Chronic high NaCl intake prolongs the cardiorenal responses to central N/OFQ and produces regional changes in the endogenous brain NOP receptor system.

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Running Head Title: High NaCl alters endogenous N/OFQ-NOP receptor systems

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Abstract

Intracerebroventricular (i.c.v.) nociceptin/orphanin FQ (N/OFQ) produces cardiovascular depressor, diuretic and renal sympathoinhibitory responses in conscious rats. These studies examined how a chronic high NaCl intake alters these peptide-evoked responses and the activity of the endogenous central N/OFQ-NOP receptor system. In normotensive Sprague-Dawley rats that were fed a chronic (3 week) high (8%) NaCl diet, i.c.v. N/OFQ (5.5 nmol) produced prolonged bradycardic, hypotensive and diuretic responses, but failed to suppress renal sympathetic nerve activity. In a separate group of rats maintained on a high NaCl diet, i.c.v. infusion of the NOP receptor antagonist, UFP-101, significantly decreased urine output. At the tissue level, high NaCl treatment of rats significantly increased NOP receptor density, without altering endogenous N/OFQ peptide levels in whole hypothalamus (control, 712±35 fmol/mg vs. 8% NaCl, 883±49 fmol/mg, P<0.05) and paraventricular nucleus (PVN). Further, in the hypothalamus, basal GTPγS binding was increased without altering the sensitivity of N/OFQ stimulated G-protein coupling. In contrast, in whole medulla and the ventrolateral medulla (VLM), high NaCl treatment decreased NOP receptor density (medulla; control, 1473±131 fmol/mg vs. 8% NaCl, 327±31 fmol/mg, P<0.05) and endogenous N/OFQ peptide levels (medulla; control, 35.3±2 fmol/mg vs. 8% NaCl, 11.9±3 fmol/mg, P<0.05), while increasing the sensitivity of G-protein signaling pathways to N/OFQ stimulation. Together, these findings suggest that during a chronic high salt intake, regional changes in the activity of the N/OFQ-NOP system in the brain may contribute to the tonic regulation of cardiovascular function and urine output, and to the altered physiological responses to exogenous central N/OFQ.
**Key words:** sodium-chloride diet, nociceptin/orphanin FQ (N/OFQ), cardiovascular function, renal excretory function, central nervous system.
Introduction

Nociceptin/Orphanin FQ (N/OFQ) is an endogenous opioid–like peptide that selectively binds to the N/OFQ peptide (NOP) receptor (24, 28). The NOP receptor is a G-protein coupled receptor (GPCR), which elicits its physiological responses via pathways involving downstream $G_{a1,2,3}$, $G_{aoA,B}$ (9, 32), $G_{az}$ (4, 13) and $G_{aq}$ subunit proteins (33). The intracerebroventricular (i.c.v.) administration of N/OFQ to conscious rats evokes a unique pattern of changes in cardiovascular (hypotension, bradycardia, inhibition of central sympathetic outflow) and renal excretory function (water diuresis) that are independent from those produced by central nervous system (CNS) activation of mu, delta, or kappa opioid systems (16, 17, 19). Although the cardiovascular and renal responses produced by central administration of N/OFQ are well characterized, the endogenous role(s) of the N/OFQ-NOP receptor system remain unclear. Both N/OFQ and NOP receptors are highly expressed in CNS sites that regulate cardiovascular and renal homeostasis (25, 34). N/OFQ has been demonstrated to play a critical modulatory role in the adaptive behavioral fear responses to stressful stimuli (5, 12); therefore it is likely that the endogenous N/OFQ-NOP receptor system plays a physiologically important role in regulating cardiovascular and renal responses to acute/chronic stressors (18).

The stress of excessive dietary sodium chloride intake can have significant impact on cardiovascular and renal function and contribute to the development of hypertension (2, 24). However, the mechanisms by which high salt intake produces or augments hypertension have yet to be clearly elucidated. Typically, in health, neural (renal sympathetic) and circulating humoral (angiotensin-aldosterone) sodium-retaining mechanisms are suppressed as a means to facilitate the renal excretion of a sodium load (2, 7). In this setting (e.g., healthy normotensive Sprague-
Dawley rats) enhanced water intake (i.e., drinking) associated with consumption of a high salt diet would be expected to occur without an increase in plasma vasopressin (AVP, antidiuretic hormone) levels as a means to facilitate an increase in urine output. This is in contrast to the increase in plasma AVP and water retention which occur in certain models of salt-sensitive hypertension (27, 30, 31). Together, in health these regulatory mechanisms operate to help maintain total body water/sodium balance and normotension.

As noted above, central NOP receptor activation causes cardiovascular depressor and diuretic responses via inhibiting central sympathetic outflow (19) and AVP secretion (15, 33), respectively. The ability of central N/OFQ to influence these regulatory pathways is of potential physiological importance. Since a chronic high NaCl intake can alter central sympathetic outflow (2, 8), basal plasma AVP levels (30) and urine output (26, 29), it is possible that the endogenous central N/OFQ system may act to oppose NaCl-induced changes in these parameters. Based on this premise it would be expected that a chronic high NaCl intake would cause changes in the activity of the endogenous N/OFQ-NOP receptor system in specific brain regions involved in the control of cardiovascular function and the renal handling of water.

