8 sections from each rat and the average mRNA value of each rat was then averaged across all rats within each experimental group. The average mRNA values from each experimental group were then converted to relative values of the main vehicle-treated control group. All the data was analyzed by one-way analysis of variance (ANOVA). Preplanned comparisons with the control group were performed by post-hoc Fisher's PLSD test or unpaired *t*-test using StatView software (Abacus, Berkeley, CA).

RESULTS AND DISCUSSION

Significant reductions in food intake and body weight occurred during the two weeks of fluoxetine administration (Fig. 1). Note that the pair-fed group showed similar amount of reduction in weight gain, compared to the fluoxetine group. These findings indicate that the reduction in body weight gain of fluoxetine treated rats is largely due

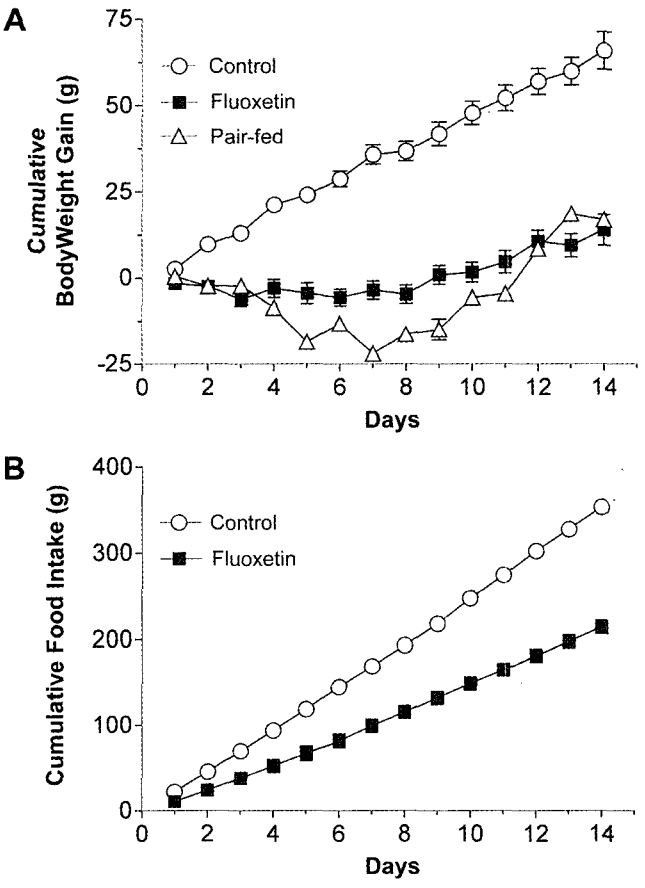

Fig. 1. Effect of fluoxetine on the weight gain of normal rat. *A*, Fluoxetine-treatment (10 mg/kg, i.p., for 14 days) significantly reduces the weight gain as compared with rats challenged with saline (the same dosage as fluoxetine). The restriction of the amount of food in pair-fed group also significantly reduced the weight gain of normal rat. *B*, Fluoxetine significantly reduces the food intake for rats as compared with saline-treated ones. The data represents the mean ± SEM of 6-16 rats per group.

to decreased food intake, suggesting that fluoxetine may exert an anorectic effect on the level of intake control. A number of papers have reported that fluoxetine increases the brain 5-HT level (Badawy *et al.*, 1996; Hervas *et al.*, 1996; Muck-Seler *et al.*, 1996). It has also been reported that the administration of 5-HT synthesis inhibitor *p*-chlorophenylalanine increases feeding (Dryden *et al.*, 1996) and 5-HT reuptake blocker fenfluramine decreases food intake (Le Feuvre *et al.*, 1991). Our data concurs with these observations by others, suggesting that the reduction in body weight of the fluoxetine treated group is due to the satiation effect, which is likely induced by an increase in brain 5-HT levels.

We examined the gene expression of feeding peptides (such as NPY and POMC) to define the molecular mechanism which allows fluoxetine to reduce food intake in the part of the brain after fluoxetine treatment. In order to assess the influences of fluoxetine on NPY and POMC in several parts of the brain other than in the hypothalamus and pituitary glands, the mRNA expression level of NPY and POMC in 12 different parts of the brain were measured by RT-PCR.

