Molecular Characterization and Biological Function of Neuroendocrine Regulatory Peptide-3 in the Rat

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Neuroendocrine regulatory peptide (NERP)-3, derived from the neurosecretory protein VGF (non- acronymic), is a new biologically active peptide identified through peptidomic analysis of the peptides secreted by an endocrine cell line. Using a specific antibody recognizing the C-terminal region of NERP-3, immunoreactive (ir)-NERP-3 was identified in acid extracts of rat brain and gut as a 30-residue NERP-3 with N-terminal pyroglutamylation. Assessed by radioimmunoassay, ir-NERP-3 was more abundant in the brain, including the posterior pituitary (PP), than in the gut. Immunohistochemistry demonstrated that ir-NERP-3 was significantly increased in the suprachiasmatic nucleus, the magnocellular division of the paraventricular nucleus, and the external layer of the median eminence, but not in the supraoptic nucleus, after dehydration. The immunoreactivity was, however, markedly decreased in all of these locations after chronic salt loading. Intracerebroventricular administration of NERP-3 in conscious rats induced Fos expression in a subset of arginine vasopressin (AVP)-containing neurons in the supraoptic nucleus and the magnocellular division of the paraventricular nucleus. On in vitro isolated rat PP preparations, NERP-3 caused a significant AVP release in a dose-related manner, suggesting that NERP-3 in the PP could be an autocrine activator of AVP release. Taken together, the present results suggest that NERP-3 in the hypothalamo-neurohypophyseal system may be involved in the regulation of body fluid balance. (Endocrinology 153: 1377–1386, 2012)

The vgf gene, originally identified in nerve growth factor-stimulated PC12 cells, encodes a 68-kDa secretory protein of 617 amino acids in rodents. The VGF (non- acronymic) protein harbors multiple consensus motifs for prohormone convertase (PC)1/3 and PC2 to produce peptides secreted via the regulated secretory pathway (1). Studies of vgf-deficient mice have received much attention because they are lean and hypermetabolic (2). These findings support the notion that VGF serves as a precursor to a series of biologically active peptides. Biologically active peptides derived from rat VGF described to date are AQEE-30, TLQP-62, TLQP-21 (3), neuroendocrine regulatory peptide (NERP)-1, and NERP-2 (4, 5).

We have recently used a mass spectrometric approach to analyze the peptides secreted by exocytosis from an endocrine cell line (6, 7). In parallel with this peptidomic survey, we have developed an ex vivo assay using tissue pieces from transgenic mice systemically expressing the Ca\(^{2+}\) indicator protein apoaequorin, considering that most neuropeptides use Ca\(^{2+}\) as a second messenger (7).
With this assay system, we have demonstrated that rat VGF (180–209) and VGF (489–507), designated NERP-3 and NERP-4, respectively, elicit a transient intracellular free calcium concentration response in the hypothalamus and pituitary tissue pieces, in addition to AQEE-30 (7). AQEE-30 has already been independently described as a bona fide VGF-derived neuropeptide, which is reported to enhance synaptic activity in a whole-cell patch clamp recording on rat hippocampal cells and induce penile erection upon injection into the paraventricular nucleus (PVN) of male rats (8). Regarding NERP peptides, NERP-3 exhibits sequence identity in more species of mammals than NERP-4, which might suggest a common biological implication attributed to NERP-3 (Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). In the present study, therefore, we characterized immunoreactive (ir)-NERP-3 using chromatography coupled with mass spectrometry and measured by RIA tissue distribution of ir-NERP-3 in central and peripheral tissues. Furthermore, immunohistochemistry of NERP-3 was also conducted on the hypothalums of colchicine-treated rats.

The hypothalamic supraoptic (SON) and the PVN are major sites for synthesizing arginine vasopressin (AVP) and oxytocin (OXT) (9). It is well known that the magnocellular neurosecretory cells in the SON and the PVN project into the posterior pituitary (PP) (termed the hypothalamo-neurohypophyseal system) and release these peptides into the systemic circulation in response to various physiological stimuli such as dehydration and hypovolemia (10, 11).

