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Nociceptin/orphanin FQ (N/OFQ)-evoked bradycardia, hypotension and diuresis are absent in N/OFQ peptide (NOP) receptor knockout ($NOP^{-/-}$) mice.

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Running title: N/OFQ-evoked changes in CV and renal function in $NOP^{-/-}$ mice

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Abbreviations: I.C.V., intracerebroventricular; HR, heart rate; MAP, mean arterial pressure; N/OFQ, nociceptin/orphanin FQ; NOP receptor, nociceptin/orphanin FQ peptide receptor; V, urine output; UNaV, urinary sodium excretion; UKV, urinary potassium excretion; C_{H20}, free water clearance; RSNA, renal sympathetic nerve activity; $NOP^{-/-}$, NOP receptor knockout; $NOP^{+/+}$, NOP receptor wild-type

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Abstract

Intracerebroventricular (i.c.v.) administration of the opioid-like peptide nociceptin/orphanin FQ (N/OFQ) produces bradycardia, hypotension and diuresis in mice. We hypothesized that these responses are solely due to selective activation of central N/OFQ peptide (NOP) receptors. To test this premise, we first examined whether i.c.v. N/OFQ produced dose-dependent diuretic and cardiovascular depressor responses in commercially available C57BL/6 mice. Next, using doses established in these studies, we examined the renal excretory and cardiovascular responses to i.c.v. N/OFQ in conscious transgenic NOP receptor knockout mice ($NOP^{-/-}$). In metabolic studies, i.c.v. N/OFQ, but not saline vehicle, dose-dependently increased urine output (V) in $NOP^{+/+}$; this response was significant at 3 nmol (N/OFQ, V=0.39±0.10 cc/2hr; saline, 0.08±0.05 cc/2hr). The N/OFQ-evoked diuresis was absent in littermate $NOP^{-/-}$ (N/OFQ, V=0.06±0.06 cc/2hr; saline, 0.03±0.03 cc/2hr). There were no significant changes in urinary sodium or potassium excretion or free-water clearance in either group. In telemetry studies, i.c.v. N/OFQ dose-dependently lowered heart rate (HR) and mean arterial pressure (MAP). At 3 nmol N/OFQ, both HR and MAP were reduced in $NOP^{+/+}$ (peak ΔHR = -217±31 bpm; peak ΔMAP = -47±7 mmHg) compared to saline (peak ΔHR = -14±5 bpm; peak ΔMAP = 2±3 mmHg). These N/OFQ-evoked bradycardic and hypotensive responses were absent in $NOP^{-/-}$ (peak ΔHR = -13±17 bpm; peak ΔMAP = -2±4 mmHg, resp.). Basal 24-hr cardiovascular and renal excretory function were not different between $NOP^{-/-}$ and $NOP^{+/+}$ mice. These results establish that the bradycardia, hypotension and diuresis produced by centrally-administered N/OFQ are mediated by selective activation of NOP receptors.

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Introduction

Nociceptin/orphanin FQ (N/OFQ) is an endogenous neuropeptide that selectively binds to and activates an opioid-like receptor called the N/OFQ peptide (NOP) receptor (previously called opioid receptor-like 1, ORL1) (Meunier et al., 1995, Reinscheid et al., 1995). Although the N/OFQ-NOP system belongs to the opioid receptor family, classical opioid receptor agonists do not display appreciable binding affinity to the NOP receptor and vice-versa (Lachowicz et al., 1995; Mollereau et al., 1999). Moreover, the N/OFQ-NOP system has pharmacological and physiological actions that are distinct from classical opioid receptor systems.

We and other investigators have established that the central administration of N/OFQ produces novel changes in cardiovascular and renal excretory function. In conscious rats (Kapusta et al., 1997; Kapusta and Kenigs, 1999; Zhang et al., 1999; Chen et al., 2002) and mice (Burmeister and Kapusta, 2007), the intracerebroventricular (i.c.v.) administration of N/OFQ concomitantly lowers heart rate (HR) and mean arterial pressure (MAP). In rats, central N/OFQ also decreases renal sympathetic nerve activity (RSNA) (Kapusta and Kenigs, 1999; Shirasaka et al., 1999). The pattern of these responses is of considerable interest since the N/OFQ-evoked hypotension is not accompanied by a characteristic baroreflex-mediated tachycardia. Moreover, concurrent with these cardiovascular depressor responses, i.c.v. N/OFQ produces an increase in urine flow rate in both rodent species (Kapusta et al., 1997; Kapusta and Kenigs, 1999; Rizzi et al., 2004; Burmeister and Kapusta, 2007), which, in rats, occurs as a selective water diuresis (e.g., concurrent diuresis, antinatriuresis and increase in free-water clearance) (Kapusta and Kenigs, 1999). The pattern of the renal excretory responses to

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central N/OFQ is also intriguing since it is unlike the diuretic and natriuretic profile produced by central alpha-2/imidazoline receptor agonists (e.g., clonidine), which also produce cardiovascular depressor and renal sympathoinhibitory effects (Szabo, 2002; Patel and Zeigler, 1993; Koepke and DiBona, 1986). Collectively, these findings suggest that the central N/OFQ-NOP system plays a novel role in regulating cardiovascular and fluid/electrolyte homeostasis.

