Recent advances in peptidomics have enabled the identification of previously uncharacterized peptides. However, sequence information alone does not allow us to identify candidates for bioactive peptides. To increase an opportunity to discover bioactive peptides, we have focused on C-terminal amidation, a post-translational modification shared by many bioactive peptides. We analyzed peptides secreted from human medullary thyroid carcinoma TT cells that produce amidated peptides, and we identified two novel amidated peptides, designated neuroendocrine regulatory peptide (NERP)-1 and NERP-2. NERPs are derived from distinct regions of the neurosecretory protein that was originally identified as a product of a nerve growth factor-responsive gene in PC12 cells. Mass spectrometric analysis of the immunoprecipitate using specific antibodies as well as reversed phase-high performance liquid chromatography coupled with radioimmunoassay analysis of brain extract demonstrated the endogenous presence of NERP-1 and NERP-2 in the rat. NERPs are abundant in the paraventricular and supraoptic nuclei of the rat hypothalamus and colocalized frequently with vasopressin but rarely with oxytocin. NERPs dose-dependently suppressed vasopressin release induced by intracerebroventricular injection of hypertonic NaCl or angiotensin II in vivo. NERPs also suppressed basal and angiotensin II-induced vasopressin secretion from hypothalamic explants in vitro. Bioactivity of NERPs required C-terminal amidation. Anti-NERP IgGs canceled plasma vasopressin reduction in response to water loading, indicating that NERPs could be potent endogenous suppressors of vasopressin release. These findings suggest that NERPs are novel modulators in body fluid homeostasis.

Peptide hormones or neuropeptides function as cell-to-cell signaling molecules to mediate a variety of physiological phenomena. These bioactive peptides are cleaved from precursor proteins via limited cleavage and often undergo post-translational modifications to perform their functions (1). Technological advancement in mass spectrometry, along with an ever increasing number of genomes being sequenced, has made it possible to study the peptidome or a whole set of endogenously processed peptides. In fact, peptidomic approaches have been applied to the analysis of peptides found in mammalian tissues or body fluids, leading to the description of a number of previously uncharacterized peptides (2–4).

In mammalian peptidomic studies, however, most peptides identified are fragments of intracellular proteins. Even in the studies designed to identify pituitary and hypothalamic peptides using specific sample preparation methods (5, 6), the peptides identified have turned out to be N-terminally or C-terminally deleted or extended fragments of relatively abundant precursors of known peptide hormones or secretory proteins. This is because mass spectrometry schemes detect only abundant molecules or easily ionized molecules, although tandem mass spectrometry has the potential to efficiently identify peptides present in complex mixtures. Thus, candidates for novel bioactive peptides present in trace amounts remain elusive in peptidomic identification studies. Another critical issue in peptidomics is that we cannot infer biological activity just from the sequence of a target peptide; it is practically impossible to synthesize and test all the peptidomic-identified peptides for assessing bioactivity.

We thought that one solution to increase the probability of identifying potentially bioactive peptides is to focus on secretory peptides with a post-translational modification characteristic of bioactive peptides. In this study, we targeted C-terminal amidation, which is shared by many known bioactive peptides or peptide hormones (7). By analyzing peptides released by a human cell line of endocrine origin, we discovered two C-terminally amidated peptides derived from the neurosecretory protein VGF (8). Although VGF has long been considered a precursor of bioactive peptides, functional studies are limited to C-terminal peptides as yet (9, 10). Biological functions of other VGF-related peptides, identified by recent peptidomic
studies, have not been investigated (11, 12). To get a clue for identifying their biological functions, we prepared antisera against these peptides and performed immunohistochemical studies to identify peptide-producing tissues and cells in the rat. We took advantage of the well documented findings on the localization and production sites of peptide hormones and deduced the possible biological functions of candidate peptides in relation to these known peptide hormones. This study would provide a new approach to the peptidomics-aided discovery of mammalian bioactive peptides.

