Actions of neuropeptide W in paraventricular hypothalamus: implications for the control of stress hormone secretion


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Neuropeptide W (NPW) is produced in neurons located in hypothalamus and brain stem, and its receptors are present in the hypothalamus, in particular in the paraventricular nucleus (PVN). Intracerebroventricular (ICV) administration of NPW activated, in a dose-related fashion, the hypothalamic-pituitary-adrenal axis, as determined by plasma corticosterone levels in conscious rats but, at those same doses, did not stimulate the release of oxytocin or vasopressin into the peripheral circulation or alter blood pressure or heart rate. The ability of ICV-administered NPW to stimulate the hypothalamic-pituitary-adrenal axis in conscious male rats was blocked by intravenous pretreatment with a corticotropin-releasing hormone antagonist. This suggested an action of NPW in the paravascular division of the PVN. Indeed, in hypothalamic slice preparations (whole cell patch recording), bath application of NPW depolarized and increased the spike frequency of the majority of electrophysiologically identified putative neuroendocrine PVN neurons. Effects on membrane potential were maintained in the presence of TTX, suggesting them to be direct postsynaptic actions on these neuroendocrine cells. Our data suggest that endogenous NPW, produced in brain, may play a physiologically relevant role in the neuroendocrine response to stress.

corticotropin-releasing hormone; corticosterone

MATERIALS AND METHODS

In vivo experiments. All procedures were approved by the Saint Louis University Animal Care and Use Committee. Adult, male Sprague-Dawley rats (250–300 g, Harlan, Indianapolis, IN) were employed. Animals were housed individually under constant conditions (25°C, lights on 12 h) and provided tap water and conventional lab chow ad libitum. Under ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA)/xylazine (TranqulVed, Vedco, St. Joseph, MO) anesthesia (60 mg/8 mg mixture per milliliter, 0.1 ml/100 g body wt, intraperitoneal injection), rats were placed in a stereotaxic device, and a 23-gauge stainless steel cannula (17 mm) was implanted into the right lateral cerebroventricle, as previously described (12). Minimally 5 days later, after the animals had returned to preimplantation body weights, an indwelling jugular vein cannula was implanted, as previously described (5), under isoflurane-induced anesthesia (IsoSo, Vedco). The jugular cannula was exteriorized at the back of the neck and sealed with heparinized saline (200 U/ml 0.9% NaCl). On the following day, an extension tubing (PE-50) was attached to the jugular cannula to facilitate blood sampling, and rats were left undisturbed for 60 min. Then an initial blood sample was withdrawn from the jugular vein without disturbing the animal. All blood samples (0.3–0.4 ml)

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were removed from conscious, unrestrained rats into heparinized syringes and replaced with an equal volume of 0.9% NaCl (37°C). Blood samples were stored on ice before plasma was separated (10,000 g, 5 min) and stored at −20°C until hormone assay (corticosterone) was conducted. Immediately after the removal of the initial (time 0) blood sample, rats were pretreated (13) with saline vehicle or vehicle containing 0.2 mg CRH antagonist [alpha-helical CRF9–41, intravenous (IV), Phoenix Pharmaceuticals, Belmont, CA]. Fifteen minutes later, a second blood sample was collected, and then all animals received a central [intracerebroventricular (ICV)] injection of 1.0 nmol NPW-23 (Phoenix Pharmaceuticals) in 2 μl saline. Blood samples were collected as before, 30, 45, and 60 min after the ICV injections.

In another group of conscious male rats, saline vehicle (2 μl) or vehicle containing 1.0 or 3.0 nmol NPW was injected ICV (as above), and rats were killed by decapitation 15 min later. Transect bloods were collected into heparinized tubes, and plasma was collected following centrifugation (4°C, 10 min, 600 g). Plasma oxytocin (OT) and vasopressin contents were determined by RIA (10, 11).