We have previously shown that i.c.v. administration of UFP-101 ([Nphe1,Arg14,Lys15]N/OFQ-NH2), a purported antagonist of the NOP receptor, prevents the cardiovascular depressor and diuretic responses to i.c.v. N/OFQ in conscious CD-1 mice (Burmeister and Kapusta, 2007) and Sprague-Dawley rats (Calo et al., 2005). As an alternative to pharmacological antagonists such as UFP-101, whose effects are acute, the use of transgenic NOP receptor knockout mice ($N/OFQ^{-/-}$) now offers a valuable research approach to study the role(s) of endogenous N/OFQ-NOP receptor pathways in the control of cardiovascular and renal function. However, before this genetic approach can be used to explore the role of endogenous N/OFQ in different chronic stressful or pathological conditions, it is essential to first establish that the cardiorenal responses to central N/OFQ are absent in $N/OFQ^{-/-}$ mice. Furthermore, it is important to determine whether $N/OFQ^{-/-}$ mice, by virtue of the genetic deletion of NOP receptors, have altered basal levels for cardiovascular function and/or daily sodium/water excretion.

Thus, the purpose of the present studies was to use $NOP^{-/-}$ mice to determine if N/OFQ-mediated bradycardia, hypotension and diuresis are due to selective activation of central NOP receptors. For this purpose, dose-response studies were initially

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performed to investigate the central N/OFQ-evoked changes in cardiovascular and renal excretory function produced by i.c.v. injection of this peptide in commercially available C57BL/6 mice (Harlan, Inc.). C57BL/6 mice were used for these initial studies since they are similar in genetic background strain onto which the transgenic $NOP^{-/-}$ mice were bred. Subsequent studies then used maximally effective doses of the peptide to examine the cardiovascular and renal excretory responses produced by i.c.v. N/OFQ in conscious $NOP^{-/-}$ mice and $NOP^{+/+}$ littermates. Related studies also examined whether there were differences in basal 24-hr cardiovascular and renal excretory function between $NOP^{-/-}$ and $NOP^{+/+}$ mice. For these investigations, cardiovascular and renal excretory function was independently assessed using radio-telemetric and metabolic function methods, respectively. Here we report that N/OFQ failed to evoke cardiodepression and diuresis following this peptide's i.c.v. injection in $NOP^{-/-}$ mice, which provides clear evidence that the responses are due to selective activation of central NOP receptors.

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Methods

Subjects

Age-matched non-transgenic C57BL/6NHsd mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) as well as transgenic C57BL/6J (Jackson Laboratory, Bar Harbor, ME) mice functionally deficient in the NOP receptor gene (knockout, $NOP^{-/-}$) and their age-matched littermates (wild-type, $NOP^{+/+}$) weighing 20-25g were used in these studies. $NOP^{-/-}$ mice were produced as described (Clarke et al., 2001) and backcrossed for at least 10 generations to C57BL/6J. $NOP^{-/-}$ and $NOP^{+/+}$ mice used for these studies were generated from mating wild-type and homozygous $NOP^{-/-}$ mice following the backcross. In previous studies, it has been shown that there is a complete absence of binding in homozygous brains from these $NOP^{-/-}$ mice, thus indicating the single gene encodes for the NOP receptor and any putative subtypes (Clarke et al., 2001).

Animals were housed and tested in a climate-controlled room set to a 12-hr dark/light cycle. Animals were fed a standard mouse chow diet (Lab Diet, Richmond, IN, product code 5015) and allowed tap water *ad libitum*. All experimental procedures were performed in accordance with LSUHSC and NIH guidelines for the care and use of experimental animals.

Surgical procedures

Mice were instrumented with a stainless steel guide cannula (Plastics One, Inc., Roanoke, VA) into the right lateral cerebral ventricle under anesthesia (ketamine, 200 mg/kg, i.p., in combination with xylazine, 10 mg/kg, i.p.). The coordinates used for cannula implantation were derived from the atlas of the mouse brain by Paxinos and

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Franklin (Paxinos and Franklin, 2003): 0.3 mm posterior to the bregma; 1.0 mm lateral to the midline; and 3.1 mm below the skull surface. Custom cut and fabricated guide, dummy (obturator) and internal cannula were also purchased from Plastics One, Inc. (Roanoke, VA). The guide cannula was fixed into position by jeweler's screws and cranioplastic cement. Verification of cannula position in the lateral cerebroventricle was made by observing spontaneous flow of cerebrospinal fluid from the tip of the cannula after removal of the obturator (after cannula implantation and before experimentation) and after completion of the experiment by intracerebroventricular dye injection and subsequent postmortem brain section verification of dye placement.

