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Neuropeptide W as a Stress Mediator in the Hypothalamus

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Neuropeptide W (NPW) was isolated and found to be an endogenous peptide ligand for the orphan receptors GPR7 and GPR8. Centrally administered NPW caused a dose-dependent increase in corticosterone levels in rats. This observation indicates that NPW may play an important role in the hypothalamic organization of the endocrine response to stress. We examined the effects of immobilization stress and cold exposure on NPW-containing neurons in the hypothalamus of the rat, using dual immunostaining for NPW and Fos. In addition, to analyze the function of NPW, we studied the effect of intracerebroventricular (icv) NPW administration on Fos protein accumulation in the brain. Double immunohistochemistry for NPW and Fos showed that the percentage of Fos expression in the NPW-immunoreactive cells of the perifornical nucleus was significantly increased by immobilization stress compared with that in nonstressed rats. Similarly, the results indicated that cold exposure activates NPW-immunoreactive neurons in the perifornical nucleus. An icv administration of NPW resulted in significant Fos expression in the paraventricular nucleus, as compared with saline-infused controls. These results suggest that NPW is related to stress-responsive signal transduction, and that NPW may modulate the hypothalamus–pituitary–adrenal axis.

Key Words: Neuropeptide W; stress; Fos; hypothalamus.

Introduction

Recently, three groups discovered identical ligands to the orphan receptors GPR7 and GPR8, which were designated neuropeptide W (NPW) *(1–3)*. NPW was isolated as two molecular forms, NPW23 and NPW30, with NPW23 being identical to the N-terminal 23-amino-acid sequence of NPW30. Immunohistochemical studies have shown that NPW immunoreactivity is found most abundantly in the hypothalamus of the rat, including the paraventricular nucleus (PVN), supraoptic nucleus, dorsal and lateral hypothalamic areas, perifornical nucleus, and arcuate nucleus *(4)*. Although both GPR7 and GPR8 are expressed in human and mouse hypothalamus, only GPR7 is expressed in the rat *(5)*. The distribution of GPR7 in some hypothalamic nuclei, including PVN, suggests that it may have a role in the modulation of neuroendocrine functions *(6)*. Baker et al. *(7)* have recently demonstrated that intracerebroventricular (icv) administration of NPW23 elevates prolactin and corticosterone, and lowers growth hormone (GH) levels in rats. No significant effects of NPW on hormone release from cultured anterior pituitary cells were observed. This suggests endogenous NPW may play a role in the hypothalamic control of stress hormone secretion. Therefore, we examined the effects of immobilization stress and cold exposure on the immediate response gene (Fos) accumulation in NPW neurons. Furthermore, we performed immunohistochemistry to investigate the effect of icv administration of NPW on Fos immunoreactivity in the hypothalamus. We report here that stress is a potent activator of NPW neurons and that NPW is an important stress mediator in the central nervous system.

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Results

Effects of Immobilization Stress and Cold Exposure on Fos Immunoreactivity in NPW Containing Neurons

NPW-immunoreactive (ir) neurons were widely distributed throughout the hypothalamus, including regions such as the PVN, the supraoptic nucleus, the retrochiasmatic part of the supraoptic nucleus, lateral hypothalamic area, perifornical nucleus, and arcuate nucleus (Figs. 1 and 2). In the hypothalamus, immobilization stress evoked Fos immunoreactivity in the parvocellular division of the PVN, the dorsomedial nucleus, the perifornical nucleus, and the lateral hypothalamic area (Figs. 2A,B). The distribution of Fos-ir cells in the hypothalamus in cold-exposed rats was essentially similar to that seen after immobilization stress, but the distribution of Fos immunoreactivity in the dorso-medial nucleus was diffused in cold-exposed rats (Figs. 2C,D). In the immobilized rats, 25.0 ± 3.0% of NPW-ir cells in the perifornical nucleus showed Fos immunorectivity (mean ± SEM), whereas in the control rats only 4.8 ± 0.8% of NPW-ir cells showed Fos immunoreactivity (Table 1). The percentage of double-labeled cells in the immobilized rats was significantly higher than that in the controls. In the cold-exposed rats, 20.3 ± 1.0% of NPW-ir cells showed Fos immunoreactivity, whereas in the control rats, only 3.9 ± 0.2% of