In a separate group of rats (n = 8) bearing lateral cerebroventricular cannulas, the left carotid artery was cannulated, as previously described (12). On the following day, rats were moved to a quiet room, and the carotid cannula was connected to a pressure transducer (Digi-Med BPA, Micro-Med, Louisville, KY). Blood pressure and heart rate (HR) were monitored for a 1-h stabilization period before ICV injection of 2-μl saline vehicle or vehicle containing 1.0 or 3.0 nmol NPW. Blood pressure [systolic, diastolic, and mean arterial pressures (MAPs)] and HRs were monitored at 10-s intervals for 30 min after peptide or vehicle administration. Treatments were administered in random order, with all rats receiving saline and both doses of NPW. At the end of all testing periods, 50 pmol angiotensin II were injected ICV to verify cannula patency (dipsogenic response and increased MAP).

RIAs. Plasma corticosterone levels were determined according to the instructions of the commercial RIA kit (rat/mouse corticosterone, ICN Biomedicals, Costa Mesa, CA), as previously described (1). The minimum detectable hormone level was 25 ng/ml, and the interassy and intra-assay coefficients of variability were <10%. Plasma vasopressin [arginine vasopressin (AVP)] and OT levels were determined by RIAs uniquely developed in our laboratory (10, 11). The lower limits of sensitivity in those assays were defined as minimally 95% of total binding for AVP (0.125 pg/ml plasma) and OT (2 pg/ml plasma). The intra- and interassay coefficients of variabilities for these assays were <7%.

Statistical analyses. For the in vivo hormone experiments, data were analyzed by ANOVA (with Scheffé’s multiple-comparison posthoc testing), both within treatment groups across time and across treatment groups at any sampling time point. Blood pressure and HR responses were analyzed similarly at 1-min intervals. Values are means ± SE. Significance was assigned to results that occurred with <5% probability.

Slice preparation. Experiments were performed by using hypothalamic slices prepared as previously described (7). Male Sprague-Dawley rats (150–250 g, Charles River, Quebec, Canada) were decapitated, and the brain was quickly removed from the skull and immersed in cold (1–4°C) artificial cerebrospinal fluid (aCSF). The hypothalamus was blocked, and 300-μm slices, including the PVN, were cut with the use of a vibrotome. Slices were incubated in oxygenated aCSF (95% O₂–5% CO₂) for at least 60 min at room temperature. Fifteen minutes before recording, the slice was transferred into an interface-type recording chamber and continuously perfused with oxygenated aCSF (see below for solution composition) at a rate of 1 ml/min. All techniques were carried out in accordance with the guidelines of the Canadian Council for Animal Care and were approved by Queen’s University Animal Care Committee.

Electrophysiology. Whole cell patch recordings were obtained from PVN neurons by using blind patch techniques, as described previously (7). Signals were processed with an Axoclamp-2B amplifier and a Ag-AgCl electrode connected to the bath solution via a KClagar bridge, which served as reference. A 3- to 8-mV (measured at end of each recording) liquid junction potential correction was applied to all data presented. Drugs were applied by switching perfusion from aCSF to a solution containing the desired drug. All signals were digitized (5 kHz) by using the CED 1401 plus interface (CED, Cambridge, UK) and stored on computer for off-line analysis. Data were collected by using Spike2 (continuous recording) packages (CED).

Solutions. The internal pipette solution contained (in mM) 140 potassium gluconate, 0.1 CaCl₂, 2 MgCl₂, 1.1 EGTA, 10 HEPES, and 2 Na₂ATP, adjusted to pH 7.25 with KOH. The aCSF composition was (in mM) 124 NaCl, 2 KCl, 1.25 KPO₄, 2.0 CaCl₂, 1.3 MgSO₄, 20 NaHCO₃, and 10 glucose. Osmolarity was maintained between 285 and 300 mosM and pH between 7.3 and 7.4. NPW (NPW-23, Phoenix Pharmaceuticals) was prepared fresh on the day of the experiment from 200-μl aliquots of a 1 μM stock solution stored at −80°C, to concentrations ranging from 100 to 1 nM in aCSF. TTX (Alomone Laboratories, Jerusalem, Israel) was used in a concentration of 1 mM to block voltage-activated sodium currents. Once dissolved in aCSF, the drug was applied directly to the slice through the bath perfusion system.