EXPERIMENTAL PROCEDURES

Mass Spectrometric Analysis—The supernatant of human medullary thyroid carcinoma TT cells (13) cultured in serum-free media for 6 h was harvested and immediately processed using a Sep-Pak C18 cartridge (Waters) as described (14). The resultant eluate was applied to a gel filtration column (Superdex Peptide PE7.5/300, GE Healthcare) to obtain peptide-rich fractions. These were subjected to reductive alkylation, desalted, and fractionated by RP-HPLC4 into 50 fractions. Each fraction was analyzed by off-line nano-electrospray ionization MS/MS with a Q-Tof II mass spectrometer (Micromass, Milford, MA) and by matrix-assisted laser desorption ionization-time of flight MS/MS with a Proteomics 4700 mass spectrometer (Applied Biosystems, Foster City, CA). Each MS/MS spectrum was used to probe the NCBI and Swiss-Prot databases with Mascot MS/MS ion search software (Matrix Science, Boston, MA) and was also interpreted by SeqMS (15).

Peptide Synthesis—All peptides were synthesized on an Abacus peptide synthesizer (Sigma Genosys) using Fmoc (N-(9-fluorenylethoxycarbonyl) strategy, purified by RP-HPLC, and verified for correct synthesis by mass spectrometry and amino acid analysis. Purity of the peptides was confirmed on separate HPLC systems. Synthetic rat NERP-Us were used in all the in vivo and in vitro administration experiments.

Antibody Preparation and Radioimmunoassay (RIA)—A C-terminal octapeptide common to human and rat NERP-1 (QGLAQVEA-NH2) was conjugated with keyhole limpet hemocyanin (Pierce) by the glutaraldehyde method. Cysteinyl C-terminal decapetides of rat NERP-2 (CQGGARQRDLG-NH2) and human NERP-2 (CQGGARQRGLG-NH2) were each coupled with maleimide-activated keyhole limpet hemocyanin (Pierce) through its thiol groups. Rabbits were immunized with each conjugate emulsified with an equal volume of Freund’s complete adjuvant. Radioimmunoassay was carried out as reported (16) using 125I-radiolabeled YLLQQGLAQVEA-NH2 (human and rat NERP-1), YLLQQGARQRDLG-NH2 (rat NERP-2), or YQGGARQRGLG-NH2 (human NERP-2). A half-maximum inhibition concentration of ligand binding in each RIA was 20 fmol/tube (human and rat NERP-1), 10 fmol/tube (rat NERP-2), or 20 fmol/tube (human NERP-2). Specificity of the three RIAs for human/rat NERP-1 and rat NERP-2 was examined with immunized peptides with C-terminal Gly extension, C-terminal rat VGF-(588–615), and human VGF-(586–615), rat VGF-(556–585), and human VGF-(554–583) corresponding to a C-terminally extended form of TLQP-21 (10) and 13 known bioactive peptides listed below, including 10 C-terminally amidated peptides. Vasopressin, neuropeptide Y, neuropeptide A, calcitonin, calcitonin gene-related peptide, calcitonin receptor-stimulating peptide, adrenomedullin, proadrenomedullin N-terminal 20-amino acid peptide, peptide histidine isoleucine, corticotropin-releasing factor, angiotensin II, leucine-enkephalin, and methionine-enkephalin-Arg-Gly-Leu.

Immunological Detection of NERPs—Rat hypothalamic tissue was extracted and condensed with a Sep-Pak C18 cartridge as described previously (17). An aliquot of cartridge eluate was examined by RIA to quantify each NERP. The remaining portion was separated by RP-HPLC and assessed by RIA to identify individual immunoreactive (ir-) NERPs. To determine major endogenous forms of rat NERPs, Sephadex G-50 gel-fractionated fractions of rat brain extracts (1.1- and 5.9- eq for NERP-1 and NERP-2, respectively) were immunoprecipitated with anti-NERP antibodies and then analyzed on a surface-enhanced laser desorption ionization mass spectrometer (Ciphergen, Fremont, CA). Immunoprecipitate from TT cell extract was prepared and mass analyzed as described (14).

Intracerebroventricular Administration—Male Wistar rats (aged 9–10 weeks, from Charles River Laboratories, Shiga, Japan) were maintained in individual cages under controlled temperature (21–23°C) and light (light on 08:00–20:00) conditions with ad libitum access to food and water. Cannulation and intracerebroventricular (icv) administration were performed as described (18). Test materials for icv administration were dissolved in 10 μl of artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 5 mM KCl, 1.3 mM MgSO4, 1.24 mM KH2PO4, 2 mM CaCl2, 25.9 mM NaHCO3, and 10 mM glucose, pH 7.3. All animal experiments were repeated three to five times and performed in accordance with the guidelines for animal care from the Japanese Physiological Society.

