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Centrally Administered Neuropeptide S Activates Orexin-Containing Neurons in the Hypothalamus and Stimulates Feeding in Rats

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Neuropeptide S (NPS) is a newly identified transmitter that modulates arousal and anxiety. To determine potential neuronal targets for NPS, we studied the pattern of neuronal activation as indicated by the expression of Fos. Centrally administered NPS increased Fos-like immunoreactivity (FLI) in the paraventricular, dorsomedial nuclei and lateral hypothalamic area (LHA) of the hypothalamus, the midline thalamic nuclei, and the amygdala, many parts of which are involved in the regulation of emotion, arousal, and feeding. In particular, we noted that Fos-immunoreactive (Fos-ir) levels were increased in orexin-expressing neurons in the LHA. We then studied whether an icv injection of NPS increased food intake. The injection of NPS (1 nmol) significantly stimulated feeding at 2 h in rats, but there was no difference in food intake at 4 h or 24 h. These results suggest that arousal and feeding induced by NPS in the central nervous system may be related to the activation of orexin-expressing neurons.

Key Words: Neuropeptide S; orexin; Fos; food intake; hypothalamus.

Introduction

A new 20-amino-acid peptide, neuropeptide S (NPS), is identified as the endogenous ligand of an orphan G protein–coupled receptor (GPCR) *(1–4)*. Mammalian NPSs and chicken NPS were deduced with high sequence similarity to human NPS *(1–4)*. Both the NPS precursor and receptor mRNA are found predominantly in the central nervous system *(1–4)*. The most prominent expression of the NPS precursor is found in a previously uncharacterized cluster of neurons in the pontine area, located between the noradrenergic locus ceruleus and Barrington's nucleus. NPS acts by activating its cognate receptor and inducing mobilization of intracellular Ca^{2+} *(1)*. The NPS receptor mRNA is widely distributed in areas with high expression levels including the cortex, hypothalamus, amygdala, and multiple midline thalamic nuclei *(1,3)*. Interestingly, its receptor is expressed within hypothalamic structures implicated in the central control of feeding behavior and arousal, i.e., the paraventricular hypothalamic nucleus (PVN), dorsomedial hypothalamic nucleus (DMN), and lateral hypothalamic area (LHA) *(1,3)*. Functionally, the central administration of NPS induces a long-lasting arousal and suppresses all stages of sleep *(1–4)*. In addition, icv administration of NPS produces an anxiolytic-like effect in animals exposed to different stressful paradigms, such as the open field, light-dark box, elevated plus maze *(1)*.

Orexin-A and -B, which are restricted to an area of the hypothalamus centered around the perifornical nucleus, are able to suppress sleep and induce profound wakefulness *(5–7)*, but have no effects on anxiety-like behavior. From the data about the NPS receptor's distribution and function, it is plausible to examine interactions with the orexin neuronal system.

In this study, we mapped the areas of the brain activated by icv administration of NPS by examining Fos-like immunoreactivity (FLI). We also examined the effects of centrally administered NPS on orexin-A-containing neurons in the lateral hypothalamic area of rats, using dual immunostaining for orexin-A and Fos. In addition, orexins have been reported to have roles in regulating feeding behavior *(7,8)*. Therefore, we determined whether the central injection of NPS altered food intake.

Results

Immunohistochemical Identification

To determine the extent of neuronal activation after the icv injection of NPS, we used the early gene product Fos as an immunohistochemical marker. The distribution of Fos-immunoreactivity was analyzed throughout the hypothalamus and thalamus. In the hypothalamus, a large number of Fos-ir cells was observed in the DMN and LHA (Fig. 1C). NPS peptide also induced a moderate number of Fos-ir cells