Animals were given 5 days to recover before the initiation of renal function studies. Upon completion of the renal function studies, a subset of the mice were again anesthetized (ketamine 200 mg/kg i.p. in combination with xylazine, 10 mg/kg i.p.) and instrumented with a TA11PC20 telemetry probe (Data Sciences International, Arden Hills, MN), which was implanted into and secured to the left common carotid artery for chronic measurement of mean arterial pressure (MAP) and heart rate (HR). Proper catheter placement was verified by the quality of the audio output signal on an FM radio. The body of the probe was placed in a subcutaneous pocket created in the right flank and the wound closed and sutured. Body temperature was maintained at 37°C using a heat lamp until sternal recumbency had been restored. Animals received a single injection of antibiotic (sterile potassium penicillin, 60,000 units/kg BW, i.m.) as well as a single i.p. injection of the α_2-agonist yohimbine (0.1 mg/kg) following all surgical procedures in order to prevent injection and facilitate recovery from surgery, respectively. Mice remained undisturbed in their home cages for seven days before the

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initiation of cardiovascular function studies. Prior to testing, basal cardiovascular function was observed in all mice and shown to have characteristic circadian rhythm indicative of recovery from surgery.

I.c.v. N/OFQ dose-response renal and cardiovascular studies conducted in non-transgenic C57BL/6 mice (Harlan, Inc.).

Renal function studies: In the first set of experiments, renal excretory function studies were performed using metabolic cages specially designed for the acute collection of urine from conscious mice. The cages were constructed of Plexiglass (walls) and stainless steel (row of bars positioned horizontally upon which the animal sat) and were designed to allow spontaneously voided urine to be collected onto a large plastic weigh boat positioned underneath the animal. On the day prior to experimentation, each mouse was removed from its home cage and "trained" by being placed individually into a metabolic cage for 20 minutes. The animals were then removed, and the lower abdominal region was gently massaged to void any urine already present in the bladder. Each animal was also touched in the region of the i.c.v. cannulae and then returned to the metabolic cages for two hours. On the experimental day, the same procedure was repeated with the exception that after the 20-min habituation period, each animal (n=13-14/group) randomly received an i.c.v. injection of either isotonic saline vehicle (2 µl) or N/OFQ (0.03, 0.3, 1, 3 or 10 nmol). Note that the i.c.v. doses of N/OFQ used in the present cardiovascular and renal function studies are within range of those previously used (0.055, 1, 3.0 or 10 nmol, i.c.v.) to test this

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peptide's antinociceptive, hyperalgesic and allodynic effects in acute thermal or mechanical tests in mice and rats (Vanderah et al., 1998).

I.c.v. injections were performed by securely placing an i.c.v. injector into each animal's indwelling i.c.v. guide cannula; the i.c.v. injector was attached via polyethylene (PE-10) tubing to a Hamilton syringe for injection of drug/vehicle. Following i.c.v. drug/vehicle injection, the animals were returned to their metabolic cages. The experimental period then involved the hourly collection of urine that was spontaneously voided from mice starting after vehicle/drug administration for two hours. Parameters analyzed were urine flow rate (V), urinary excretion of sodium (UNaV) and potassium (UKV) and free-water clearance (C_{H20})**.** Animals were not allowed access to food and water during testing and were returned to their home cages upon completion of each study.

Cardiovascular function studies: In a second set of experiments, a subset of the same animals that had completed the renal excretory function studies (described above) were randomly selected and assigned to be surgically instrumented with radio telemetry probes in order to determine if diuretic doses of N/OFQ also altered cardiovascular function. Upon recovery seven days later (i.e., restoration of the diurnal cycle), the conscious, telemetered mice were tested in their home cages. Telemetry probes were turned on with the least possible disturbance to the animals for the measurement of baseline HR and MAP (sampling frequency = 500 Hz; sampling interval = 2-sec). Following baseline sampling, an i.c.v. injector was securely attached to each animal's indwelling cannula; the i.c.v. injector was attached via PE-10 tubing to a Hamilton syringe for injection of drug/vehicle. Animals were then returned to their home

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cages. The PE tubing was extended outside of the cages so as to keep disturbance to the animals to a minimum and to prevent it from being gnawed and damaged. After stabilization and then additional baseline sampling (i.e., two 10-min pre-drug control periods), N/OFQ (0.03, 0.3, 1 or 3 nmol) or isotonic saline vehicle (2 µl) was randomly delivered i.c.v. via the Hamilton syringe injector assembly. MAP and HR were then measured continuously during a 120-min experimental period (twelve consecutive 10-min periods). Parameters analyzed were the MAP and HR responses to i.c.v. N/OFQ and isotonic saline control.

Renal and cardiovascular function studies conducted in transgenic C57BL/6J NOP$^{-/-}$ mice and wild-type littermates.

