Title

Neuroendocrine regulatory peptide-1 and -2 (NERPs) inhibit the excitability of magnocellular neurosecretory cells in the hypothalamus

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Abstract

Neuroendocrine regulatory peptide (NERP)-1 and NERP-2 (NERPs) are novel carboxy-terminally amidated peptides derived from the neurosecretory protein VGF. NERPs are colocalized with vasopressin in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus, and suppress vasopressin secretion evoked by intracerebroventricular administration of angiotensin II (AngII) and hypertonic saline or bath administration of AngII. Magnocellular neurosecretory cells (MCNs) of the hypothalamus release vasopressin and oxytocin from their dendrites and soma. The two cell types have common electrophysiological properties in response to glutamate and AngII. We investigated the mechanisms underlying the suppressive effects of NERPs on MCNs. Microdialysis of the PVN demonstrated that NERPs suppressed glutamate release induced by AngII. A whole-cell patch-clamp study of the SON showed that NERPs suppressed the potentiation of excitatory postsynaptic currents (EPSCs) evoked by AngII without affecting the amplitude, indicating that NERPs suppressed EPSCs by a presynaptic mechanism. The suppressive effect of NERP-2, but not NERP-1, was blunted in the presence of tetrodotoxin and bicuculline, a γ -aminobutyric acid (GABA) A receptor antagonist. These results indicate that NERP-1 suppresses presynaptic glutamatergic neurons connected to MCNs, whereas NERP-2 activates GABAergic interneurons, which suppress presynaptic glutamatergic neurons; thus, both peptides suppress vasopressin release. This study demonstrates that NERPs function as inhibitory modulators of vasopressin release.

1. Introduction

Neuroendocrine regulatory peptide (NERP)-1 and NERP-2 (NERPs) were identified as 25- and 38-amino-acid long amidated peptides, respectively, produced by human medullary thyroid carcinoma TT cells in a strategy for discovering endogenous bioactive peptides (Yamaguchi et al., 2007). NERPs are derived from distinct regions of the neurosecretory protein VGF (non-acronymic), which was identified as a product of the nerve growth factor-responsive gene *vgf* in rat pheochromocytoma PC12 cells and was secreted through the regulated pathway from neurons and endocrine cells (Lev et al., 1985; Possenti et al., 1989; Hahm et al., 1999). Immunostaining of NERPs in the hypothalamus has been found in the magnocellular neurosecretory cells (MNCs) of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) (1). MNCs regulate plasma osmolality by releasing vasopressin and oxytocin (a reproductive hormone) (Poulain and Wakerley, 1982). Immunohistochemical observations have shown that NERPs colocalized with vasopressin, and to a lesser extent with oxytocin, in the storage granules (Yamaguchi et al., 2007).

The release of vasopressin is closely related to the electrical activity of vasopressin neurons, which in turn are regulated by neuronal excitatory glutamatergic and inhibitory γ -aminobutyric acid (GABA)ergic inputs (Wuarin and Dudek, 1993; Kabashima et al., 1997; Leng et al., 1999). Our previous studies using a whole-cell patch-clamp technique revealed that a mannitol-induced hyperosmotic condition (Yokoyama et al., 2010) and several molecules, such as nitric oxide (Ozaki et al., 2000), angiotensin II (AngII) (Ozaki et al., 2010), and ghrelin (Yokoyama et al., 2009), modulate the electrophysiological properties of vasopressin neurons. Vasopressin release induced

by AngII and hypertonic saline in rats was suppressed in a dose-dependent manner by intracerebroventricular (icv) injection of NERPs (Yamaguchi et al., 2007). The immunoneutralization by icv administration of anti-NERP-1 IgG or anti-NERP-2 IgG after intraperitoneal (ip) administration of water eliminated the plasma vasopressin reduction in response to water loading in rats deprived of water (Yamaguchi et al., 2007). This result suggests that the blockade of endogenous functions of NERPs failed to reduce vasopressin release in a hypoosmotic condition. To explore the mechanisms underlying the suppressive effects of NERPs on vasopressin secretion, we examined glutamate (Glu) release from the rat PVN by *in vivo* microdialysis, in addition to examining the electrophysiological properties of MNCs in the SON by whole-cell patch-clamp techniques.