NERP-1 and NERP-2, also derived from VGF, are hypothalamic peptides involved in the control of body fluid homeostasis by regulating AVP release (4). We then asked whether NERP-3 as well would take part in osmotic control in the hypothalamo-neurohypophyseal system. Therefore, we investigated the role of NERP-3 in body fluid homeostasis as follows: 1) the effects of osmotic stimuli (water deprivation for 2 d and 2% salt loading for 5 d) on ir-NERP-3 levels in the rat hypothalamo-neurohypophyseal system, 2) the effects of intra-cerebroventricular (icv) administration of NERP-3 on Fos expression in AVP and OXT neurons, and 3) the effects of NERP-3 on AVP release from freshly isolated rat PP.

**Materials and Methods**

**Animals**

For measurements of tissue concentration and characterization of ir-NERP-3, male Sprague Dawley rats were used (250–300 g; SLC, Shizuoka, Japan) after a 1-wk taming period. Histological and physiological experiments were performed on adult male Wistar rats weighing 180–300 g, which were housed in standard cages at 23–25 °C under a 12-h light, 12-h dark cycle (lights on at 0700 h). All experiments were carried out in accordance with the protocols of the Physiological Society of Japan and under the control of the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health and National Cerebral and Cardiovascular Center Research Institute, Japan.

**Peptide synthesis**

NERP-3 (QQETAAETETRTHLTRYNLESPGPERVW with N terminal pyroglutamylation) and its tyrosinyl C-terminal nonapeptide YESPGPERVW for radioiodination were custom synthesized using the 9-fluorenlymethoxy carbonyl strategy and purified by reverse-phase (RP)-HPLC as described previously (4). Peptide sequences were confirmed by mass spectrometry and amino acid analysis.

**Antibody preparation and RIA**

An antiserum against NERP-3 was prepared as reported (7), and the RIA for NERP-3 was carried out as described previously (4) using the antiserum (no. 586-3, 1:300,000). Specificity of the RIA was examined with the following peptides: rat and human NERP-1, NERP-2, NERP-4 (7), AQEE-30, and 20 known peptides [angiotensin II, adrenomedullin, α-atrial natriuretic peptide, calcitonin, calcitonin gene related peptide, calcitonin receptor stimulating peptide, glucagon, Leu-enekephalin, melanocyte concentrating hormone, Met-enkephalin-Arg-Gly-Leu, neuropeptide Y (NPY), neurotensin, peptide histidine isoleucine-27, preademulin N-terminal 20 amino acid peptide, corticotropin-releasing factor, neuregulin U, secretin, somatostatin, AVP, β-melanocyte stimulating hormone]. No peptide showed more than 0.01% cross-reactivity.

The RIA for the rat C-terminal end of VGF protein (VGF-C), rat NERP-1, and NERP-2 was performed as reported (4, 12). These antibodies have no cross-reactivity with other peptides derived from VGF protein, including NERP-3.

**Extraction and characterization of ir-NERP-3 in tissues**

Rat brain, pituitary, and peripheral tissues were collected, dissected on ice, weighed, and extracted, as described in our previous reports (12, 13). After lyophilization, tissue extracts were dissolved in an RIA standard buffer and used in RIA for NERP-3, NERP-1, NERP-2, and VGF-C. Brain (15 g) and jejunum/ileum (9.7 g) extracts were separated with a Sephadex G-50 gel filtration column equilibrated with 1 M acetic acid (fine, 1.8 × 135 cm; GE Healthcare, Buckinghamshire, UK) at a flow rate of 8 ml/h with a fraction size of 6 ml/tube. NERP-3 immunoreactivities were pooled and separated on an RP-HPLC column (Symmetry 300 C18 5 μm, 4.6 × 250 mm; Waters, Milford, MA) using gradient elution of acetonitrile from 10–60% in 0.1% trifluoroacetic acid over 60 min at a flow rate of 1 ml/min. The immunoreactivity of each fraction was assessed by RIA for NERP-3. Major ir-NERP-3 in the gel filtration of brain extracts (2.5 g equivalents) was immunoprecipitated with antiserum against NERP-3 (antiserum no. 586-3) and analyzed on a mass spectrometer (PBS IIc; Ciphergen, Fremont, CA) (14).
Implantation of a guide cannula for icv administration

Adult male Wistar rats weighing 250–300 g were anesthetized (sodium pentobarbital, 50 mg/kg body weight) by ip injection and then placed in a stereotaxic frame in a prone position. A stainless steel guide cannula (550 μm outer diameter, 10 mm length) was implanted stereotaxically. 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 rats were then returned to their cages and allowed to recover for at least 7 d. They were then handled every day and housed in cages before experiments.

