Centrally administered neuropeptide W-30 activates magnocellular neurosecretory cells in the supraoptic and paraventricular nuclei with neurosecretion in rats

Makoto Kawasaki^{1,2}, Tatsushi Onaka³, Masamitsu Nakazato⁴, Jun Saito^{1,2}, Takashi Mera¹, Hirofumi Hashimoto¹, Hiroaki Fujihara¹, Nobukazu Okimoto², Hideo Ohnishi², Toshitaka Nakamura² and Yoichi Ueta¹

Departments of ¹Physiology and ²Orthopaedics, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan

³Department of Physiology, Jichi Medical School, Tochigi 329-0498, Japan

⁴Department of Third Internal Medicine, Miyazaki University, Miyazaki 889-1692, Japan

(Requests for offprints should be addressed to Y Ueta; Email: yoichi@med.uoeh-u.ac.jp)

Abstract

We examined the effects of i.c.v. administration of neuropeptide W-30 (NPW30) on plasma arginine vasopressin (AVP) and plasma oxytocin (OXT) using RIA. The induction of *c-fos* mRNA, AVP heteronuclear (hn)RNA, and c-Fos protein (Fos) in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of rats were also investigated using *in situ* hybridization histochemistry for *c-fos* mRNA and AVP hnRNA, and immunohistochemistry for Fos. Both plasma AVP and OXT were significantly increased at 5 and 15 min after i.c.v. administration of NPW30 (2.8 nmol/rat). *In situ* hybridization histochemistry revealed that the

induction of *c-fos* mRNA and AVP hnRNA in the SON and PVN were significantly increased 15, 30, and 60 min after i.c.v. administration of NPW30 (1.4 nmol/rat). Dual immunostaining for Fos/AVP and Fos/OXT revealed that both AVP-like immunoreactive (LI) cells and OXT-LI cells exhibited nuclear Fos-LI in the SON and PVN, 90 min after i.c.v. administration of NPW30 (2.8 nmol/rat). These results suggest that central NPW30 may be involved in the regulation of secretion of AVP and OXT in the magnocellular neurosecretory cells in the SON and PVN.

Journal of Endocrinology (2006) **190**, 213–223

Introduction

Neuropeptide W-23 (NPW23) and neuropeptide W-30 (NPW30), 23- and 30-amino acid neuropeptide respectively, were originally isolated from porcine hypothalamus (Shimomura *et al.* 2002). The amino acid sequence of NPW23 is identical to that of the 23 residues of the N-terminal in NPW30 (Shimomura *et al.* 2002). These neuropeptides were discovered as endogenous ligands of G protein-coupled orphan receptors, GPR7 and GPR8, which were originally identified by the cloning of opioid-somatostatin-like receptor genes from human genomic DNA (O'Dowd *et al.* 1995). NPWs and GPR7 are widely distributed in the central nervous system (CNS) (Lee *et al.* 1999, Dun *et al.* 2003).

The i.c.v. administration of NPW23 and NPW30 change secretion of anterior pituitary hormones, such as prolactin and growth hormone, and behavior, such as feeding and drinking (Shimomura *et al.* 2002, Baker *et al.* 2003, Mondal *et al.* 2003, Ishii *et al.* 2003, Levine *et al.* 2005, Taylor *et al.* 2005). NPW23 and NPW30 bind to and activate their receptors at similar effective doses (Mondal *et al.* 2003).

NPW23-like immunoreactive (LI) cells and GPR7 are detected in the parvocellular divisions of the paraventricular nucleus (PVN), which contain neurosecretory cells that produce corticotropin-releasing hormone (CRH) in rats (Lee *et al.* 1999, Dun *et al.* 2003). In rats, i.c.v. administration of NPW23 stimulated the secretion of corticosterone and activated the putative CRH-producing neurons in the parvocellular division of the PVN (Baker *et al.* 2003, Taylor *et al.* 2005). Endogenous NPW may have physiological roles in the neuroendocrine response to stress (Taylor *et al.* 2005).

The overall distribution of Fos-LI cells after i.c.v. administration of NPW23 was consistent with the distribution of GPR7 receptors (Levine *et al.* 2005). In detail, Fos-LI cells after i.c.v. administration of NPW23, NPW23-LI cells, and GPR7 are detected in the supraoptic nucleus (SON), which contains magnocellular neurosecretory cells (MNCs) that produce arginine vasopressin (AVP) and oxytocin (OXT) in rats (Lee *et al.* 1999, Dun *et al.* 2003, Levine *et al.* 2005). However, there are few studies that examine the effects of NPWs on the secretion of AVP and OXT in plasma and MNCs in the SON (Taylor *et al.* 2005).

In the present study, we first examined the effects of i.c.v. administration of NPW30 on plasma concentrations of AVP and OXT in rats using RIA. Secondly, we examined whether i.c.v. administration of NPW30 activated MNCs in the SON and PVN using *in situ* hybridization histochemistry for *c-fos* mRNA. Finally, whether i.c.v. administration of NPW30 selectively activated AVP- and OXT-producing MNCs in the SON and PVN was determined using dual immunostaining for Fos/AVP and Fos/OXT. The expression of the *c-fos* gene has been widely used to detect the neuronal activity in the CNS, in particular, the MNCs in the SON and PVN (Sagar *et al.* 1988, Ceccatelli *et al.* 1989, Sharp *et al.* 1991, Handa *et al.* 1993, Larsen & Mikkelsen 1995, Kawasaki *et al.* 2005). The MNCs in the PVN as well as in the SON are known to synthesize AVP and OXT, which are released into general circulation through terminal axons located in the posterior pituitary. In addition, intronic *in situ* hybridization histochemistry for AVP heteronuclear (hn)RNA was performed to detect the effects of i.c.v. administration of NPW30 on the transcription of the AVP gene in the SON and PVN because it has been used to detect rapid changes in AVP gene transcription (Herman *et al.* 1991, Priou *et al.* 1993, Arima *et al.* 1999, Kawasaki *et al.* 2005).

