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Fos Expression in CRF-containing Neurons in the Rat Paraventricular Nucleus after Central Administration of Neuromedin U

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We examined the effects of centrally administered neuromedin U (NMU) on corticotrophin-releasing factor (CRF)-containing neurons in the hypothalamic paraventricular nucleus (PVN) of rats, using double immunohistochemistry for CRF and Fos. Almost all CRF-containing neurons in the parvocellular divisions of the PVN expressed Fos-like immunoreactivity 90 min after intracerebroventricular administration of NMU (3 nmol/rat). This results suggest the possibility that central NMU may be involved in stress-induced activation of CRF-containing neurons in the PVN.

Keywords: CRF; Fos; Neuromedin U; Paraventricular nucleus

Neuromedin U (NMU), a smooth muscle-contracting peptide, was first isolated from the porcine spinal cord (Minamino *et al.*, 1985). Recent studies have demonstrated that NMU is an endogenous ligand of G-protein coupled orphan receptors, NMU1R and NMU2R (previously called FM-3 and FM-4, respectively) (Fujii *et al.*, 2000; Howard *et al.*, 2000; Kojima *et al.*, 2000; Szekeres *et al.*, 2000). NMU1R is expressed abundantly in the small intestine with little expression in the rat brain (Fujii *et al.*, 2000; Howard *et al.*, 2000; Kojima *et al.*, 2000; Szekeres *et al.*, 2000). On the other hand, NMU2R is expressed in specific regions of the rat brain, including the paraventricular nucleus (PVN) (Howard *et al.*, 2000). NMU is widely distributed in peripheral tissues and the central nervous system (CNS) (Domin *et al.*, 1986; Honzawa *et al.*, 1987; Ballesta *et al.*, 1988). Peripheral administration of NMU increases arterial blood pressure (Minamino *et al.*, 1985), alters ion transport in the gut (Brown & Quito, 1988) and regulates adrenocortical function (Malendowicz *et al.*, 1994). Central administration of NMU caused stress-related responses such as inhibition of feeding, increased gross locomotor activity, hyperthermia and activation of the sympathetic nervous system (Chu *et al.*, 2002; Howard *et al.*, 2000; Ivanov *et al.*, 2002; Nakazato *et al.*, 2000).

Our previous study demonstrated that intracerebroventricular (icv) administration of NMU caused elevation of the plasma concentration of adrenocorticotropic hormone (ACTH) and corticosterone as well as of arginine-vasopressin (AVP) and oxytocin (OXT) in rats (Ozaki *et al.*, 2002). Microinjection of NMU into the PVN caused increases in plasma ACTH and corticosterone concentrations in rats (Wren *et al.*, 2002). Incubation of NMU stimulated secretion of corticotrophin-releasing factor (CRF) and AVP from the hypothalamic explant *in vitro* (Wren *et al.*, 2002). A recent electrophysiological study demonstrated that NMU selectively depolarized some parvocellular neurons in the PVN of rats (Qiu *et al.*, 2003). These results indicate that icv administration of NMU may excite CRF-containing neurons in the CNS, in particular the parvocellular neurons in the PVN. Our previous study showed that after icv administration of NMU many Fos-like immunoreactive (LI) cells were observed in the parvocellular division of the PVN, but AVP-LI cells did not express Fos-LI in this division (Ozaki *et al.*, 2002). Thus, we speculate that Fos-LI cells in the parvocellular division of the PVN after icv administration of NMU may be identical to CRF-containing neurons. However, there is no direct evidence to confirm that centrally administered NMU activates CRF-containing neurons in the CNS, in particular

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the parvocellular division of the PVN. In the present study, we examined whether CRF-containing neurons in the parvocellular divisions of the PVN express Fos after icv administration of NMU in rats, using double immunohistochemistry for Fos and CRF. The expression of the c-*fos* gene has been widely used as a useful marker to detect the activation of neurons in the CNS (Sagar *et al.*, 1988).

Adult male Wistar rats, weighing between 240 and 250 g, were housed in threes in plastic cages on a 12 h light (07:00–19:00), 12 h dark cycle, in an air-conditioned room (24–25°C). Food and tap water were available *at libitum* for 1 week before the experiment. All experiments in this study were approved by the Ethics Committee of the University of Occupational and Environmental Health, Japan.