Intracerebroventricular administration and tissue preparation for immunohistochemistry

A stainless steel cannula (500 μm outer diameter, 11 mm length) was introduced through the cannula to a depth of 1.0 mm beyond the end of the guide. Artificial cerebrospinal fluid was used as the vehicle or solvent for colchicine (Nacalai Tesque Inc., Kyoto, Japan) and NERP-3. The total volume of solution injected into the lateral ventricle was 10 μl, as in previous studies (15–18). For NERP-3 immunohistochemistry, rats received an icv administration of vehicle or colchicine (100 μg per 10 μl) (n = 6 in each group) (19).

Two days after the treatment, the rats were anesthetized independently with an ip injection of sodium pentobarbital (50 mg/kg body weight) followed by perfusion for tissue fixation. For Fos, AVP, and OXT immunohistochemistry, the rats were centrally administered with vehicle or NERP-3 (5 nmol per 10 μl) (n = 6 in each group), and 2 h later anesthetized and perfused as described above.

Water deprivation and salt loading

Male Wistar rats weighing 250–300 g (n = 9) were used. In one group (n = 3), the rats were deprived of water for 2 d. In another group (n = 3), 2% saline was given as drinking water for 5 d. Rats (n = 3) that were allowed free access to tap water and dry food were used as controls. For each experimental protocol, the rats were anesthetized followed by perfusion, and then the fixed brains were used for NERP-3 immunohistochemistry.

Immunohistochemistry for NERP-3 and dual detection of ir-Fos and ir-AVP/or OXT

Deeply anesthetized animals were perfused transcardially with 0.1 M PBS (pH 7.4) containing heparin (1000 U/liter), followed by 4% paraformaldehyde in 0.1 M PBS. The brains were then removed and divided into blocks that included the hypothalamus. The blocks were postfixed with 4% paraformaldehyde in 0.1M PBS for 48 h at 4 C. The tissues were then cryoprotected in 20% sucrose in 0.1M PBS for 48 h at 4 C. For immunostaining, serial sections (30 μm thick, 60 μm apart) were cut with a cryostat (REM-700; Yamato Kohki Industrial Co., Saitama, Japan). The sections were rinsed twice with 0.1 M PBS containing 0.3% Triton X-100 with 1% hydrogen peroxidase for 60 min, followed by two rinses with 0.1 M PBS containing 0.3% Triton X-100. The floating sections were incubated with NERP-3 (dilution 1:5000) or Fos primary antibody (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:500) in 0.1 M PBS containing 0.3% Triton X-100 at 4 C for 3 d. After washing for 20 min in 0.1 M PBS solution containing 0.3% Triton X-100, the sections were further incubated for 2 h with a biotinylated secondary antibody solution (dilution 1:250) and finally with an avidin-biotin peroxidase complex (Vectorstain ABC kit; Vector Laboratories, Burlingame, CA) for 2 h. Peroxidase in the sections was visualized with 0.02% diaminobenzidine in a Tris buffer containing 0.05% hydrogen peroxidase for 5–7 min. In a preabsorption test of NERP-3 staining, three different amounts of NERP-3 peptide (0.1, 1, and 10 nmol) were separately incubated with NERP-3 antibody (2 μl at a dilution of 1:5000) at 4 C for 1 d, and the supernatants after centrifugation were used for experiments as described above. NERP-3 staining of brain sections from vgf knockout mice (generation of the mice will be described elsewhere) was similarly performed, except that no colchicine treatment was applied and that the antibody was used at a dilution of 1:2000 and incubated with sections for 16 h.

During the dual staining for AVP or OXT, the sections were incubated sequentially with AVP antibody (INCSTAR, Stillwater, MN; dilution 1:10,000) or OXT antibody (CHEMICON International, Temecula, CA; dilution 1:5,000) for 2 d 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 mounted with a coverslip. The presence of a brown label that appeared in round structures was judged to be indicative of ir-Fos positive nuclei and a violet label that appeared in spindle-shaped structures was judged to be indicative of ir-AVP/or OXT.