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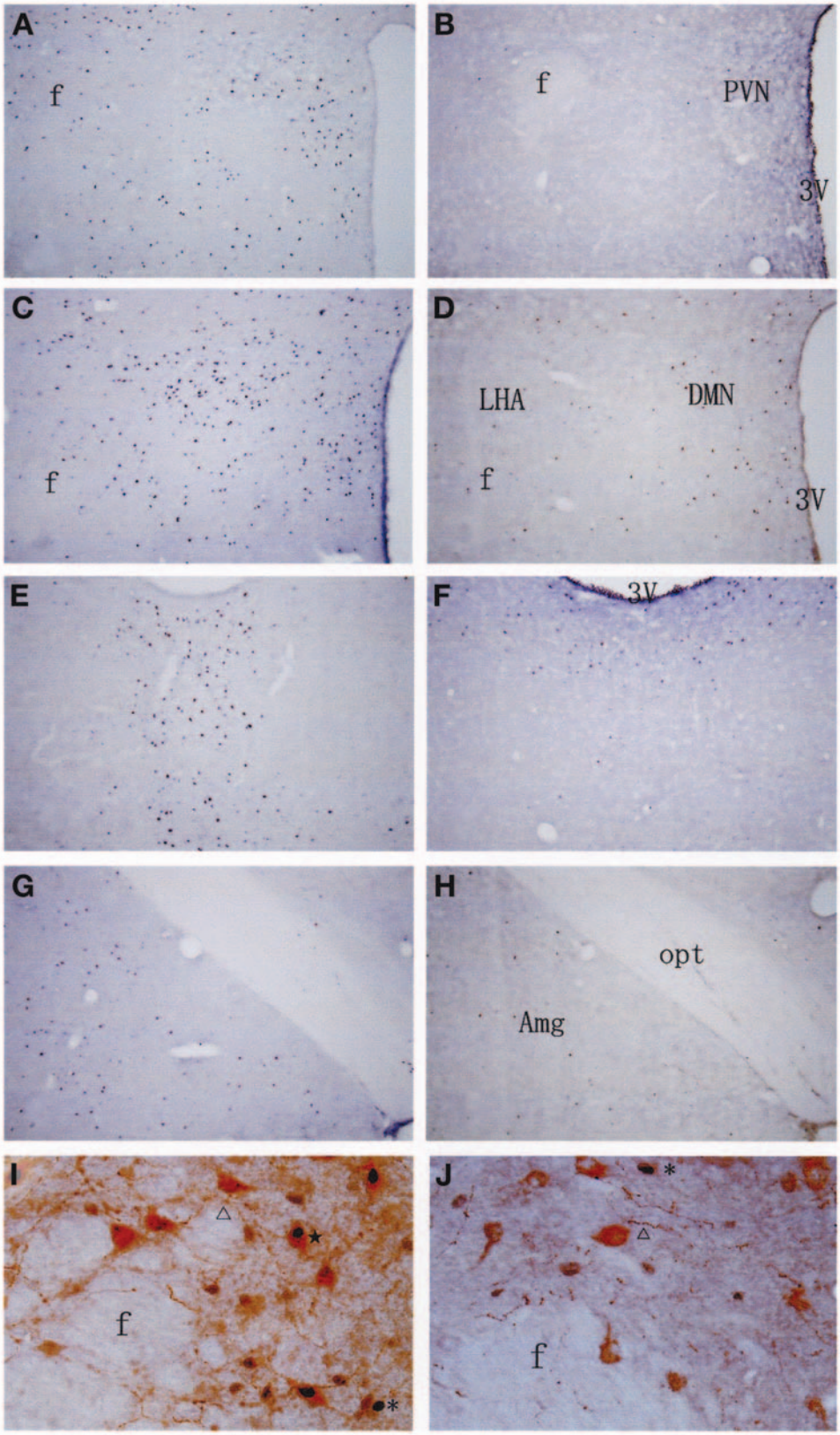

Fig 1. Photomicrographs showing Fos-ir cells in the (**A**) paraventricular hypothalamic nucleus (PVN), (**C**) dorsomedial nucleus (DMN) and lateral hypothalamic area (LHA), (**E**) midline thalamic nuclei, and (**G**) amygdala (Amg) after an icv administration of NPS; photomicrographs of Fos-ir cells in the (**B**) PVN, (**D**) DMN and LHA, (**F**) midline thalamic nuclei, and (**H**) Amg after an icv administration of saline. Magnification: ×100. Many orexin-ir cells in the LHA expressed Fos in NPS-injected animals (**I**) or controls (**J**). The symbols represent cells stained for Fos only (*), NPS only (△), and NPS+Fos (★). Magnification: ×400. f, fornix; 3V, third ventricle; opt, optic tract.