Cell identification. Cells were functionally characterized in accordance with previous studies demonstrating that specific electrophysiological fingerprints are associated with magnocellular [transient potassium currents (18)], preautonomic [low threshold calcium currents (15)], or NE neurons [neither of the above (8); see Fig. 2D]. In view of our specific focus in these studies on the effects of NPW on CRH neurons, our recordings were obtained from neurons in the medial parvocellular subdivision (macroscopically identified) with the purpose of primarily recording from NE parvocellular neurons.

Analysis. All neurons included in this analysis maintained action potentials of at least 60 mV in magnitude throughout the recording period and maintained a stable baseline membrane potential for at least 100 s before application of NPW. Following peptide application, responses were characterized as follows: depolarization (>3-mV increase in membrane potential followed by a return to baseline), hyperpolarization (>3-mV decrease in membrane potential), or no response (<3-mV change in membrane potential). Changes were evaluated by measuring the peak membrane potential change maintained for a minimum of 10 s (this excludes transient changes such as action potentials/postsynaptic potentials) following peptide application.

RESULTS

We verified our laboratory’s earlier report (1) of the ability of NPW to act in brain to elevate plasma corticosterone levels (Fig. 1). We pretreated animals with vehicle or the CRH antagonist (IV to compromise CRH function in the anterior pituitary gland) and then administered the minimum effective dose of the GPR7 ligand ICV to determine whether the stimulation of the HPA axis was due to increased release of CRH into the portal vessels. Saline vehicle pretreatment did not significantly alter plasma corticosterone levels before NPW administration (0-min levels vs. 15-min levels, P = 0.99). Furthermore, no significant differences in plasma corticosterone levels were observed between the two groups before IV infusion of vehicle or CRH antagonist or 15 min later before ICV NPW administration. In animals pretreated with saline vehicle, significant elevations [F(4,40) = 8.65, P < 0.001] in plasma corticosterone levels were observed 30 (P < 0.001) and 45 min (P < 0.05) following ICV administration of 1.0 nmol NPW (Fig. 1).
The ability of centrally administered NPW to activate the HPA axis was significantly diminished in rats pretreated with the CRH antagonist. As observed in prior studies in our laboratory (13), IV administration of the CRH antagonist did not significantly alter basal corticosterone levels 15 min after infusion (P = 0.99); however, CRH antagonist pretreatment significantly reduced the ability of centrally administered NPW to elevate circulating corticosterone levels. Although a residual effect was observed, with levels present 30 min after NPW administration being significantly greater than those following antagonist treatment before NPW infusion (P < 0.05), the elevations observed in the saline vehicle-pretreated control group greatly exceeded those observed in the antagonist-pretreated animals at all subsequent sampling times (P < 0.05).

It is possible that the residual corticosterone elevation in the CRH antagonist (IV)-pretreated, NPW (ICV)-administered animals was due to an effect of the peptide on AVP or OT secretion, because both of these peptides, under certain conditions, can act in the pituitary gland as corticotropin-releasing factors (19). However, doses of NPW (1.0 and 3.0 nmol ICV) that significantly elevated plasma corticosterone levels failed to significantly alter plasma levels of vasopressin or OT in conscious male rats (Table 1).

MAPs and HRs were not significantly different between treatment groups before ICV injections. No significant changes in MAP and HR were observed following saline vehicle injection ICV (within-group ANOVA). Similarly, neither dose of NPW significantly altered MAP or HR in these animals, when values were compared within group or between groups compared with control. However, MAP did significantly rise in these animals following ICV administration of 50 pmol angiotensin II (Table 2). HRs declined following angiotensin II administration, reflecting, in all likelihood, reflex bradycardia in response to the pressor effect of the peptide, although significance was not obtained.

Results from the NE protocol suggested that NPW exerts its effects on the HPA axis by an action in brain and in particular in the parvocellular PVN, site of the CRH-producing neurons that project to median eminence. We then sought to investigate that possibility by examining the effects of bath application of NPW on the activity of single electrophysiologically identified neurons in the parvocellular region of PVN in the hypothalamic slice preparation using patch-clamp recording techniques.