Immunohistochemistry—Brains were removed from colchicine (200 μg)-treated rats following perfusion with either 2% paraformaldehyde (PFA) or 4% PFA containing 0.1% glutaraldehyde, respectively, for immunofluorescence microscopy or electron microscopy. Immunofluorescence staining and immunogold electron microscopy were performed as described previously (19, 20). For light microscopy, peripheral tissues from rats perfused with 2% PFA were stained with antibodies against NERP-1 (1:2,500), rat NERP-2 (1:5,000), oxytocin (1:15,000; Chemicon, Temecula, CA), and vasopressin (1:80,000; Peninsula Laboratories, Torrance, CA). Samples were visualized as described (21). Control studies were done with normal rabbit serum or NERP antisera that had been pretreated with 10 μg of synthetic NERPs.

In Situ Hybridization—VGF and vasopressin mRNA levels in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) from rats deprived of water for 48 h were examined by in situ hybridization with 35S-labeled deoxoyguanosine triphosphate probes specific for VGF (complementary to bases 1741–1785

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4 The abbreviations used are: RP-HPLC, reversed phase-high performance liquid chromatography; All, angiotensin II; ir, immunoreactive; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NERP, neuroendocrine regulatory peptide; PFA, paraformaldehyde; PVN, paraventricular nucleus; RIA, radioimmunoassay; SON, supraoptic nucleus; TOF, time-of-flight; RP-HPLC, reversed phase-high performance liquid chromatography; aCSF, artificial cerebrospinal fluid.
and 1825–1870 of rat VGF nucleotides; GenBank™ accessions number M74223 and vasopressin (complementary to bases 1843–1868 of rat vasopressin nucleotides) as described (18). Autoradiographic images were analyzed on an MCID imaging analyzer (18). VGF mRNA intensity is expressed relative to that of control rats drinking water ad libitum (n = 5 per group).

**Vasopressin Secretion and Measurements**—Rats (n = 8–14 per group) received an icv injection of test peptide 5 min before icv injection of either hypertonic NaCl (8.5 µmol of NaCl/10 µl of aCSF) or AII (0.1 nmol/10 µl of aCSF). Plasma vasopressin was measured using an RIA kit (Mitsubishi Chemical, Tokyo, Japan) in blood samples taken 10 min after the hypertonic saline or AII injection. Static incubation of PVN and SON explants punched out from the hypothalamus was performed as reported previously with minor modifications (22). They were sequentially stimulated (each at a final concentration of 10⁻⁶ M for 5 min) as indicated in Fig. 5. Stimulation periods were separated by 5-min recovery periods. At the end of each experiment, KCl was added at a final concentration of 6 × 10⁻² M to confirm depolarization-induced secretion. Perifusion assays were replicated 5–8 times.

**Effects of NERPs on Plasma Vasopressin in Rats**—Rats (n = 8 per group) deprived of water for 48 h were decapitated 10 min after icv administration of NERP-1 or NERP-2 (Gly (1 nmol/rat) to measure plasma vasopressin. Prior to immunoneutralization studies of NERPs, water (5 ml/100 g body weight) was loaded to rats (n = 8 per group) by oral injection through a stomach tube. Fifteen minutes after water loading, rats received an icv injection of anti-NERP-1-IgG (0.1 µg), anti-NERP-2-IgG (0.1 µg), or control IgG (0.1 µg). Rats were decapitated 45 min after immunoneutralization, and plasma vasopressin was measured.

**Statistical Analysis**—All data are expressed as means ± S.E. Groups of data were compared with analysis of variance and the noneutralization, and plasma vasopressin was measured.

