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Tonic nociceptinergic inputs to neurons in the hypothalamic paraventricular nucleus contribute to sympathetic vasomotor tone and water and electrolyte homeostasis in conscious rats

Zbigniew K. Krowicki and Daniel R. Kapusta

Department of Pharmacology and Experimental Therapeutics,

Louisiana State University Health Sciences Center, New Orleans, Louisiana

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Corresponding author:

Zbigniew K. Krowicki, M.D., Ph.D.

Department of Pharmacology and Experimental Therapeutics

LSU Health Sciences Center

New Orleans, LA 70112

Phone: 504-568-6336 Fax: 504-568-2361 Email: zkrowi@lsuhsc.edu

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Abbreviations: AVP, [Arg^{8}]-vasopressin; N/OFQ, nociceptin/orphanin FQ; NOP receptor, nociceptin/orphanin FQ peptide receptor; PVN, hypothalamic paraventricular nucleus; RSNA, renal sympathetic nerve activity; UFP-101, [$Nphe^{1}$,Arg^{14},Lys^{15}]N/OFQ-NH_2.

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Abstract

Central administration of nociceptin/orphanin FQ (N/OFQ) produces bradycardia, hypotension, diuresis and antinatriuresis in rats. Since N/OFQ peptide (NOP) receptors exist in the paraventricular nucleus (PVN) of the hypothalamus, we hypothesized that N/OFQ acts in the PVN to alter cardiovascular and renal function. To test this premise, N/OFQ (10 and 100 pmol) or artificial cerebrospinal fluid (vehicle) was microinjected into the right PVN of conscious, chronically instrumented rats infused intravenously (i.v.) with isotonic saline. Following injection, N/OFQ, but not vehicle, dose-dependently decreased renal sympathetic nerve activity (RSNA) and increased urine flow rate. At 100 pmol, N/OFQ also decreased urinary sodium and potassium excretion and increased free water clearance. In separate groups, the diuretic response to N/OFQ injection into the PVN was blunted in chronic bilaterally renal denervated rats and abolished in intact rats continuously infused i.v. with [Arg^{8}]-vasopressin (60 fmol/kg/min). Finally, in other studies bilateral microinjection of the NOP receptor antagonist [$Nphe^{1}$,Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-101; 300 pmol) into the PVN increased heart rate and RSNA and decreased urine flow rate without altering electrolyte excretion. Pretreatment of separate rats with UFP-101 (300 pmol, PVN) blocked the N/OFQ-evoked (100 pmol) cardiovascular, renal sympathetic nerve and renal excretory responses. Together, these findings demonstrate that in conscious rats activation of NOP receptors in the PVN by N/OFQ produces bradycardia, renal sympathoinhibition and water diuresis. Moreover, UFP-101 blocks a tonically active inhibitory influence of endogenous N/OFQ on central sympathetic outflow and vasopressin pathways which arise from the PVN to affect heart rate and urine output.

Introduction

Nociceptin/orphanin FQ (N/OFQ) is an endogenous neuropeptide which selectively binds to and activates an opioid-like receptor referred to as the N/OFQ peptide (NOP) receptor (previously called opioid receptor-like 1) (Meunier et al., 1995; Reinscheid, 1995; Cox et al., 2000). Although structurally similar to the classical opioid family of peptides and receptors (e.g., the endorphins), the N/OFQ-NOP peptide system has been established as a distinct neurochemical entity. Anatomical distribution of N/OFQ and NOP receptor immunoreactivity and mRNA expression in rodent and human brain regions including those involved in the regulation of cardiovascular function and fluid/electrolyte balance is well characterized (Isgor et al., 2003; Witta et al., 2004).

Following intracerebroventricular (i.c.v.) administration in conscious rats, N/OFQ produces a significant decrease in mean arterial pressure, heart rate and renal sympathetic nerve activity (RSNA; Kapusta et al., 1997; Kapusta and Kenigs, 1999; Shirasaka et al., 1999). In addition, i.c.v. N/OFQ evokes a dose-dependent diuretic and antinatriuretic response (Kapusta et al., 1997; Kapusta and Kenigs, 1999) and decrease in circulating plasma levels of vasopressin (AVP; Kakiya et al., 2000). Despite these findings, the specific brain sites and mechanisms by which N/OFQ affects systemic cardiovascular function and the renal handling of water and electrolytes in conscious rats are not completely known.

The hypothalamic paraventricular nucleus (PVN) is a brain site involved in the regulation of neuroendocrine function and activity of the autonomic nervous systems. The magnocellular neurons of the PVN synthesize the neurohypophysial hormones

AVP and oxytocin and project to the posterior pituitary. The parvocellular PVN neurons project to the anterior pituitary to modulate activity of the hypothalamic pituitary-adrenal axis and to other autonomic centers involved in the central nervous system (CNS) regulation of sympathetic activity (Kenney et al., 2003). Functional studies also implicate the PVN in the regulation of cardiovascular function and the control of central sympathetic outflow (Coote, 2005). NOP receptors and N/OFQ-positive fibers have been identified in the mammalian PVN (Isgor et al., 2003) and N/OFQ has been shown to modulate the activity of magnocellular and parvocellular PVN neurons *in vitro* through activation of an inwardly rectifying K^+ channel (Shirasaka et al., 2001). However, whether N/OFQ acts within the PVN to contribute to the central actions of N/OFQ on cardiovascular and renal function *in vivo* has not been previously examined.

Therefore, the present studies were performed to investigate the cardiovascular and renal responses produced by microinjection of the opioid-like peptide, N/OFQ, into the PVN of conscious rats. Studies were then performed to examine the role of the renal nerves and vasopressin in mediating the cardiovascular and renal responses to N/OFQ administration into this brain site. Participation of the renal sympathetic nerves in mediating N/OFQ-induced changes in renal excretory function was examined by directly measuring RSNA and by repeating the experimental protocol in rats that had the influence of the renal nerves on kidney function removed by chronic bilateral renal denervation. Separate studies were also performed in rats continuously infused i.v. with AVP to establish whether N/OFQ might act in the PVN to affect renal excretory function via a pathway that involves alteration in the circulating levels of this hormone. Finally, studies were performed in which rats were microinjected into the PVN with the selective

NOP receptor antagonist, UFP-101 (Calo et al., 2005) alone, or as a pretreatment prior to N/OFQ injection. These studies examined whether native N/OFQ-NOP receptor systems in the PVN might elicit a tonic influence on cardiovascular and/or renal function and tested the NOP receptor selectivity of N/OFQ's action in this brain region, respectively.

Methods

Animal preparation and data collection

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 285-400 g were housed in a controlled environment and allowed free access to laboratory rat chow and water. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Louisiana State University Health Sciences Center Animal Care and Use Committee.

Surgery

Aseptic technique was used for all surgeries. For microinjection of drugs (N/OFQ alone or following UFP-101 pretreatment), a chronic cannula was placed in the right PVN 5-8 days before the experimental day. For studies involving bilateral UFP-101 microinjection a chronic cannula was also implanted into the right PVN. Briefly, rats were anesthetized with an i.m. injection of ketamine and xylazine mixture (50 mg/kg ketamine plus 5 mg/kg xylazine, 1 ml/kg) and placed in a stereotaxic apparatus (Kopf, Tujunga, CA). A 2 cm incision was made to expose the scalp and connective tissue was removed to expose the cranial sutures. A 26-gauge stainless steel cannula was directed at the PVN at a 10^0 angle from the vertical plane through a burr hole located streotaxically 1.6–1.7 mm posterior to the bregma, 1.8–1.9 mm laterally to the midline, and 7.8 mm ventral from the skull surface. Inner stylets were inserted into the guide cannulae to prevent obstruction. The guide cannulae were secured to the skull with stainless steel machine screws and dental acrylic. To prevent post surgery infection all

rats received ampicillin (50 mg/kg, s.c.). The animals were then placed in individual plastic cages and allowed at least 5 days to recover from the stereotaxic surgery.