Whole cell current clamp recordings were obtained from 28 electrophysiologically characterized NE PVN neurons (8, 15, 18). These NE neurons were tested for the effects of bath application of NPW on the activity of single electrophysiologically identified neurons in the parvocellular region of PVN in the hypothalamic slice preparation using patch-clamp recording techniques.

### Table 1. Plasma vasopressin and oxytocin levels (pg/ml) in trunk bloods collected 15 min after intracerebroventricular administration of saline vehicle or vehicle containing 1.0 or 3.0 nmol NPW in conscious male rats

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>AVP, pg/ml</th>
<th>OT, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>27</td>
<td>2.0±0.5</td>
<td>23.5±2.0</td>
</tr>
<tr>
<td>1.0 nmol NPW</td>
<td>12</td>
<td>3.4±1.2</td>
<td>18.6±2.8</td>
</tr>
<tr>
<td>3.0 nmol NPW</td>
<td>19</td>
<td>3.2±0.7</td>
<td>23.7±2.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. NPW, neuropeptide W; AVP, arginine vasopressin; OT, oxytocin.

### Table 2. Comparison of the effects of intracerebroventricular administration of saline vehicle, angiotensin II (50 pmol), or NPW (1.0 or 3.0 nmol) on mean arterial pressures and heart rate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time After ICV Injection, min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Mean arterial pressure, mmHg</strong></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>135±4.5</td>
</tr>
<tr>
<td>1.0 nmol NPW</td>
<td>133±5.1</td>
</tr>
<tr>
<td>3.0 nmol NPW</td>
<td>132±7.8</td>
</tr>
<tr>
<td>50 pmol Angiotensin II</td>
<td>139±5.3</td>
</tr>
</tbody>
</table>

**Heart rate, beats/min**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>385±40</td>
<td>384±14</td>
<td>385±8</td>
<td>397±10</td>
</tr>
<tr>
<td>1.0 nmol NPW</td>
<td>362±5</td>
<td>357±10</td>
<td>359±17</td>
<td>355±10</td>
</tr>
<tr>
<td>3.0 nmol NPW</td>
<td>350±6</td>
<td>365±12</td>
<td>369±13</td>
<td>348±15</td>
</tr>
<tr>
<td>50 pmol Angiotensin II</td>
<td>398±13</td>
<td>392±17</td>
<td>356±13</td>
<td>348±15</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 rats. ICV, intracerebroventricular. *P < 0.05 vs. preinjection values within group.
Fusion (unpaired t-test, \( P < 0.001 \)) (Fig. 2E). The remaining NE neurons tested showed either no response (\(-0.8 \pm 0.9 \text{ mV, } n = 5\)) or hyperpolarizations (\(-9.9 \pm 1.7 \text{ mV, } n = 6\)) in response to NPW. At the higher concentrations of NPW, complete return to baseline membrane potential often did not occur within the 30-min postapplication recording period, whereas recovery was normally observed within 15 min of return to aCSF following lower concentrations of NPW (100 pM, 1 nM), as illustrated in Figs. 2A and 3.

In addition to the depolarizing effect of NPW on NE neurons, statistically significant increases in spike frequency were also observed with mean spike frequency changing from control values of \(0.054 \pm 0.016\) to \(0.287 \pm 0.025\) Hz during NPW treatment, followed by a return to baseline \(0.047 \pm 0.007\) Hz (ANOVA followed by Newman Keuls, \( P < 0.001 \) compared with control and recovery), as illustrated in Fig. 2F. In an additional five NE cells, depolarizing effects were still observed in response to NPW during bath perfusion with TTX (1 mM), with three of five NE cells tested (similar proportion of responsive neurons), showing a mean depolarization of \(13.7 \pm 4.2\) mV (Fig. 2, C and E), effects that were not statistically
different to those observed in response to NPW in normal aCSF (t-test, \( P > 0.2 \)).

