Endocrine, *vol. 16, no. 3, 201–206, December 2001* 0969–711X/01/16:201–206/$11.50 © 2001 by Humana Press Inc. All rights of any nature whatsoever reserved.

Central Administration of Neuromedin U Activates Neurons in Ventrobasal Hypothalamus and Brainstem

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Neuromedin U (NMU) is a peptide isolated from the porcine spinal cord. Recently, two receptors for NMU have been identified and characterized. A recent study indicated that NMU is an anorectic chemical in the brain. The present study shows that NMU has an action in the brain to inhibit food intake in rats. Intracerebroventricular injection of NMU inhibited dark-phase feeding. Animals injected with NMU showed a strong increase in Fos-immunoreactive nuclei in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, and in the parabrachial nucleus of the brain stem. Double immunohistochemistry revealed that a high number of oxytocin-immunoreactive neurons in the PVN and SON contained Fos after intracerebroventricular injection of NMU. In addition, a small proportion of vasopressinergic cells within the PVN and SON were found to contain Fos. The effect of NMU on the hypothalamus and brain stem contributes to the inhibitory effects of NMU on feeding behavior.

Key Words: Neuromedin U; food intake; Fos; oxytocin; vasopressin; immunohistochemistry.

Introduction

Neuromedin U (NMU) is a brain-gut peptide originally isolated from the porcine spinal cord by Minamino et al. *(1)*. The amino acid sequences of NMU-like peptides from mammalian tissues have been determined *(2–5)*. Rat NMU is an amidated 23 amino acid peptide that is synthesized as part of a much larger precursor protein containing five pairs of dibasic amino acids *(6)*. It is widely distributed in the central nervous system, pituitary gland, and gastrointestinal tract. Recently, cognate receptors for NMU were identified *(7–10)*. FM-3, designated NMU1R, is abundantly expressed in peripheral tissues, whereas FM-4, designated NMU2R, is expressed in specific regions of the brain such as the hypothalamic paraventricular nucleus (PVN), along the wall of the third ventricle in the hypothalamus, and the CA1 region of the hippocampus *(7)*.

NMU's physiologic role is not entirely understood. NMU potently contracts smooth muscles, increases arterial blood pressure, and modifies intestinal ion transport. In addition, complex effects on steroid secretion from the adrenal cortex have been reported *(11)*.

Regarding the pathophysiologic relevance of NMU in the brain, Howard et al. *(7)* presented a hypothesis that NMU is a new anorectic peptide because intracerebroventricular (icv) administration of NMU inhibits overnight food intake and fasting-induced feeding, while fasting reduces the expression of NMU mRNA in the arcuate nucleus (ARC) of the hypothalamus. Nakazato et al. *(12)* also showed that centrally injected NMU decreased food intake and body weight and increased energy expenditure in free-feeding rats.

The present study showed that NMU acts in the brain to suppress dark-phase food intake. Furthermore, we used immunohistochemistry for the Fos protein to map the potential neuronal structures activated after icv administration of NMU. In addition, using immunohistochemical double staining for Fos and oxytocin (OT) or Fos and vasopressin (AVP), we characterized the hypothalamic neurons activated by central injection of NMU.

Results

Effects of NMU on Food Intake

Cumulative food intake in the first 2 h of the dark phase after icv NMU (5 nmol/10 µL) injection in rats was significantly reduced by 54% compared with controls, but the effect of NMU (1 nmol/10 µL) was not different from that of controls (Fig. 1).

Immunohistochemical Identification

In NMU-injected (5 nmol) animals, many Fos-immunoreactive (Fos-ir) cells were present in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, and in the superior lateral parabrachial nucleus (PBN) of the brain stem (Figs. 2 and 3). Figure 2A shows that magnocellular cells expressed Fos, while few parvicellular

Received August 31, 2001; Revised November 6, 2001; Accepted December 6, 2001.