Immunoreactive NERP-3 in the suprachiasmatic nucleus (SCN) (bregma ~1.30 mm), the SON (bregma ~1.30 mm), the PVN (bregma ~1.80 mm), the median eminence (ME) (bregma ~3.14 mm), and the arcuate nucleus (Arc) (bregma ~2.30 mm) was observed by light microscopy (Eclipse E600; Nikon, Tokyo, Japan). The images were captured using a camera unit (Digital Sight DS-Fi1 and L2; Nikon) connected with the microscope. Immunoreactivities were quantified using Image J software (National Institutes of Health, Bethesda, MD). Each location was delineated using the captured images. The mean OD for each unilateral brain region in each animal was taken from the integrated density per area. Intensity calibration was performed with densitometric standards (Kodak no. 3 control scale tablet; Kodak, Rochester, NY). To count double-labeled cells of ir-Fos and ir-AVP/or OXT in the SON and the PVN, three serial and equidistant sections (30 μm thick, 60 μm apart) including the SON per animal and two similarly prepared sections including the PVN per animal were stained with Fos antibody and AVP antibody or OXT antibody and counted under a light microscope.

Measurement of peptide release from the posterior pituitary

For each experiment, six adult male Wistar rats (180–220 g) were killed by decapitation with a guillotine. After removal of the brains, PPs, including the pars intermedia, were dissected from the whole pituitaries, and the pars intermedia was removed mechanically from the PP. The isolated PPs were transferred to normal Locke’s (NL) buffer in individual 1.5-ml flat-bottomed
tubes and maintained at 37 °C throughout the experiments. The NL buffer contained (in millimoles) NaCl, 140; KCl, 5; MgCl₂, 1.2; CaCl₂, 1.8; glucose, 10; HEPES, 10; pH 7.25 with Tris. The osmolality of the solution was 300 mOsm/kg. The tissues were constantly washed with NL buffer every 10 min for 90 min before collecting samples for AVP measurements. After the preincubation period, the samples were collected every 10 min until the end of the experiment. NERP-3 (1, 5, and 10 μM) with 0.05% BSA in NL buffer or 50 mM high-potassium solution [(in millimoles) NaCl, 95; KCl, 50; HEPES, 10; glucose, 10; MgCl₂, 1.2; CaCl₂, 2] was applied for 20 min (20–30 min and 30–40 min). The collected samples were immediately frozen and stored at −20 °C until the RIA. The amount of AVP release in each 10-min period was calculated by subtracting the amount of AVP released under basal conditions (mean of AVP amounts released in 0–10 min and 10–20 min from six PPs) from that observed before, during, and after stimulation with NERP-3 or 50 mM K⁺ solution (20). The released AVP content was assayed by a RIA using a specific antibody with high sensitivity (half-maximum inhibition concentration = 2 fmol/tube) (21).

Data analysis
Data were also analyzed by one-way ANOVA followed by a Tukey-Kramer-type adjustment for multiple comparisons. For release experiments, a one-way repeated-measures ANOVA was used. All data are provided as the mean ± SEM unless otherwise noted. P < 0.05 was considered statistically significant.

Results
Antisera against NERP-3
The amino acid sequence of NERP-3 is completely conserved among mammals including human, chimpanzee, rat, mouse, and cow (Supplemental Fig. 1). We first developed a RIA system using an antibody specific to NERP-3 prepared by immunizing with its C-terminal 9-residue peptide (7). This antibody specifically recognized the C-terminal peptide with high sensitivity (half-maximum inhibition concentration, 12 fmol/tube) and reacted equally with the 30-residue NERP-3 but not with NERP-1, NERP-2, AQEE-30, NERP-4 (7), or 20 other known biologically active peptides.

Chromatographic characterization and mass spectrometric identification of ir-NERP-3
Ir-NERP-3 in brain extracts was eluted in gel filtration as a single peak at the position of synthetic 30-residue NERP-3 (Fig. 1A). This ir-NERP-3 behaved identically with the synthetic NERP-3 on RP-HPLC (Fig. 1B). To perform molecular identification of this ir-NERP-3, fractions containing the ir-NERP-3 peak in the gel filtration were pooled and immunoprecipitated with the anti-NERP-3 antibody and analyzed by mass spectrometry. The molecular mass of the main component was determined to be 3389.7 Da, which corresponded to rat VGF (180–209) with N-terminal pyroglutamylation having a monoisotopic molecular mass of 3389.66 Da (Fig. 1C). Similar findings were obtained with jejunum/ileum extract (Supplemental Fig. 2). It was thus concluded that ir-NERP-3 in acid extracts of the rat brain and gut is composed of 30-residue NERP-3 with N terminal pyroglutamylation.