Therefore, the present studies were performed to determine how a chronic (3 weeks) high (8%) NaCl intake alters the cardiovascular and renal excretory responses to central administration of N/OFQ in conscious Sprague-Dawley rats. As a correlate, we examined how a chronic high NaCl stress alters endogenous levels of the N/OFQ peptide, NOP receptor expression and activity (GTPγS binding) in the hypothalamus and the medulla of the brain. In addition, high salt-induced changes in N/OFQ peptide levels and NOP receptor expression were
measured in the ventrolateral medulla (VLM) and the paraventricular nucleus (PVN), these being key brain regions involved in the central neural control of cardiovascular function and AVP synthesis/release, respectively. Finally, experiments were performed to investigate the premise that endogenous central N/OFQ systems are activated as an adaptive mechanism to counter the influence of chronic high NaCl on cardiovascular function and/or urine output in rats. In these studies, i.c.v. administration of the selective NOP receptor antagonist, UFP-101 (4, 34) was used to pharmacologically block a potential ongoing tonic influence of central N/OFQ on a given cardiovascular or renal excretory parameter.
Methods

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN), 275-300 g, were housed individually under a 12 h light/dark cycle with free access to food and water. All procedures were conducted in accordance with National Institutes of Health and Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee guidelines for the Care and Use of Animals.

Surgical and Experimental Methods

Sprague-Dawley rats were maintained for 3 weeks on either a normal rodent diet (0.4% NaCl) plus tap drinking water, or as an experimental model of chronic high salt intake (7), a high (8%) NaCl diet (TestDiet, Richmond, IN). One week before experimentation all rats were anesthetized (30 mg/kg i.m. ketamine in combination with 2 mg/kg i.m. xylazine; 17, 19), and stereotaxically implanted with a stainless steel cannula into the right lateral cerebral ventricle. Verification of cannula position was established via observation of CSF and/or placement of dye following i.c.v. injection (17, 19). On the day of study, rats were anaesthetized with sodium methohexital (20 mg/kg i.p., supplemented with 10 mg/kg i.v. as required; 16, 17, 19) and instrumented with catheters in the left femoral artery, left femoral vein and bladder as described previously for measurement of arterial blood pressure, administration of drugs/saline and collection of urine, respectively (17, 19, 20). In certain studies, rats (still anesthetized with sodium methohexital) were then implanted with a recording electrode on a renal nerve bundle for direct measurement of multifiber renal sympathetic nerve activity (RSNA) (19, 20). Following surgical preparation, rats were placed in a rat holder and an i.v. infusion of isotonic saline (55 µl/min) was started and continued for the duration of the experiment. The experimental protocol
commenced after the animal regained full consciousness, and cardiovascular and renal excretory functions stabilized (4-6 h). Mean arterial pressure (MAP), heart rate (HR) and RSNA were continuously recorded using computer-driven BIOPAC data acquisition software (MP100 and AcqKnowledge 3.8.2). Because of the limitations of comparing values for multifiber RSNA between animals, RSNA data was expressed as percentage of control with the control values for each animal taken as 100% (20). Urine volume was determined gravimetrically. Urine sodium concentration was measured by flame photometry (model 943; Instrumentation Laboratories, Lexington, MA) and expressed as urinary sodium excretion.

**Central N/OFQ studies** Experiments were performed to determine the changes in systemic cardiovascular and renal excretory function and RSNA produced by i.c.v. N/OFQ in conscious Sprague-Dawley rats that were chronically (3 weeks) fed either a normal (0.4%) or high (8%) NaCl diet. On the day of the experiment, cardiovascular function and urine output were initially measured in rats during a 20-min control period. Following this, N/OFQ (5.5 nmol) (16) or isotonic saline vehicle (5 µl) was injected i.c.v. (N=8 per group). The dose of N/OFQ used in these studies does not represent either the EC50 or maximally effective dose; instead the dose of 5.5 nmol has been previously demonstrated to produce consistent significant reproducible effects on the physiological parameters under investigation (16). Cardiovascular function was then measured and urine samples collected during a 90-min experimental period (consecutive 10-min periods).

**Central UFP-101 antagonist studies** Studies were performed to determine the changes in systemic cardiovascular and renal excretory function produced by acute blockade of central NOP
receptors in conscious Sprague-Dawley rats maintained for 3 weeks on either a normal (0.4%) or high (8%) NaCl chow. On the day of the experiment, systemic cardiovascular function was measured and urine collected during a 20-min control period. Next, rats then received an i.c.v. infusion of isotonic saline vehicle (5 µl/hr; N=6/group) or the selective NOP receptor antagonist, UFP-101 (18 nmol/5µl/h; N=6/group) (4, 34), which was continued for the duration of study. Cardiovascular function was then measured and urine samples collected during a 90-min experimental period (consecutive 10-min periods).

**AVP measurement**

Naïve Sprague-Dawley rats were fed a control diet (0.4% NaCl) or high salt (8% NaCl) diet for three weeks (7) (N=6 per group). Animals were then decapitated and plasma AVP was determined using an AVP ELISA kit as per manufacturers’ instruction (Assay Designs Inc, MI) and expressed as pg/ml.

**Brain tissue collection**

Naïve Sprague-Dawley rats were fed a control diet (0.4% NaCl) or high salt (8% NaCl) diet for three weeks (7). Animals were then sacrificed by decapitation and brain tissue was dissected on ice and stored at -80°C. Brain tissue was taken from the frontal cortex, the whole hypothalamus and medulla as identified visually using morphological landmarks (28). In separate groups of rats, whole brains were removed, wrapped in aluminum foil and frozen at -80°C. Brain cortex, PVN and VLM tissue samples were then taken from frozen brain sections cut on a cryostat using a brain punch tool (Stoelting, IL). Brain cortex and PVN samples were taken using a punch diameter of 1.00 mm; VLM samples were taken using a punch diameter of
0.76 mm and were stored at -80°C. The location of the PVN and VLM was determined using visual landmarks (27), and by identification of neuron populations in sections examined under a light microscope.