**RESULTS**

**Peptidomic Identification of NERPs from Culture Supernatant of TT Cells**—We analyzed peptides secreted from human medullary thyroid carcinoma TT cells, because this cell line is known to actively secrete the C-terminally amidated peptide calcitonin gene-related peptide α and calcitonin (13). The supernatant of the cell line cultured in a serum-free medium for 6 h was concentrated and subjected to gel filtration chromatography to obtain a peptide-rich fraction, whose cysteine residues were then converted to carboxyamidomethyl cysteine using dithiothreitol and iodoacetamide. This peptide fraction was separated by conventional RP-HPLC to 50 fractions (Fig. 1A), each of which was analyzed with tandem mass spectrometric techniques for identification. We identified 19 C-terminally amidated peptides (Fig. 1B), of which 15 peptides were the entire or partial sequences corresponding to calcitonin gene-related peptide α and calcitonin. The identification of a series of 13 calcitonin gene-related peptide α-derived (CRGα) peptides with sequential N-terminal deletions is consistent with the fact that this cell line produces this peptide α at higher levels than calcitonin (13). Of note, we discovered two novel amidated peptides with monoisotopic masses of 2677.4 and 4062.2, both of which were derived from distinct regions of the neurosecretory protein VGF; one is from human VGF-(281–306), and the is other from VGF-(310–347) (NCBI accession number gi|17136078) (Fig. 1, C and D). Shorter fragments of both peptides were also identified (Fig. 1B). Based on their localization and physiological role described below, we designated these peptides as neuroendocrine regulatory peptide peptide (NERP)-1 and NERP-2.

**Antisera against NERP-1 and NERP-2**—The rat VGF sequences registered in the NCBI database, as represented by gi|13591864 and gi|1352860, suggest that the rat precursor comprised of 617 amino acids generates amidated peptides as well. To characterize rat endogenous peptides, we prepared antibodies specific to the C-terminal region of each peptide; an octapeptide common to human and rat NERP-1 (QGLAQVEA-NH₂) and decapetides of rat NERP-2 (QGGARQGDLG-NH₂) and human NERP-2 (QGGARQQGDLG-NH₂) were used for immunization. We confirmed that each antiserum strictly recognizes the C-terminal amide structure but does not show more than 0.1% cross-reactivity with its C-terminally Gly-extended peptide or another NERP. Furthermore, the antiserum did not recognize rat C-terminal VGF-(588–617) peptide or human C-terminal VGF-(586–615) peptide even at 10 µM, indicating that they do not detect the intact VGF precursor.

**Characterization and Identification of NERP-1 and NERP-2**—In the Sephadex G-50 gel filtration of rat brain extracts, ir-NERP-1 and ir-NERP-2 were observed as distinctive peaks in the region of relative molecular mass <6 kDa (data not shown). These NERP-1- and NERP-2-immunoreactive fractions were further characterized by mass analysis of immunoprecipitates using these antibodies (Fig. 2, A and B). Based on the observed mass of the immunoprecipitates, we concluded that rat NERP peptides are derived from the VGF precursor (gi|13591864, Gly at residue 342), with the major endogenous forms of rat NERP-1 and NERP-2 being 25 and 38 amino acids long, respectively (Fig. 1E). The rat hypothalamus ir-NERPs behaved identically to synthetic rat NERP-1 or NERP-2 on RP-HPLC (Fig. 2C). Immunoprecipitation experiments with TT cell extract also showed the dominant peaks that correspond to human NERP-1 (2677 Da) and NERP-2 (4062 Da). These findings suggest that the processing and amidation of NERPs occur intracellularly before secretion, as is known with amidated bioactive peptides secreted by endocrine cells (7).
Neuroendocrine Regulatory Peptides, NERPs

Immunogold electron microscopy revealed the colocalization of NERPs with vasopressin in storage granules (Fig. 3, A and C, insets). Based on these results, we supposed that we could elucidate a biological function of NERPs in the context of vasopressin physiology.

VGF mRNA levels in both the PVN and SON were up-regulated upon water deprivation in rats (Fig. 4, A and B), accompanied by the up-regulation of vasopressin mRNA levels (PVN, 153.0 ± 13.6%; SON, 161.9 ± 12.4%; % of controls, p < 0.01). These in vivo and immunocytochemical observations suggest that NERPs are involved in the central control of body fluid balance. Consistent with previous reports (25, 26), icv injection of hypertonic NaCl or AII increased plasma vasopressin levels in rats (Fig. 4C, 2nd