Tissue distribution of ir-NERP-3
Immunoreactive NERP-3 occurred at higher levels in the brain, excluding the cerebellum, and pituitary than in the peripheral tissues examined (Fig. 2 and Supplemental Fig. 3). In the brain, the highest concentration of ir-NERP-3 was observed in hypothalamus and midbrain/thalamus, the second highest in cerebral cortex, and the lowest in cerebellum. This distribution profile was almost similar to those for ir-NERP-1, ir-NERP-2, and ir-VGF-C, and it is noted that midbrain/thalamus and cerebral cortex contain relatively high levels of ir-NERP-3. Throughout
the brain regions examined, ir-NERP-3 was dominant over ir-NERP-1 and ir-NERP-2. In the gastrointestinal tract, the neural component of which expresses VGF (12), lower intestinal tract had a higher level of ir-NERP-3. The ir-NERP-3 level was a little higher than but almost comparable with those of ir-NERP-1 and ir-NERP2. Immunoreactive VGF-C was measured to estimate the total amount of VGF protein. Given the ratios between the amounts of ir-VGF-C and ir-NERP, VGF protein appears to undergo proteolytic processing to a greater extent in the gastrointestinal tract than in the central nervous system (CNS). In the anterior pituitary and adrenal and thyroid glands, ir-NERP-3 was less abundant relative to ir-NERP-1 or ir-NERP-2, whereas no remarkable preference among the three entities was observed in the PP, including the pars intermedia.

Effect of icv administration of colchicine on ir-NERP-3 in the hypothalamus

Before the experiments, we performed a preabsorption test in which increasing amounts of NERP-3 peptide, incubated with a given concentration of the primary antibody, resulted in a dose-dependent decrease in staining of the rat SCN, SON, and PVN (Supplemental Fig. 4). The antibody specificity was further confirmed by the lack of significant staining on the SON and PVN slices from vgf knockout mice (Supplemental Fig. 5).

In vehicle-administered rats, NERP-3-ir cells and fibers were observed in the dorsomedial parts of the SCN, the SON, and the magnocellular division of the PVN (Fig. 3, A–C). NERP-3-ir fibers were also observed in the external layer of the ME (Fig. 3D). No positive cells were found in the Arc in vehicle treatment (Fig. 3E). In colchicine treatment, the density of ir-neurons and fibers was markedly increased in the SCN and the SON (Fig. 3, F and G), whereas it was not increased in the magnocellular division of the PVN (Fig. 3H). Positively stained fibers in the external and internal layers of the ME were increased after colchicine treatment (Fig. 3I). Interestingly, ir-neurons were also observed in the Arc after the treatment (Fig. 3J).

Effect of osmotic stimuli on ir-NERP-3 in the hypothalamus

NERP-3-ir cells and fibers were observed in the SCN, the SON, the magnocellular division of the PVN, and the external layer of the ME (Fig. 4, A–D) in the control animals. In 2-d dehydrated rats, the density of positively-stained neurons and fibers was increased in the SCN, the magnocellular division of the PVN, and the ME but not in the SON (Fig. 4, E–H). After 5-d salt loading, NERP-3-ir structures were decreased in the SCN, SON, PVN, and ME (Fig. 4, I–L). Quantitative and statistical analysis supported the above observations (Fig. 4, M–P).

Dual detection of ir-Fos and ir-AVP/ir-OXT in the SON and the PVN after icv administration of NERP-3 or vehicle

After icv administration of NERP-3 to conscious rats, Fos-ir cells were observed in the SON and the PVN, whereas few Fos-ir cells were observed after vehicle administration (data not shown). Then we performed dual staining of Fos and AVP/OXT in the SON and the PVN; ir-Fos was detected in AVP-ir cells but not in OXT-ir cells (Fig. 5, A–D). In a magnified view, ir-Fos was stained brown in round structures (Fig. 5E), and cytosolic ir-AVP was stained violet (Fig. 5F). Taking the total AVP-ir cells counted in the SON or the PVN as 100%, the proportion of Fos-ir cells was 30.5 ± 5.1% in the SON and 28.3 ± 8.2% in the PVN after icv administration of NERP-3. After icv administration of vehicle, the percentage of Fos-ir cells in the AVP-ir cells was almost negligible (0%) (Fig. 5G). Similarly, no Fos-ir cell was observed in the
OXT-ir cells after icv administration of NERP-3 or vehicle (data not shown).