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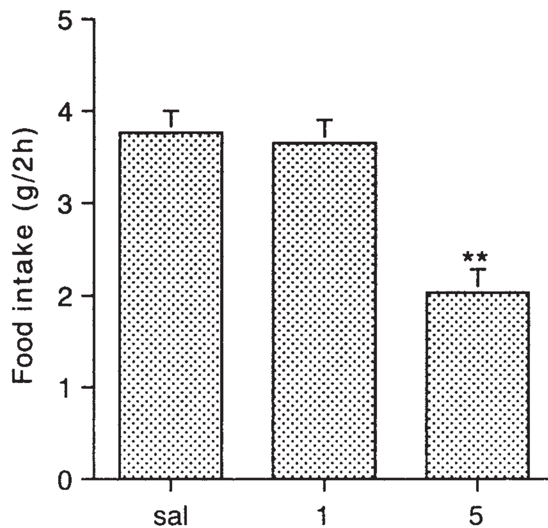

Fig. 1. Effects of icv injection of NMU (1 or 5 nmol) or saline on dark-phase food intake (g/2 h). The histogram represents the mean ± SEM (n = 5 rats/group). **p < 0.001 vs saline (sal) group.

cells expressed Fos in the PVN. A few Fos-ir cells were seen in the lateral preoptic area, dorsomedial hypothalamic nucleus (DMN), and paraventricular thalamic nucleus. Moreover, the distribution patterns of Fos-positive neurons in the hypothalamus and brain stem in the rats treated with a lower dose of NMU (1 nmol) were almost the same (Fig. 3). Fos expression in the PVN increased in a dose-related manner after icv administration of NMU (1 and 5 nmol). In rats injected with saline as control, Fos-ir neurons were present consistently in very low numbers in several areas of the hypothalamus and brain stem.

Double staining revealed that Fos was present in many OT-containing neurons of the PVN and SON in the NMU-treated group (Figs. 4 and 5). The percentage of OT neurons double labeled for Fos was 31.1 ± 2.2% in PVN and 45.9 ± 3.8% in SON in the NMU-treated group. In the saline-treated control rats, the proportion of OT neurons coexpressing Fos was 2.3 ± 0.4% in PVN and 2.6 ± 0.4% in SON. In addition, a small portion of vasopressinergic cells within the PVN and SON was found to contain Fos (Figs. 4 and 5). AVP-containing neurons in the hypothalamus showed double positivity 2 h after administration of NMU (7.4 ± 2.0% in PVN, 10.0 ± 2.4% in SON), while only very little Fos expression was observed in AVP-containing neurons in the PVN (1.0 ± 0.1%) and SON (1.4 ± 0.2%) after administration of saline.

Discussion

The present study confirmed that central administration of NMU (5 nmol) potently suppressed dark-phase food intake in free-feeding rats. However, a low dose of NMU (1 nmol) did not show a significant inhibitory effect. Conversely, when NMU was injected at a dose of 3 or 10 μg (about 1.1 or 3.7 nmol) in rats, overnight food intake was significantly decreased *(7)*. Furthermore, Kojima et al. *(10)* reported that icv injection of 1 or 5 nmol of NMU to free-feeding rats during the dark phase (12 h) decreased food intake by 31 and 67%, respectively. We do not know yet the reason that suppression of dark-phase feeding was not observed in rats treated with central injection of 1 nmol of NMU. This might be owing to the method of injection or stress. We expect that the appetite-suppressing effect of NMU was relatively weak compared with other well-known appetite-suppressing neuropeptides, such as α-melanocyte-stimulating hormone or cocaine- and amphetamine-regulated transcript.

Furthermore, we used Fos immunohistochemistry as an index of stimulated neuronal activity *(13)*. Central administration of NMU (1 or 5 nmol) stimulated Fos in neurons located in the PVN and SON of the hypothalamus. Although Fos-ir cells were observed in the PVN after the administration of 1 nmol of NMU, these were fewer than after 5 nmol. The high dose of NMU used inhibited feeding. Based on these findings, we speculate that PVN is the most potent site correlating with suppression of food intake. This was supported by the localization of NMU2R, which is expressed in PVN of the hypothalamus *(7)*. On the other hand, it was reported that NMU mRNA is discretely located, with the most abundant signals in the ARC of the hypothalamus *(7)*. NMU-immunoreactive nerve fibers were widely distributed in the anterior hypothalamus, containing PVN, SON, and DMN *(14)*. These results suggest that NMU neurons of the ARC may project to the PVN, SON, and DMN and have synaptic contacts with them. The ARC-PVN pathways may be activated in response to signals associated with a satiation.

Central administration of NMU induced Fos in a high proportion of OT-ir neurons in the PVN and SON. This result shows that the OT pathway may be one of the pathways of NMU-induced feeding behavior. Interestingly, central infusion of OT has been shown to reduce food intake *(15,16)*. It has been estimated that OT neurons comprise 11–16% of the PVN projection to the medulla and spinal cord in rats *(17)*. We consider that NMU mediates its inhibitory action on feeding via brain stem projecting OT-containing neurons.