**N/OFQ RIA**

Peptide extracts were prepared from brain regions (brain cortex, hypothalamus, medulla), and specific brain regions (PVN, VLM) (N=6 0.4% NaCl diet; N=6 8% NaCl diet), and quantified for protein content (21). N/OFQ peptide levels were quantified using a RIA kit (Phoenix Pharmaceuticals Inc, Burlingame, CA), and expressed as fmol N/OFQ per mg of protein.

**[leucyl-³H]N/OFQ saturation binding assay**

Sprague-Dawley CNS tissue membrane preparations from brain cortex, whole hypothalamus and medulla (N=6 0.4% NaCl diet; N=6 8% NaCl diet) were prepared and quantified for protein content (21). Samples were not prepared from PVN and VLM punches owing to tissue limitations of total protein content obtained from brain tissue punch samples. 100 µg of tissue membranes were incubated with varying concentrations (~ 0.002pM – 2nM) of [leucyl-³H] N/OFQ for 60 min at room temperature in 500 µl of binding buffer supplemented with a 10 µM peptidase inhibitor cocktail (containing amastatin, bestatin, captopril and phosphoramidon; 10 µM each) (23). Non-specific binding was determined in the presence of 1 µM unlabeled N/OFQ. Reactions were terminated via vacuum filtration through polyethylenimine (0.5%) pre-soaked Whatman GF/B filters using a Brandel-harvester (23). Radioactivity was determined following filter extraction using liquid scintillation spectroscopy.
NOP receptor levels are expressed as fmol NOP per mg of protein, concentration response curves were analyzed using computer-assisted curve fitting with Graphpad Prism 4, with data fitted to a one-site binding model, and subjected to non-linear regression using a sigmoidal dose response (variable slope) model, to produce affinity (pK_D) and receptor density (B_max) values.

**NOP receptor Immunoblotting**

Brain punch samples (BC, PVN, VLM), whole hypothalamus and medulla were taken from Sprague-Dawley rats maintained on control (0.4% NaCl) or high (8% NaCl) salt diets for 3 weeks (N=6 per group). Tissue lysates, containing both membrane and cytoplasmic fractions, were then prepared and protein levels were quantified (21). Lysates were resolved on SDS-PAGE gels and transferred to nitrocellulose membrane (GE Healthcare, Piscataway, NJ). NOP receptor levels were determined using anti-KOR3 antibody (Santa Cruz, CA) (1:1000), protein levels were normalized to GAPDH (anti-GAPDH 1:1000, Abcam, MA, 34). Chemiluminescent immunoreactive bands were detected by horseradish peroxidase-conjugated secondary antibody. Data was imaged and analysed using Bio-Rad Quantity One software.

**GTPγS assay**

Sprague-Dawley brain tissue membrane preparations from frontal cortex, whole hypothalamus and medulla were prepared (N=6 0.4% NaCl diet; N=6 8% NaCl diet) and quantified for protein content (21). Samples were not prepared from PVN and VLM punches owing to tissue limitations of total protein content of brain punch samples. A N/OFQ stimulated GTPγS binding assay was performed using 20 µg of tissue membranes. Membranes were incubated for 1 h at 30°C with gentle shaking in 500 µl of incubation buffer (pH 7.4) containing
Tris (50mM), EGTA (0.2mM), NaCl (100mM), BSA (1mg/ml), bacitracin (0.15mM), amastatin (10µM), bestatin (10µM), captopril (10µM), phosphoramidon (10µM), GDP (100µM) and ~ 150 pM GTPγ35S. Non-specific binding was determined in the presence of 10 µM unlabeled GTPγS, N/OFQ was added over the concentration range log 10⁻⁵ – 10⁻¹² (M). Reactions were terminated via vacuum filtration through Whatman GF/B filters using a Brandel-harvester (23). Radioactivity was determined following filter extraction using liquid scintillation spectroscopy. Concentration response curves were analyzed using computer-assisted curve fitting with Graphpad Prism 4, with data subjected to non-linear regression analysis using a sigmoidal dose response (variable slope) model to produce functional potency (pEC₅₀) and efficacy (Stimulation factor). Data are presented as stimulation factor, which is the ratio between N/OFQ stimulated GTPγ35S binding and basal specific binding, and pEC50, which is the negative logarithm to base 10 of the agonist (N/OFQ) molar concentration that produces 50% of the maximal possible effect, and un-stimulated basal binding, expressed as total DPM, representing endogenous GTP γ35S binding (4, 23).

**Statistical Analysis**

All data are expressed as mean ± SEM. The magnitude of the changes in cardiovascular and renal excretory parameters at different time points after i.c.v. injection of drugs were compared with respective group control values by a one-way repeated-measures analysis of variance (ANOVA) with subsequent Dunnett’s test. Differences occurring between treatment groups (e.g., 0.4 % and 8% NaCl) were assessed using two-way repeated measures ANOVA with treatment being one fixed effect and time the other, with the interaction included. The time (min) was then the repeated factor. Post hoc analysis was performed using Bonferroni’s test.
Where appropriate, a Student’s $t$ test was also used to compare means between two groups. In each case, statistical significance was defined as $p < 0.05$.