Effect of NERP-3 on AVP release from the PP

To determine whether NERP-3 has a direct physiological effect on AVP release, we isolated PP without the pars intermedia from control animals and challenged them with different concentrations of NERP-3 (1, 5, and 10 μM). NERP-3 treatment caused an increase in AVP release (Fig. 6). The amount of AVP released was significantly increased when the NERP-3 concentration was increased from 1 μM to 5 or 10 μM (P < 0.01). High potassium (50 mM) significantly stimulated AVP release and was used as a positive control.

Discussion

Rat VGF protein is recognized as a large precursor (594 residues) for biologically active peptides and has many processing motifs for PC1/3 and PC2. Consistent with these facts, VGF protein undergoes complex proteolytic processing to generate a series of peptides (1, 7, 12). We have recently identified a novel biologically active peptide, NERP-3, among the VGF-derived peptides using a peptidomic strategy, in conjunction with an ex vivo calcium assay performed on apoaequorin transgenic mouse tissues (7). In rat brain and gut acid extracts, ir-NERP-3 was identified as a 30-residue NERP-3 with N-terminal pyroglutamination (Fig. 1 and Supplemental Fig. 2). Assessed by specific RIA, ir-NERP-3 as well as ir-NERP-1 and ir-NERP-2 was similarly distributed in the rat brain, with the tissue concentration of ir-NERP-3 being higher than that of ir-NERP-1 and ir-NERP-2 (Fig. 2). In view of the highest concentration of three distinct NERP in the PP, as-yet-unknown functions may be ascribed to these peptides. Although significant, the tissue concentration of ir-NERP-3 in the gut was much lower than in the brain or
pituitary. The ratios of the concentrations of ir-NERP-3 to ir-NERP-1 to ir-NERP-2 were different in each tissue or brain region. The N-terminal end of NERP-3 is flanked by dibasic amino acids (H11002AKR/QQE-H11002), whereas the C-terminal end is not (H11002RVW/RASW-H11002). Thus, the possible quantitative difference in processing enzymes expressed in brain and endocrine tissues, such as PC1/3, PC2, and furin, may explain the differential peptide levels of NERP-3 and NERP-1/NERP-2. These findings suggest that NERP-3 represents a major peptide with important physiological roles in the rat CNS and PP.

We examined the anatomical distribution of ir-NERP-3 in the rat hypothalamus by immunohistochemistry after icv administration of colchicine (22). In colchicine treatment, NERP-3-ir neurons and fibers were strongly observed in the SCN, SON, PVN, and Arc. Positively stained fibers in both the internal and external zones of the ME were also increased after the treatment (Fig. 3).

The occurrence of ir-NERP-3 in the magnocellular neurons of the SON and the PVN suggests that NERP-3 co-localizes with AVP as reported for NERP-1 and NERP-2 (4). Positively stained fibers in the internal layer of the ME were observed after colchicine treatment. Because these SON and PVN neurons are known to project axons to the PP via the internal layer of the ME (10), it is reasonable to deduce that NERP-3 is transported from the neurons to the PP. Colchicine treatment resulted in contrasting changes between these magnocellular neurons: up-regulation of ir-NERP-3 in the SON and no alteration in the PVN. This finding may indicate a greater contribution of NERP-3 in the SON to the PP.

Although colchicine treatment did not alter the density of ir-NERP-3 neurons in the parvocellular division of the PVN, positively stained fibers in the external layer of the ME were increased. The parvocellular neurons in the PVN project axons to the external layer of the ME and regulate the anterior pituitary functions (10, 23). As with cortico-

![Vehicle](https://example.com/vehicle.png) ![NERP-3](https://example.com/nerp-3.png)

**Fig. 5.** Actual labeling of ir-Fos and ir-AVP in the SON and PVN after icv administration of vehicle (A and C) and NERP-3 (B and D). Insets, Magnified views of the boxed regions. Scale bar, 500 μm. OT, Optic tract. Representative immunostainings (E and F) are shown. Open arrowheads, ir-Fos without ir-AVP; filled arrowheads, ir-Fos with ir-AVP. Scale bar, 20 μm. The percentage of Fos-ir cells in AVP-ir cells is shown (G). Values are presented as means ± SEM.