2. Results

2.1. Effects of NERPs on AngII-induced Glu release in the PVN

Basal levels of glutamate concentration ranged from 8.6×10^{-9} M to 11.2×10^{-9} M. An icv administration of AngII significantly increased Glu release from the PVN compared with control vehicle administration (Fig. 1A and 1C) and returned to basal levels at 30–40 min (data not shown). This stimulation was suppressed by an icv injection of NERP-1 or NERP-2 (Fig. 1B(a), 1B(c), and Fig. C), but not NERP-1-Gly or NERP-2-Gly (Fig. 1B(b), 1B(d), and Fig. C). The second administration of AngII at the end of experiment stimulated Glu release, indicating that the PVN responded to AngII. Neither NERP-1 nor NERP-2 administration affected Glu release in the basal

condition (data not shown).

2.2. Effects of NERPs on AngII-evoked EPSCs in MNCs of the SON

We used whole-cell patch-clamp recordings of SON slice preparations to examine the effect of NERPs on synaptic inputs to MNCs. Spontaneous synaptic currents were recorded from a total of 128 MNCs in the SON from 23 rats (one neuron/one slice). EPSCs were observed under a basal condition (without any stimuli). AngII (10^{-7} M) potentiated spontaneous EPSCs (Fig. 2A and 2E). The effect of AngII was selective on the frequency of EPSCs, and the amplitude remained unaffected. Preadministration of NERP-1 reversibly reduced the frequency of AngII-evoked EPSCs without altering the amplitude (Fig. 2A and 2E). NERP-2 also reduced the frequency of AngII-evoked EPSCs without altering the amplitude (Fig. 2C and 2F). Neither NERP-1-Gly nor NERP-2-Gly affected the frequency of AngII-evoked EPSCs (Fig. 2B, 2D, 2E, and 2F). NERP-1-induced suppression of the frequency of EPSCs was unaffected by the Na^+ -channel blocker TTX (Fig. 3A and 3D), whereas NERP-2-induced suppression was abolished by both TTX and bicuculline (Fig. 3B, 3C, and 3E).

3. Discussion

NERPs, suppressors of vasopressin release, are abundant in the PVN and SON of the rat hypothalamus and colocalized with vasopressin in storage granules, but not with oxytocin (Yamaguchi et al., 2007), suggesting that NERPs seems to regulate the activation of vasopressin

neurons. This study demonstrates that NERPs function as inhibitory modulators of vasopressin release by using *in vivo* microdialysis and whole-cell patch-clamp technique.

Glutamate release from the presynaptic axon terminal causes an immediate and direct change in the membrane conductance of dendrites of vasopressin neurons by activating the non-*N*-methyl-D-aspartate (non-NMDA) receptor (Sladek et al., 1998), which in turn stimulates vasopressin release. AngII potentiates the glutamatergic synaptic inputs to vasopressin neurons via the AT1 receptor expressed on the presynaptic terminals of glutamatergic neurons (Ozaki et al., 2004). GABA inhibits glutamate release from the presynaptic axon terminal via the ionotropic GABA_A receptor, thereby causing reduction of the frequency of EPSCs in vasopressin neurons (Kabashima et al., 1997). The present *in vivo* microdialysis analysis demonstrated that AngII injection increased Glu concentration in the PVN. In addition to the PVN, NERP-1 and NERP-2 suppressed AngII-induced Glu release in the SON (Supplemental Figure). The voltage-clamp mode of the whole-cell patch-clamp technique showed that both NERP-1 and NERP-2 suppressed AngII-induced EPSCs in vasopressin neurons, indicating that NERPs inhibited AngII-induced quantal glutamate release. Importantly, the Na⁺-channel blocker TTX did not modulate the effect of NERP-1 on EPSCs, while it cancelled that of NERP-2 on EPSCs. The GABA_A receptor antagonist bicuculline abolished the effect of NERP-2. These results showed that NERP-1 directly suppressed glutamatergic inputs to vasopressin neurons, whereas NERP-2 might modulate inhibitory GABAergic inputs to glutamatergic neurons projecting to MNCs (Fig. 4). This may underlie the difference between the direct and indirect suppression of presynaptic glutamatergic neurons.