Basal 24-hr renal function studies: Following recovery from i.c.v. cannula implantation (5 days), NOP$^{+/+}$ and NOP$^{-/-}$ C57BL/6J mice were individually placed in metabolic cages for 5 days for assessment of basal 24-hr renal excretory function in each group. For these studies, consecutive 24-hr water intake, urine/sodium/potassium excretion, and free water clearance were measured. Following the completion of these studies, these same mice were then used in renal function experiments that involved the i.c.v. injection of N/OFQ or vehicle (protocol below).

I.c.v. N/OFQ renal excretory function studies: The experimental approach used for these experiments were the same as those described above for the i.c.v. N/OFQ dose-response studies (renal excretory) performed in non-transgenic mice, with the exception that transgenic NOP$^{-/-}$ and littermate NOP$^{+/+}$ C57BL/6J mice served as the

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experimental subjects. For these investigations, the i.c.v. doses of N/OFQ tested in $NOP^{-/-}$ and $NOP^{+/+}$ mice were those previously determined to have significant and maximal effects on renal excretory and cardiovascular function in the dose-response studies performed in commercially available (i.e., non-transgenic) C57BL/6 mice (Harlan, Inc.) (protocols described above).

Basal 24-hr cardiovascular function studies: Following completion of renal function studies (basal measurements and renal responses to i.c.v. N/OFQ/vehicle), a subgroup of the same $NOP^{-/-}$ and $NOP^{+/+}$ C57BL/6J mice were instrumented with telemetry probes and allowed to recover (7 days). After recovery of circadian rhythm, basal 24-hr MAP and HR was measured over a 24-hr period in $NOP^{-/-}$ and $NOP^{+/+}$ C57BL/6J mice while housed in their home cages.

I.c.v. N/OFQ cardiovascular function studies: Following the completion of the basal 24-hr cardiovascular function studies, these same telemetered mice were then used in cardiovascular function experiments that involved the i.c.v. injection of N/OFQ or vehicle (protocol same as described above for non-transgenic mice).

Analytical techniques and data analysis

Urine volume was determined gravimetrically. Urine sodium and potassium concentrations were measured by flame photometry (model 943, IL Labs). Urine osmolality was analyzed using a vapor pressure osmometer (Wescor, model 5500). Telemetry data was compiled and analyzed using Dataquest A.R.T. Software (DSI, v.

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2.3) and Microsoft Excel. The output from the radio telemetry probes was recorded (500 Hz) using receiver plates placed beneath the home cages. Data was sent to a consolidation matrix before being stored on a personal computer. Results were plotted and graphed using GraphPad Prism software (v. 4.00).

Statistics

Results are expressed as the 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 one-way repeated measures analysis of variance (ANOVA) with subsequent Dunnett's test. Differences occurring between treatment groups (e.g., multiple doses of drug) were assessed by two-way repeated measures ANOVA with treatment being one fixed effect and time the other, with the interaction included. The time (minutes) was the repeated factor. Post-hoc analysis was performed using Bonferonni's test. Where appropriate, a Student's t-test was also used to compare means between two groups. Data were verified for normality of distribution and equality of variances. In each case, statistical significance was defined as $p<0.05$.

Drugs

N/OFQ (FGGFTGARKSARKLANQ) was synthesized and graciously provided by Drs. Remo Guerrini, Severo Salvadori and Girolamo Calo' (University of Ferrara, Ferrara, Italy). Stock solutions of the peptide were prepared fresh and stored frozen. N/OFQ/vehicle was administered i.c.v. in a volume of 2 µl.

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Results

Non-transgenic C57BL/6NHsd mouse (Harlan, Inc.) studies

The renal excretory responses produced by the i.c.v. administration of N/OFQ in conscious C57BL/6 mice (Harlan, Inc.) are presented in Figure 1a (cumulative urine output data) and Figure 1b (time-course renal excretory data). As shown in Fig. 1a, i.c.v. N/OFQ (tested at 0.03, 0.3, 1, 3 and 10 nmol) elicited a significant and dose-dependent increase in urine output (V) over a 2-hr period. At the 1 nmol i.c.v. dose, urine output was significantly and maximally elevated (V=0.33±0.04 cc/2hr) in comparison to the change in urine output produced by i.c.v. isotonic saline vehicle (V=0.13±0.04 cc/2hr). The 3 nmol i.c.v. dose produced a diuresis comparable to that of the 1 nmol dose. However, there was no urine excreted from animals following 10 nmol N/OFQ, most likely due to the marked hypotension caused by this high dose, which also caused sedation. Analysis of the time-course data revealed that at 1 nmol, the i.c.v. N/OFQ-evoked diuresis occurred primarily during the second hour of the 2-hr time course (Fig. 1b); there were no significant changes in urinary sodium (UNaV) or potassium (UKV) excretion or free-water clearance (C_{H20}) (Fig. 1b).