**Results**

**Cardiovascular and renal excretory responses to i.c.v. N/OFQ**

In control rats fed a normal diet (0.4% NaCl), i.c.v. N/OFQ (5.5 nmol), but not i.c.v. saline vehicle (5 µl), produced characteristic reductions in HR, MAP and urinary sodium excretion and an increase in urine flow rate (Fig 1). To determine whether chronic salt loading alters these responses, changes in cardiovascular and renal excretory function produced by i.c.v. N/OFQ were examined in rats maintained for 3 weeks on a high salt diet (8% NaCl chow plus tap drinking water). In these studies, levels for baseline systemic hemodynamics and urine output were not statistically different from those in rats maintained on a normal NaCl intake. However, basal urinary sodium excretion was significantly increased in rats that consumed a high salt diet (Fig 1). As depicted (Fig 1), in high NaCl-treated rats the duration of cardiovascular depressor responses (bradycardia, hypotension) to i.c.v. N/OFQ were significantly prolonged. In contrast to control diet animals in which HR and MAP returned to pre-drug levels by 40-min, the bradycardia and hypotension persisted for 60 and 80-min respectively in the 8% NaCl group (Fig 1). There was a delayed diuresis in response to i.c.v. N/OFQ in both control and high NaCl groups, with increased urinary flow detectable within 30-min. However, the diuretic response was significantly prolonged in animals fed an 8% NaCl diet (70-min urine flow rate: control diet, 56 ± 12 µl/min vs. 8% NaCl diet, 123 ± 32 µl/min, $P<$0.05) resulting in significantly greater cumulative urine output (control diet 4869 ± 324 µl, 8% NaCl diet 5900 ± 226 µl, $P<$0.05). Similarly, the antinatriuresis to i.c.v. N/OFQ tended to be of longer duration than that obtained in the control diet group.
Additional experiments were performed in rats chronically maintained (3 weeks) on a normal (0.4%; N=8) or high (8%; N=8) NaCl diet to examine the influence of high salt intake on N/OFQ induced changes in renal sympathetic nerve activity (RSNA). In control diet animals, RSNA did not significantly change over the first 20-min following i.c.v. N/OFQ despite a drug-induced reduction in MAP (Fig 2). However, by 30-min post-drug injection (a time in which MAP tended to return to control level), RSNA was significantly (P<0.05) decreased (30-min RSNA, 76 ± 6% predrug control level; N=8) and remained depressed for the duration of the experimental protocol (90-min RSNA, 70 ± 9% predrug control level; N=8). In contrast, in rats chronically maintained on an 8% NaCl diet i.c.v. N/OFQ caused a more prolonged hypotensive response, but did not significantly alter RSNA throughout the protocol (30-min RSNA, 85 ± 6% predrug control level; N=8; 90-min RSNA, 95 ± 7% predrug control level; N=8; and Fig 2).

UFP-101 antagonist studies

The continuous central infusion of UFP-101 (18 nmol/h/5µl) did not alter cardiovascular or renal excretory function in Sprague-Dawley rats maintained on a 0.4% NaCl diet (Fig 3). In this control group, HR, MAP, urine output and urinary sodium excretion remained constant over the duration of the experimental period in which UFP-101 was continuously infused. In contrast to the lack of response observed in rats maintained on a normal salt diet, i.c.v. infusion of UFP-101 significantly altered urine output in animals chronically maintained on a high NaCl-intake (Fig 3). In particular, urine flow rate significantly decreased 30 and 40-min after starting the UFP-101 infusion (control, C, urine flow rate, 60 ± 2.8 µl/min vs. 40-min urine flow rate, 44 ± 3.9 µ/min, P<0.05), after which levels for urine flow rate returned to pre-drug, baseline control
levels. In rats maintained on a high NaCl-intake, i.c.v. infusion of UFP-101 also produced a small increase in MAP which occurred approximately 50-min after start of i.c.v. antagonist infusion which persisted for the duration of the experimental protocol (Fig 3). However, this central UFP-101 evoked increase in mean arterial pressure did not achieve statistical significance. I.c.v. UFP-101 infusion did not alter heart rate or urinary sodium excretion over the course of the 90-min experimental protocol.

**Plasma AVP levels**

In control male Sprague-Dawley rats maintained on a normal (0.4%) NaCl diet, the level of endogenous plasma AVP was 1.54 ± 0.16 pg/ml. Maintenance of rats on a chronic (3 week) high (8%) NaCl diet did not alter the endogenous level of plasma AVP (1.58 ± 0.17 pg/ml; Fig 4).

**Brain N/OFQ peptide levels**

In control male Sprague-Dawley rats maintained on a normal (0.4%) NaCl diet, N/OFQ levels in brain cortex tissue (1.9 ± 0.4 fmol/mg) were significantly lower than that observed in the hypothalamus and medulla (40 ± 7 and 35 ± 2 fmol/mg respectively). Tissue samples from the PVN and VLM had higher endogenous levels of N/OFQ (57 ± 4 and 49 ± 6 fmol/mg, resp.) as compared to peptide levels present in hypothalamic and medulla samples (Fig 5). As compared to animals on a normal chow, maintenance of rats on a chronic (3 week) high (8%) NaCl diet did not significantly alter endogenous N/OFQ levels in the brain cortex, hypothalamic or PVN samples. However, in high NaCl-treated rats (Fig 5), there was a significant reduction in N/OFQ peptide levels in both the whole medulla and VLM (medulla; control diet, 35.3 ± 2 fmol
Brain NOP receptor expression