After recovery, rats were anesthetized with methohexital sodium (Brevital; 20 mg/kg i.p., supplemented with 10 mg/kg as needed; Lilly, Indianapolis, IN) and polyethylene catheters (PE-50; Becton Dickinson, Sparks, MD) were implanted into the left femoral artery and vein for recording of arterial pressure and infusion of isotonic saline or drugs, respectively. Through a suprapubic incision, a flanged polyethylene cannula (PE-240, Becton Dickinson) was inserted into the urinary bladder for collection of urine. The bladder catheter was then exteriorized and secured by suturing to adjacent muscle, tissue, and skin.

Rats (still anesthetized with methohexital sodium) were then implanted with a recording electrode on a renal nerve bundle for direct measurement of multifiber RSNA using techniques described previously (Kapusta and Kenigs, 1999). In brief, a left renal nerve bundle was dissected carefully via a retroperitoneal approach and freed from the surrounding tissue under a dissecting microscope (x25). The nerve was placed on e bipolar electrode made of Teflon-coated platinum wire (Cooner Wire, Chatsworth, CA). Spike potentials were amplified (x10,000-50,000) and filtered (low, 30 Hz; high, 3,000 Hz) with a Grass P511 Bandpass Amplifier (Grass Instrument, Quincy, MA). The amplified and filtered signal was channeled to a Grass model 7DA polygraph for visual evaluation, to an audio amplifier-loudspeaker (Grass model AM 8 Audio Monitor) for auditory evaluation, to a rectifying voltage integrator (Grass model 7P10) and was continuously recorded on the Grass polygraph and a commercially available data acquisition system (Model MP100 and Acknowlege version 4.7.2 software, Biopac

Systems, Inc., Santa Barbara, CA). The quality of the renal sympathetic nerve signal was assessed by its pulse synchronous rhythm. When an optimal RSNA signal was observed, the recording electrode was fixed to the renal nerve branch with and covered with a silicone-based impression material (Coltene President, Alstatten, Switzerland). The electrode cable was then secured in position by suturing it to the abdominal trunk muscles. Finally, the electrode cable was exteriorized, and the flank incision was closed in layers.

In separate studies the role of intact renal nerves in mediating the cardiovascular and renal excretory responses produced by the injection of N/OFQ into the PVN was examined by repeating the above protocol in rats (n=6) having undergone chronic bilateral renal denervation 10-13 days before experimentation. Renal denervation was performed via bilateral flank incisions and stripping the renal arteries and veins of adventitia, cutting the renal nerve bundles, and coating the vessels with a solution of 10% phenol in absolute ethanol as previously described (DiBona and Sawin, 1983). This renal denervation procedure prevents the renal vasoconstrictor response to suprarenal lumbar sympathetic nerve stimulation, prevents the antinatriuretic response to environmental stress, reduces renal catecholamine histofluorescence to undetectable levels, and reduces renal tissue noradrenaline concentration to <5% of control for up to 15 days post-denervation (DiBona and Sawin, 1983). Because our laboratory has previously and repeatedly verified that this renal denervation procedure completely removes the influence of the renal nerves on kidney function (Kapusta and Kenigs, 1999), verification of renal denervation was not performed in these studies. Six to seven

days later the same rats were anesthetized with ketamine/xylazine and implanted with a chronic PVN cannula as previously described.

After surgical preparation and recovery from anesthesia, the rat was placed in a rat holder (a chamber of stainless steel rods connected by plexiglas ends; the metal rods formed an inverted U shape and a flat base in which the rat would sit) to minimize movement and damage to the renal nerve electrode preparation and to permit steady-state urine collection. An infusion (50 μl/min i.v.) of isotonic saline was then started and continued for the duration of the experiment. After stabilization of renal excretory parameters (4-6 hrs after recovery), the arterial catheter was flushed and attached to a pressure transducer (Viggo-Spectramed, model P23XL, Oxnard, CA), and the urinary bladder catheter was lead to a collection vial. Heart rate was derived from the pulse pressure by a tachograph (Grass model 7 P4H), and mean arterial pressure, heart rate and RSNA were recorded on a Grass model 7 polygraph and the Biopac data acquisition system (MP100 and Acknowledge 3.7.2 software; Santa Barbara, CA).

Experimental protocols

Studies were performed to examine cardiovascular and renal excretory responses to microinjection of N/OFQ into the PVN of conscious animals. After stabilization of cardiovascular, renal excretory, and RSNA parameters, urine was collected during a 20-min control period. After that, a 33-gauge injection needle connected to 10-μl microsyringe was inserted into the guide cannula so that the tip of the injection needle extended beyond the guide cannula by 0.5-0.75mm and reached the PVN. Vehicle or N/OFQ (10 or 100 pmol, n=7-8 per group) was injected into the

PVN in a volume of 60 nl over 10 sec. Artificial cerebrospinal fluid (aCSF) of the following composition was used as vehicle (all concentrations are expressed as millimolar concentrations): NaCl, 140; KCl, 3.35; $MgCl_2$, 1.15; $CaCl_2$, 1.26; Na_2HPO_4, 1.2; and NaH_2PO_4, 0.3; pH 7.4. A 30-sec period was allowed for diffusion. Then, the injection cannula was withdrawn. Immediately after PVN administration, urine was collected during eight consecutive 10-min experimental urine samples. The doses of N/OFQ used in the present study were derived from our previous dose-response study performed in chloralose-anesthetized rats (Krowicki et al., 1997). In this study, microinjection of N/OFQ into the dorsal vagal complex produced consistent reductions in heart rate and arterial blood pressure.

Since our initial experiments demonstrated that free-water clearance increased in response to N/OFQ in the PVN (Fig. 3), we investigated whether the renal excretory responses produced by the peptide were caused by decreases in the systemic circulating levels of AVP. For these experiments the microinjection study described above was repeated with the exception that the cardiovascular and renal responses produced by the injection of N/OFQ into the PVN were examined in rats that were continuously infused with isotonic saline containing AVP [85 pg (60 fmol)/kg/min, i.v.]. Because AVP has a relatively short half-life of approximately 2-min in rats, steady-state plasma concentration of AVP should be achieved within a 15-min period. And indeed, antidiuretic and natriuretic responses to continuous i.v. infusion of AVP (63 fmol/kg/min) have been shown to require 20-min to reach plateau and persist for the duration of AVP infusion (Smith, 1993). Therefore, in our previous (Kapusta and Dzialowski, 1995) and present studies AVP was allowed to infuse for 30-min and then consecutive urine

samples (10-min ea.) were collected before (control) and after the microinjection of N/OFQ (100 pmol) into the PVN.

Additional studies were performed to evaluate whether the N/OFQ-NOP receptor system in the PVN plays a tonic role in the regulation of cardiovascular and renal function. For these studies the selective NOP receptor antagonist, UFP-101 (300 pmol; Calo et al., 2005) was microinjected into the PVN 10 min before vehicle or N/OFQ (100 pmol). Cardiovascular and renal function was measured during consecutive 10-min periods before (control) and after drug injection (experimental) for 80-min.

At the end of all microinjection experiments, the site of drug injection in the brain was examined functionally and histologically. The microinjection of L-glutamate (15 nmol) was used to confirm localization of the tip of an injection needle in the PVN. Typical responses to L-glutamate in the PVN are increases in heart rate and mean arterial pressure (Martin and Haywood, 1992). In addition, at the end of the experiments, rats received injections of 60 nl of pontamine sky blue dye (1%) to mark the injection site(s).