The data in Fig. 2 illustrate the mRNA expression level of NPY and POMC in 12 different parts of rat brains. The mRNA levels of β-actin showed an equi-quantitative expression in 12 different parts of rat brain of both vehicle- and fluoxetine-treated groups. All the mRNAs were purified from equal tissue amounts and the cDNAs were synthesized and linearly amplified in parallel under the same conditions. Hence, the amounts of the 408 b.p. NPY and 676 b.p. POMC cDNAs in each lane represent the total amounts of NPY and POMC mRNAs in the original

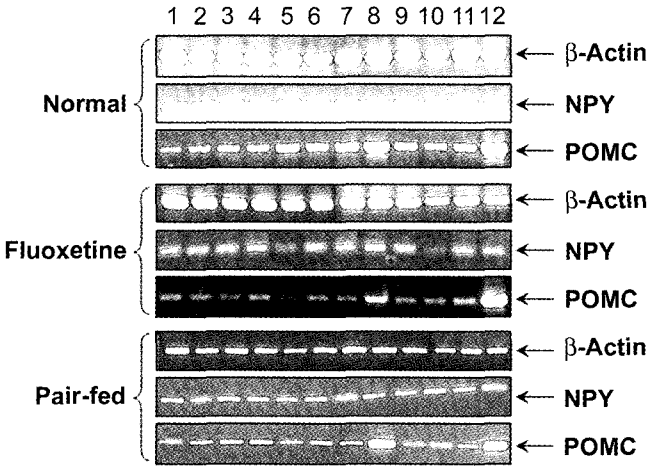

Fig. 2. Quantitative RT-PCR analysis of NPY and POMC mRNA expression. RT-PCR amplification of β-actin 852 b.p., NPY 408 b.p., and POMC 676 b.p. fragment from 12 different parts of rat brain: 1, frontal cortex; 2, cerebral cortex; 3, striatum; 4, hippocampus; 5, thalamus; 6, brain stem; 7, cerebellum; 8, hypothalamus; 9, septum; 10, amygdale; 11, preoptic area; 12, pituitary gland

preparations. An analysis of the intensities of these cDNA fragments revealed that all parts of the brain including the hypothalamus and pituitary glands, contain significant amounts of NPY and POMC transcripts. The administration of fluoxetine caused a decrease in the mRNA expression level of NPY all around of rat brain parts as compared with saline-treated group. A reduction in the mRNA expression level of POMC was observed throughout brain except the pituitary gland. The reduced pattern of both NPY and POMC expressions in fluoxetine-treated groups was similar to the pair-fed group.

There are two main concerns. First, the fluoxetine administration influences on the expressions of NPY and POMC not only in the hypothalamus, but in all regions of brain as well. Since both NPY and POMC have many diverse physiological actions in different parts of the brain, these results suggest that the sustained treatment of fluoxetine may cause an alteration of physiological actions of NPY and POMC in different parts of the brain. Second, the data showed that fluoxetine decreased the expression levels of NPY and POMC in hypothalamus. These results suggest that fluoxetine may block the hyperphagic effects of NPY in the hypothalamus by reducing NPY levels. A decrease in POMC levels in the hypothalamus by fluoxetine might correlate with the rapid consumption of POMC to release α-MSH. Our preparation for RT-PCR experiments contains undefined whole hypothalamic tissues and does not reflect the microanatomical features of mRNA expression in either ARC or PVN of hypothalamus. To explain which parts of the hypothalamus are critical for the reduction in body weight gain and food intake by chronic fluoxetine administration, we have carried out a more delicate method for the measurement of mRNA levels in brain using *in situ* hybridization.

NPY mRNA levels appeared to increase in the ARC of fluoxetine treated rats, compared to the vehicle controls (Fig. 3A). However, a densitometric measurement of the *in situ* signals on X-ray films showed no significant changes (Fig. 3B). The arcuate mRNA levels of NPY in the pair-fed group were not different from the control group, suggesting that this amount of food restriction may not significantly influence the arcuate NPY expression. These results concur with a previous report that chronic fluoxetine administration does not alter the NPY mRNA expression in the ARC of hypothalamus in lean Zucker rats (Gutierrez *et al.*, 2002). Thus, we concluded that fluoxetine-induced anorexia may not be mediated by changes in the ARC expression of NPY. However, in the RT-PCR data, as seen in Fig. 2, NPY mRNA expression levels decreased in hypothalamic tissues of the fluoxetine treated group, compared to the saline-treated control group. This contradiction can be explained by technical difficulties, (i.e. a variety of neurons expressing NPY are