The table below shows the sequence, observed mass, theoretical mass, and precursor of NERPs:

| Sequence | Obs. mass | Theor. mass | Precursor |
|----------|-----------|-------------|-----------|
| PESALLGGSAGELLQQGLAQVEA-NH₂ | 2521.3 | VGF |
| =EAAQAQRLAILASDLILLQQGGLAQLGLG-LH₂ | 2677.4 | VGF |
| =EAETRQAAEGERLADSLLLQQGLAQVEA-NH₂ | 4062.2 | VGF |
| VPTN(V8K)F-AP | 1164.6 | CTGRP |
| NVP(V8K)F-AP | 1287.7 | CTGRP |
| NVP(V8K)F-AP | 1529.8 | CTGRP |
| GGVVKNPV(K8KF)F-AP | 1755.9 | CTGRP |
| GGVVKNPV(K8KF)F-AP | 1832.9 | CTGRP |
| GGVVKNPV(K8KF)F-AP | 1902.0 | CTGRP |
| GGVVKNPV(K8KF)F-AP | 2076.0 | CTGRP |
| LSRLRRGK(V8KF)F-AP | 2276.0 | CTGRP |
| GVVKNPV(K8KF)F-AP | 2466.4 | CTGRP |
| GVVKNPV(K8KF)F-AP | 2630.0 | CTGRP |
| CGNL5CTMGTYTQDFNKHFTPQ7AIGVAP-NH₂ | 3531.7 | CT |

A and C, insets). Based on these results, we supposed that we could elucidate a biological function of NERPs in the context of vasopressin physiology.

VGF mRNA levels in both the PVN and SON were up-regulated upon water deprivation in rats (Fig. 4, A and B), accompanied by the up-regulation of vasopressin mRNA levels (PVN, 153.0 ± 13.6%; SON, 161.9 ± 12.4%; % of controls, p < 0.01). These in vivo and immunocytochemical observations suggest that NERPs are involved in the central control of body fluid balance. Consistent with previous reports (25, 26), icv injection of hypertonic NaCl or AII increased plasma vasopressin levels in rats (Fig. 4C, 2nd and
11th lanes). This stimulation was dose-dependently suppressed by icv injection of NERP-1 before injection of the vasopressin secretagogues (Fig. 4C). Similar effects were observed with NERP-2, but its potency was weaker than that of NERP-1 because 0.3 nmol of NERP-2 was not effective. Neither nonamidated NERP-1 (NERP-1-Gly) nor nonamidated NERP-2 (NERP-2-Gly) suppressed vasopressin secretion (Fig. 4C). The increase in plasma vasopressin levels caused by water deprivation was also suppressed by icv-administered NERP-1 or NERP-2 (Fig. 4D). Furthermore, icv administration of anti-NERP-1 IgG or anti-NERP-2 IgG significantly reversed plasma vasopressin suppression induced by acute water loading (Fig. 4E), suggesting that NERPs function as endogenous peptides to regulate vasopressin secretion. Next, we examined the in vitro effect of NERPs on vasopressin secretion using hypothalamic explants. NERP-1 suppressed basal and AII-induced vasopressin secretion from the PVN and SON (Fig. 5). NERP-2 was likewise effective, but NERP-1-Gly or NERP-2-Gly was not (Fig. 5).

**DISCUSSION**

In this study, to expedite the identification of potentially bioactive peptides, we analyzed peptides present in the supernatant of cultured cells, rather than analyzing peptides extracted from tissues. In addition, the benefit of our approach is that we examined an endocrine cell line that secretes C-terminally amidated peptide hormones such as calcitonin or calcitonin gene-related peptide at a high rate. Because C-terminal amidation is a post-translational modification most often shared by bioactive peptides (7), we thought that potentially bioactive peptides could be identified easier using this chemical feature. This tag has been used to discover a series of bioactive peptides such as...
galanin or neuropeptide Y (27). Using this “peptide first” approach, it remains difficult to infer biological activity just from the sequence of a peptide even if it is C-terminally amidated. Fortuitously, localization of NERPs in specific hypothalamic nuclei allowed us to speculate on their possible biological roles in relation to known peptide hormone, vasopressin, and to analyze them with in vitro and in vivo experiments described here.