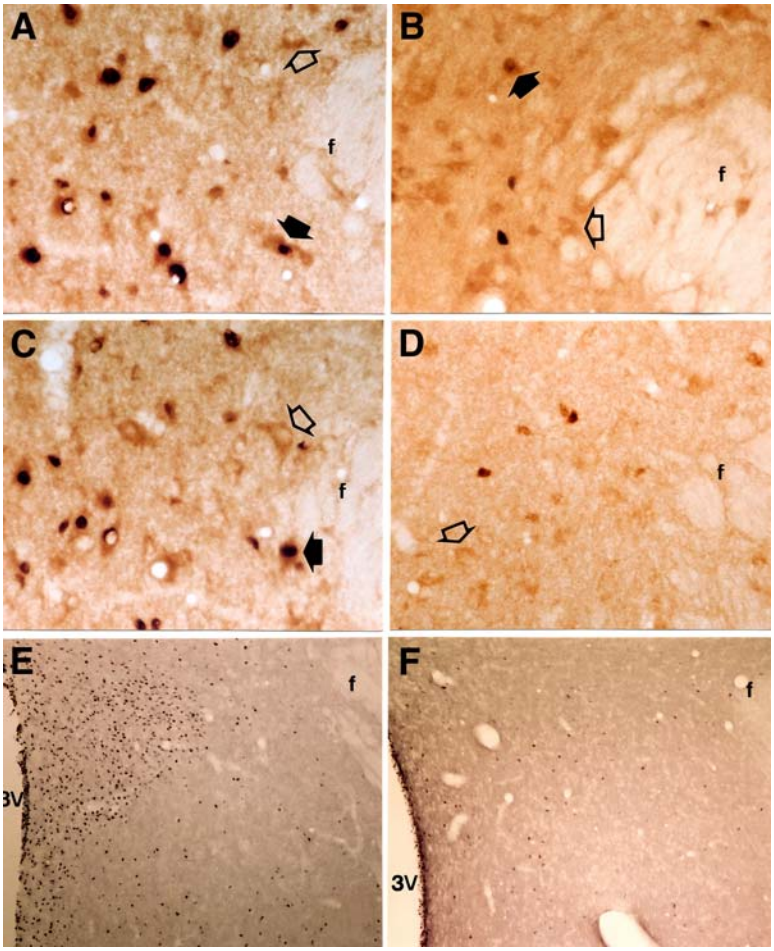

Fig. 1. A representative section of the perifornical nucleus stained immunocytochemically for NPW (cytoplasm) and Fos (nucleus) in immobilized rats (**A**) or controls (**B**). Many NPW-ir cells in the perifornical nucleus expressed Fos in cold-exposed rats (**C**) or controls (**D**); solid arrows indicate double-labeled cells; open arrows indicate NPW neurons without Fos signals. Magnification ×400. 3V; third ventricle, f; fornix. Photomicrograph of Fos-ir cells in the PVN after icv administration of NPW (**E**), or saline (**F**). Magnification ×100.

NPW-ir cells showed Fos immunoreactivity. The percentage of double-labeled cells in cold-exposed rats was significantly higher than that in the controls. The localization of cells containing both Fos immunoreactivy and NPW immunoreactivity was similar in the immobilized and cold-exposed rats.

Effect of icv Administration of NPW on Fos Immunoreactivity in the Hypothalamus

Fos expression in the PVN and the supraoptic nucleus increased significantly after administration of NPW, as compared with the saline infused controls (Figs. 1 and 3).

Discussion

In this study, we demonstrated that immobilizing stress and cold exposure activated NPW-containing neurons in the hypothalamus of rats. Stress is considered to be a modulator of the autonomic nervous system. Central administration of corticotrophin-releasing hormone (CRH) increases sympathetic and decreases central parasympathetic outflow *(8)*. In our study, the rats were immobilized. Immobilization stress increases parasympathetic outflow, and is a predominantly emotional stressor *(9,10)*. In contrast, cold stress is considered mainly to increase sympathetic outflow *(11)*. These results suggest that NPW may participate in the regulation of both the sympathetic and parasympathetic parts of the autonomic nervous system.