It was evident that icv injection of NMU triggered Fos expression in a small proportion of AVP-containing perikarya within the PVN and SON. Howard et al. *(7)* demonstrated that water intake, as well as food intake, was suppressed in rats by NMU. These findings suggest that the AVP pathway may also be one of the pathways of NMU-induced feeding and drinking behavior.

Intracerebroventricular infusion of NMU also induced Fos in the PBN of the brain stem. However, the PBN neuronal activation observed might be owing to the activation

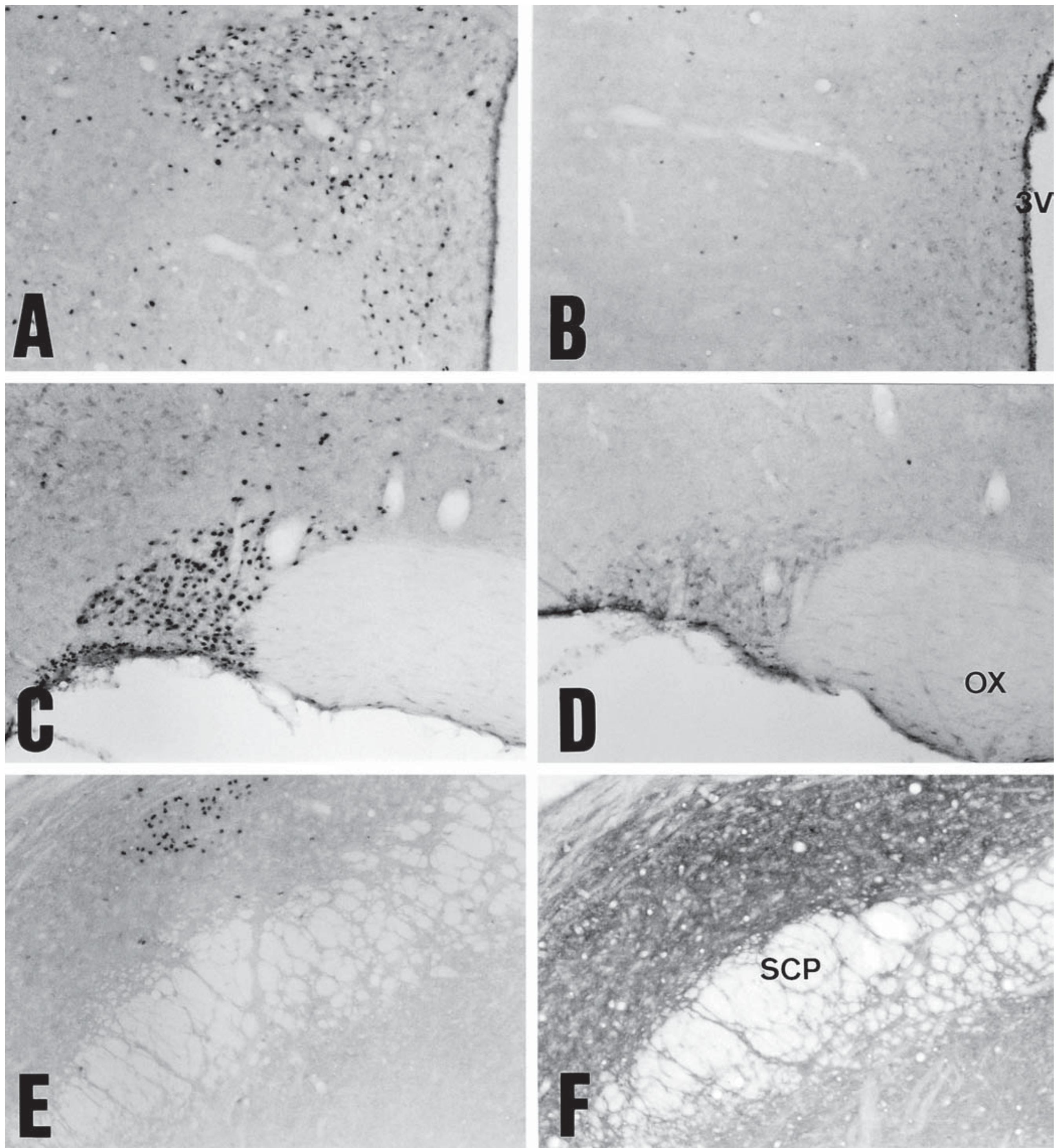

Fig. 2. Photomicrographs of Fos-ir cells in the (**A**) PVN, (**C**) SON, and (**E**) PBN after icv administration of NMU; photomicrographs of Fos-ir cells in the (**B**) PVN, (**D**) SON, and (**F**) PBN after icv administration of saline. 3V, third ventricle; OX, optic chiasm; SCP, superior cerebellar peduncle. Magnification: ×100.