NOP receptor expression was determined via radioligand binding, using membrane preparations, and immunoblotting using tissue homogenates containing both membrane and cytoplasmic fractions. Owing to tissue limitations, NOP receptor expression was not determined via radioligand binding in brain punch samples and was instead examined through immunoblotting. In control rats fed a normal (0.4%) NaCl diet, the highest NOP receptor levels were observed in the medulla and VLM, followed by hypothalamic and PVN tissue. Lower expression levels were detected in brain cortex samples (Table 1, Fig 6a). Chronic (3 week) intake of high (8%) NaCl produced regional changes in NOP receptor density within the brain of Sprague-Dawley rats (Table 1, Fig 6a, 6b). Data from radioligand binding studies revealed no significant difference between $pK_D$ values across brain regions (Table 1) following high salt-intake, indicating chronic high salt-intake does not alter the affinity of N/OFQ for the NOP receptor (Table 1). Following high salt-intake NOP receptor levels were elevated in both the hypothalamus and PVN; when assessed by immunoblotting these changes were significant in the PVN (Fig 6a). When assessed by the more sensitive method of radioligand binding a statistically significant increase in NOP receptor expression was observed in the whole hypothalamus (Table 1). In contrast to the increase in NOP expression in the hypothalamus and PVN, endogenous NOP receptor levels were significantly reduced in animals maintained on a high salt diet in the brain cortex and medulla (medulla; control diet, 1473 ± 131 fmol NOP/mg of protein vs. 8% NaCl diet, 327 ± 31 fmol NOP /mg of protein, P<0.05) as assessed by radioligand binding (Table
1) and immunoblotting (Fig 6a). Additionally, correlating with a reduced level of NOP receptor expression in the whole medulla, NOP receptor levels were significantly reduced in the VLM (VLM; NOP control diet, 2.5 ± 0.1 optical density units/mm² normalized to GAPDH vs. 8% NaCl diet, 0.87 ± 0.06 optical density units/mm² normalized to GAPDH, P<0.05) (Fig 6a, 6b).

**Brain N/OFQ stimulated GTPγS binding activity**

N/OFQ evoked GTPγS binding activity (an indicator of G-protein activation) was determined in brain regions using radioligand binding to investigate if chronic high salt intake alters N/OFQ stimulated G-protein activity. Brain tissue punches were not used in these studies owing to insufficient levels of total protein. In rats fed a normal (0.4% NaCl) diet, maximal N/OFQ stimulated GTPγS activity was present in the hypothalamus and medulla. Medullary tissue was the least sensitive to N/OFQ stimulation (medulla; pEC₅₀ 7.48 ± 0.26) with brain cortex displaying the greatest sensitivity (brain cortex; pEC₅₀ 9.49 ± 0.25) (Table 2). Maintenance of rats on a chronic (3-week) high (8%) NaCl diet significantly, and differentially, altered the sensitivity of brain tissues to N/OFQ stimulation (Table 2). Medullary tissue exhibited increased sensitivity (medulla; control diet, pEC₅₀ 7.48 ± 0.26 vs. 8% NaCl diet, 8.33 ± 0.26, P<0.05) in contrast to a reduction in sensitivity to N/OFQ stimulation in brain cortical tissue (brain cortex; control diet, pEC₅₀ 9.49 ± 0.25 vs. 8% NaCl diet, 8.21 ± 0.09, P<0.05). Chronic high salt-intake significantly increased basal GTPγS binding approximately 2.5-fold in the hypothalamus, with no change in basal binding observed in the medulla or brain cortex (Table 2). Maximal N/OFQ stimulation of GTPγS binding was unaltered by high salt-intake in the brain cortex and medulla, in contrast a significant reduction in maximal stimulation was observed in
the hypothalamus (hypothalamus stimulation factor; control diet, 2.14 ± 0.16 vs. 8% NaCl diet 1.43 ± 0.08, P<0.05, Table 2).
Discussion

The central administration of N/OFQ to conscious rats significantly decreases HR and MAP and produces a water diuresis via central pathways that involve inhibition of central sympathetic outflow and AVP secretion, respectively (17, 18, 19). The findings of the present studies demonstrate that a chronic high NaCl diet significantly increases the duration of the cardiovascular depressor responses and the magnitude and duration of diuresis evoked by central N/OFQ in Sprague-Dawley rats. In particular, in chronic high NaCl-treated rats, the central N/OFQ-evoked bradycardia and hypotension persisted for approximately 60- and 80-min, respectively. This is in contrast to a typical recovery time of 30-40-min that is observed in Sprague-Dawley rats fed a control NaCl diet (present study and 17, 19). While a high NaCl diet affected the duration of the cardiovascular depressor responses, the peak bradycardic and hypotensive responses were not significantly different than those produced in rats maintained on a normal NaCl diet. In addition to sustained cardiovascular depressor responses, the chronic treatment of rats with a high NaCl diet also altered the pattern of diuresis elicited by central N/OFQ. As compared to rats fed a normal chow, animals fed a chronic high NaCl diet displayed a diuresis that was significantly delayed in onset, of greater duration (70-min), and of greater cumulative output. Together, these findings clearly demonstrate that chronic high salt intake can markedly modify the cardiovascular and diuretic responses to the exogenous central administration of N/OFQ.