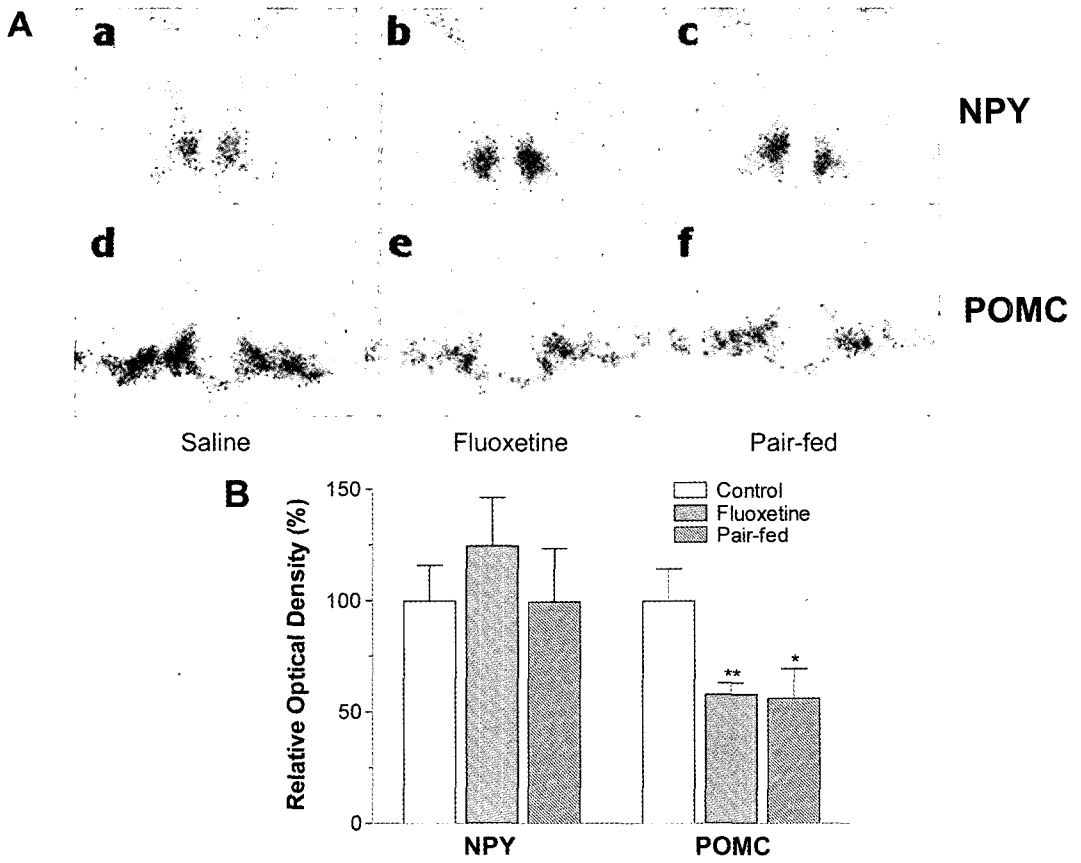

Fig. 3. *In situ* hybridization analysis of NPY and POMC mRNA expression. *A*, Autoradiography of NPY (a, b, c) and POMC (d, e, f) *in situ* signals in the arcuate nucleus of rats. NPY mRNA level appeared to be increased in the fluoxetine treated rat (b) compared with the vehicle control (a) or the pair-fed group (c). POMC mRNA expression decreased both in the fluoxetine (e) and the pair-fed rats (f), compared to the vehicle control (d). *B*, Relative optical density of the *in situ* signals on X-ray films. Fluoxetine treatment did not significantly alter NPY expression level in the arcuate nucleus, but markedly decreased POMC mRNA level. $^{*}p < 0.05$ and $^{**}p < 0.001$ vs. the vehicle control.

closely located each other in the hypothalamic regions). This made it difficult for investigators to isolate just the ARC region for RT-PCR analysis. Therefore, it is possible that some hypothalamic regions other than the ARC might have been included in the hypothalamic preparation for the RT-PCR analysis.

The POMC expression in the ARC of the hypothalamus was significantly reduced both in the fluoxetine-treated and pair-fed groups, compared to the freely-fed vehicle control (Fig. 3A and 3B). The size of reduction was similar in both groups (Fig. 3B). This result indicates that a reduction of POMC expression levels in the hypothalamic ARC of the fluoxetine-treated group may have resulted from long-term food restriction. POMC is an anorectic molecule and the ARC expression of POMC is decreased by food deprivation or food restriction (Mizuno *et al.*, 1998; Kim *et al.*, 1996; Schwartz *et al.*, 1997). POMC expressed in the ARC is converted to α-MSH, and then α-MSH is released to the PVN (Kim *et al.*, 2002), and reduces food intake (Yaswen *et al.*, 1999; Marsh *et al.*, 1999). These reports support our results that the reduction in ARC POMC mRNA levels of fluoxetine-treated rats may be due to decreased food intake. Hence, this suggests that fluoxetine-induced anorexia may not be mediated by changes in the ARC expression of POMC.

In conclusion, these results show that the chronic administration of fluoxetine leads to a reduction in body weight and food intake. It also decreases the expression levels of both NPY and POMC in all parts of the brain including the ARC of the hypothalamus. However, fluoxetine-induced anorexia may not be mediated by changes in the ARC expression of either NPY or POMC. Possibly, a raised level of 5-HT by fluoxetine may act on the downstream of POMC (α-MSH) to play an inhibitory role in the orectic action caused by a reduced expression of ARC POMC.

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