Materials and Methods

Animals

Adult male Wistar rats, weighing 295 ± 2.0 g (mean $\pm$ S.E.M.), were used in all experiments. They were housed individually in standard plastic cages in an air-conditioned room (23–25 °C) under a 12 h light (0700–1900 h):12 h darkness (1900–0700 h) cycle. Food and water were available *ad libitum*.

Surgical procedures

For i.c.v. administration of NPW30 or saline, the animals were anesthetized (sodium pentobarbital, 50 mg/kg body weight) by i.p. injection and then placed in a stereotaxic frame in a prone position. A stainless steel guide cannula (550 μ m outer diameter, 10 mm length) was implanted stereotactically, following coordinates given by Paxinos & Watson (1986). These coordinates were 0.8 mm posterior to the bregma, 1.4 mm lateral to midline, and 2.0 mm below the surface of the right cortex, so that the tip of the cannula was 1.0 mm above the right cerebral ventricle. Two stainless steel anchoring screws were fixed to the skull, and the cannula was secured in place by acrylic dental cement. The rodents were then returned to their cages and allowed to recover for at least 7 days. They were then handled every day and housed in cages before the start of the experiments.

Central administration of NPW30 or vehicle

For i.c.v. administration of NPW30, a stainless steel injector (300 μ m outer diameter) was introduced through the cannula

to a depth of 1.0 mm beyond the end of the guide. The total volume of solution injected into the lateral ventricle was 10 μ l. This volume was also used in previous studies (Serino *et al.* 1999, Shen & Krukoff 2001, Ozaki *et al.* 2002, Hashimoto *et al.* 2005). Rat NPW30 was purchased from the Peptide Institute (Osaka, Japan). The quality of rat NPW30 has been confirmed by Peptide Institute (supplementary data in the online version of Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol190/issue2/>). NPW30 was dissolved in sterile pyrogen-free 0.9% saline solution (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan).

Experimental procedures

In the first experiment, i.c.v. administration of NPW30 (1.4 and 2.8 nmol/rat) or 0.9% saline (vehicle) was performed ($n=6$ in each group). The rodents were decapitated 5, 15, 30, and 60 min after i.c.v. administration. The trunk blood was collected in plastic tubes containing 0.1 ml of heparin (200 U/ml; Sigma) and then spun (3000 r.p.m.) at 4 °C for 10 min before the plasma concentrations of AVP and OXT were measured using a RIA.

In the second experiment, NPW30 (0.3, 1.4, and 2.8 nmol/rat) or vehicle was also administered i.c.v. ($n=6$ in each group). Thirty minutes after i.c.v. injection, the rodents were decapitated. Brains were removed and placed on powdered dry ice for *in situ* hybridization histochemistry for *c-fos* mRNA.

In the third experiment, i.c.v. administration of NPW30 (1.4 nmol/rat) or vehicle was performed ($n=6$ in each group). The rodents were decapitated 5, 15, 30, 60, or 180 min after i.c.v. administration. The time-course of this experiment was determined by our previous studies (Ozaki *et al.* 2002, Hashimoto *et al.* 2005, Kawasaki *et al.* 2005). Brains were removed and placed on powdered dry ice for *in situ* hybridization histochemistry for *c-fos* mRNA and AVP hnRNA.

In the final experiment, NPW30 (2.8 nmol/rat) or vehicle was administered i.c.v. ($n=5$ in each group). Ninety minutes after i.c.v. administration of the solution, the rodents were anesthetized deeply with an i.p. injection of sodium pentobarbital (75 mg/kg body weight) following perfusion and then fixed brains were used for immunohistochemistry for Fos, AVP, and OXT.

All experimental procedures in this study were performed in accordance with guidelines on the use and care of laboratory animals as set out by the Physiological Society of Japan and under the control of the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan.

RIA for AVP and OXT

Plasma concentrations of AVP and OXT were determined by RIA with specific anti-AVP (Sakurai *et al.* 1985) and anti-OXT (Higuchi *et al.* 1985) antiserum as described previously

(Onaka & Yagi 1990). The inter- and intraassay coefficients of variation were 14 and 7% for AVP, and 10 and 6% for OXT respectively. The minimum detection limits were 0.48 and 7.8 pg/ml for AVP and OXT respectively.

In situ hybridization histochemistry for c-fos mRNA and AVP hnRNA

All rodents were decapitated between 1400 and 1700 h, the brains were removed rapidly, frozen on dry ice, and stored at -80°C . Frozen sections were cut at $12\text{ }\mu\text{m}$ -thickness in a cryostat at -20°C and mounted onto gelatin/chrome alum-coated slides. The SON and PVN were localized by referring to the standard atlas of the rat brain by Paxinos & Watson (1986). The sections including the SON were chosen from plate 23 in the atlas. The sections including the PVN were chosen from plate 25 in the atlas. The localization of the sections obtained from each rat was checked for similar positions by microscopic observation. The SON and PVN consist of four sections, i.e., eight sites per rat, were used to measure the density of signals by autoradiography. The slides were warmed to room temperature and allowed to dry for 10 min and then fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 5 min. They were then washed twice in PBS and incubated in 0.9% NaCl containing 0.25% acetic anhydride (v/v) and 0.1 M triethanolamine at room temperature for 10 min. The sections were then dehydrated using a series of 70% (1 min), 80% (1 min), 95% (2 min), and 100% (1 min) ethanol solutions consecutively and dilapidated in 100% chloroform for 5 min. The slides were then rehydrated partially first in 100% (1 min) and then 95% (1 min) ethanol and allowed to air dry briefly.