In conscious Sprague-Dawley rats, i.c.v. N/OFQ produces cardiovascular depressor responses and an inhibition of renal sympathetic nerve activity (19, 32). Moreover, the bradycardia to i.c.v. N/OFQ is blocked by systemic pre-treatment of rats with propranalol, but
not atropine (32). Since these findings indicate that central N/OFQ elicits cardiovascular depressor responses primarily by decreasing central sympathetic outflow (19, 32), we also examined whether a chronic high NaCl diet would alter the ability of central N/OFQ to inhibit RSNA. In the present investigations, i.c.v. injection of N/OFQ to rats maintained on a chronic high NaCl diet failed to suppress RSNA over the entire time course studied (90-min post-drug injection). This finding is of considerable interest since our laboratory and others have demonstrated a temporal correlation between the cardiovascular and renal nerve responses produced by central N/OFQ (19, 32). More specifically, while central N/OFQ can directly (and rapidly) inhibit RSNA (e.g., as demonstrated in sinoaortic denervated rats, 19), in intact animals the renal sympathoinhibitory response is delayed (30-min) and only observed after the decrease in MAP produced by this peptide returns to pre-drug injection levels (present study and 19, 32). These findings indicate that during the peptide-evoked hypotension observed in intact rats, central N/OFQ selectively blocks the baroreflex-induced sympathoexcitatory effect to the heart (and possibly blood vessels), but not to the kidneys (18, 19, 32). Analogous to these findings, our present results strongly suggest that in high salt-treated rats, the prolonged hypotension produced by central N/OFQ counters/masks the characteristic decrease in RSNA by a pathway that involves the baroreflex. Although RSNA can have significant impact on the renal excretion of water and sodium (16, 17, 20) our previous studies have shown the diuretic (and antinaturetic) response to central N/OFQ can occur via a renal nerve-independent pathway (19) involving the suppression of AVP release into the systemic circulation (15, 34). This may explain why i.c.v. N/OFQ continued to produce a diuretic response in animals maintained on a chronic high NaCl diet.
As noted above, a chronic high NaCl diet significantly altered the pattern of cardiovascular, renal excretory and renal nerve responses to i.c.v. N/OFQ in conscious rats. Based on these findings, it may be speculated that a high salt intake may alter the activity of the endogenous N/OFQ-NOP receptor system as a mechanism(s) to counter the effects of high salt on systemic cardiovascular and renal excretory regulatory systems. In studies performed in Sprague-Dawley rats maintained on a normal salt-intake the continuous i.c.v. infusion of the selective NOP receptor antagonist, UFP-101 (34), failed to produce a change in any systemic cardiovascular or renal excretory parameter. This suggests that under conditions of normal NaCl intake the endogenous N/OFQ-NOP system does not appear to play a major role in the control of cardiovascular function or urine output. In contrast, the present studies demonstrated that when Sprague-Dawley rats were maintained on a chronic high NaCl intake the pharmacological blockade of the central NOP receptor system with UFP-101 resulted in a significant, but transient reduction in urine output, suggesting that this antagonist blocked an ongoing inhibitory influence of endogenous N/OFQ on AVP secretion. This is of interest since in the present studies we observed that the chronic treatment of a high NaCl diet to Sprague-Dawley rats did not alter plasma AVP levels, presumably this is mediated in part by activation of a pathway involving the endogenous central N/OFQ system. This is in contrast to the increase in plasma AVP which occurs in several hypertensive models (2, 9). These data provide evidence for an endogenous role of the N/OFQ-NOP system, likely at the level of the hypothalamic PVN, in inhibiting the secretion of AVP and thus enhancing urine output when animals are faced with the physiological stress of a high salt challenge. The involvement of the native N/OFQ-NOP system in the excretion of water during stressful conditions has also been demonstrated in NOP receptor
knockout mice which exhibited an impaired ability to excrete urine following an acute water load when compared to wild type littermates (3).

In addition to changes in urine output, the continuous i.c.v. infusion of UFP-101 produced a slight, but persistent elevation in MAP in Sprague-Dawley rats maintained on a chronic NaCl diet. Although these changes were not statistically significant, these data suggest that the NOP receptor system may, under certain conditions, play a contributory role in countering the hypertensive effects of high salt on MAP.

Based on evidence from in vivo studies, we also examined how a chronic high NaCl intake alters the central N/OFQ-NOP receptor system at the tissue level. Chronic high salt loading did not alter endogenous N/OFQ peptide levels or N/OFQ stimulated G-protein signaling sensitivity in the hypothalamus. Instead there was a significant increase in NOP receptor expression in the whole hypothalamus and PVN, coupled with a significant increase in basal hypothalamic GTPγS binding and a reduction in hypothalamic GTPγS stimulation factor. These findings are of particular interest since activation of NOP receptors within the hypothalamus, particularly in the PVN, results in a water diuresis by inhibiting the release of AVP (15, 20). During high salt challenge, the modified N/OFQ system would favor greater endogenous hypothalamic and PVN N/OFQ signal transduction via an increased number of NOP receptors. Furthermore, the observed decrease in GTPγS stimulation factor, which is likely due to the observed increase in basal hypothalamic GTPγS activity, is hypothesized to reflect increased basal hypothalamic signaling pathways that are acting to suppress the release of AVP during the stress of high salt challenge. Together, these salt-induced changes in the NOFQ-NOP system
reflect an endogenous mechanism functioning at the whole animal level to counter the effects of high salt intake on increased AVP release and subsequently, water retention.