Figure 2 illustrates the changes in HR and MAP produced by i.c.v. injection of N/OFQ (0.3, 1 or 3 nmol) or isotonic saline vehicle in conscious telemetered C57BL/6 mice (Harlan, Inc.). As shown (Fig. 2), the i.c.v. administration of isotonic saline did not alter HR or MAP at any point throughout the study. However, following i.c.v. injection of N/OFQ HR began to decrease within 30-sec to 1-min, although the bradycardia was only statistically significant at the 3 nmol dose. Concurrent with the bradycardia, i.c.v. N/OFQ also reduced MAP at the 1 and 3 nmol doses. At 3 nmol, the highest dose

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tested, N/OFQ decreased HR from 587±19 bpm (C) to 428±37 bpm (20-min) and MAP from 106±3 mmHg (C) to 78±8 mmHg (20-min) (Fig. 2). The peak reductions in each parameter occurred 20-min after central drug injection, and each response returned to baseline between 30- and 40-min post-injection, respectively.

Transgenic C57BL/6J NOP$^{-/-}$ and NOP$^{+/+}$littermate mouse studies

Studies were performed to compare basal 24-hr levels for renal excretory and cardiovascular function in transgenic NOP$^{-/-}$ and NOP$^{+/+}$ mice (Clarke et al., 2001). As depicted in Table 1, there were no significant differences in basal 24-hr HR and MAP (telemetry studies) or 24-hr urine output, sodium/potassium excretion and free water clearance (metabolic studies) between NOP$^{+/+}$ and NOP$^{-/-}$ mice.

In subsequent studies in which mice were tested during acute experimental protocols, the baseline control levels for urine output (Fig. 3, depicted as 'saline 2-hr') and MAP (depicted as 'C' in Figs. 4a and 4b) observed in NOP$^{-/-}$ mice were not different than respective levels for each parameter observed in NOP$^{+/+}$ littermate controls. Further, in these studies both the renal excretory (Fig. 3) and cardiovascular (Figs. 4a and 4b) responses elicited by i.c.v. N/OFQ were absent in NOP$^{-/-}$ mice as compared to responses produced by this ligand in NOP$^{+/+}$ control littermates. More specifically, in NOP$^{+/+}$ animals i.c.v. injection of 3 nmol N/OFQ produced a significant diuresis (V=0.39±0.10 cc/2hr) compared to that elicited by isotonic saline vehicle administration (V=0.03±0.03 cc/2hr; in this case only 1 mouse urinated over the 2-hr study). In contrast, in NOP$^{-/-}$ animals urine output was not different between groups of animals treated with this dose of N/OFQ (V=0.06±0.06 cc/2hr; only one mouse urinated) or

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isotonic saline vehicle (V=0.08±0.05 cc/2hr). In other studies in which these same animals were instrumented with telemetry probes, N/OFQ evoked a significant bradycardia (Fig. 4a) in C57BL/6 $NOP^{+/+}$ mice (1 nmol, peak ΔHR = -135±53 bpm, nadir 20-min: 3 nmol, -217±31 bpm, nadir 30-min) as compared to isotonic saline vehicle treated animals (peak ΔHR = -14±5 bpm, nadir 20-min). At each of these doses (1 and 3 nmol), as the central N/OFQ-evoked bradycardia completely recovered, this was followed by a significant rebound increase in HR (Fig. 4a).

In contrast, the bradycardia to central N/OFQ was abolished in $NOP^{-/-}$ animals (Fig. 4b; 1 nmol, peak ΔHR = 34±10 bpm; 3 nmol, -13±17 bpm) and did not differ from that produced by i.c.v. isotonic saline vehicle (peak ΔHR: -3±7 bpm). In these studies, i.c.v. N/OFQ (Fig. 4a) also produced a significant hypotensive response in $NOP^{+/+}$ animals (1 nmol, peak ΔMAP = -20±10 mmHg, nadir 30-min; 3 nmol, -47±7 mmHg, nadir 20-min) as compared to that produced by isotonic saline vehicle (peak ΔMAP = 2±3 mmHg, nadir 20-min). The N/OFQ-evoked hypotension was completely absent in $NOP^{-/-}$ animals (Fig. 4b; 1 nmol, peak ΔMAP = 0±1 mmHg; 3 nmol, -2±4 mmHg) and was similar to the MAP response produced by isotonic saline vehicle (Fig. 4b; peak ΔMAP = 2±1 mmHg).

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Discussion

The results of these studies confirm our previous observations made in ICR-CD1 mice (Burmeister and Kapusta, 2007) and show that i.c.v. N/OFQ also elicits marked cardiovascular depressor and diuretic responses in conscious C57BL/6 mice. In addition, it is clear that central N/OFQ mediates these cardiovascular and renal excretory responses via selective activation of a NOP receptor pathway since the characteristic bradycardic, hypotensive and diuretic responses to i.c.v. N/OFQ were absent in transgenic $NOP^{-/-}$ mice. This observation is of interest since it indicates that the cardiovascular and renal responses to central N/OFQ are elicited through a NOP receptor or splice variant (Pan et al., 1998) in the brain that is derived from a single NOP gene.