Hybridization was carried out overnight at 37°C in 45 μl hybridization buffer containing 50% formamide and $4\times$ saline sodium citrate (SSC; $1\times$ SSC = 150 mM NaCl and 15 mM sodium citrate), which contained 500 $\mu\text{g}/\text{ml}$ sheared salmon sperm DNA (Sigma), 250 $\mu\text{g}/\text{ml}$ baker's yeast total RNA (Boehringer Mannheim GmbH, Mannheim, Germany), $1\times$ Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), and 10% dextran sulfate (molecular mass 500 000 Da; Sigma) under a Nescofilm (Bando Chemical IMD, Ltd, Osaka, Japan) coverslip. The probes used were 3'-end ^{35}S -oligodeoxynucleotides complementary to mRNA coding for *c-fos* (probe sequence = 5'-CAG CGG GAG GAT GAC GCC TCG TAG TCC GCG TTG AAA CCC GAG AAC ATC-3') and AVP hnRNA (probe sequence = 5'-GCA CTG TCA GCA GCC CTG AAC GGA CCA CAG TGG TAC-3'). The specificity of each probe has been described previously (Nomura *et al.* 1999, Serino *et al.* 1999). Total counts of 1×10^6 c.p.m./slide were used. After hybridization, sections were washed for 1 h at intervals of 15 min in $1\times$ SSC at 55°C , and for a further 1 h at intervals of 30 min in $1\times$ SSC at room temperature. All independent experimental sections were treated simultaneously to minimize the variable effects of hybridization and wash stringency. Hybridized sections containing the

SON and PVN were apposed to autoradiography film (Hyperfilm; Amersham) for 10 days for each probe. The autoradiographic images were quantified using an MCID imaging analyzer (Imaging Research, Inc., St Catherines, ON, Canada). The images were captured by charge-coupled device camera (DAGE-MTI, Inc., Michigan City, IN, USA) with $40\times$ magnification.

The locations of the magnocellular division of the SON and the magnocellular and parvocellular divisions of the PVN were chosen from the atlas. The areas of the SON and PVN were enclosed using captured images. The edges of the SON and PVN were determined using plates 23 and 25 of the atlas respectively (Paxinos & Watson 1986). The areas of the PVN contained both the magnocellular and parvocellular divisions. The mean optical density of autoradiographs was measured by comparing it with simultaneously exposed [^{14}C] microscale samples (Amersham). The standard curve was fitted by the optical density of the [^{14}C] microscale in the same film. Slides hybridized with the probes of the *c-fos* mRNA and AVP hnRNA were dipped into a nuclear emulsion (K-5, Ilford, Cheshire, UK) and exposed for a further 30 days. After development, the sections that stained by cresylecht violet were observed by microscopy.

Dual detection of Fos-LI and AVP-/OXT-LI

Deeply anesthetized animals were perfused transcardially with 0.1 M phosphate buffer (PB; pH 7.4) containing heparin (1000 U/l) followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB. The brains were then removed and divided into three blocks that included the hypothalamus. The blocks were post-fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB for 60 h at 4°C . The tissues were then cryoprotected in 20% sucrose in 0.1 M PB for 48 h at 4°C . Serial sections of $30\text{ }\mu\text{m}$ for dual staining for Fos and AVP/OXT were cut using a microtome. The sections were rinsed twice with 0.1 M PBS containing 0.3% Triton X-100 with 1% hydrogen peroxidase for 60 min. They were then rinsed twice with 0.1 M PBS containing 0.3% Triton X-100. The floating sections were incubated with a primary Fos antibody (sc-52, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by a dilution ratio of 1:500 in 0.1 M PBS containing 0.3% Triton X-100 at 4°C for 3 days. After washing for 20 min in 0.1 M PBS solution containing 0.3% Triton X-100, the sections were further incubated for 120 min with a biotinylated secondary antibody solution (1:250), and finally with an avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) for 120 min. The peroxidase in the sections was visualized with 0.02% diaminobenzidine in a Tris buffer containing 0.05% hydrogen peroxidase for 5–7 min. During the dual staining for AVP or OXT, the sections were incubated sequentially in AVP antibody (INCSTAR Corp., Stillwater, MN, USA; diluted 1:10 000) or OXT antibody (CHEMICON International, Inc., Temecula, CA, USA;

diluted 1:5000) for 2 days each at 4 °C. The avidin–biotin peroxidase complex was made visible using nickel sulfate.

The sections were then mounted onto gelatin-coated slides, air dried, dehydrated in 100% ethanol, cleared with xylene, and coverslipped. The presence of a dark-brown label that appeared in round structures was judged as indicative of Fos-LI positive nuclei and a violet label that appeared in spindle-shaped structures was judged as indicative of AVP-/OXT-LI. Details of the immunohistochemistry have been published elsewhere (Ison *et al.* 1993, Onaka *et al.* 1995). To count the double-labeled cells, three serial sections including the SON per animal and two serial sections including the PVN per animal were chosen and counted under a light microscope.

Statistical analysis

A mean deviation from control (percentage) $\pm$ S.E.M. was calculated from the results of the *in situ* hybridization histochemistry. Each group within an experiment was compared to the control group. The results of RIA and immunohistochemical staining were also expressed as the mean $\pm$ S.E.M. The data were analyzed using a one-way fractional ANOVA followed by a Fisher's-protected least significant difference type adjustment test for multiple comparisons. The statistical significance was set at $P < 0.05$.

Results

Effects of i.c.v. administration of NPW30 on plasma concentrations of AVP and OXT

The concentrations of plasma AVP and OXT were measured after i.c.v. administration of NPW30 (1.4 and 2.8 nmol/rat)

or vehicle. In comparison to vehicle, the concentrations of both plasma AVP and OXT were significantly increased in a dose-related manner, 5 min after i.c.v. administration of NPW30 (1.4 and 2.8 nmol/rat), and were also significantly increased 15 min after i.c.v. administration of NPW30 (2.8 nmol/rat; Fig. 1; $n = 6$ in each group).