Histology

The animals were euthanized with an overdose of methohexital sodium and perfused transcardially with saline and 4% paraformaldehyde. Their brains were removed, post-fixed in the same solution, sectioned at 50 µm and counterstained with neutral red dye for histological confirmation of injection sites. Injections were considered acceptable if the cannula tip was shown to be within a distance of 0.5 mm of the PVN

boundaries (Lessard and Bachelard, 2002). Several sites around the PVN served as anatomical controls for the N/OFQ injections.

Data analysis

Urine volume was determined gravimetrically. Urine sodium and potassium concentration was measured by flame photometry (model 943, Instrumentation Laboratories, Lexington, MA) and expressed as urinary sodium ($U_{Na}V$) and urinary potassium (U_KV) excretion. Urine osmolality was measured by vapor pressure osmometer (model 5500, WESCOR, Logan, UT). Free water clearance (C_{H2O}) was calculated as a difference between the rate of urine volume (µl) per minute and the osmolar clearance. Cardiovascular function and RSNA was measured through a computer driven data acquisition software (Acknowledge 3.7.2, Biopac Systems, Santa Barbara, CA). Integrated RSNA was expressed as microvolt-seconds per 1-sec intervals. For each 10-min control and experimental period, the values for integrated RSNA were sampled over the entire collection period and the numbers were averaged. The data for RSNA were expressed as the percent of the baseline value obtained during the control period with this being expressed as 100% for each animal. After completion of each experiment the level of post-mortem background noise was measured and the value was then subtracted from all control and experimental values of RSNA. Because of the limitations of comparing values for multi-fiber RSNA between animals, the data are expressed as percentage control with the control values for each animal taken as 100%.

Drugs

N/OFQ (1-17) was obtained from Phoenix Pharmaceuticals (Mountain View, CA) and AVP was purchased from Bachem California, Inc. (Torrance, CA). [Nphe(1), Arg(14), Lys(15)] N/OFQ-NH2 (UFP-101) was synthesized and graciously provided by Dr. Remo Guerrini (University of Ferrara, Ferrara, Italy). Stock solutions of peptides were prepared fresh and stored frozen.

Statistics

Results are expressed as the mean ± SEM. The magnitude of the changes in cardiovascular and renal parameters at different time points after injection of drugs in the PVN 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., intact versus renal denervated; infusion with vehicle versus AVP) were assessed by a two-way repeated measures ANOVA with treatment being one fixed effect and time another, with the interaction included. The time (minutes) was the repeated factor and post-hoc analysis was performed using the Holm-Sidak or Bonferroni's test. Data were verified for normality of distribution and equality of variances. If needed, data were normalized using a log10 transformation for the purpose of statistical analyses; however, results are reported in standard units. Statistical testing was carried out using Prism and SigmaStat programs. In each case, statistical significance was defined as $P < 0.05$.

Results

Cardiovascular and renal effects of N/OFQ microinjected into the PVN of conscious rats.

Figure 1 illustrates the cardiovascular, RSNA and renal excretory responses produced by microinjection of N/OFQ (10 or 100 pmol), or aCSF (vehicle) in conscious Sprague-Dawley rats. When N/OFQ was microinjected into the PVN at a dose of 10 pmol (n=7), RSNA decreased, whereas mean arterial pressure and heart rate did not change. The reduction in RSNA was gradual and became significantly different from control levels at time points 30 to 50-min after injection with a nadir at the 40-min time point (C, control, 100 %; 40-min: 76.0±5.1 %, P<0.01). The 10 pmol dose of N/OFQ also produced a significant increase in urine flow rate but urinary sodium and potassium excretion remained unchanged. The diuretic response to N/OFQ at the low dose achieved statistical significance 20-min after injection and reached a maximum at the 30-min time point (C, 33±4 μl/min, 30-min, 104±22 μl/min; p<0.01; Fig. 1) before returning to control levels. In contrast to these responses, when microinjected at the 100 pmol dose (n=8), N/OFQ produced an immediate reduction in RSNA that was significant over the entire experimental period (time points 10 to 80-min) and which reached a nadir at the 50-min time point (50-min, 72.2±7.0 % of C; p<0.01). The magnitude of renal sympathoinhibition produced by the 10 and 100 pmol doses of N/OFQ was significantly different (p<0.0001; 2-way ANOVA). The 100 pmol dose of N/OFQ in the PVN also decreased heart rate (time points 20-40 min) without altering mean arterial pressure. At the 100 pmol dose, N/OFQ also significantly elevated urine flow rate 20 to 50-min after injection, with a peak response observed at the 40-min time point (C, 42±6

μl/min; 40-min, 164±31 μl/min; p<0.01). The magnitude of diuresis produced by the 10 and 100 pmol doses of N/OFQ was significantly different (p<0.01; 2-way ANOVA). Concurrent with the diuresis, the high dose of N/OFQ in the PVN decreased urinary sodium (C, 7.3±0.7 μeq/min; 30-min nadir, 3.6±1.4 μeq/min; p<0.001) and potassium (C, 3.4±0.1 μeq/min; 30-min nadir, 1.9±0.1 μeq/min; p<0.05) excretion. Microinjection of aCSF (vehicle) into the PVN (n=7) did not significantly alter any cardiovascular, renal sympathetic nerve or renal excretory parameter over the course of the experiment (Fig. 1).

Figure 2 depicts the changes in free water clearance produced by the microinjection of N/OFQ (100 pmol) and aCSF into the PVN for the same rats depicted in Fig. 1. As shown, N/OFQ evoked a significant increase in free water clearance over time points 20-50-min with the peak response observed 40-min after injection (C, -52±7 ml/min; 40-min, 49±21 ml/min; p<0.001). Microinjection of aCSF did not significantly alter free water clearance over the course of the study.

The histological identified sites into which N/OFQ (10 and 100 pg) was microinjected into the PVN of animals depicted in Fig. 1 are shown in Fig. 3. Injections were considered acceptable if the cannula tip was within a distance of 0.5 mm of the PVN boundaries (Lessard and Bachelard, 2002). Several sites around the PVN served as anatomical controls for the N/OFQ injections. No significant responses were elicited from these microinjections (data not shown).

Cardiovascular and renal effects of N/OFQ microinjected into the PVN of rats infused with AVP.

The cardiovascular and renal responses produced by the microinjection of N/OFQ into the PVN of conscious rats continuously infused i.v. with isotonic saline containing AVP (85 pg/kg/min) are shown in Fig. 4. For comparison, superimposed are data from Fig. 1 which illustrate the cardiovascular and renal responses to microinjection of N/OFQ into the PVN of rats infused with isotonic saline vehicle alone. Although not depicted, continuous infusion of AVP in rats during the first 40-min (stabilization period) evoked a decrease in V, HR, and RSNA accompanied with an increase in MAP and no apparent change in UNaV. In separate animals, it was shown that all these changes tended to reach plateau within the first 30-min and remained relatively unchanged until the AVP infusion was stopped at the end of the experiment (i.e., 80-min post aCSF injection). As illustrated in Figure 4, the i.v. infusion of AVP completely prevented the diuretic response produced by the injection of N/OFQ into the PVN of conscious rats. Similarly, in contrast to rats infused with vehicle alone, the injection of N/OFQ into the PVN of AVP-infused animals did not significantly alter urinary sodium or potassium excretion. While administration of N/OFQ into the PVN produced similar patterns of cardiovascular and RSNA responses between groups, N/OFQ did evoke a slight, but statistically significant reduction in mean arterial blood pressure in the AVP-treated group.

Cardiovascular and renal effects of N/OFQ microinjected into the PVN of renal-denervated rats.