As a means to understand the physiological role(s) of the endogenous N/OFQ-NOP receptor system, transgenic $NOP^{-/-}$ mice have been phenotyped in numerous biological and behavioral paradigms including pain sensitivity (Bertorelli et al., 2002), locomotor activity (Nishi et al., 1997), morphine dependence and tolerance (Mamiya et al., 2001; Ueda, 2004), and antinociceptive response to morphine- and kappa-opioid peptides (Noda et al., 1999). Stress and anxiety (Gavioli et al., 2007), hyperalgesia and allodynia (Okuda-Ashitaka et al., 2006), auditory function (Nishi et al., 1997), learning and memory (Manabe et al., 1998), and depression (Gavioli et al., 2003; Gavioli et al., 2004) have all also been examined in $NOP^{-/-}$ mice. These investigators reported that endogenous N/OFQ mediates a tonic influence on certain biological processes via NOP receptor activation since basal levels for these respective systems are significantly altered in $NOP^{-/-}$ mice.

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To date, studies have not previously explored whether genetic deletion of the NOP receptor influences basal cardiovascular and/or renal excretory function. Despite the pronounced effects produced by exogenous N/OFQ (Madeddu et al., 1997; Burmeister and Kapusta, 2007), the results of the present study demonstrate that under resting conditions, $NOP^{+/+}$ and $NOP^{-/-}$ mice have similar cardiovascular and renal excretory phenotypes, with no major differences in 24-hr basal level for any cardiorenal parameter measured. These findings indicate that the endogenous N/OFQ-NOP system does not appear to tonically influence the mechanisms that regulate daily basal levels for MAP, HR or the renal excretion of water/sodium. This is in agreement with our recent findings that the i.c.v. administration of the selective NOP receptor antagonist UFP-101 did not elicit a change in any of these parameters, which would be expected to be opposite to those typically produced by i.c.v. N/OFQ (Burmeister and Kapusta, 2007). However, our present findings are in contrast to those in which we demonstrated that when microinjected directly into the paraventricular nucleus of the hypothalamus (PVN) of conscious Sprague-Dawley rats, the NOP antagonist UFP-101 increased heart rate and renal sympathetic nerve activity and decreased urine flow rate without altering electrolyte excretion (Krowicki and Kapusta, 2006). Thus, in these previous studies UFP-101 was shown to block 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 (Krowicki and Kapusta, 2006). There are likely to be several reasons underlying the discrepancy of a tonic role for N/OFQ-NOP in the previous and present studies. These are likely to involve differences between rat and mouse strains, locus of brain site of drug action, and genetic versus

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pharmacological approaches used to block the native N/OFQ-NOP system. In regards to this latter point, it should be clarified that the transgenic $NOP^{-/-}$ mice used in the present investigations express a whole body gene deletion for the NOP receptor. This is in contrast to performing similar phenotype studies in which there has been a selective removal of the NOP receptor from within the brain (or specific nucleus) of an adult animal (e.g., cre-loxP technology).

Despite there being no differences in cardiovascular and renal excretory function between $NOP^{+/+}$ and $NOP^{-/-}$ mice under basal conditions, it is possible that the N/OFQ-NOP system may be activated and affect these systems during stress or pathology. This is suggested since other endorphin systems have typically been shown to remain quiescent without influence on a biological system until they are activated by a particular stress or pathology (Carr and Verrier, 1991; Kapusta and Obih, 1995). While transgenic $NOP^{-/-}$ mice now provide an alternative approach in which to explore these possibilities, it was the purpose of these investigations to first determine whether or not the cardiovascular and renal excretory responses to central N/OFQ are unequivocally absent in transgenic $NOP^{-/-}$ mice as compared to responses observed in conscious littermate $NOP^{+/+}$ mice and non-transgenic C57BL/6 mice, a genetic background strain similar to the C57BL/6 mice onto which $NOP^{-/-}$ mice were backcrossed.

Here we report that i.c.v. N/OFQ produced a dose-dependent bradycardia, hypotension and diuresis in non-transgenic (Harlan, Inc) and $NOP^{+/+}$ mice (Jackson Laboratories, Inc.). While i.c.v. N/OFQ evoked a similar directional change in cardiovascular function (i.e., decrease) and urine output (increase) in both groups, the magnitudes of the bradycardia and hypotension produced by this peptide were strain-