Effects of i.c.v. administration of NPW30 on the expression of the *c-fos* gene in the SON and PVN

In situ hybridization histochemistry revealed that the expression of the *c-fos* gene in the SON and PVN increased 30 min after i.c.v. administration of NPW30, in all the doses (0.3, 1.4, and 2.8 nmol/rat; Fig. 2). Thirty minutes after administration of NPW30 (0.3 nmol/rat), the expression of the *c-fos* gene increased significantly in the SON ($143 \pm 11\%$ of control; $n = 6$ in each group; $P = 0.03$; Fig. 2A) and PVN ($395 \pm 17\%$ of control; $n = 6$ in each group; $P < 0.01$; Fig. 2B). Thirty minutes after i.c.v. administration of NPW30 (1.4 nmol/rat), the expression of the *c-fos* gene increased significantly in the SON ($330 \pm 16\%$ of control; $n = 6$ in each group; $P < 0.05$; Fig. 2A) and PVN ($523 \pm 22\%$ of control; $n = 6$ in each group; $P < 0.01$; Fig. 2B). Thirty minutes after i.c.v. administration of NPW30 (2.8 nmol/rat), the expression of the *c-fos* gene increased significantly in the SON ($358 \pm 19\%$ of control; $n = 6$ in each group; $P < 0.05$; Fig. 2A) and PVN ($596 \pm 27\%$ of control; $n = 6$ in each group; $P < 0.01$; Fig. 2B).

In situ hybridization histochemistry also revealed that the i.c.v. administration of NPW30 (1.4 nmol/rat) caused significant increases in the expression of the *c-fos* gene from 15 to 60 min in the SON and PVN (Fig. 3). The i.c.v. administration of NPW30 induced the expression of the *c-fos* gene to the greatest degree at 30 min in the SON ($450 \pm 48\%$ of control at 5 min; $n = 6$ in each group; $P < 0.01$; Fig. 3A)

Figure 1 Time-course effects of i.c.v. administration of neuropeptide W-30 (NPW30; 1.4 and 2.8 nmol/rat) or 0.9% saline (vehicle) on plasma concentrations of (A) arginine vasopressin (AVP) and (B) oxytocin (OXT) in conscious rats. Data for plasma concentrations of AVP and OXT are expressed as means $\pm$ S.E.M. (each group, $n = 6$). * $P < 0.01$, compared with vehicle-administered rats. † $P < 0.05$, ‡ $P < 0.01$, compared with NPW (1.4 nmol/rat)-administered rats.

Figure 2 Dose-response effects of i.c.v. administration of NPW30 (0.3, 1.4, and 2.8 nmol/rat) or vehicle on *c-fos* transcript prevalence in the (A) SON and (B) PVN. Values represent means $\pm$ s.e.m. (each group, $n=6$). * $P<0.05$, † $P<0.01$, compared with vehicle-administered rats.

and PVN ($666 \pm 68\%$ of control at 5 min; $n=6$ in each group; $P<0.01$; Fig. 3B). The expression of the *c-fos* gene after i.c.v. administration of vehicle did not change significantly. Microscopic observation revealed that the *c-fos* gene-expressing cells were distributed throughout the SON (Fig. 4A and C) and in the parvocellular and magnocellular divisions of the PVN throughout the ependymal cells of the third ventricle (Fig. 4B and D). The sections stained by cresylecht violet also emphasized the distributions of the *c-fos* gene-expressing cells in the SON and PVN (Fig. 4E and F).

Dual detection of Fos-LI and OXT-/AVP-LI in the SON and PVN

Numerous Fos-LI cells in the SON after i.c.v. administration of NPW30 (2.8 nmol/rat) were concomitantly labeled with one of either antibody for AVP or OXT (Fig. 5). In the magnocellular part of the PVN, numerous Fos-LI cells were also concomitantly labeled with one of either antibody for AVP or OXT (Fig. 6). Taking the total AVP-LI-positive cells counted in each nucleus as 100%, the percentage of Fos-LI-positive cells was $59.3 \pm 5.4\%$ in the SON and $71.9 \pm 2.6\%$ in the PVN after i.c.v. administration of NPW30

Figure 3 Time-course effects of i.c.v. administration of NPW30 (1.4 nmol/rat) or vehicle on *c-fos* transcript prevalence in (A) SON and (B) PVN. Values represent means $\pm$ s.e.m. (each group, $n=6$). * $P<0.01$, compared with vehicle-administered rats.

Figure 4 Representative dark-field photomicrographs (A)–(D) of emulsion-dipped slides hybridized to ^{35}S -labeled oligodeoxynucleotide probe complementary to *c-fos* mRNA in the SON (A) and (C) and PVN (B) and (D) 30 min after i.c.v. administration of NPW30 (1.4 nmol/rat) (C) and (D) or vehicle (A) and (B). After development, these sections (C) and (D) were stained by cresylecht violet as representative bright-field photomicrographs (E) and (F). Bars = 100 μm . 3V, third ventricle; OX, optic chiasma.

(2.8 nmol/rat, $n=5$). After i.c.v. administration of vehicle, the percentage of Fos-LI-positive cells of AVP-LI-positive cells was $6.77 \pm 3.7\%$ in the SON and $19.3 \pm 5.2\%$ in the PVN ($n=5$). Similarly, the percentage of Fos-LI-positive cells

Figure 5 Coexistence of Fos-like immunoreactivity (LI) and AVP-/OXT-LI in the SON 90 min after i.c.v. administration of NPW30 (2.8 nmol/rat). (A) Coexistence of Fos-LI (brown) and AVP-LI (violet). (B) Enlargement from the boxed areas in (A). (C) Coexistence of Fos-LI (brown) and OXT-LI (violet). (D) Enlargement from the boxed areas in (C). Black arrowheads indicate coexistence of nuclear Fos-LI and AVP-/OXT-LI. White arrowhead indicates AVP-LI without Fos-LI. Bars = 100 μm . OX, optic chiasma.

Figure 6 Coexistence of Fos-LI and AVP-/OXT-LI in the PVN 90 min after i.c.v. administration of NPW30 (2.8 nmol/rat). (A) Coexistence of Fos-LI (brown) and AVP-LI (violet). (B) Enlargement from the boxed areas in (A). (C) Coexistence of Fos-LI (brown) and OXT-LI (violet). (D) Enlargement from the boxed areas in (C). Black arrowheads indicate coexistence of nuclear Fos-LI and AVP-/OXT-LI. White arrowhead indicates AVP-LI without Fos-LI. Bars = 100 μm . 3V, third ventricle.

of OXT-LI was $85.4 \pm 3.3\%$ in the SON and $90.1 \pm 1.5\%$ in the PVN, after i.c.v. administration of NPW30 (2.8 nmol/rat, $n=5$). After i.c.v. administration of vehicle, the percentage of Fos-LI-positive cells of OXT-LI was $9.40 \pm 0.99\%$ in the SON and $29.5 \pm 1.3\%$ in the PVN ($n=5$). The distributions of the dual-stained cells are shown in Fig. 7 and 8. Although the Fos-LI cells were detected throughout the SON and PVN, the Fos-LI-positive cells of AVP-LI cells were found throughout the SON and mainly in the magnocellular divisions of the PVN. The Fos-LI-positive cells of OXT-LI cells were also found throughout the SON and in the anterior magnocellular and ventral medial parvocellular divisions of the PVN.