Microinjection of N/OFQ (100 pmol) into the PVN of renal-denervated rats (n=6) caused a transient increase in urine flow rate with a peak at 30 min after injection (C,

55±6 μl/min; 30-min, 98±4 μl/min; P<0.05; Fig. 5). However, in comparison to the responses observed in rats with intact kidneys, the diuresis produced by this dose of N/OFQ into the PVN was significantly (p<0.001) blunted. In renal denervated rats N/OFQ also decreased urinary sodium and potassium excretion with nadirs attained 30 min after injection (C, 8.7±0.3 μeq/min, 30-min, 5.4±0.8 μeq/min; P<0.01) and (C, 3.0±0.1 μeq/min, 30-min, 1.8±0.2 μeq/min; P<0.05; Fig. 5), respectively.

Cardiovascular and renal effects of UFP-101 microinjected into the PVN of conscious rats.

Figure 6 illustrates the cardiovascular and renal responses produced by the unilateral microinjection of aCSF or N/OFQ into the PVN of rats pretreated in the same brain site with the selective NOP receptor antagonist, UFP-101. When microinjected into the PVN at a dose of 300 pmol with subsequent aCSF (n=6), UFP-101 caused a significant increase in RSNA with a peak 30-min after injection (C, 100%; 30-min, 129±12 %; P<0.05). In this same group of animals UFP-101 did not alter mean arterial pressure, but heart rate was significantly elevated (time periods 40-60 min) as compared to the respective group control value. In regards to renal excretory responses, microinjection of UFP-101 into the PVN reduced urine flow rate and urinary sodium excretion, although only the reduction in urine output reached statistical significance (C, 46±6 μl/min; 30-min, 25±4 μl/min; p<0.05). As also shown in Fig 6, the pretreatment (10-min) of UFP-101 (300 pmol, left PVN) completely prevented the cardiovascular and renal excretory responses produced by the subsequent

microinjection of N/OFQ (100 pmol) into the PVN (compare to N/OFQ responses in Fig. 1).

As shown in Fig. 7, the bilateral microinjection of UFP-101 also produced significant increases in heart rate and RSNA and a reduction in urine flow rate, but the magnitude of the sympathoexcitatory and antidiuretic responses were of greater magnitude than that produced by unilateral UFP-101 injection (compare to responses depicted in Fig. 6).

Discussion

The results of the present investigations demonstrate that microinjection of N/OFQ into the PVN of conscious rats evokes a dose-dependent increase in urine flow rate and free water clearance. Of interest, while microinjection of a low dose of N/OFQ (10 pmol) into the PVN selectively increased the renal excretion of water, a 10-fold higher dose of N/OFQ (100 pmol) produced a concurrent reduction in urinary sodium and potassium excretion. In contrast, microinjection of aCSF into the PVN or N/OFQ (100 pmol) outside of the PVN had no effect on renal excretory (or cardiovascular) function. Considering that i.c.v. injection of N/OFQ produces diuretic, antinatriuretic and antikaliuretic responses (Kapusta and Kenigs, 1999), the present findings suggest that the PVN of the hypothalamus is a brain site where N/OFQ acts to produce a selective water diuresis (e.g., aquaresis) following central administration (e.g., i.c.v. injection) or endogenous release of the peptide.

In addition to altering renal excretory function, the microinjection of N/OFQ into the PVN of conscious rats reduced heart rate and RSNA but did not significantly change mean arterial pressure. Similarly, i.c.v. administration of N/OFQ evokes slight hypotension (10-15 mmHg) and a pronounced bradycardia and renal sympathoinhibitory response. However, the reduction in RSNA is delayed (30-min) until mean arterial pressure recovered toward pre-drug baseline levels (Kapusta and Kenigs, 1999; Shirasaka et al., 1999). Thus, while the N/OFQ-induced activation of NOP receptors in the PVN causes a reduction in central sympathetic outflow to the kidneys (renal sympathoinhibition) and heart (bradycardia), it appears that mean arterial pressure is either not significantly affected by drug administration into this site or that

other compensatory mechanisms (e.g., involving the baroreflex) contributed to maintain mean arterial blood pressure constant. It should be noted that in the experiments of the present investigations basal mean arterial pressure was observed to be slightly elevated in N/OFQ and vehicle-treated rats with all groups having essentially the same starting mean arterial pressure. However, we strongly feel that the neurohumoral status of these animals was not altered to an extent that would have compromised cardiovascular and/or renal function from being studied particularly since the hypotensive effect of drugs in animals with elevated blood pressure are typically greater (Irvine et al., 1997).

Studies were conducted to determine the roles of AVP and the renal sympathetic nerves in mediating the renal excretory responses evoked by administration of N/OFQ into the PVN of conscious rats. Although plasma levels of AVP were not measured, the results of the present study suggest that the N/OFQ-induced diuresis (UFP-101 sensitive) was secondary to suppression of circulating AVP levels. Support for this premise is demonstrated by the observation that a continuous low-dose infusion of AVP abolished the diuretic response to injection of N/OFQ in the PVN. This finding is in accord with previous reports demonstrating that N/OFQ acts in the PVN to inhibit firing of AVP neurons (Olszewski et al., 2000). Further, while changes in renal function were not measured, other studies have shown that i.c.v administration of N/OFQ suppresses circulating plasma levels of AVP in dehydrated rats and blunts the increase in plasma AVP to either hyperosmolality or hypovolemia (Kakiya et al., 2000). These observations strongly imply that NOP receptor activation in the PVN by N/OFQ produces diuresis by a pathway (e.g., reduction in plasma AVP levels) in which the hydroosmotic effect of AVP in the collecting duct is reduced. However, the present data also suggests that in

addition to AVP, the renal sympathetic nerves contribute in mediating the N/OFQ-evoked diuresis. This possibility is first supported by the observation that the microinjection of N/OFQ into the PVN significantly reduced RSNA over the course of the diuresis. Moreover, as compared to the pattern observed in intact rats, the diuretic (but not antinatriuretic or antikaliuretic) response produced by N/OFQ injection into the PVN of chronic bilateral renal denervated animals was markedly decreased in magnitude and time course. Together, these findings indicate that stimulation of NOP receptors in the PVN produces a selective water diuresis by suppressing AVP secretion/release and RSNA. In accord with these observations, the activation of PVN neurons (e.g., via bicuculline microinjection and disinhibition) has been shown to produce antidiuresis by pathways involving augmentation of AVP and renal sympathetic nerve pathways (Haselton and Vari, 1998).

As demonstrated by the findings discussed above and from previous investigations (Coote, 2005), the PVN can influence renal function via the renal nerves. However, in the present study it is clear that the renal nerves did not contribute to the PVN-mediated affects of N/OFQ on urinary sodium excretion. This is supported by the fact that the microinjection of N/OFQ into the PVN decreased urinary sodium excretion instead of producing natriuresis, the latter of which would have been expected if the drug's affect on urinary sodium excretion had been due to renal sympathoinhibition (DiBona, 2001). In addition, chronic bilateral renal denervation did not alter the antinatriuresis produced by N/OFQ injection into the PVN. Therefore, while the mechanisms have yet to be determined, it appears that N/OFQ acts in the PVN via pathways other than the renal nerves to influence the renal handling of sodium and

potassium. While yet to be tested, there is the possibility that N/OFQ may act from the PVN to evoke antinatriuresis and antikaliuresis by decreasing circulating levels of oxytocin, a hypothalamic hormone that produces natriuresis and kaliuresis (Sjoquist et al., 1999). Oxytocin has been suggested to have an important role in renal sodium homeostasis under basal conditions (Conrad et al., 1993). Moreover, N/OFQ inhibits rat oxytocin and vasopressin supraoptic nucleus neurons *in vitro* (Doi et al., 1998).