We also demonstrated that centrally administered NPW-stimulated Fos in neurons located in the PVN of the hypothalamus. This was supported by the localization of GPR7, which is expressed in the PVN of the hypothalamus *(6)*. The NPW-ir fibers have been observed to be abundant in the PVN *(4)*. The electrophysiological study revealed that PVN neurons are activated by NPW in in vitro brain-slice preparations *(12)*. In addition, they demonstrated that the inhibition of the NPW-induced increase in corticosterone by a CRH antagonist, α-helical CRH, indicates that corticosterone secretion is induced through CRH. Therefore, NPW may be an important regulator of the hypothalamus–pituitary–adrenal (HPA) axis during stress.

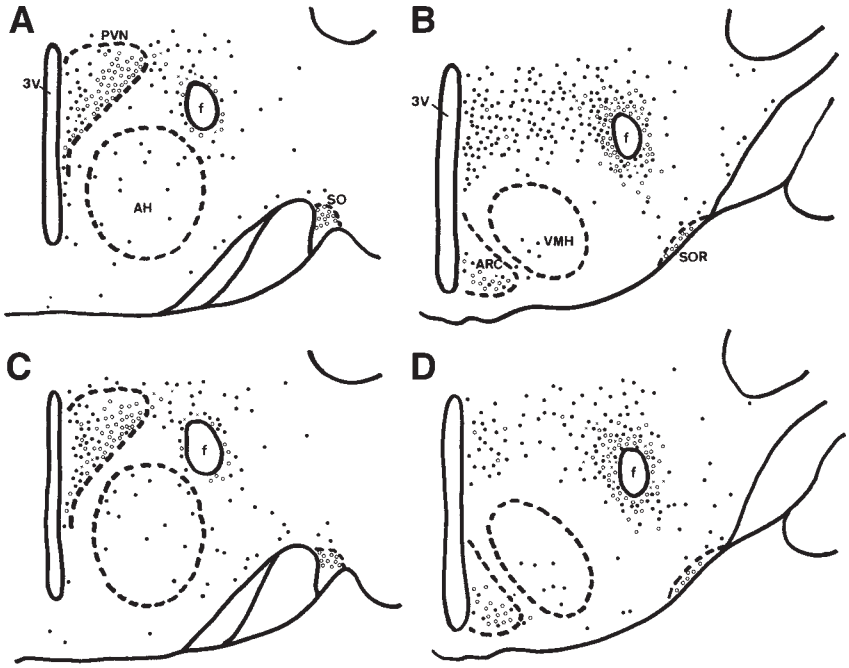

Fig. 2. Localization of neurons containing NPW immunoreactivity and Fos immunoreactivity 90 min after immobilization stress (**A,B**) or cold exposure (**C,D**). Fos-ir, NPW-ir, and these colocalized cells are indicated by dots, open circles, and crosses, respectively. 3V, third ventricle; f, fornix; PVN, paraventricular nucleus; AH, anterior hypothalamic area; SO, supraoptic nucleus; SOR, supraoptic nucleus, retrochiasmatic part; VMH, ventromedial hypothalamic nucleus; ARC, arcuate nucleus.

Table 1
Percentage of Fos-Immunoreactive (ir) Cells in NPW-ir Cells in the Perifornical Nucleus 90 min after Immobilization Stress for 30 min, or 90 min After Cold Exposure for 30 min

	Fos-ir	NPW-ir	Double-labeled: Fos-ir/NPW-ir	Double-labeled: (%) Fos-ir + NPW-ir/ Total NPW-ir
Immobilized stress	47 ± 3.7[a]	67 ± 4.6	17 ± 1.1[b]	25.0 ± 3.0[b]
Control	19 ± 2.0	56 ± 4.0	2 ± 0.6	4.8 ± 0.8
Cold exposure	39 ± 5.2[a]	68 ± 3.8	14 ± 1.7[b]	20.3 ± 1.0[b]
Control	16 ± 1.8	58 ± 5.2	2 ± 0.3	3.9 ± 0.2

Data are expressed as the mean ± SEM ($n = 3$). [a]$p < 0.001$, [b]$p < 0.0001$ vs controls.