![Accumulated AVP release vs Time](https://example.com/avp-release.png)

**Fig. 6.** Effect of NERP-3 or high potassium (K+)(50 mM) on AVP release from freshly isolated PP. The graph represents the accumulated AVP release during the course of experiments measured at 10-min intervals. AVP levels before and after stimulation with NERP-3 or high K+ (50 mM) were measured at 10-min intervals. Each AVP value was calculated by subtracting the mean amount of hormone released during basal periods (0–10 and 10–20 min) from the observed amount of AVP released after stimulation. Diamond, High K+; triangle, 1 μM NERP-3; square, 5 μM NERP-3; circle, 10 μM NERP-3. Values are presented as means ± SEM. **, P < 0.01, ††, P < 0.01, ##, P < 0.01, and ‡‡, P < 0.01 compared with those during the basal period from 0 to 20 min in each experimental set.
tropin-releasing factor, NERP-3 may be transported from the neurons in the PVN to the external layer of the ME and regulate anterior pituitary hormones such as ACTH. This possibility should be examined in the future study.

NERP-3-ir neurons appeared in the Arc after colchicine treatment (Fig. 3). The Arc is one of the regulatory centers of feeding behavior in which feeding-regulatory peptides such as NPY/agouti-related protein (AgRP) neurons and proopiomelanocortin/cocaine- and amphetamine-regulated transcript neurons are localized (24). NPY/AgRP neurons are localized in the ventromedial part of the Arc, whereas proopiomelanocortin/cocaine- and amphetamine-regulated transcript neurons are localized in the ventrolateral part of the Arc. It is worth noting that ir-NERP-3 neurons as well as NPY/AgRP neurons were localized in the ventromedial part. NERP-3 may be implicated in feeding regulation through the Arc and other relevant hypothalamic nuclei. Recently we reported that NERP-2-ir neurons are found in the Arc and lateral hypothalamus (12), and this peptide stimulates feeding behavior in rodents (5). NERP-3 is worthy of further investigation for its possible role in feeding regulation and energy metabolism.

The SCN is a regulatory center of the circadian rhythm initiated by light-induced stimuli (25). AVP neurons in the dorsomedial part of the SCN are responsible for circadian information output (25, 26) rather than input triggered by light-induced stimuli. Given its distribution pattern in the SCN (Figs. 3 and 4), NERP-3 may colocalize with AVP and take part in the process of circadian information output as well. Furthermore, we found that ir-NERP-3 levels were increased in the SCN of dehydrated rats and decreased in salt-loaded rats (Fig. 4, A, E, I, and M). Although the underlying mechanism remains to be explained, these osmotic challenges may influence the circadian rhythm using NERP-3 as a mediator. Because vgf-deficient mice showed a shorter circadian period (27), the function of NERP-3 in the SCN should be clarified in the future study.

Fos has been commonly used as a neuronal activity marker in the CNS (28). Centrally administered NERP-3 induced Fos expression in the SON and the PVN. More specifically, dual immunohistochemistry confirmed that NERP-3 stimulated about 30% of AVP-ir cells, but not OXT-ir cells, in both the SON and the magnocellular division of the PVN (Fig. 5). Thus, the presence of these NERP-3-responsive AVP neurons may point to a direct involvement of the peptide in the regulation of water-salt balance and some other physiological phenomena.

In dehydrated animals, the density of NERP-3-ir neurons and fibers was increased in the magnocellular division of the PVN, one of the responsible regions of osmoregulation, but not in that of the SON (Fig. 4). In response to dehydration, in which the body fluid decreases and the osmotic pressure increases, VGF mRNA levels in the SON and PVN are up-regulated along with AVP mRNA (4). These findings suggest that both NERP-3 peptide and VGF mRNA levels in the PVN are increased by the osmotic stimulus, whereas these two levels are not coordinated in the SON. In the external layer of the ME, a pathway from the parvocellular division of the PVN to the anterior pituitary (10, 23), ir-NERP-3 levels were also elevated, whereas not in the internal layer of the ME. Taken together, NERP-3 may work as a mediator of dehydration stress, although its role in the anterior pituitary remains to be clarified. It is established that chronic salt ingestion causes a continued osmotic stimulation, resulting in the secretion and depletion of AVP content in the SON and PVN cell bodies and in the PP, even though its mRNA levels were up-regulated in the SON and PVN (29). With regard to VGF, ir-NERP-3 in salt-loaded rats was decreased similarly in the SON, the magnocellular division of the PVN, and the ME. Mahata et al. (30) reported that VGF mRNA levels are up-regulated in the SON and PVN of salt-loaded rats. We reasoned by analogy that a mechanism similar to that of AVP may explain the opposite changes in NERP-3 peptide and VGF mRNA levels in the salt-loaded rats.