Effects of i.c.v. administration of NPW30 on AVP hnRNA in the SON and PVN

In situ hybridization histochemistry revealed that the i.c.v. administration of NPW30 (1.4 nmol/rat) caused significant increases in the expression level of AVP hnRNA from 15 to 60 min in the SON and PVN (Fig. 9). The i.c.v. administration of NPW30 induced the expression level of AVP hnRNA to the greatest degree at 30 min in the SON ($167 \pm 7.4\%$ of control at 5 min; $n=6$ in each group; $P < 0.01$; Fig. 9A) and PVN ($205 \pm 11\%$ of control at 5 min, $n=6$ in each group, $P < 0.01$; Fig. 9B). The expressing cells of AVP hnRNA were observed throughout the SON (Fig. 10A and C) and in the magnocellular and parvocellular divisions of the PVN (Fig. 10B and D). The sections stained by cresylecht violet also emphasized the distributions of the expressing cells of AVP hnRNA in the SON and PVN (Fig. 10E and F).

Figure 7 Topographical mapping of Fos-LI and AVP/OXT-LI in the SON 90 min after i.c.v. administration of vehicle (A) and (B) or NPW30 (2.8 nmol/rat) (C) and (D). (A) and (C) Coexistence of Fos- and AVP-LI (closed square). (C) and (D) Coexistence of Fos- and OXT-LI (closed triangle). In each panel, three 30 µm thick coronal sections from the anterior to posterior SON were selected. The open circle indicates a Fos-LI-positive cell, the open square indicates an AVP-LI-positive cell, and the closed square, a cell immunoreactive for both Fos and AVP. The open triangle indicates an OXT-LI-positive cell, and the closed triangle, a cell immunoreactive for both Fos and OXT. Bar indicates 100 µm. OX, optic chiasma.

Discussion

The present study demonstrated that centrally administered NPW30 caused significant increases in the plasma AVP and OXT levels in conscious rats. Centrally administered NPW30 also induced the *c-fos* gene expression throughout the SON and the magnocellular and parvocellular neurons of the PVN. Dual immunohistochemistry confirmed that most AVP- and OXT-LI cells expressed Fos-LI in the SON and PVN, after central administration of NPW30.

The plasma concentrations of AVP and OXT were increased significantly 5 and 15 min after i.c.v. administration of NPW30 (2.8 nmol/rat) and returned to normal levels since then (Fig. 1). Ozaki *et al.* (2002) also demonstrated that

Figure 8 Topographical mapping of Fos-LI and AVP/OXT-LI in the PVN 90 min after i.c.v. administration of vehicle (A) and (B) or NPW30 (2.8 nmol/rat) (C) and (D). (A) and (C) Coexistence of Fos-LI and AVP-LI (closed square). (C) and (D) Coexistence of Fos-LI and OXT-LI (closed triangle). In each panel, two 30 µm thick coronal sections from the anterior to posterior PVN were selected. The open circle indicates a Fos-LI-positive cell, the open square, an AVP-LI-positive cell, and the closed square, a cell immunoreactive for both Fos and AVP. The open triangle indicates an OXT-LI-positive cell, and the closed triangle, a cell immunoreactive for both Fos and OXT. Bar indicates 100 µm. 3V, third ventricle.

centrally administered neuromedin U (NMU), a peptide that suppresses food intake, caused significant increases in the plasma concentration of OXT from 15 to 60 min after i.c.v. administration of NMU in conscious rats. The effect of i.c.v. administration NMU on plasma OXT levels appeared later than those of NPW30. Later elevations of plasma OXT levels after i.c.v. administration of NMU may be mediated, at least in part, by secondary effects, such as changes in behavior (Howard *et al.* 2000), hyperthermia (Nakazato *et al.* 2000), and increased arterial blood pressure (Minamino *et al.* 1985). Relatively rapid changes in plasma AVP and OXT levels after i.c.v. administration of NPW30 indicate that centrally administered NPW30 may activate AVP- and OXT-secreting neurons directly or the neural pathways to these neurons.

Figure 9 Time-course effects of i.c.v. administration of NPW30 (1.4 nmol/rat) or vehicle on AVP heteronuclear (hn)RNA transcript prevalence in (A) SON and (B) PVN. Values represent the means $\pm$ s.e.m. (each group, $n=6$). * $P<0.01$, compared with vehicle-administered rats.

In addition, it may be expected that degradation and/or clearance of NPW30 in the cerebrospinal fluid may be shorter and/or faster than that of NMU, because increase in plasma AVP and OXT levels was rapid and transient (5–15 min) after i.c.v. administration of NPW30 compared with those results obtained from i.c.v. administration of NMU. The secondary effects, such as possible changes of arterial blood pressure and/or plasma osmolality, may affect the plasma AVP and OXT levels after i.c.v. administration of NPW30, however, those possibilities can be excluded, because Taylor *et al.* (2005) showed centrally administered NPW23 did not alter arterial blood pressure or heart rate, and we have demonstrated that the plasma osmolality at any time (5, 15, 30, and 60 min) in the time-course study and any dose (1.4 and 2.8 nmol) in the dose–response study used here did not change significantly after i.c.v. administration of vehicle or NPW (data not shown).