UFP-101 is a peptide antagonist with nanomolar affinity and high selectivity for NOP receptors (Calo et al., 2005). In the present studies we demonstrated that the pretreatment of conscious rats in the PVN with UFP-101 completely blocked the cardiovascular and renal excretory responses produced by the subsequent injection of N/OFQ into the same brain site. Therefore, these studies, which were performed with unilateral drug (UFP-101 and N/OFQ) administration, clearly demonstrate the selectivity of NOP receptors in mediating N/OFQ's responses. Of additional interest, it was observed that the unilateral administration of UFP-101 alone produced an increase in heart rate and RSNA and a slight but statistically significant reduction in urine flow rate. These UFP-101-mediated responses are of significance since they suggest that endogenous N/OFQ may play a tonic role in the PVN in the regulation of these physiological parameters. To further examine this possibility additional cardiovascular and renal function studies were performed in which UFP-101 was microinjected bilaterally into the PVN of conscious rats. The results of these experiments confirmed this premise and in fact revealed that bilateral injection of UFP-101 into the PVN produced of even greater magnitude tachycardia, renal sympathoexcitatory and antidiuretic responses. Thus, considering that the microinjection of the native ligand

N/OFQ into the PVN produced bradycardia, renal sympathoinhibition and diuresis, these findings with bilateral UFP-101 provide strong evidence that the N/OFQ-NOP receptor system in the rat PVN provides tonic inhibitory influences on central sympathetic outflow to the heart (e.g., to produce bradycardia) and on the secretion/release of AVP and RSNA, which together affect the renal handling of water (e.g., to produce diuresis). These observations are of interest since the existence of a population of neurons in the PVN that can inhibit RSNA has been demonstrated (Deering and Coote, 2000). Moreover, Badoer et al. (2002) provided evidence that renal sympathoinhibitory neurons in the PVN are tonically active.

In summary, the results of the present investigations demonstrate that microinjection of N/OFQ into the PVN of the hypothalamus of conscious rats produces significant reductions in systemic cardiovascular function and RSNA and a water diuresis. These N/OFQ-evoked responses were shown to be mediated by activation of NOP receptors since they were completely blocked by PVN pretreatment with the selective NOP receptor antagonist, UFP-101. In other studies, microinjection of the antagonist UFP-101 alone produced tachycardia, renal sympathoexcitation and antidiuresis. Together, these findings provide evidence that the PVN is a brain site where centrally administered and endogenously released N/OFQ mediate cardiovascular depressor, renal sympathoinhibitory and water diuretic responses in conscious rats. Moreover, UFP-101 blocks a tonically active inhibitory influence of endogenous N/OFQ on central sympathetic outflow and AVP pathways which arise from the PVN to affect heart rate and urine output.

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Footnotes

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Figure Legends

Fig. 1. Changes in cardiovascular and renal function produced by microinjection of aCSF or N/OFQ into the PVN of conscious rats. The values are mean ± S.E.M. and illustrate the systemic cardiovascular, renal sympathetic nerve and renal excretory responses produced by the microinjection of 10 pmol N/OFQ (Δ, n=7), 100 pmol N/OFQ (O, n=8) or aCSF vehicle (□, n=7) into the PVN of rats. Urine samples were collected during control (C, 20-min) and immediately after drug/vehicle microinjection for 80-min, denoted as time periods 10 to 80 min (consecutive 10-min samples). MAP, mean arterial pressure; HR, heart rate, RSNA, renal sympathetic nerve activity; V, urinary flow rate; $U_{Na}V$, urinary sodium excretion; U_KV, urinary potassium excretion. *, P<0.05 and **, P<0.01 when compared with respective pre-drug control (time C).

Fig. 2. Effect of N/OFQ (O, 100 pmol, n=7) and aCSF (□, n=5) in the PVN on free water clearance (C_{H2O}) for the same rats depicted in Fig. 1. Graph represents mean values for each 10-min collection period ± SE. **, P<0.01 when compared with respective pre-drug control (time C).

Fig. 3. Schematic representations of serial coronal sections from the rostral (-1.4 mm) to the caudal (-2.12 mm) extent of the region of the PVN. Coordinates are in reference to bregma using standard sections from the atlas of Paxinos and Watson (1998). Filled squares (N/OFQ 10 pmol) and filled circles (N/OFQ 100 pmol), shown in the vicinity of right PVN, represent sites of termination of injections considered to be within the PVN boundaries. Open circles, shown in the vicinity of left PVN for clarity, illustrate sites of injection of aCSF, whereas shaded circles represent termination sites outside the PVN at which N/OFQ (100 pmol) failed to elicit responses. AH, anterior hypothalamic area, f, fornix; PVN, paraventricular nucleus; 3V, third ventricle.

Fig. 4. Changes in cardiovascular and renal function produced by microinjection of N/OFQ into the PVN of conscious rats continuously infused i.v. with AVP (●, n=5) compared to responses in rats infused with saline (O, n=8). Values are means ± S.E.M. and illustrate the cardiovascular, renal sympathetic nerve and renal excretory responses produced by microinjection of N/OFQ (100 pmol, n=5) during continuous AVP (85 pg/kg/min, i.v.) or isotonic saline infusion (55 µl/min). AVP or isotonic saline alone was infused for 40-min, the last 10-min of which served as pre-drug control (time C) for each group. Next, N/OFQ was microinjected into the PVN and cardiovascular and renal function immediately measured for 80-min, denoted as time periods 10 to 80 min (consecutive 10-min samples). Abbreviations are the same as in Figure 1. *, $P<0.05$ and **, $P<0.01$ when compared with respective pre-drug control (time C).

Fig. 5. Changes in cardiovascular and renal function produced by microinjection of N/OFQ (100 pmol) into the PVN of rats with intact or chronically denervated kidneys. Values are means ± S.E.M. and illustrate the cardiovascular, renal sympathetic nerve and renal excretory responses produced by microinjection of N/OFQ (100 pmol) into the PVN of intact (O, n=8) or chronic bilateral renal denervated rats (●, n=6). Urine samples were collected before (C, 20-min) and after the microinjection of N/OFQ for 80-min, denoted as time periods 10 to 80 min (consecutive 10-min samples). Abbreviations are the same as in Figure 1. *, P<0.05 and **, P<0.01 when compared with respective pre-drug control (time C).

Fig. 6. Cardiovascular and renal responses produced by microinjection of N/OFQ or aCSF in conscious rats pretreated with UFP-101. Values are means ± S.E.M. and illustrate the cardiovascular, renal sympathetic nerve and renal excretory responses produced by microinjection of N/OFQ (100 pmol) or aCSF into the right PVN of rats that received prior (10-min) PVN pretreatment (ipsilateral) with the selective NOP receptor antagonist, UFP-101 (300 pmol). A 20-min urine sample was collected during control (C, 20-min). Then either UFP-101 was injected into the PVN and allowed 10-min to distribute. N/OFQ (●, n=6) or aCSF (O, n=6) was then microinjected into the PVN of each group and urine samples were immediately collected for 80-min, denoted as time periods 10 to 80 min (consecutive 10-min samples). Abbreviations are the same as in Figure 1. *, P<0.05 and **, P<0.01 when compared with respective pre-drug control (time C).

Fig. 7. Changes in cardiovascular and renal function produced by the bilateral microinjection of UFP-101 into the PVN of conscious rats. Values are means ± S.E.M. and illustrate the cardiovascular, renal sympathetic nerve and renal excretory responses produced by the bilateral microinjection of UFP-101 (300 pmol/site) into the PVN of conscious rats. Urine samples were collected before (C, 20-min) and after the microinjection of UFP-101 for 80-min, denoted as time periods 10 to 80 min (consecutive 10-min samples). Abbreviations are the same as in Figure 1. *, $P<0.05$ and **, $P<0.01$ when compared with pre-drug control (time C).