VGF, originally identified in rat pheochromocytoma PC12 cells as a nerve growth factor-responsive gene, encodes a 617-amino acid protein in rodents (8). Immunohistochemical studies in PC12 cells revealed that the protein is stored in dense core granules and secreted through the regulated pathway (28, 29). Because VGF harbors several paired basic amino acid residues targeted by prohormone convertases, it has long been considered a precursor for several bioactive peptides (9). Using antibodies raised against the C terminus of the intact VGF protein, some VGF-derived peptides have been reported and shown to possess biological activity; C-terminal peptides TLQP-62 and AQEE-30 enhance synaptic activity in a whole-cell patch clamp recording on rat hippocampal cells (30), and AQEE-30 and LQEQ-19 enhance penile erection (31). More recently, the study has extended to the identification of TLQP-21 that increases energy expenditure by stimulating autonomic activation of adrenal medulla and adipose tissues (10).

In contrast to these conventional approaches, several peptidomics studies have reported the identification of VGF-derived peptides in brain tissues or cerebrospinal fluid (11, 12). Some VGF peptides are reported to be a marker for Alzheimer disease (32, 33). Except for the aforementioned VGF peptides, however, no VGF-related peptides have been demonstrated to be bioactive. Current peptidomic studies use tandem mass spectrometric techniques for peptide identification, in which C-terminal amidation is considered as a possible modification that target peptides could undergo. Because mass spectrometry schemes tend to detect abundant peptides or easily ionized peptides, it would make sense that no C-terminally amidated peptides from VGF have been reported to date.

It should also be mentioned that NERP-2 might have escaped in silico prediction of bioactive peptides that principally takes into account only paired basic amino acid residues for processing sites; human and rat NERP-2 are cleaved at the amino acid residues 345GLG2GRG350 and 348DLG2GRG353, respectively. The identification of these processing sites also demonstrates a methodological advantage of a peptidomic approach, and in silico prediction in turn may also be reinforced by accumulating data about endogenous peptides that peptidomics is going to provide.

Vasopressin synthesized in the PVN and SON magnocellular neurosecretory cells is packed in the secretory granules. Axons from these cells terminate in the posterior pituitary from which vasopressin is secreted into the systemic circulation to control renal excretion of water. NERPs were frequently colocalized with vasopressin in the secretory granules of the PVN and SON. VGF mRNA levels increased along with vasopressin mRNA in response to water deprivation, suggesting that NERPs participate in the hypothalamic control of plasma osmolarity balance. Both NERP-1 and NERP-2 suppressed vasopressin release stimulated by icv administration of hypertonic saline or All in vivo. In in vitro experiments, NERPs also abolished AII-induced vasopressin release from the PVN and SON. All these actions were observed with C-terminally amidated forms only. Both anti-NERPs IgGs canceled plasma vasopressin reduction in

FIGURE 5. NERPs suppress vasopressin secretion from PVN (A and B) and SON (C and D) explants. Black, gray, shaded, and white bars indicate the NERPs, NERPs-Gly, All, and KCl administration periods, respectively. *, p < 0.05; **, p < 0.01; NS, not significant.
response to water loading, indicating that NERPs could be potent endogenous suppressors of vasopressin release.

Vasopressin release is regulated by the electrical activity of vasopressin neurons, which are modulated by various neurotransmitters and neuromodulators (25, 26). The major neural signals to vasopressin neurons are excitatory and inhibitory postsynaptic currents generated by presynaptic release of glutamate and γ-aminobutyric acid, respectively. All and NaCl potentiate excitatory postsynaptic currents in vasopressin neurons, thereby stimulating vasopressin secretion (25). Although cell-surface receptors or target proteins of NERPs have not been identified yet, the actions of NERP to suppress AI1- and NaCl-induced vasopressin release from the hypothalamus may suggest that they presynaptically inhibit the glutamatergic inputs or enhance GABAergic inputs to vasopressin neurons. Further investigation using whole-cell patch clamp recordings of PVN or SON slice preparations to examine the effect of NERPs on synaptic inputs to vasopressin neurons should elucidate the mechanisms by which NERPs modulate vasopressin release.

In conclusion, NERPs are novel bioactive peptides involved in body fluid homeostasis; they appear to modulate the actions and secretions of other neuropeptides. This study exemplifies the ability of focused peptidomics to facilitate the discovery of mammalian bioactive peptides. Further studies of NERPs and their receptors will pave the way for elucidating unknown extracellular signaling mechanisms as well as understanding the physiological roles of NERPs in body fluid homeostasis.

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