In contrast to the changes observed in the hypothalamus, Sprague-Dawley rats treated with a chronic high salt intake demonstrated reduced levels of both the N/OFQ peptide and NOP receptor in the medulla and VLM, whilst increasing the sensitivity of the medulla to N/OFQ stimulated G-protein activation. These salt-induced changes likely reflect an altered influence of endogenous N/OFQ activity on central cardiovascular regulatory mechanisms in the medulla/VLM which contribute to maintaining normotension in the face of high NaCl stress. The data obtained indicate that, despite a reduction in both the endogenous N/OFQ peptide and receptor in the whole medulla and VLM, a given concentration of N/OFQ (exogenous or endogenously released) is able to stimulate greater G-protein activity, and presumably downstream signaling. The summation of these effects is likely to translate at the whole animal level to the prolonged bradycardic and hypotensive responses to i.c.v. N/OFQ observed in high salt-treated Sprague-Dawley rats. These findings are of merit considering that increased dietary NaCl intake is known to increase neuronal activity in CNS sites that regulate sympathetic control of cardiovascular function including the medullary nucleus tractus solitarius (1, 22) and the rostral ventrolateral medulla (RVLM) (11, 12). High NaCl intake increases the sensitivity, and subsequently the cellular properties of medullary tissue (RVLM sympathoexcitatory neurons), which may explain how several classes of compounds including glutamate (11, 12) GABA\textsubscript{A} and GABA\textsubscript{B} antagonists (7) and N/OFQ (present study) are able to exert either greater, or more prolonged cardiovascular depressor effects in animals maintained on a high NaCl diet. Finally, the tendency for MAP to increase in high NaCl-treated rats during central UFP-101 infusion may
reflect an ongoing activity of endogenous N/OFQ pathways in the medulla that are contributing
to maintain constant MAP in the face of the stress of high salt intake.

**Perspectives and Significance**

Together, these data demonstrate that the stress of a chronic high NaCl diet alters the
pattern in which central N/OFQ (native or exogenous), influences cardiovascular function and
the renal excretion of water, presumably in part because central N/OFQ inhibits the same central
neural (sympathetic) and humoral (AVP) regulatory pathways that are influenced by high NaCl.
Additionally, this study provides evidence that endogenous N/OFQ peptide levels, NOP receptor
density and the sensitivity of N/OFQ stimulated G-protein activation are selectively and
differentially altered by high NaCl in brain regions/sites involved in the regulation of systemic
cardiovascular function and the renal handling of water. Together, these studies link altered
physiological responses to central N/OFQ observed at the whole animal level during high salt
stress to modifications in the endogenous N/OFQ-NOP receptor system which occur at the brain
tissue level. Finally, studies with the NOP antagonist, UFP-101, revealed that endogenous central
N/OFQ-NOP receptor pathways contribute, presumably via inhibiting AVP secretion, to
enhancing urine output in Sprague-Dawley rats maintained on a chronic high NaCl diet. Based
on these findings it may be speculated that the endogenous N/OFQ-NOP receptor system in the
VLM and/or PVN (and potentially other brain regions) may be involved in the long-term
regulation of cardiovascular function and body fluid homeostasis in the face of chronic high salt
challenge.
Grants

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Conflicts of Interest/Disclosure(s) Statement

None

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References


Figure legends

Figure 1: Cardiovascular and renal responses produced by i.c.v. N/OFQ (5.5 nmol/5 µl) (N=8) or i.c.v. isotonic saline vehicle (5 µl) (N=8) in conscious male Sprague-Dawley rats maintained on a control diet (0.4% NaCl) or a high (8%) NaCl diet for 3 weeks (N=8 per group). HR, heart rate; MAP, mean arterial pressure; V, urine flow rate; UNaV, urinary sodium excretion. The results are the mean ± standard error of the mean. *P<0.05, compared with control value at respective time points, τ P<0.05, between control diet and 8% NaCl content diet groups at respective time points.

Figure 2: Representative tracing obtained from the BIOPAC data acquisition system illustrating typical heart rate (HR), arterial pressure (AP), mean arterial pressure (MAP), and integrated renal sympathetic nerve activity (RSNA), responses produced by the intracerebroventricular (i.c.v.) injection of N/OFQ (5.5 nmol/5 µl) in (A) a conscious Sprague–Dawley rat maintained on a control diet (0.4% NaCl) and (B) a conscious Sprague-Dawley rat maintained chronically (3-weeks) on an 8% NaCl diet.

Figure 3: Effect of continuous i.c.v. UFP-101 infusion (18 nmol/5 µl/h) on cardiovascular and renal excretory function in conscious male Sprague-Dawley rats maintained on either a control (0.4%) NaCl diet or a high (8%) NaCl diet for 3-weeks (N = 8/group). The values are means ± SEM and illustrate the cardiovascular and renal effects of central UFP-101 infusion in 6 conscious rats/group. HR, heart rate; MAP, mean arterial pressure; V, urine flow rate; UNaV, urinary sodium excretion. *P<0.05 compared with respective group control value (designated C). †P<0.05 sig. diff. compared to control diet group at respective time points.
Figure 4: Effect of chronic high NaCl intake in conscious male Sprague-Dawley rats maintained on either a control (0.4%) NaCl diet or a high (8%) NaCl diet for 3-weeks (N = 6/group), on endogenous plasma AVP levels, expressed as pg/ml. Values are means ± SEM.