For icv administration of NMU (3 nmol/rat) ($n = 7$) or saline ($n = 6$), a stainless steel guide cannula (550 μm external diameter) was implanted stereotaxically (Paxinos and Watson, 1986) after the rats were anesthetized with an intraperitoneal (ip) administration of sodium pentobarbital (50 mg/kg). These coordinates were 0.8 mm posterior to bregma, 1.4 mm lateral to the midline and 2.0 mm below the surface of the left cortex, such that the tip of the cannula was 1.5 mm above the left lateral cerebral ventricle. Two stainless steel anchoring screws were fixed to the skull and the cannula was secured in place using dental acrylic cement. The rats were then transferred to their cages and allowed to recover for at least 5 days. The rats were handled and housed individually before the experiment. For icv administration of NMU or saline, a stainless steel injector (300 μm, od) was introduced through the cannula at a depth of 1.5 mm beyond the end of the guide. NMU (Peptide Institute, Minoh, Japan) was dissolved in sterile 0.9% saline. The total volume injected into the lateral ventricle was 7.9 μl.

Ninety minutes after icv administration of NMU or saline, the rats were anesthetized deeply with an ip administration of sodium pentobarbital (50 mg/kg). Deeply anesthetized animals were perfused transcardially with 100 ml of 0.1 M phosphate buffer (PB) (pH 7.4) containing heparin (1,000 U/l) and 150 ml of fixative containing 4% paraformaldehyde (PFA) and 0.2% picric acid in 0.1 M PB. The brains were then removed and divided into blocks including the hypothalamus. These blocks were postfixed with 4% PFA and 0.2% picric acid in 0.1 M PB for 48 h at 4°C. The tissues were then cryoprotected in 20% sucrose in 0.1 M PB for 24 h at 4°C. For immunostaining, serial sections (30 μm thick) were cut with a microtome. The sections were rinsed twice with 0.1 M phosphate buffered saline (PBS) and washed in 0.1 M Tris buffer (pH 7.6) containing 0.3% Triton X-100. Floating sections were incubated with 1% hydrogen peroxide for 60 min, followed by a rabbit polyclonal anti-Fos protein antiserum (#sc-52, Santa Cruz, Santa Cruz, CA, USA) and then diluted 1:1000 in 0.1 M PBS containing 0.3% Triton X-100 at 4°C for 4 days. After washing for 20 min in 0.3% Triton X-100/PBS, the sections were further incubated for 120 min with a biotinylated secondary antibody solution (1:250) and finally with an avidin–biotin peroxidase complex (Vector Labs, Burlingame, CA, USA) for 120 min. The peroxidase in the sections was visualized with 0.02% diaminobenzidine (DAB) in a Tris buffer containing 0.05% hydrogen peroxidase for 20–30 min. For immunostaining for CRF, sections were sequentially incubated with rabbit antisera for CRF antibody (provided by Dr W. Vale: Salk Institute, USA) diluted 1:5000 in 0.1 M PBS containing 0.3% Triton X-100 at 4°C for 5 days. The avidin–biotin peroxidase complex was visualized with diaminobenzidine using nickel sulfate enhancement. Absorption tests were performed with anti-Fos and anti-CRF. The sections were mounted onto glass slides coated with gelatin, air dried, dehydrated in 100% ethanol, cleared with xylene and covered with a coverslip. The number of neurons containing Fos-LI and CRF-LI in the PVN was evaluated under a light microscope by two independent investigators. Three to five sections per rat were used for counting the number of neurons with Fos-LI and CRF-LI. To analyze the division of the PVN representative sections were stained with cresyl violet. The percentage of neurons exhibiting Fos-LI in CRF-LI cells after icv administration of NMU was analyzed by one-way fractional ANOVA followed by Fisher adjustment for multiple comparisons. A value of $P < 0.05$ was considered to be significant.

Fos-LI was observed as dark brown-labeled nuclei in the neurons, whereas CRF-LI neurons were identified by a violet cytoplasmic and axonal precipitate (Fig. 1). In the PVN, CRF-LI neurons were mainly localized in the parvocellular divisions of the PVN (Fig. 1). After absorption tests with anti-Fos and anti-CRF there was no specific immunostaining in the sections.