Materials and Methods

Animals

All experiments were performed on adult male Sprague–Dawley (SD) rats, weighing 250–300 g. They 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

Rats were put in a plastic tube and prevented from moving for 30 min, and then the immobilization stress was examined. For the double immunostaining for Fos and NPW, immobilized rats ($n = 3$) and controls ($n = 3$) were deeply anesthetized with intraperitoneal (ip) injections of sodium pentobarbital (40 mg/kg) 90 min after undergoing immobilization stress.

The rats were put in a refrigerator at 4°C for 30 min for the exposure to cold. For double immunostaining for Fos and NPW, the cold-exposed rats ($n = 3$) and controls ($n = 3$) were deeply anesthetized with ip injections of sodium pentobarbital (40 mg/kg) 90 min after the cold exposure. All experiments were performed between 09:00 and 11:00. The rats were transcardially perfused 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.

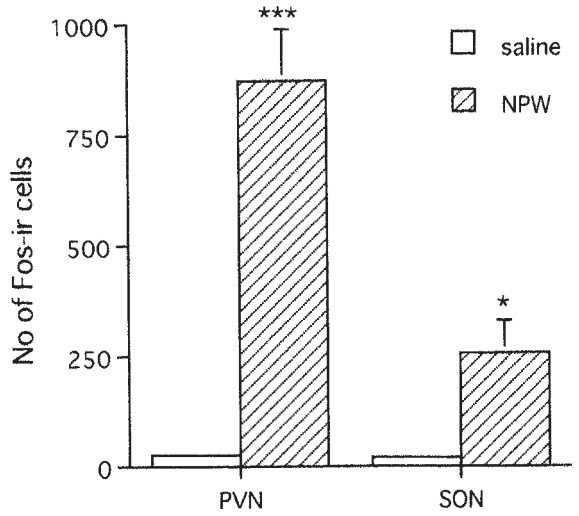

Fig. 3. Effects of icv administration of NPW (5 nmol) or saline on the number of Fos-ir cells in the PVN and supraoptic nucleus (SON). Results represent the mean number of blue-black-stained nuclei per three sections of the PVN and SON of three rats per group. Data are expressed as the mean ± SEM. ***$p < 0.001$, *$p <$ 0.05 vs saline group.

Experiment 2

Animals were anesthetized with sodium pentobarbital (40 mg/kg ip) and a lateral ventricle cannula was implanted according to a previously described method *(13)*. After surgery, all rats were placed in individual cages and were handled for about 10 min/d. Seven days after the operation, NPW (5 nmol/5 µL in saline) 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 paraformaldehyde in phosphate buffer. 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 *(13,14)*. For Fos and NPW double immunohistochemistry, sections were first stained for Fos (rabbit polyclonal IgG, Oncogene Science, US; 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 nickel ammonium sulfate, 10 mg DAB, 10 µL 0.3% hydrogen peroxide in 10 mL 0.05 *M* Tris-HCl, pH 7.6). Before immunostaining for NPW, 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-rat NPW IgG (purchased from Phoenix Pharmaceuticals, Inc., CA, 1:200) containing 4% normal goat serum. After an approx 48-h incubation with the primary antibody, the sections were rinsed in PBS and the subsequent procedure was carried out according to the Vector protocol. Peroxidase activity linked to NPW was visualized with 3,3'-diaminobenzidine and hydrogen peroxide. Preadsorption of the NPW antibody with 10 µ*M* rat NPW (purchased from Peptide Institute) completely blocked the NPW immunoreactivity.

In the immobilized and cold exposed rats, immunoreactive cells (Fos, NPW, Fos + NPW) were counted bilaterally on three sections per animal throughout the perifornical nucleus containing these neural populations, and a mean value for one side was calculated ($n = 3$). In addition, in three NPW-treated and control rats, the number of Fos-ir cells were counted in three coronal sections throughout the PVN and supraoptic nucleus. Counting was performed on sections from identical retro-caudal levels, making as direct comparison between the animals as possible.

Statistic Analysis

The percentage of neurons containing Fos immunoreactivity in NPW-ir cells after immobilization stress and cold exposure was analyzed with one-way ANOVA followed by Fisher's PLSD test. Cell count data in the second experimental group were also analyzed with one-way ANOVA followed by Fisher's PLSD test. Data are presented as the mean ± SEM.

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