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dependent. More specifically, i.c.v. N/OFQ (3 nmol) produced a peak (20-min) decrease in HR of -159±14 bpm in non-transgenic C57BL/6NHsd mice (Harlan, Inc.). This same i.c.v. dose of N/OFQ lowered HR to a peak (30-min) of -218±12 bpm in C57BL/6J $NOP^{+/+}$ mice. Related to this issue, we have previously reported (Burmeister and Kapusta, 2007) that in CD1 mice (Harlan, Inc.), i.c.v. N/OFQ (3 nmol) also produced a pronounced bradycardia (-261±19 bpm), but the magnitude was even greater than that observed in the $NOP^{+/+}$ mice tested in the present study. Similar to HR responses, i.c.v. N/OFQ produced differences in the peak magnitude hypotensive response observed across groups of mouse strains. In non-transgenic C57BL/6NHsd mice (Harlan, Inc.), i.c.v. N/OFQ (3 nmol) significantly reduced MAP by -28±3 mmHg 20-min post drug injection. In comparison, i.c.v. N/OFQ (3 nmol) lowered MAP by -47±3 mmHg in C57BL/6J $NOP^{+/+}$ mice, (Jackson Laboratories, Inc.), which was similar to the decrease (-46±5 mmHg) in MAP produced 20-min after i.c.v. N/OFQ (3 nmol) as reported in ICR-CD1 mice (Harlan, Inc.) (Burmeister and Kapusta, 2007). Of interest, in the present study the durations of both the hypotension and bradycardia to i.c.v. N/OFQ were greatest in $NOP^{+/+}$ mice.

It is likely that underlying genetic differences between the strains of mice tested contribute to the variations in the magnitude and duration of the systemic cardiovascular depressor responses to central N/OFQ. Numerous studies have reported differences in the specific pattern (e.g., magnitude and time course) of changes in a given physiological parameter among different strains of the same species or sub-strains generated from the same background (e.g., C57BL/6) (Stiedl et al., 1999; Faulx et al., 2005). Although entirely speculative, it is possible that the mouse strains

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we studied have differences in basal sympathetic neural tone, which governs resting HR and MAP. Significant differences in neurotransmission and cellular stress-related gene expression patterns to environmental stimuli have been reported between C57BL/6J and C57BL/6NHsd mice (Grottick et al., 2005). In this case, it would be predicted that mice with a greater sympathetic influence (e.g., potentially C57BL/6J $NOP^{+/+}$ mice) would display a more profound and prolonged reduction in MAP and HR. This is suggested since central N/OFQ is recognized to produce hypotension and bradycardia primarily via inhibition of central sympathetic outflow (Kapusta and Kenigs, 1999). Under these conditions (i.e., hypotension due to sustained central sympathoinhibition), it may also be predicted that following central N/OFQ injection, alternative hormonal mechanisms such as angiotensin II and epinephrine may be triggered in an attempt to restore MAP and HR to normal; In this case the overshoot of the rebound HR and MAP responses observed in C57BL/6J $NOP^{+/+}$ mice in the present study could be explained by slower process for hormonal pathways to tightly and rapidly regulate these systemic cardiovascular hemodynamic parameters to normal (pre-drug) levels.

Despite sub-strain phenotypic variations, a major finding of the present investigations is that, in direct opposition to the enhanced systemic cardiovascular responses observed in $NOP^{+/+}$ mice, the transgenic $NOP^{-/-}$ mice completely failed to elicit any change in HR, MAP or urine output to i.c.v. N/OFQ (1 or 3 nmol). Thus, data from these studies unequivocally demonstrates that central N/OFQ activates a single NOP receptor pathway to mediate this peptide's cardiovascular depressor and diuretic responses.

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In summary, the endogenous N/OFQ-NOP system does not appear to be involved in the tonic regulation of cardiovascular and renal excretory function in conscious mice since there were no differences in basal 24-hr HR, MAP, and water/sodium excretion between $NOP^{-/-}$ mice and their wild-type littermates. Further, it is clear that the cardiovascular (bradycardia and hypotension) and renal excretory (diuresis) responses to i.c.v. N/OFQ are mediated by selective activation of central NOP receptors as demonstrated by the failure of i.c.v. N/OFQ to change cardiovascular or renal excretory function in transgenic $NOP^{-/-}$ mice. Together, these findings provide an important foundation for the use of $NOP^{-/-}$ mice in future studies aimed toward elucidating a potential physiological role of the endogenous N/OFQ-NOP system in the central control of cardiovascular and renal function during acute or chronic states in which cardiovascular and/or renal function may be altered (stress) or compromised (pathology).

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Footnotes

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Legends for Figures

Fig. 1a. Changes in urine output produced by the i.c.v. injection of N/OFQ in conscious, non-transgenic C57BL/6 mice (Harlan, Inc). The values are mean ± S.E.M. and illustrate the changes in cumulative urine output (V) produced by the i.c.v. injection of N/OFQ (n=13), 0.3 nmol N/OFQ (n=13), 1 nmol N/OFQ (n=13), 3 nmol N/OFQ (n=14) 10 nmol N/OFQ (n=13) or isotonic saline vehicle (2 µl, n=13). Data shown in Fig. 1a represents mean values for cumulative urine collected throughout the entire 120-min experimental protocol beginning immediately after vehicle/drug administration. *, $p<0.05$ compared to isotonic saline vehicle-treated mice.