Factors secreted from postsynaptic dendritic terminal, such as endocannabinoids, nitric oxide, and dopamine can retrogradely modulate neurotransmitter release from presynaptic terminal (Alger, 2002; Ludwig and Pittman, 2003; Branco and Staras, 2009). This local feedback communication has been demonstrated in glutamate onto vasopressin neurons (Chevaleyre et al., 2000; Chevaleyre et al., 2002). Via whole-cell patch-clamp recording of vasopressin neurons in the PVN, Iremonger and Bains (2009) verified that an opioid peptide dynorphin was released from dendritic vesicles of vasopressin neurons in response to postsynaptic activity and acted in a retrograde manner in presynaptic release of glutamate. NERP-1 and -2 synthesized in vasopressin neurons may also retrogradely modulate the activity of vasopressin neurons in two different pathways to suppress presynaptic glutamatergic neurons. Taken together with our previous finding that NERP-2, but not NERP-1, activated the orexin system in the regulation of feeding (Toshinai et al., 2010), there likely are target proteins specific for respective NERP-1 and NERP-2. Additionally, we identified to inhibit vasopressin release from the isolated PVN and SON in the basal condition (Yamaguchi et al., 2007), suggesting that NERPs may regulate vasopressin release from the axon terminal in addition to inactivation of the soma. NERPs may be multifaceted functional peptides for suppression of vasopressin release.

The granin family proteins, including chromogranins, secretogranins, and VGF, share many properties, such as acidic isoelectric point, binding to calcium, and propensity to form large dense-core vesicles in secretory granules of endocrine cells (Bartolomucci et al., 2004). The majority of these proteins are the precursors of biologically active peptides modulating, for example, feeding

behavior, secretions of catecholamine and insulin, and blood pressure. Mammalian VGF orthologs have 10–11 conserved clusters of basic residues, many of which are processed in endocrine, neuroendocrine, and neuronal cells in a tissue-specific manner, generating a number of peptide fragments (Levi et al., 2004). The precise mechanisms of cognate receptors for a number of granin-derived peptides remain elusive. No established data have shown binding of any granin-derived peptides to a known or orphan G protein–coupled receptor, whereas downstream pathways associated with G protein signaling are activated in response to treatment of chromogranin A–derived peptides (Severini et al., 2008; Riedl et al., 2009; Koshimizu et al., 2011). Noncompetitive interaction between granin-derived peptides and G protein–coupled receptors, at sites distinct from those that interact with known ligands would be a possible mechanism. Receptor-independent triggering of signal transduction pathways by granin-derived peptides has been proposed as an alternative possible mechanism of action (Bartolomucci et al., 2011). Future studies of characterization of receptor-dependent and receptor-independent signaling mechanisms of NERPs will be challenging.

The present study shows that NERPs may function as inhibitory retrograde modulators of vasopressin release: NERP-1 suppresses presynaptic glutamatergic neurons connected to vasopressin neurons, whereas NERP-2 activates GABAergic interneurons. These presynaptic actions of NERPs may provide a means to suppress excessive excitation of vasopressin neurons. These findings will provide a better understanding of the molecular mechanisms of vasopressin release and regulation of body fluid homeostasis.

4. Experimental Procedure

4.1. Animals

Male Wistar rats (aged 9–10 weeks, Charles River Laboratories, Yokohama, 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. All animal experiments were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

4.2. Peptide synthesis

NERP-1, NERP-2, a form of NERP-1 bearing a COOH-terminal glycine (Gly) extension (NERP-1-Gly), and NERP-2-Gly were synthesized on an Abacus peptide synthesizer (Sigma Genosys Japan K.K., Ishikari, Japan) using an Fmoc (N-(9-fluorenyl) methoxycarbonyl) strategy. They were then purified by RP-HPLC, and verified for correct synthesis by mass spectrometry and amino acid analysis. The purity of the peptides was confirmed on separate HPLC systems.

4.3. Microdialysis

In vivo microdialysates were analyzed using HPLC with electrochemical detection (HTEC-500, Eicom, Kyoto, Japan) as described previously (Ohara et al., 2009). Dialysates were assayed with HPLC coupled with an autosampler every 20 min for the latter 10 min (10–20 min). Rats were anaesthetized with sodium pentobarbital (50 mg/kg), and a guide cannula (AG-12, Eicom) was