Figure 5: Endogenous N/OFQ peptide levels in brain cortex, whole hypothalamus, whole medulla, PVN and VLM tissue isolated from male Sprague-Dawley rats maintained 3-weeks on either a control diet (0.4 % NaCl) or an 8% NaCl diet (N = 6/group) expressed as fmol N/OFQ/mg protein. The results are the mean ± standard error of the mean. * P< 0.05, statistically different from normal respective chow value.

Figure 6: (a) NOP receptor protein expression as optical density units per mm² normalised to GAPDH in brain cortex, whole hypothalamus, whole medulla, PVN and VLM tissue from male Sprague-Dawley rats maintained for 3 weeks on a control diet or an 8% NaCl diet (N = 6/group). The results are the mean ± standard error of the mean. *P< 0.05 control vs 8% NaCl diet. (b) Representative immunoblots of GAPDH and NOP receptor protein expression in brain regions from male Sprague-Dawley rats maintained for 3 weeks on a control diet or an 8% NaCl diet. Samples were loaded as tissue lysates at a concentration of 20 µg total protein.
Figure 1

- **HR (bpm)**
  - Graph showing changes over time with annotations for statistical significance.

- **MAP (mmHg)**
  - Graph showing changes over time with annotations for statistical significance.

- **V (μl/min)**
  - Graph showing changes over time with annotations for statistical significance.

- **UNaV (μeq/min)**
  - Graph showing changes over time with annotations for statistical significance.

**Legend:**
- ○ Vehicle (5 μl), 0.4% NaCl diet (n=8)
- ▲ N/OFQ (5.5 nmol), 0.4% NaCl diet (n=8)
- ♦ N/OFQ (5.5 nmol), 8% NaCl diet (n=8)
Fig. 2

(A) i.c.v. N/OFQ (5.5 nmol)

(B) i.c.v. N/OFQ (5.5 nmol)
Figure 3

- **HR (bpm)**
- **MAP (mmHg)**
- **V (µl/min)**
- **UNaV (µeq/min)**

**i.c.v.:**
- UFP-101 infusion (18 nmol/h), 0.4% NaCl diet
- UFP-101 infusion (18 nmol/h), 8% NaCl diet
Figure 4

[Bar graph showing plasma AVP (pg/ml) for two treatment groups: 0.4% NaCl diet and 8% NaCl diet. The graph indicates a higher plasma AVP level in the 8% NaCl diet group compared to the 0.4% NaCl diet group.]
Figure 6a

Graph showing the expression of NOP receptor protein in different brain regions (BC, H, PVN, M, VLM) normalized to GAPDH under 0.4% NaCl diet (white bars) and 8% NaCl diet (black bars). Significant differences are indicated by asterisks (*).
<table>
<thead>
<tr>
<th>$pK_D$</th>
<th>B$_{\text{max}}$ (fmol NOP receptor/mg protein)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Normal diet</td>
</tr>
<tr>
<td></td>
<td>Normal (0.4% NaCl)</td>
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<tr>
<td>Brain Cortex</td>
<td>9.09 ± 0.03</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>9.43 ± 0.04</td>
</tr>
<tr>
<td>Medulla</td>
<td>9.14 ± 0.12</td>
</tr>
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Table 1: [leucyl-$^3$H]N/OFQ binding in CNS tissues isolated from male Sprague-Dawley rats maintained on either a chronic (3-week) a normal (0.4%) or high (8%) NaCl chow. Data are mean ± SEM from six individual experiments. ** $p < 0.01$, * $p < 0.05$, statistically different from normal chow value.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>pEC50 Normal diet (0.4% NaCl)</th>
<th>pEC50 High Salt diet (8% NaCl)</th>
<th>Stimulation Factor Normal diet (0.4% NaCl)</th>
<th>Stimulation Factor High Salt diet (8% NaCl)</th>
<th>Un-stimulated basal binding (Total DPM) Normal diet (0.4% NaCl)</th>
<th>Un-stimulated basal binding (Total DPM) High Salt diet (8% NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain Cortex</td>
<td>9.49 ± 0.25</td>
<td>8.21 ± 0.09 *</td>
<td>1.16 ± 0.03</td>
<td>1.30 ± 0.03</td>
<td>3816 ± 59</td>
<td>4899 ± 390</td>
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<td>Hypothalamus</td>
<td>8.18 ± 0.47</td>
<td>8.83 ± 0.14</td>
<td>2.14 ± 0.16</td>
<td>1.43 ± 0.08 *</td>
<td>1774 ± 156</td>
<td>4164 ± 100 *</td>
</tr>
<tr>
<td>Medulla</td>
<td>7.48 ± 0.16</td>
<td>8.33 ± 0.26 *</td>
<td>1.67 ± 0.06</td>
<td>1.50 ± 0.11</td>
<td>6318 ± 348</td>
<td>5871 ± 398</td>
</tr>
</tbody>
</table>

Table 2: GTPγ<sup>35</sup>S activity in CNS tissues isolated from male Sprague-Dawley rats maintained on either a chronic (3-week) normal (0.4%) or high (8%) NaCl chow. Data are presented as stimulation factor, which is the ratio between N/OFQ stimulated GTPγ<sup>35</sup>S binding and basal specific binding, pEC50, which is the negative logarithm to base 10 of the agonist (N/OFQ) molar concentration that produces 50% of the maximal possible effect, and un-stimulated basal binding, expressed as total DPM, representing endogenous GTP γ<sup>35</sup>S binding. Data are mean ± SEM from six individual experiments. *p< 0.05, statistically different from normal chow value.