Ninety minutes after icv administration of NMU (3 nmol/rat), 91 ± 3% of CRF-LI cells showed Fos-LI (mean ± SEM, $n = 7$) in the parvocellular divisions of the PVN (Fig. 2), whereas only 3 ± 1% of CRF-LI cells showed Fos-LI in vehicle-treated rats ($n = 6$) (Fig. 2). The number of neurons with Fos-LI in the PVN after icv administration of saline or NMU (3 nmol/rat) were 194 ± 50 and 1930 ± 236 per rat, respectively. The number of neurons with Fos-LI without CRF-LI after icv administration of saline or NMU per rat were 188 ± 50 (97 ± 26% of total number of Fos-LI cells) and 1350 ± 217 per rat (70 ± 11% of total number of Fos-LI cells), respectively. Many Fos-LI cells without CRF-LI after icv administration of NMU were observed in the magnocellular and parvocellular divisions of the PVN (Fig. 2).

The present study demonstrated that icv administration of NMU caused activation of almost all CRF-containing neurons in the parvocellular divisions of the PVN.

These results suggest that ACTH secreted from the anterior pituitary after icv administration of NMU may be a result of activation of CRF-containing neurons in the PVN and subsequent secretion of CRF in the median eminence. As CRF from the dorsomedial parvocellular division of the PVN stimulates the secretion of ACTH from the anterior

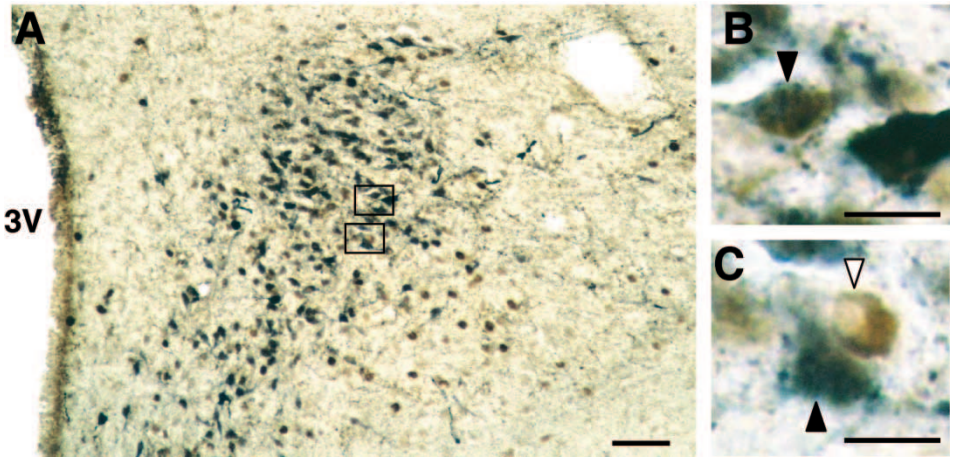

FIGURE 1 Co-existence of Fos-like immunoreactivity (LI) and CRF-LI in the paraventricular nucleus (PVN) 90 min after intracerebroventricular (icv) administration of NMU (3 nmol/rat). Panel **A** shows co-existence of Fos-LI (brown) and CRF-LI (violet). Panel **B** and **C** are enlargements from the boxed areas in panel **A**. Black arrowheads indicate co-existence of nuclear Fos-LI and CRF-LI. White arrowhead indicates Fos-LI without CRF-LI. Scale bar indicates 50 μm in **A** and 10 μm in **B** and **C**. Third ventricle-3V.

pituitary, the present results are in agreement with previous endocrine studies of central effects of NMU on ACTH release (Ozaki *et al.*, 2002; Wren *et al.*, 2002).

In the present study, NMU was administered into a lateral cerebral ventricle. Thus, it is difficult to determine whether NMU activated CRF-containing neurons in the parvocellular division of the PVN directly or indirectly. However, it is speculated that centrally administered NMU may activate CRF-containing neurons directly because PVN neurons express NMU2R (Howard *et al.*, 2000), intra-PVN injection of NMU stimulates secretion of ACTH (Wren *et al.*, 2002) and NMU depolarizes the parvocellular neurons of the PVN in an *in vitro* slice preparation (Qiu *et al.*, 2003).