Taylor *et al.* (2005) have demonstrated that plasma concentrations of AVP and OXT were not increased significantly, 15 min after i.c.v. administration of NPW23 (1 and 3 nmol/rat, 2 μ l volume of total solutions) in conscious rats. They have also demonstrated that the release of AVP and OXT were not significantly increased 5 and 15 min after i.c.v. administration of neuropeptide B (NPB), which was also discovered as endogenous ligand of GPR7 (Samson *et al.* 2004). It is possible to explain why the effects of NPW23 and NPW30 in the present study, and NPB on the concentrations of AVP and OXT in plasma were different. One possibility is the biochemical difference among NPW23, NPW30, and NPB. Although NPWs and NPB are expressed from two different genes, these peptides share approximately 60% sequence homology (Fujii *et al.* 2002). NPW23 and NPW30 bind to and activate their

receptors at similar effective dose, and also, NPWs and NPB bound with similar affinity to their receptors (Mondal *et al.* 2003, Tanaka *et al.* 2003). Biochemical differences alone cannot explain this discrepancy; the reason for the

Figure 10 Representative dark-field photomicrographs of emulsion-dipped slides hybridized to 35 S-labeled oligodeoxynucleotide probe complementary to AVP hnRNA in SON (A) and (C), and PVN (B) and (D), 30 min after i.c.v. administration of NPW30 (1.4 nmol/rat) (C) and (D) or vehicle (A) and (B). After development, these sections (C) and (D) were stained by cresylecht violet as representative bright-field photomicrographs (E) and (F). Bars=100 μ m. 3V, third ventricle; OX, optic chiasma.

early elevations of plasma AVP and OXT after i.c.v. administration of NPW30 remains unclear. Another possibility is the difference in the volume of centrally administered solutions. Although they used a small volume (2 μ l) for i.c.v. administration, we used a relatively large volume (10 μ l). We cannot exclude the possibility that a relatively large volume of centrally administered solution may reach and activate wide brain sites, such as the brainstem, which is known to terminate their axons to the MNCs in the SON and PVN.

Although i.c.v. administration of NPW30 caused an increase in plasma AVP in conscious rats, its physiological meaning is not clear. A previous study demonstrated that central administration of NPW23 stimulated drinking in rats (Baker *et al.* 2003). It is postulated that NPW in the CNS may be involved in the regulation of AVP secretion, drinking behavior, and body fluid balance. OXT is a peptide, which has various kinds of physiological functions; however, we could not suggest in this study the physiological relevance to the transient elevation of plasma OXT. Further physiological and behavioral studies need to be performed to elucidate the relationship between NPW-induced elevation of OXT and physiological/behavioral changes.

Dual immunostaining showed that Fos-LI was seen in both AVP-LI and OXT-LI cells throughout the SON and in the magnocellular and parvocellular divisions of the PVN. A previous study demonstrated that GPR7, which is the receptor bound to and activated by NPW30, was observed in the various brain regions, including the SON and parvocellular division of the PVN (Lee *et al.* 1999). Our results showed that the distribution of the Fos-LI cells appears to overlap with that of GPR7 mRNA in the SON and the parvocellular division of the PVN. An electrophysiological study demonstrated that NPW acted directly on the post-synaptic membrane of the parvocellular neurosecretory cells in the PVN, using the whole-cell patch clamp technique by perfusion with tetrodotoxin (Taylor *et al.* 2005). Our results showed that Fos-LI cells were detected not only in the parvocellular division, but also in the magnocellular division of the PVN. As Fos is directly and/or indirectly induced by an activation of neuronal activity, the parvocellular neurosecretory cells in the PVN are activated directly, and the MNCs in the PVN may be activated indirectly after i.c.v. administration of NPW30. The primary site activated by i.c.v. administration of NPW30 in the CNS is not clear, except for the parvocellular neurosecretory cells in the PVN. The large number of Fos/AVP cells and Fos/OXT MNCs were observed in the SON and PVN after i.c.v. administration of NPW30. These results are consistent with the results that elevations in plasma concentrations of both AVP and OXT were observed after i.c.v. administration of NPW30.

In the present study, we demonstrated that the expression levels of the *c-fos* mRNA and Fos-LI in the SON and PVN were increased after i.c.v. administration of NPW30. The marked increase in the *c-fos* mRNA in the SON and PVN was observed 30 min after i.c.v. administration of NPW30.

The increase in the expression level of *c-fos* mRNA should reflect the neuronal activation either directly and/or indirectly after i.c.v. administration of NPW30. The neuronal activity in the SON and PVN may be modulated by the NPW30 released in an autocrine and/or paracrine manner, because NPW-LI cells were detected in the SON and PVN (Dun *et al.* 2003). Also, neural networks may be involved in the activation of the AVP- and OXT-producing cells in the SON and PVN. It is worth noting that the expression level of *c-fos* mRNA and the number of Fos-LI in the PVN were greater than those in the SON after i.c.v. administration of NPW30 (in each dose; Fig. 2, 7, and 8). One possibility why the PVN cells were more activated than the SON cells is that the data obtained from the PVN may reflect the total changes of parvocellular cells and magnocellular cells, because the magnocellular and parvocellular divisions of the PVN were not considered for the measurement of the *c-fos* mRNA levels by quantitative imaging analysis. The other possibility is that the signal in the ependymal cells may contaminate the data obtained from the PVN, because the area of ependymal cells is close to the PVN. In addition, the activation of the *c-fos* gene and Fos expression in the PVN by NPW-administration may be more sensitive than that in the SON, because Levine *et al.* (2005) demonstrated in the Fos study, that the regional density of Fos-LI in the PVN was much higher than that of Fos-LI in the SON after i.c.v. administration of NPW. It is also worth noting that the *c-fos* gene was expressed throughout the ependymal cells of the third ventricle after i.c.v. administration of NPW30. The expression levels of the *c-fos* mRNA after i.c.v. administration of NPW30 were relatively stronger than those after i.c.v. administration of the vehicle. It may be possible that ependymal cells around the third ventricle may be activated by endogenous NPW30 in an autocrine and/or paracrine manner, because the NPW-LI ependymal cells and fibers were exhibited in lining the third ventricle of rats (Dun *et al.* 2003). However, as there are no previous reports regarding the presence of GPR7 in the ependymal cells, the possibility of a non-specific effect by i.c.v. administration of NPW30 cannot be excluded.