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Fig. 1

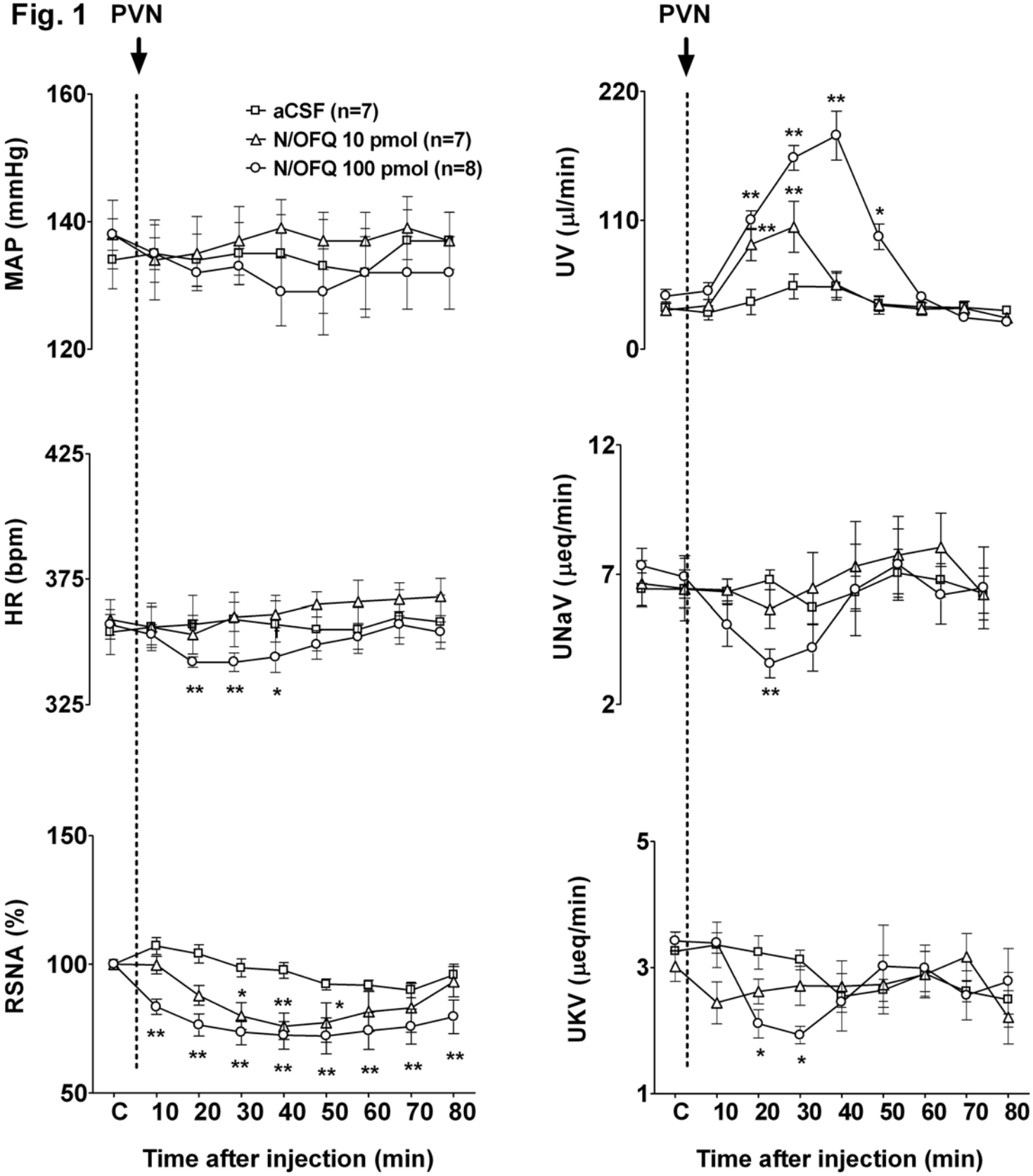

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Fig. 2

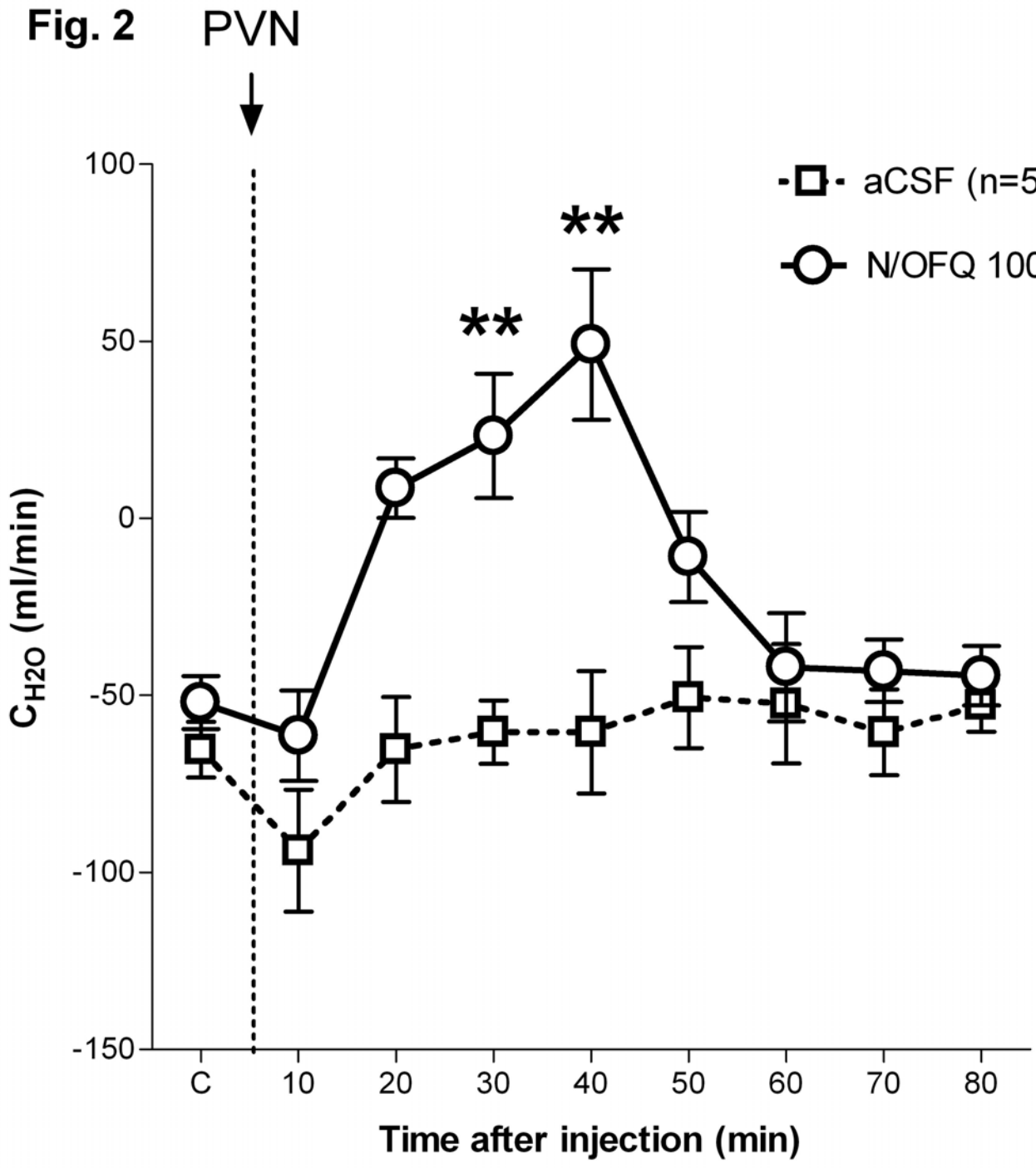