Table 1
Percentage of Fos-Immunoreactive (IR) Cells
in Orexin-IR (OX-IR) Cells in the Lateral Hypothalamic
Area after ICV Administration of NPS (1 nmol) or Saline

	Fos-ir	OX-ir	Double-labeled: Fos-ir/OX-ir	%Double-labeled: Fos-ir +OX-ir/ total OX-ir
NPS	542 ± 46.9[a]	467 ± 35.2	100 ± 14.6[b]	21.0 ± 1.8[a]
saline	202 ± 21.5	462 ± 33.4	9 ± 1.4	1.9 ± 0.3

Data are expressed as the mean ± SEM ($n = 4$). [a]$p < 0.0001$, [b]$p < 0.05$ compared with saline-treated rats.

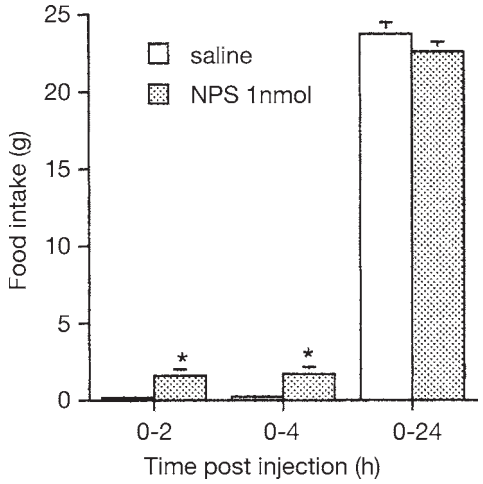

Fig. 2. Effect of NPS (1 nmol, icv) on food intake in rats. Data are expressed as the mean ± SEM for $n = 5$ per group. *$p < 0.05$ versus saline-treated group.

in the PVN (Fig.1A), anterior hypothalamic area, midline thalamic nuclei (Fig 1E), and amygdala (Fig.1G). The brains of rats treated with saline had a few scattered Fos-stained nuclei, but were mostly devoid of immunoreactivity (Figs. 1B,D,F,H).

To determine whether NPS activates orexin-expressing neurons, we carried out double staining for Fos and orexin. Double staining revealed that FLI was present in many orexin-containing neurons of the LHA in the NPS-treated group (Fig. 1I). The percentage of these neurons double-labeled for FLI was 21.0 ± 1.8% in the NPS-treated group (Table 1). In the saline-treated control rats, the proportion of orexin-containing neurons coexpressing Fos was 1.9 ± 0.3% in the LHA.

Effects of NPS on Food Intake

Food intake was significantly increased by 1 nmol NPS at 2 h after the injection compared to saline-treated animals (Fig. 2). This effect was no longer evident at the 4 h and 24 h time points.

Discussion

The present study demonstrated that the icv injection of NPS induced neuronal activation in several regions of the central nervous system as revealed by the induction of Fos expression 2 h after the administration. The overall distribution of FLI is consistent with the distribution of the NPS receptor. Fos-positive cells were located mainly in the DMN and LHA. The regions activated by the injection of NPS are in accordance with nuclei that have been suggested to be involved in the regulation of wakefulness, anxiety, and the energy balance. Notably, the LHA has been shown to be involved in maintenance of the awake state *(9)*. Recent studies suggested that orexin-expressing neurons are distinct neuronal populations in the LHA *(7,8,10–12)*. Orexins have been reported to have roles in regulating the sleep–wake cycle and feeding behavior *(8,12)*. An interesting observation of the present study was the activation of orexin-containing neurons in response to the injection of NPS. These results suggest that NPS neurons innervate the orexin neuronal system. However, whether the recruitment of Fos-positive cells occurred through mono- or polysynaptic connections still remains to be determined.

Although the acute orexigenic action induced by NPS was similar to that seen after the injection of orexins, its effect only lasted for 2 h. The effect of NPS on food intake may be mediated through the activation of the orexin neuronal system. It is well recognized that the orexin neuronal system is closely related with monoaminergic systems *(11)*. These interactions may be involved in the emotional aspects underlying NPS-induced feeding behavior.

A moderate induction of Fos expression was observed in the PVN. As the PVN is known to be an integrative site for the coordination of neuroendocrine and autonomic functions *(13)*, centrally administered NPS may activate the PVN neurons that project to the regions of the brainstem and the spinal cord related to sympathetic outflow. The midline nuclei of the thalamus is known to act as a relay between arousal centers of the brainstem and cortex *(14)*. The NPS-induced expression of Fos in the thalamic midline nuclei

suggests that arousal-promoting functions may be regulated by NPS. The amygdala also expresses Fos following the administration of NPS icv. The amygdala is centrally involved in regulating responses associated in the processing of emotional behavior *(15)*. Thus, it is possible that NPS influences emotional behavior. In fact, Reinscheid et al. found that NPS induced anxiolytic-like behavior in mice *(1–4)*.