Previous studies also described that centrally administered NPW23 increased the release of corticosterone (Baker *et al.* 2003, Taylor *et al.* 2005). They suggested that centrally administered NPW activated the hypothalamo-pituitary-adrenal axis. We have demonstrated that *c-fos* mRNA and Fos-LI were detected throughout the SON and in the magnocellular and parvocellular divisions of the PVN after i.c.v. administration of NPW30. These results indicate that the release of corticosterone may be stimulated by both AVP and CRH.

The present study also showed that i.c.v. administration of NPW30 caused a rapid increase in the expression of AVP hnRNA in the SON and PVN. As the analysis of AVP hnRNA using intronic *in situ* hybridization histochemistry can detect a significant change in AVP gene transcription in the SON and PVN after stress, such as osmotic change, AVP hnRNA is a sensitive indicator for AVP gene transcription

(Herman *et al.* 1991, Priou *et al.* 1993, Arima *et al.* 1999, Kawasaki *et al.* 2005). In this study, the elevation in AVP hnRNA levels is consistent with the result of elevated plasma AVP after i.c.v. administration of NPW30. According to the representative dark- and bright-field photomicrographs (Fig. 10), the expressing cells of AVP hnRNA were observed throughout the SON and in the magnocellular and parvocellular divisions of the PVN. Several studies demonstrated that AVP hnRNA levels were rapidly and sensitively increased in response to acute stress (Kovacs & Sawchenko 1996, Ma *et al.* 1997a, 1997b). Thus, NPW, which induces AVP gene expression, may be involved in stress-related responses in the CNS.

In conclusion, centrally administered NPW30 activates AVP- and OXT-producing MNCs and stimulates secretion of AVP and OXT in conscious rats.

Acknowledgements

We thank Akio Takeuchi for his technical assistance. This paper was supported in part by UOEH Grant for Advanced Research, Grant-in-Aid for Science Research (B), no. 16390061 for Y U and Grant-in-Aid for Science Research (C), no. 16591518 for H O from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Arima H, Kondo K, Kakiya S, Nagasaki H, Yokoi H, Yambe Y, Murase T, Iwasaki Y & Oiso Y 1999 Rapid and sensitive vasopressin heteronuclear RNA responses to changes in plasma osmolality. *Journal of Neuroendocrinology* **11** 337–341.
- Baker JR, Cardinal K, Bober C, Taylor MM & Samson WK 2003 Neuropeptide W acts in brain to control prolactin, corticosterone, and growth hormone release. *Endocrinology* **144** 2816–2821.
- Ceccatelli S, Villar MJ, Goldstein M & Hokfelt T 1989 Expression of c-Fos immunoreactivity in transmitter-characterized neurons after stress. *PNAS* **86** 9569–9573.
- Dun SL, Brailoiu GC, Yang J, Chang JK & Dun NJ 2003 Neuropeptide W-immunoreactivity in the hypothalamus and pituitary of the rat. *Neuroscience Letters* **349** 71–74.
- Fujii R, Yoshida H, Fukusumi S, Habata Y, Hosoya M, Kawamata Y, Yano T, Hinuma S, Kitada C, Asami T *et al.* 2002 Identification of a neuropeptide modified with bromine as an endogenous ligand for GPR7. *Journal of Biological Chemistry* **277** 34010–34016.
- Handa RJ, Nunley KM & Bollnow MR 1993 Induction of c-fos mRNA in the brain and anterior pituitary gland by a novel environment. *Neuroreport* **4** 1079–1082.
- Hashimoto H, Hyodo S, Kawasaki M, Mera T, Chen L, Soya A, Saito T, Fujihara H, Higuchi T, Takei Y *et al.* 2005 Centrally administered adrenomedullin 2 activates hypothalamic oxytocin-secreting neurons, causing elevated plasma oxytocin level in rats. *American Journal of Physiology. Endocrinology and Metabolism* **289** E753–E761.
- Herman JP, Schafer MK, Watson SJ & Sherman TG 1991 *In situ* hybridization analysis of arginine vasopressin gene transcription using intron-specific probes. *Molecular Endocrinology* **5** 1447–1456.
- Higuchi T, Honda K, Fukuoka T, Negoro H & Wakabayashi K 1985 Release of oxytocin during suckling and parturition in the rat. *Journal of Endocrinology* **105** 339–346.
- Howard AD, Wang R, Pong SS, Mellin TN, Strack A, Guan XM, Zeng Z, Williams DL, Jr., Feighner SD, Nunes CN *et al.* 2000 Identification of receptors for neuromedin U and its role in feeding. *Nature* **406** 70–74.
- Ishii M, Fei H & Friedman JM 2003 Targeted disruption of GPR7, the endogenous receptor for neuropeptides B and W, leads to metabolic defects and adult-onset obesity. *PNAS* **100** 10540–10545.
- Ison A, Yuri K, Ueta Y, Leng G, Koizumi K, Yamashita H & Kawata M 1993 Vasopressin- and oxytocin-immunoreactive hypothalamic neurones of inbred polydipsic mice. *Brain Research Bulletin* **31** 405–414.
- Kawasaki M, Yamaguchi K, Saito J, Ozaki Y, Mera T, Hashimoto H, Fujihara H, Okimoto N, Ohnishi H, Nakamura T *et al.* 2005 Expression of immediate early genes and vasopressin heteronuclear RNA in the paraventricular and supraoptic nuclei of rats after acute osmotic stimulus. *Journal of Neuroendocrinology* **17** 227–237.
- Kovacs KJ & Sawchenko PE 1996 Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. *Journal of Neuroscience* **16** 262–273.
- Larsen PJ & Mikkelsen JD 1995 Functional identification of central afferent projections conveying information of acute “stress” to the hypothalamic paraventricular nucleus. *Journal of Neuroscience* **15** 2609–2627.
- Lee DK, Nguyen T, Porter CA, Cheng R, George SR & O’Dowd BF 1999 Two related G protein-coupled receptors: the distribution of GPR7 in rat brain and the absence of GPR8 in rodents. *Brain Research. Molecular Brain Research* **71** 96–103.
- Levine AS, Winsky-Sommerer R, Huitron-Resendiz S, Grace MK & de Lecea L 2005 Injection of neuropeptide W into paraventricular nucleus of hypothalamus increases food intake. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **288** R1727–R1732.
- Ma XM, Levy A & Lightman SL 1997a Rapid changes in heteronuclear RNA for corticotrophin-releasing hormone and arginine vasopressin in response to acute stress. *Journal of Endocrinology* **152** 81–89.
- Ma XM, Levy A & Lightman SL 1997b Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: a study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. *Endocrinology* **138** 4351–4357.
- Minamino N, Kangawa K & Matsuo H 1985 Neuromedin U-8 and U- novel uterus stimulating and hypertensive peptides identified in porcine spinal cord. *Biochemical and Biophysical Research Communications* **130** 1078–1085.
- Mondal MS, Yamaguchi H, Date Y, Shimbara T, Toshinai K, Shimomura Y, Mori M & Nakazato M 2003 A role for neuropeptide W in the regulation of feeding behavior. *Endocrinology* **144** 4729–4733.
- Nakazato M, Hanada R, Murakami N, Date Y, Mondal MS, Kojima M, Yoshimatsu H, Kangawa K & Matsukura S 2000 Central effects of neuromedin U in the regulation of energy homeostasis. *Biochemical and Biophysical Research Communications* **277** 191–194.
- Nomura M, Ueta Y, Serino R, Yamamoto Y, Shibuya I & Yamashita H 1999 Effects of centrally administered pituitary adenylate cyclase-activating polypeptide on c-fos gene expression and heteronuclear RNA for vasopressin in rat paraventricular and supraoptic nuclei. *Neuroendocrinology* **69** 167–180.
- O’Dowd BF, Scheidele MA, Nguyen T, Cheng R, Rasmussen JS, Marchese A, Zastawny R, Heng HH, Tsui LC, Shi X *et al.* 1995 The cloning and chromosomal mapping of two novel human opioid-somatostatin-like receptor genes, GPR7 and GPR8, expressed in discrete areas of the brain. *Genomics* **28** 84–91.
- Onaka T & Yagi K 1990 Differential effects of naloxone on neuroendocrine responses to fear-related emotional stress. *Experimental Brain Research* **81** 53–58.
- Onaka T, Luckman SM, Guevara-Guzman R, Ueta Y, Kendrick K & Leng G 1995 Presynaptic actions of morphine: blockade of cholecystokinin-induced noradrenaline release in the rat supraoptic nucleus. *Journal of Physiology* **482** 69–79.
- Ozaki Y, Onaka T, Nakazato M, Saito J, Kanemoto K, Matsumoto T & Ueta Y 2002 Centrally administered neuromedin U activates neurosecretion and induction of c-fos messenger ribonucleic acid in the paraventricular and supraoptic nuclei of rat. *Endocrinology* **143** 4320–4329.