It is worth noting that many Fos-LI cells without CRF-LI were observed in the magnocellular and the parvocellular division of the PVN. The parvocellular divisions of the PVN contains heterogeneous populations of neurosecretory neurons such as CRF, AVP and thyrotropin-relasing hormone (TRH). Thus, central administration of NMU may also activate neurosecretory neurons other than CRF-containing neurons, because Fos-LI was observed in neurons without CRF-LI. AVP in addition to CRF is known to be a potent stimulant of secretion of ACTH from the anterior pituitary (Swanson *et al.*, 1983; Palkovits *et al.*, 1985; Cai *et al.*, 1999). However, our previous study demonstrated that AVP-LI cells do not express Fos-LI in the parvocellular division of

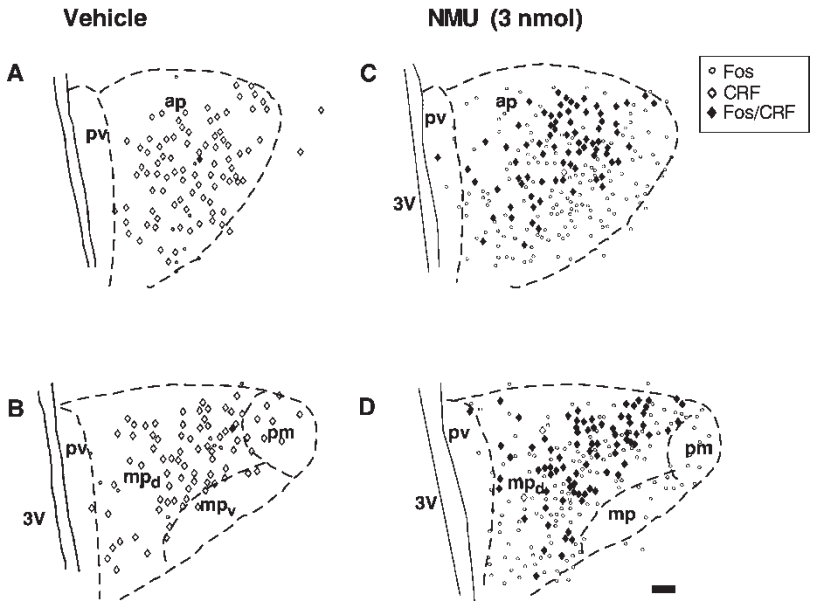

FIGURE 2 Topographical mapping of Fos-like immunoreactivity (LI) and CRF-LI in the paraventricular nucleus (PVN) 90 min after intracerebroventricular (icv) administration of vehicle (saline) (**A**, **B**) or NMU (3 nmol/rat) (**C**, **D**). Panels **A**, **B**, **C** and **D** show co-existence of Fos-LI and CRF-LI (closed diamond). The open circle indicates a Fos-LI positive cell and the open diamond indicates a CRF-LI positive cell. Scale bar indicates 50 μm. 3V, third ventricle; ap, anterior parvocellular PVN; pm, posterior magnocellular PVN; mpv, ventral medial parvocellular PVN; mpd, dorsolateral medial parvocellular PVN; pv, periventricular nucleus. Coronal sections were cut at 30 μm.

the PVN after icv administration of NMU (Ozaki *et al*., 2002).

Our finding that Fos-LI was observed in the magnocellular division of the PVN after icv administration of NMU is in agreement with recent studies, which showed a large number of Fos/OXT positive cells in the PVN and SON after icv administration of NMU, but only a small proportion of Fos/AVP positive cells (Niimi *et al.*, 2001; Ozaki *et al.*, 2002). These results are consistent with the finding that the plasma concentration of OXT, rather than AVP, is predominantly increased after icv administration of NMU (Ozaki *et al.*, 2002). A recent study demonstrated that NMU activates noradrenergic neurons in the brainstem and stimulates the secretion of OXT via activation of β adrenoreceptors (Rokkaku *et al.*, 2003). It is interesting that pretreatment with a β antagonist did not attenuate the increase in plasma ACTH concentration after icv administration of (NMU Rokkaku *et al*., 2003).

NMU-induced stress responses are greatly reduced by pretreatment with an antagonist of CRF, alpha-helical CRF, or anti-CRF IgG. In addition, NMU does not induce locomotor activity in CRH knockout mice (Hanada *et al.*, 2001). Thus, central NMU may play an important role in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis. Further studies should clarify whether stress activates NMU-containing neurons in the CNS.

In conclusion, centrally administered NMU activates CRF-containing neurons in the parvocellular parts of the PVN of rats. NMU in the CNS may be one of the potent neuropeptide candidates that are involved in stress-related responses such as activation of the HPA axis.

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