of descending projections from the hypothalamus. In this regard, the PBN receives projections from the PVN *(18,19)*. Interestingly, this area is composed primarily of cholecystokinin (CCK) neurons projecting to the ventromedial and dorsomedial nuclei *(20)*. Cholecystokinin is believed to inhibit food intake. Double labeling of Fos-activated cells in the PBN would provide useful information for further understanding the NMU action mechanism in the brain.

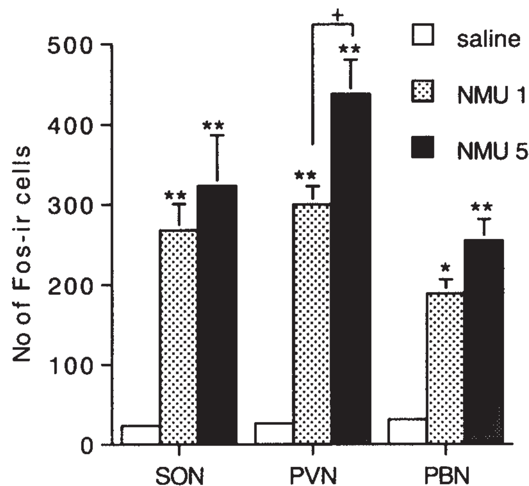

Fig. 3. Effects of icv administration of NMU (1 and 5 nmol) or saline on the number of Fos-ir cells in the SON, PVN, and PBN. Results represent the mean number of blue-black-stained nuclei per four sections of the SON, PVN, and PBN (both sides) of four rats per group. $^{**}p < 0.0001$ vs saline group; $^{*}p < 0.01$ vs saline group; $^{+}p < 0.01$.

Materials and Methods

Animals

All experiments were performed on adult male Sprague-Dawley rats, weighing 300–350 g. They were kept under controlled lighting (lights on 6:00 AM to 6:00 PM) and temperature (22ºC). Free access to laboratory chow and tap water was provided.

Experiment 1

A lateral ventricle cannula was implanted in each rat, as described previously *(21)*. Following surgery, rats were placed in individual cages and handled for about 10 min/d between 5:00 PM and 6:00 PM. Ten days after the operation, the rats were divided into three groups and food was withdrawn at 5:40 PM. Approximately 10 min before the beginning of the dark phase, rats were given icv infusions (10-µL vol manually infused with a Hamilton syringe over 60 s) of either saline ($n = 5$), 1 nmol of NMU ($n = 5$), or 5 nmol of NMU ($n = 5$). Rat NMU-23 was purchased from the Peptide Institute, Japan. After injection, the rats were returned to their home cages, which contained a known amount of rat chow. Two hours after injection, the remaining food was weighed.

Experiment 2

The rats implanted with an icv cannula were handled daily as described in experiment 1. Ten days after the operation, the rats were divided into three groups and placed in individual sampling cages at 5:30 PM. Food was withheld, but water was available at all times during this experiment. NMU (1 and 5 nmol/10 µL in saline) or 10 µL of saline was administered intracerebroventricularly into each free-moving rat at 6:00 PM. All rats were left in their cages for 2 h following the infusion. The possibility that feeding could influence NMU-induced Fos-ir patterns was minimized by removing all food for 2.5 h. The rats were anesthetized with sodium pentobarbital and perfused transcardially with 4% paraformaldehyde. The brains were removed, postfixed overnight in the same fixative, and then placed in 15% sucrose in phosphate-buffered saline (PBS) for 24 h. The brains were frozen, 25-µm transverse sections were cut with a cryostat, and every second section was collected in PBS.

Immunohistochemistry

All reactions were carried out on free-floating sections. The protocol and data for single immunohistochemistry for Fos have been described in detail previously *(21,22)*.