- Paxinos G & Watson C 1986 *The Rat Brain in Stereotaxic Coordinate*, Edition 4, Sydney, Australia: Academic Press.
- Priou A, Oliver C & Grino M 1993 *In situ* hybridization of arginine vasopressin (AVP) heteronuclear ribonucleic acid reveals increased AVP gene transcription in the rat hypothalamic paraventricular nucleus in response to emotional stress. *Acta Endocrinologica* **128** 466–472.
- Sagar SM, Sharp FR & Curran T 1988 Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science* **240** 1328–1331.
- Sakurai H, Kurimoto F, Ohono H, Kanai A, Nomura K, Demura H & Shizume K 1985 A simple and highly sensitive radioimmunoassay for 8-arginine vasopressin in human plasma using a reversed-phase C18 silica column. *Nippon Naibunpi Gakkai Zasshi* **61** 724–736.
- Samson WK, Baker JR, Samson CK, Samson HW & Taylor MM 2004 Central neuropeptide B administration activates stress hormone secretion and stimulates feeding in male rats. *Journal of Neuroendocrinology* **16** 842–849.
- Serino R, Ueta Y, Hara Y, Nomura M, Yamamoto Y, Shibuya I, Hattori Y, Kitamura K, Kangawa K, Russell JA *et al.* 1999 Centrally administered adrenomedullin increases plasma oxytocin level with induction of c-fos messenger ribonucleic acid in the paraventricular and supraoptic nuclei of the rat. *Endocrinology* **140** 2334–2342.
- Shan J & Krukoff TL 2001 Intracerebroventricular adrenomedullin stimulates the hypothalamic-pituitary-adrenal axis, the sympathetic nervous system and production of hypothalamic nitric oxide. *Journal of Neuroendocrinology* **13** 975–984.
- Sharpe FR, Sagar SM, Hicks K, Lowenstein D & Hisanaga K 1991 *c-fos* mRNA, Fos, and Fos-related antigen induction by hypertonic saline and stress. *Journal of Neuroscience* **11** 2321–2331.
- Shimomura Y, Harada M, Goto M, Sugo T, Matsumoto Y, Abe M, Watanabe T, Asami T, Kitada C, Mori M *et al.* 2002 Identification of neuropeptide W as the endogenous ligand for orphan G-protein-coupled receptors GPR7 and GPR8. *Journal of Biological Chemistry* **277** 35826–35832.
- Tanaka H, Yoshida T, Miyamoto N, Motoike T, Kurosu H, Shibata K, Yamanaka A, Williams SC, Richardson JA, Tsujino N *et al.* 2003 Characterization of a family of endogenous neuropeptide ligands for the G protein-coupled receptors GPR7 and GPR8. *PNAS* **100** 6251–6256.
- Taylor MM, Yuill EA, Baker JR, Ferri CC, Ferguson AV & Samson WK 2005 Actions of neuropeptide W in paraventricular hypothalamus: implications for the control of stress hormone secretion. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **288** R270–R275.

Received in final form 26 April 2006

Accepted 12 May 2006

Made available online as an Accepted Preprint 29 May 2006