implanted into the PVN (1.8 mm posterior and 0.6 mm lateral from the bregma, and 6.6 mm dorsal from the surface of the brain) and the lateral cerebral ventricle for injections of AngII and NERPs as previously described (Nakazato et al., 2001). Five days after surgery, a dialysis probe (1 mm membrane in length, AI-12-1, Eicom) was inserted into the PVN through a guide cannula. Ringer solution was perfused in the PVN at a flow rate of 1 μ l/min, and fractions of the outflow were collected. After Glu concentration stabilized for 40 min, we estimated Glu concentrations in 10–20-min dialysate after administration of aCSF (2.5 μ l) as a control. Rats ($n = 6$ /group) were administered aCSF, NERP-1 (1 nmol/2.5 μ l aCSF), NERP-1-Gly (1 nmol/2.5 μ l aCSF), NERP-2 (3 nmol/2.5 μ l aCSF), or NERP-2-Gly (3 nmol/2.5 μ l aCSF) icv 5 min before the administration of AngII (100 pmol/2.5 μ l aCSF). Data were indicated as relative changes to the aCSF-induced Glu concentration. A second administration of AngII was conducted 80 min after each experiment to verify the responsiveness to AngII. To confirm that the PVN surgeries were successful, the brains were immersed in 4% paraformaldehyde for 72 h at 4°C after the microdialysis tests, and the locations of the injection site were observed from brain sections stained with hematoxylin–eosin (Diagnostic BioSystems, Pleasanton, CA) using light microscopy. Only the subjects with correct placement of the dialysis probe into the PVN were used for the experiments.

4.4. Electrophysiology

The major neural signals to the SON are those from excitatory glutamatergic inputs and inhibitory GABAergic inputs. In addition, the interneurons within the SON presumably mediate the

projections from other regions in order to regulate excitatory and inhibitory inputs into the SON neurons (Wuarin, 1997). In the magnocellular hypothalamo-hypophysial neurosecretory system of the rat, vasopressin and oxytocin are synthesized in separate neurons (Vandesande and Dierickx, 1975). The postsynaptic currents of vasopressin neurons mostly localising at the ventral part of the SON can be recorded, using the whole-cell patch clamp technique in the thin hypothalamic slices containing only the SON (Yokoyama et al., 2009). In brief, rat hypothalamic SON slices (150 μ m thickness) from 83 rats were fixed in a recording chamber (volume: 1 ml) and continuously perfused (flow rate: 1.4 ml/min) with aerated (95% O₂/5% CO₂) aCSF. Excitatory postsynaptic currents (EPSCs) from SON MNCs were selectively recorded by setting the holding potential at -70 mV and -20 mV. The AC component of the synaptic current was high-pass filtered at 1-Hz and analyzed quantitatively using AxoGraph v.3.6.1 (Axon Instruments, Foster Hill, CA, USA). Spontaneous events were automatically screened using an amplitude threshold of 15 pA and then were visually accepted or rejected based upon the short latency and the fast rise and decay time course of EPSCs. The perfusion medium contained the following: 124 mM NaCl; 5 mM KCl; 1.24 mM KH₂PO₄; 2 mM CaCl₂; 25.9 mM NaHCO₃; and 10 mM glucose. The pH was adjusted to 7.3, and the osmolality of all solutions ranged between 298 and 303 mOsmol/kg. Patch pipettes were filled with a pipette solution containing 140 mM potassium gluconate, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA, and 2 mM Mg-ATP at a pH 7.3, and the osmolality was 285 mOsmol/kg. Recordings of postsynaptic currents were begun 5 min after membrane rupture when the currents reached a steady state. Two min before AngII administration, NERP-1, NERP-2, NERP-1-Gly, or

NERP-2-Gly was perfused into the chamber. The total incubation times of rat NERPs and AngII were 7 min and 3 min, respectively. The currents after the administration of NERPs were recorded for at least 15 min. The final concentrations of NERPs and AngII in the chamber were 10^{-6} M and 10^{-7} M, respectively. In a subset of the experiments, tetrodotoxin (TTX, 10^{-6} M, Sigma, St. Louis, MO, USA) or bicuculline methiodide (10^{-5} M, Sigma) was applied with the perfusate throughout the entire recording. The frequency of EPSCs gradually increased upon AngII application and reached the maximum level within 2–8 min, which gradually returned to the basal level within 15 min (Ozaki et al., 2000). Accordingly, we set the 5-min sampling time window of AngII reaction 3 min after AngII application. To minimize the possibility that data are influenced by the rundown of synaptic currents during the course of measurements, the effects of drugs were expressed as percentage of basal state for 5 min before drug application. The averaged values of frequency and amplitude during the 5 min following AngII application were normalized to basal state for every recording. The numbers of data for each condition were 6–8. A slice exposed once to any of the drugs was not re-used in future experiments.