For Fos and OT double immunohistochemistry the sections were first stained for Fos. Fos primary antiserum (1:3000) (Ab-2, rabbit polyclonal IgG; Oncogene) was used for 48 h at 4ºC. The subsequent procedure for the immunohistochemistry followed Vector's protocol (Vectastain ABC kit, Vector) using previously described methods *(22)*. Fos was visualized after the addition of 3,3'-diaminobenzidine, nickel ammonium sulfate, and 0.03%hydrogen peroxide in 50 m*M* Tris. Before staining for OT, the sections were incubated with 3% hydrogen peroxide to prevent any further reaction of the Fos-linked peroxidase. Sections were then incubated in normal goat serum and rabbit anti-OT serum (1:1000) (Chemicon). After approx 24 h of incubation with the primary antibody, the sections were rinsed with PBS and Vector's protocol was carried out. Peroxidase linked to OT was visualized with 3,3'-diaminobenzidine and hydrogen peroxide. Preadsorption of OT antiserum with 10 µ*M* OT antigen (purchased from Peptide Institute) completely blocked OT-immunoreactivity (OT-ir). In addition, the sections were stained for Fos and AVP in the manner already described. Anti-AVP serum raised in rabbits was obtained commercially (Biomeda) and used at a concentration of 1:500. Preadsorption of AVP antiserum with 10 µ*M* AVP (purchased from Peptide Institute) blocked AVP-ir.

The numbers of Fos-ir nuclei in the OT-ir or AVP-ir neurons were counted in four coronal sections throughout the hypothalamic region containing these neuronal populations. Counting was performed on sections from identical retrocaudal levels, making direct comparison between animals possible. For each field, the number of Fos-ir nuclei belonging to OT-ir or AVP-ir neurons was scored.

Statistical Analyses

Data are presented as the mean ± SEM. The food intake and the number of Fos-ir cells were analyzed using one-way analysis of variance followed by Fisher PLSD test. The percentages of dual-labeled cells in the experimental group were analyzed using the unpaired student's *t*-test.

Fig. 4. Representative section of the (**A**) PVN and (**B**) SON stained immunocytochemically for OT (cytoplasm) and Fos (nucleus) in NMU-injected group (A,B); solid arrows indicate double labeled cells, and open arrows indicate OT-ir, but Fos-negative cells. Magnification: ×200. (**C,D**) A few AVP-containing neurons in the PVN and SON expressed Fos in the NMU-injected group; black arrows indicate double labeled cells, and open arrows indicate AVP-ir, but Fos-negative cells. Magnification: ×200. 3V, third ventricle; OX, optic chiasm.

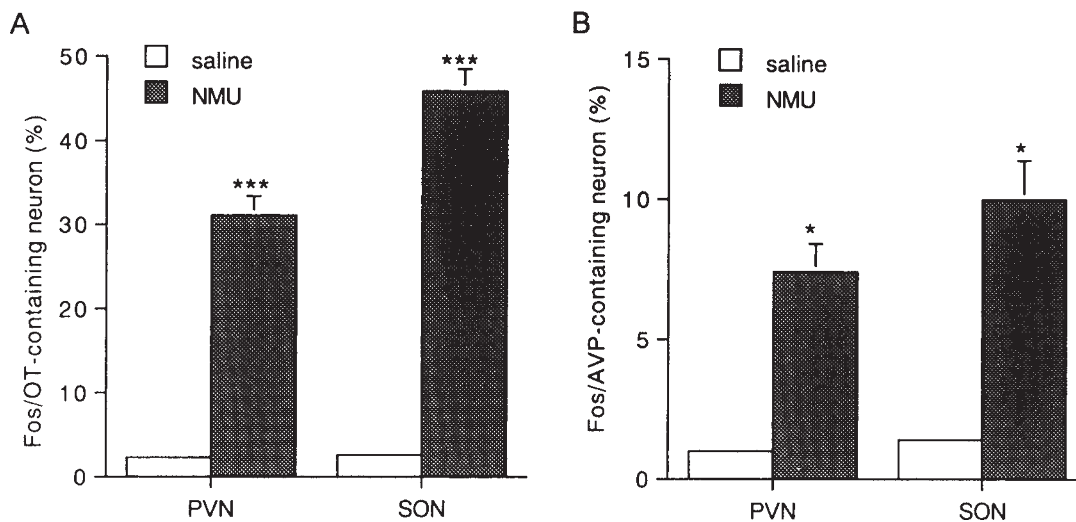

Fig. 5. (**A**) Percentage of OT-ir cells in PVN and SON with nuclear Fos immunostaining. Data are expressed as mean ± SEM ($n = 4$). ***$p < 0.0001$ vs saline control. (**B**) Percentage of AVP-ir cells in SON and PVN with nuclear Fos immunostaining. Data are expressed as mean ± SEM ($n = 4$). *$p < 0.05$ vs saline control.

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