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Fig. 3

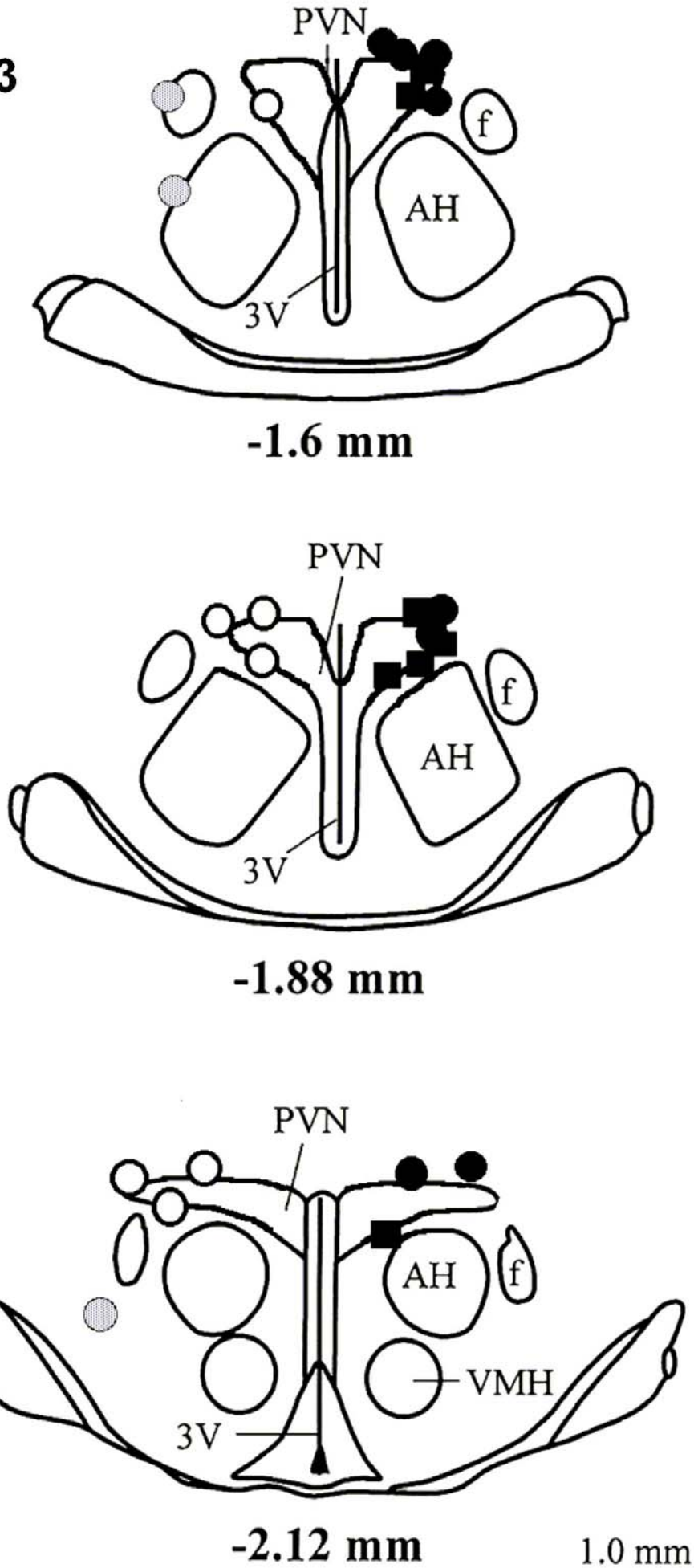

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Fig. 4

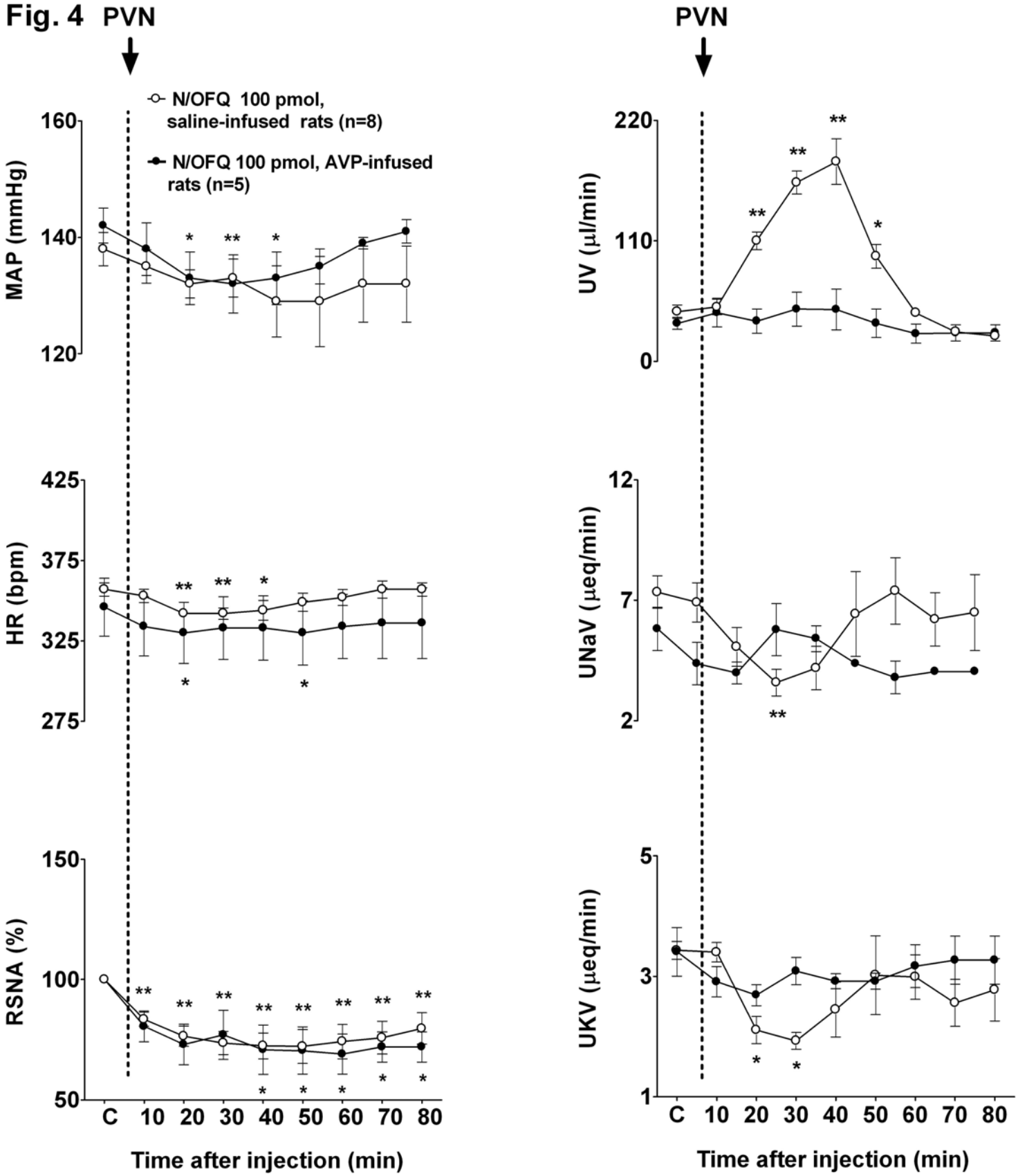

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Fig. 5