4.5. Statistical analysis

In microdialysis data, statistical comparisons of absolute value between pre-treatment and post-treatment were performed with Wilcoxon signed-rank test. These data were indicated as relative changes to the pre-treatment value. Groups of relative data were compared using ANOVA and Fisher's post-hoc test. Electrophysiological data are normalized to basal level. Statistical

comparisons were performed with Student's *t* test. Results were expressed as the mean $\pm$ S.E.M. *P*-values < 0.05 were considered to be statistically significant.

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Figure legends

Figure 1. Suppressive effects of NERPs on AngII-induced Glu release from the rat PVN. A, Figures shown are representative Glu concentration in response to icv administrations of aCSF (a) and AngII (b). Arrows indicate the elution positions of Glu in HPLC. B, Figures shown are representative changes of Glu concentrations represented by shapes of HPLC chart before (pre) and 20 min after (post) icv administration of NERP-1 (1 nmol) 5 min prior to AngII (100 pmol) injection (a), AngII + NERP-1-Gly (1 nmol) (b), AngII + NERP-2 (3 nmol) (c), and AngII + NERP-2-Gly (3 nmol) (d). C, Intracerebroventricular administration of NERP-1, but not NERP-1-Gly, to rats 5 min prior to AngII injection completely abrogated AngII-induced Glu release. NERP-2, but not NERP-2-Gly, also suppressed AngII-induced Glu release. Data are shown as relative changes to the pre-treatment of test peptide (mean $\pm$ s.e.m). The significant differences between pre- and post-treatment were obtained from absolute values, $*p < 0.05$ (vs. pre-treatment). The significant differences between groups were obtained from relative increases from pre-treatment, $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ (vs. aCSF-induced Glu release).

Figure 2. NERPs suppress the frequency of AngII-evoked EPSCs in vasopressin neurons in rats. A, Representative whole-cell patch-clamp recording showing the inhibitory effect of NERP-1 on AngII-induced potentiation of EPSC frequency in SON vasopressin neurons. EPSCs during pre-administration (a), NERP-1 + AngII administration (b), washout (c), and AngII administration (d) are shown in an expanded time scale in the lower box. Effect of NERP-1-Gly (B), NERP-2 (C), or

NERP-2-Gly (D) on AngII-evoked EPSCs in a slice preparation from the SON. Summary data for the effect of NERP-1 (E) or NERP-2 (F) on the frequency of EPSCs. Data are shown as mean $\pm$ s.e.m. * $p < 0.05$.

Figure 3. Effects of NERPs on AngII-evoked EPSCs during TTX or bicuculline administration. A, Whole-cell patch-clamp recording shows that NERP-1 reduces AngII-induced potentiation of EPSC frequency in SON vasopressin neurons during TTX administration. B and C, NERP-2 fails to suppress AngII-evoked EPSCs in the SON slice preparation during administration of TTX (B) and bicuculline (C), respectively. Summary data for the effects of NERP-1 (D) or NERP-2 (E) on the frequency of EPSCs. Data are shown as mean $\pm$ s.e.m. NS; not significant, * $p < 0.05$.

Figure 4. Hypothesized mechanisms by which NERPs regulate MNCs.

Vasopressin and oxytocin synthesized in the PVN and SON is transported to the posterior pituitary. Excitability of MCNs are regulated by excitatory glutamatergic input and inhibitory GABAergic input. AngII acts on the AngII type-1 receptor at the presynaptic terminal of glutamatergic neurons and increases spontaneous glutamate release, thereby activating the non-NMDA receptor. The GABA_A receptor-mediated response suppresses the excitation of glutamatergic neurons. NERPs synthesized in vasopressin neurons, but not oxytocin neurons, suppress AngII-induced vasopressin release (1). NERP-1 suppresses presynaptic release of glutamate, whereas NERP-2 may activate GABAergic interneurons in the immediate vicinity of the SON; however, both suppress presynaptic

glutamatergic neurons. The putative target protein specific for NERP-1 or NERP-2 is supposed to be expressed on the presynaptic terminal of glutamatergic neurons or GABAergic interneurons, respectively.

Figure 1

Figure 2

Figure 3

Figure 4