To assess the in vitro effect of NERP-3 on AVP release from isolated PP preparations, we used 1- to 10-μM doses of NERP-3 (Fig. 6). This dose range is based on that derived from the icv studies of NERP-1 and ~2 (1-5 nmol/ rat, corresponding to 1-5 μM) (4), as in previous icv studies using salusin-β (23), galanin-like peptide (31), and pituitary adenylate cyclase-activating polypeptide (32). A significant AVP release was observed with 1 μM NERP-3, with a pronounced release observed with 5 and 10 μM, the effect stronger than that with 50 mM K+. This potassium concentration is not a maximum depolarizing stimulus to evoke AVP or OXT release from the neurohypophysis (20, 33). It should also be noted that NERP-3 is nearly as potent as other peptides, such as relaxin (34) and endothelin (35); they evoke a much larger AVP or OXT release under physiological conditions than that evoked by 50 mM K+. Thus, our data support the notion that NERP-3 is a potential physiological modulator of AVP. At the single neuron/terminal level, most neuropeptides are effective at a lower concentration (i.e. nanomolar to micromolar range) in vitro. Likewise, NERP-3 may act in the subnanomolar range in such a context.

The water-salt balance in mammals is known to be principally maintained by the action of AVP, which is synthesized in the magnocellular system of the SON and PVN, and transported to neurotermini of the PP and secreted...
into blood stream. Many biologically active substances are colocalized with AVP neurons in the SON and PVN (36). However, their actual role in the regulation of hydroelectrolyte homeostasis remains unclear. Examples of such peptides include cholecystokinin, dynorphin, endothelin-1, neurokinin B, and NPY (36). In contrast, peptides such as angiotensin II, apelin, and galanin have established roles in the osmotic function (36). Although an in vivo effect of NERP-3 has not been examined in this study, we think that it is possible to propose a potential physiological role for NERP-3 in the water-salt balance.

Because NERP-3 directly stimulated AVP release from the PP in vitro and was abundantly present in the hypothalamic-hypophyseal system, the peptide may activate AVP neurons in the SON and PVN and neuroterminals in the PP. The important aspect to consider in this investigation is the receptors through which NERP-3 acts. Although we have not identified such receptors, we got evidence that NERP-3’s target resides in the hypothalamus and pituitary, using our ex vivo calcium assay system (7), a finding that will be helpful to receptor identifcation. There is also a possibility that NERP-3 regulates blood pressure and body fluid balance via control of AVP release. Both the neurohypophysial and somatodendritic release of AVP from the SON and the PVN evoked by various biologically active substances has been extensively studied under many physiological conditions (22, 37, 38). Because AVP release is known to be regulated by the electrical activity of AVP neurons in the SON and the PVN (39, 40), NERP-3 may alter the electrical activity of these neurons as well.

VGF protein undergoes complex proteolytic processing to generate more than 15 peptides and longer intermediates (7, 12). Among them, NERP-1 and NERP-2 suppress the AVP release stimulated by icv administration of hypertonic saline or angiotensin II in vivo as well as that from tissues of the SON and PVN in vitro (4). In the present study, NERP-3 was found to have an opposite effect on the AVP release. Given the complexity of the total profile of VGF-related peptides released simultaneously, the overall effect elicited by the peptides in vivo may be different from the effect observed in experimental settings involving a single peptide application.

In conclusion, the present study indicates that NERP-3 in the hypothalamus seems to be an important multifunctional peptide that regulates AVP secretion, body water-salt balance, circadian rhythm, and feeding.

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The authors are grateful to Dr. A. Mochiduki (National Cerebral and Cardiovascular Center) for the vgf knockout mice study. This work was supported in part by an University of Occupational and Environmental Health grant for Advanced Research of the University of Occupational and Environmental Health, the Intramural Research Fund of the National Cerebral and Cardiovascular Center, and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science. G.D. is supported by the Centre National de la Recherche Scientifique, France, and the Japan Society for the Promotion of Science Fellowship Program (Grants FY2008 and S-08216).

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Disclosure Summary: The authors have nothing to disclose.

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