DISCUSSION

The identification of the endogenous ligands for several G-protein-coupled orphan receptors allowed new insight into the hypothalamic mechanisms controlling appetite, metabolism, and NE function (3, 17). Recently, two endogenous ligands for the orphan receptor GPR7, previously demonstrated to be in high abundance in hypothalamus (6), were identified. One, NPW, appears to be produced predominantly in brain stem nuclei (17), but immunoreactivity was also detected in parvocellular paraventricular hypothalamic nucleus and the supraoptic nucleus (2). The presence of GPR7 mRNA in hypothalamic sites known to be important in the control of anterior pituitary function and appetite regulation suggested to us that NPW might act within those sites to regulate NE function. Indeed, in their initial description of the identity of NPW, Shimomura and colleagues (14) reported that single doses of centrally administered NPW stimulated feeding and PRL secretion. We have extended those findings (1) to include a description of the dose-related stimulation of PRL secretion by centrally administered NPW and demonstrated the concomitant increase in plasma corticosterone levels and the decrease in GH levels in those animals. Because no significant effects of NPW on the in vitro releases of PRL, ACTH, or GH were observed in anterior pituitary cell cultures, we have hypothesized that NPW, acting within the hypothalamus, controls stress hormone secretion by increasing the release of CRH and somatostatin and decreasing the release of dopamine and GHRH into the median eminence and thus the hypophysial portal vasculature.

Our present study demonstrates that the ability of centrally administered NPW to activate the HPA axis is likely mediated via an increase of CRH release in median eminence for delivery to the corticotrophs of the anterior pituitary gland. Indeed, pretreatment of animals with a dose of CRH antagonist previously demonstrated by us to abrogate CRH actions in the pituitary gland (13) significantly attenuated the ability of exogenous NPW to activate the HPA axis. Our electrophysiological data, in addition, support the conclusion that NPW exerts these effects as a result of activation of CRH neurons in the parvocellular division of the PVN rather than potential actions on nerve terminals in the median eminence. Our demonstration that NPW effects are still observed when these neurons are placed in synaptic isolation by bath perfusion with TTX also supports the conclusion that such effects are, in fact, a result of direct actions of NPW on the postsynaptic membrane of these neurons. Whereas we recognize that electrophysiologically identified NE neurons are not homogenous in their chemical phenotype, available evidence suggests that the majority (16) of these neurons are, in fact, CRH producing, supporting the conclusion that the majority of NE cells recorded in these studies are CRH-producing neurons (18). Interestingly, a small proportion of NE-type PVN cells was hyperpolarized by NPW, observations that may correlate with the previously suggested mechanisms for NPW effects on PRL (inhibition of dopamine neurons) (1, 14) or GH (inhibition of GH-releasing hormone neurons) (1) secretion. It will be important for us to develop the methodology to definitively identify the peptide phenotype of the neurons in our slice preparations that respond directly to NPW, and we are working toward that goal.

Selectivity of the effect of NPW is indicated by the failure of this peptide to alter the release from magnocellular PVN neurons of AVP or OT, two additional stress hormones originating in hypothalamus, that also, under select circumstances, can act as releasing factors to stimulate ACTH release (19). It appears that the actions of NPW are expressed in hypothalamus and not after “leakage” into the portal vasculature, because no significant effects of the peptide on basal or releasing factor-stimulated ACTH secretion were observed in primary cell cultures (1). Thus the function of GPR7 in anterior pituitary (9) remains unclear.

In summary, these results support our hypothesis that endogenous NPW plays a role in the afferent signals controlling CRH release and further suggest that endogenous NPW may be a physiologically relevant messenger in the brain networks that activate the HPA axis in response to stress. Our data indicate that the depolarizing effects of NPW in vitro are direct and are not mediated via interneurons. This would suggest that NPW receptors are present on cells in parvocellular PVN and perhaps on CRH-producing neurons in the nucleus. It will be important to develop the methodologies to identify NPW-responsive neurons on the basis of chemical content. Just the same, we now can suggest that the central action of NPW to activate the HPA axis is, in the end, mediated by increased activity of CRH neurons. We must now identify the conditions under which NPW is released in the PVN and the consequences of loss of NPW function or production on stress-induced ACTH and corticosterone release.

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