Finally, pharmacological intervention directed at the NPS receptor may prove to be an attractive pathway toward the discovery of novel therapeutics for diseases such as anorexia nervosa and sleep disorders.

Materials and Methods

Animals

All experiments were performed on adult male Sprague–Dawley (SD) rats, weighing 250–300 g. The animals were kept under controlled lighting (lights on 6:00 AM to 6:00 PM) and temperature (23°C). Free access to laboratory chow and tap water was provided. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kagawa Prefectural College of Health Sciences.

Experiment 1

For icv administration of NPS ($n = 4$) or saline ($n = 4$), a lateral ventricular cannula was implanted streotaxically. After surgery, all rats were placed in individual cages and were handled for about 10 min/d. Seven days after the operation, NPS 1 nmol (5 μL) (purchased from Peptide Institute, Osaka, Japan), or 5 μL of saline was administered intracerebroventricularly into each free-moving rat at 9:00. Two hours later, the rats were anesthetized with sodium pentobarbital (40 mg/kg ip) and perfused transcardially with 4% paraformaldehyde in 0.12 *M* phosphate buffer. The brains were removed, postfixed overnight in the same fresh fixative, and then placed in 15% sucrose in phosphate-buffered saline (PBS) for 24 h at 4°C. The brains were frozen, and 25-μm transverse sections were cut on a cryostat and collected in PBS. Immunohistochemical detection of Fos was carried out.

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 *(16)*.

Brain sections were processed for double-label immunohistochemistry as reported previously *(17,18)*. For Fos and orexin-A double immunohistochemistry, sections were first stained for Fos (rabbit polyclonal IgG, Oncogene Science, NY; 1:200) for 48 h at 4°C. The subsequent procedure for the immunohistochemistry followed Vector's protocol (Vectastain ABC kit, Vector). Fos-immunoreactivity was visualized with nickel-diaminobenzidine (Ni-DAB) chromogen (10 mg of nickel ammonium sulfate, 10 mg of DAB, and 10 μL of 0.3% hydrogen peroxide in 10 mL of 0.05 *M* Tris-HCl, pH 7.6). Before the immunostaining for orexin-A, the sections were incubated in 3% hydrogen peroxide in PBS to eliminate the Fos-linked peroxidase activity. The sections were then incubated with rabbit anti-human orexin-A IgG (purchased from Peptide Institute, Osaka, Japan, 1:2500) containing 4% normal goat serum. After an approx 24-h incubation with the primary antibody, the sections were rinsed in PBS and the subsequent procedure was carried out according to Vector's protocol. Peroxidase activity linked to orexin-A was visualized with 3,3'-diaminobenzidine and hydrogen peroxide.

In the NPS-treated and control rats, immunoreactive cells (Fos, orexin, Fos + orexin) were counted bilaterally on five sections per animal throughout the LHA containing these neural populations, and a mean value for the two sides were calculated ($n = 4$). Counting was performed on sections from identical retrocaudal levels, making as direct a comparison between the animals as possible.

Experiment 2

A lateral ventricle cannula was implanted in each rat, as in Experiment 1. Following surgery, rats were placed in individual cages and were handled for about 10 min/d between 9:00 AM and 11:00 AM. Seven days after the operation, the rats were divided into two groups and food was withdrawn at 8:30 AM. Half of the rats ($n = 5$) were injected intracerebroventricularly with NPS 1 nmol (5 μL), and the other half ($n = 5$) received saline (5 μL) alone at 9:00 PM. After the injection, the rats were returned to their home cages, which contained a known amount of rat chow. At 2, 4, and 24 h postinjection, the remaining food was weighed.

Statistic Analysis

The percentage of neurons with Fos-immunoreactivity in orexin-ir cells after the administration of NPS or saline was analyzed with a one-way ANOVA followed by Fisher's PLSD test. Food intake was also analyzed using a one-way ANOVA followed by Fisher's PLSD test. Data are presented as the mean ± SEM.

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