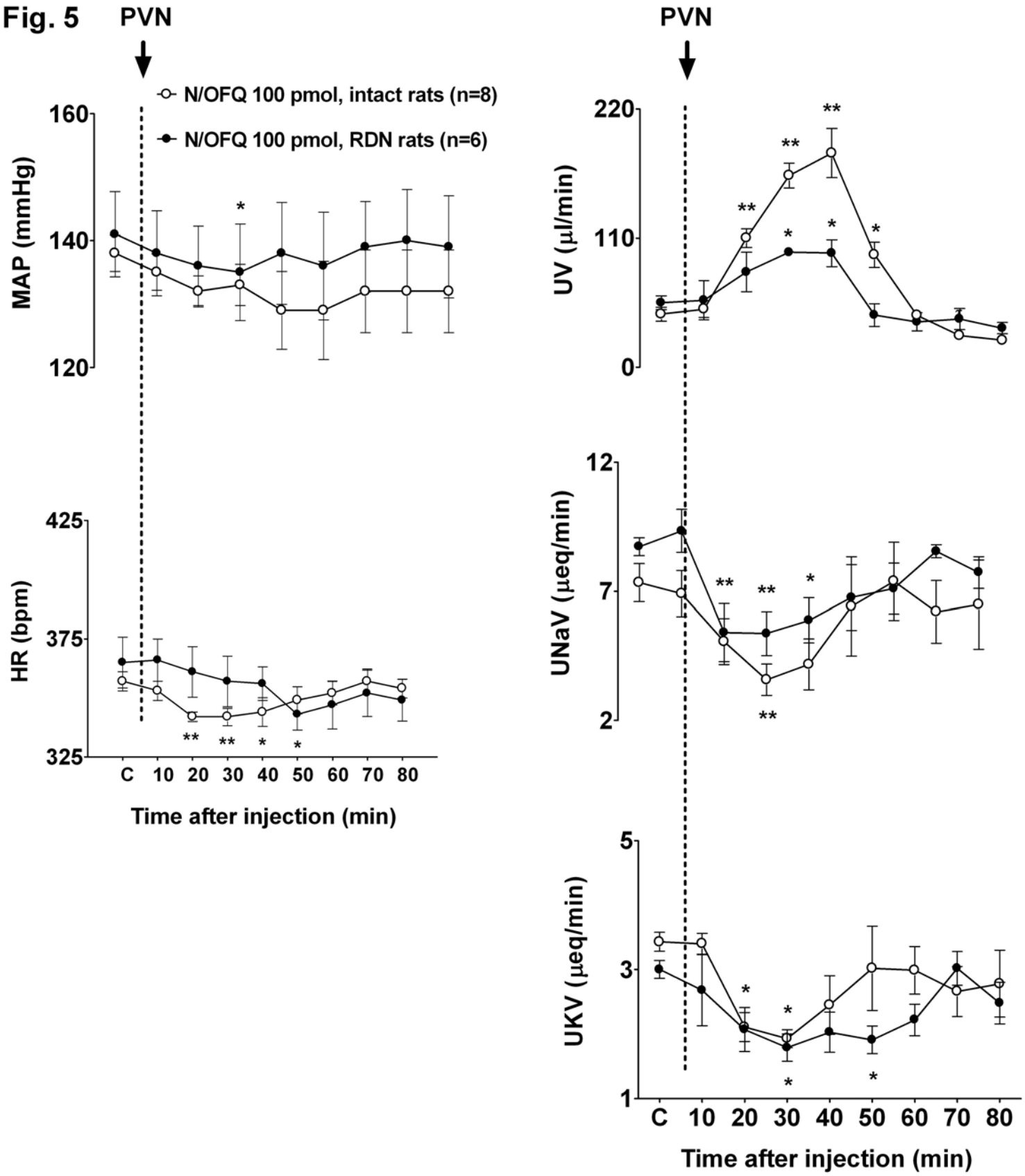

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Fig. 6

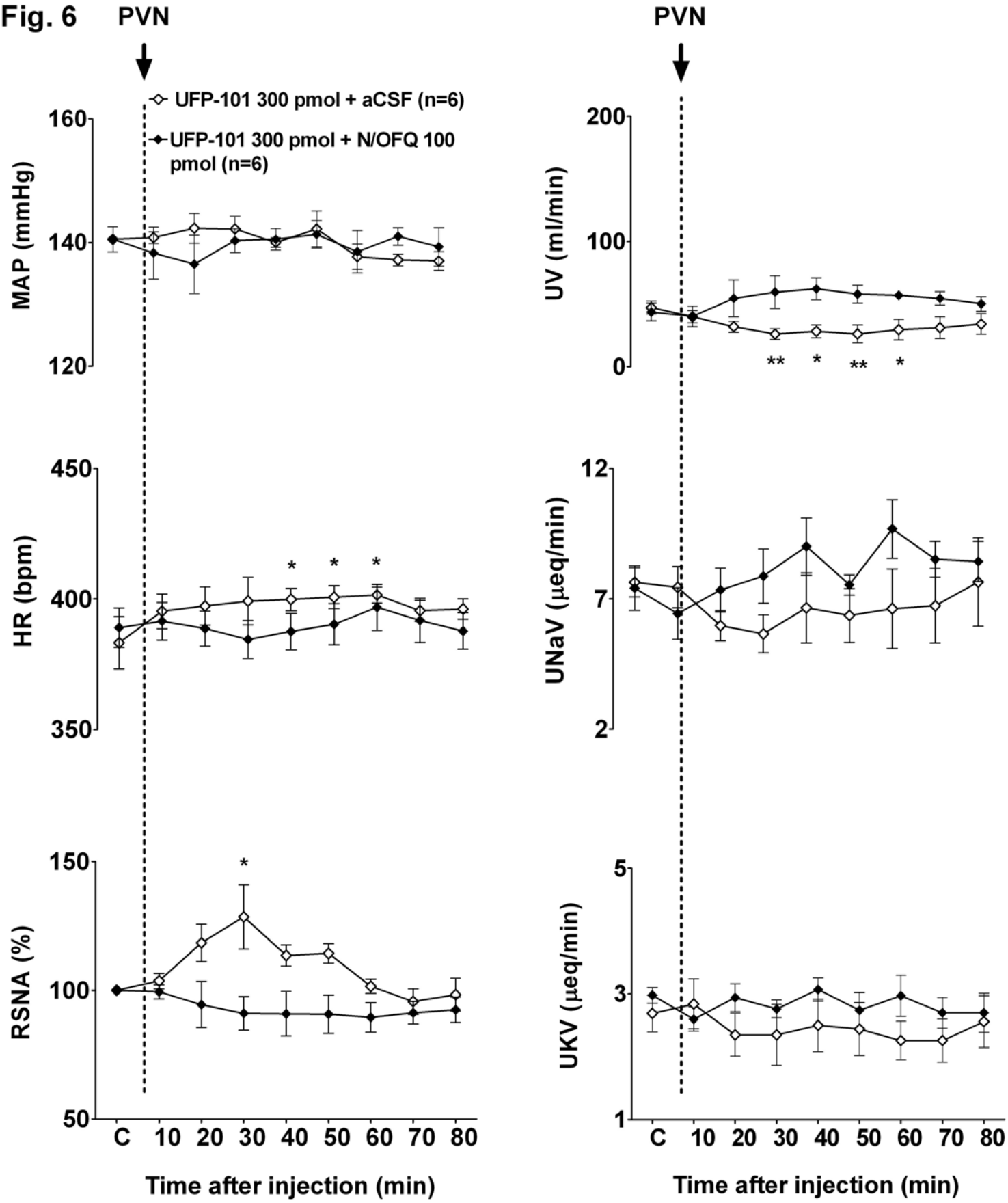

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Fig. 7