Fig. 1b. **Time-course of the renal excretory responses produced by the i.c.v. injection of N/OFQ in conscious, non-transgenic C57BL/6 mice.** The values are mean ± S.E.M. and illustrate the renal excretory responses produced by the i.c.v. injection of 1 nmol N/OFQ (n=20) or isotonic saline vehicle (n=19) during the first and second hours following i.c.v. saline/drug injection. Time-course data presented in Fig. 1b for N/OFQ (1 nmol) and saline are for the same mice in which renal excretory data are depicted in Fig. 1a. V, urine output; UNaV, urinary sodium excretion; UKV, urinary potassium excretion; C_{H20}, free-water clearance. *$p<0.05$ compared to isotonic saline vehicle-treated mice.

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Fig. 2. Time-course of the cardiovascular responses produced by the i.c.v. injection of N/OFQ in conscious, non-transgenic C57BL/6 mice (Harlan, Inc). The values are mean ± S.E.M. and illustrate the systemic cardiovascular responses produced by the i.c.v. injection of 0.3 nmol N/OFQ (●, n=7), 1 nmol N/OFQ (▲, n=7), 3 nmol N/OFQ (■, n=9) or isotonic saline vehicle (○, n=7). Heart rate (HR) and mean arterial pressure (MAP) were measured during control (C, 10-min) and immediately after i.c.v. drug/vehicle injection for 120-min denoted as time periods 10 to 120 min (consecutive 10-min periods). *, $p<0.05$ when compared to respective pre-drug group control (time C).

Fig. 3. Changes in urine output produced by the i.c.v. injection of N/OFQ in conscious transgenic C57BL/6 NOP$^{+/+}$ and NOP$^{-/-}$ mice. The values are mean ± S.E.M. and illustrate the changes in cumulative urine output (V) produced by the i.c.v. injection of 1 nmol N/OFQ (n=8/group), 3 nmol N/OFQ (n=8/group) or isotonic saline vehicle (2 µl , n=8/group) in NOP$^{+/+}$ and NOP$^{-/-}$ mice. Data shown in Figure 3 represents mean values for cumulative urine collected throughout the entire 120-min experimental protocol beginning immediately after vehicle/drug administration. *, $p<0.05$ compared to isotonic saline vehicle-treated mice.

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Fig. 4a. Time-course of the cardiovascular responses produced by the i.c.v. injection of N/OFQ in conscious, telemetered C57BL/6J NOP$^{+/+}$ control littermate mice. The values are mean ± S.E.M. and illustrate the systemic cardiovascular responses produced by the i.c.v. injection of 1 nmol N/OFQ (▲, n=5), 3 nmol N/OFQ (■, n=5) or isotonic saline vehicle (2 µl, ○, n=6) in C57BL/6J NOP$^{+/+}$ mice. Heart rate (HR) and mean arterial pressure (MAP) were measured during control (C, 10-min) and immediately after i.c.v. drug/vehicle injection for 120-min denoted at time periods 10 to 120 min (consecutive 10-min periods). *, $p<0.05$ when compared to respective pre-drug group control (time C).

Fig. 4b. Time-course of the cardiovascular responses produced by the i.c.v. injection of N/OFQ in conscious, telemetered C57BL/6J NOP$^{-/-}$ mice. The values are mean ± S.E.M. and illustrate the systemic cardiovascular responses produced by the i.c.v. injection of 1 nmol N/OFQ (▲, n=5), 3 nmol N/OFQ (■, n=5) or isotonic saline vehicle (2 µl, ○, n=5) in C57BL/6J NOP$^{+/+}$ mice. Heart rate (HR) and mean arterial pressure (MAP) were measured during control (C, 10-min) and immediately after i.c.v. drug/vehicle injection for 120-min denoted at time periods 10 to 120 min (consecutive 10-min periods). *, $p<0.05$ when compared to respective pre-drug group control (time C).

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Tables

TABLE 1

BASAL 24-HR RENAL EXCRETORY AND CARDIOVASCULAR FUNCTION IN $NOP^{+/+}$ AND $NOP^{-/-}$ MICE.						
	RENAL EXCRETORY FUNCTION (n=9/group)				CARDIOVASCULAR FUNCTION (n=5/group)	
	V (cc)	UNaV (µEq)	UKV (µEq)	C_{H2O} (µl)	HR (bpm)	MAP (mmHg)
$NOP^{+/+}$	2.1±0.02	480±46	513±85	-15±1	617±37	117±5
$NOP^{-/-}$	2.1±0.03	513±85	615±99	-16±2	602±32	110±9

V, urine output; UNaV, urinary sodium excretion; UKV, urinary potassium excretion; C_{H2O}, free-water clearance; HR, heart rate; MAP, mean arterial pressure. Values for renal excretory function are basal 24-hr mean ± SEM averaged over 5 consecutive days (metabolic studies). Values for cardiovascular function are the mean ± SEM averaged over one 24-hr period (separate telemetry studies).

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Figure 1a

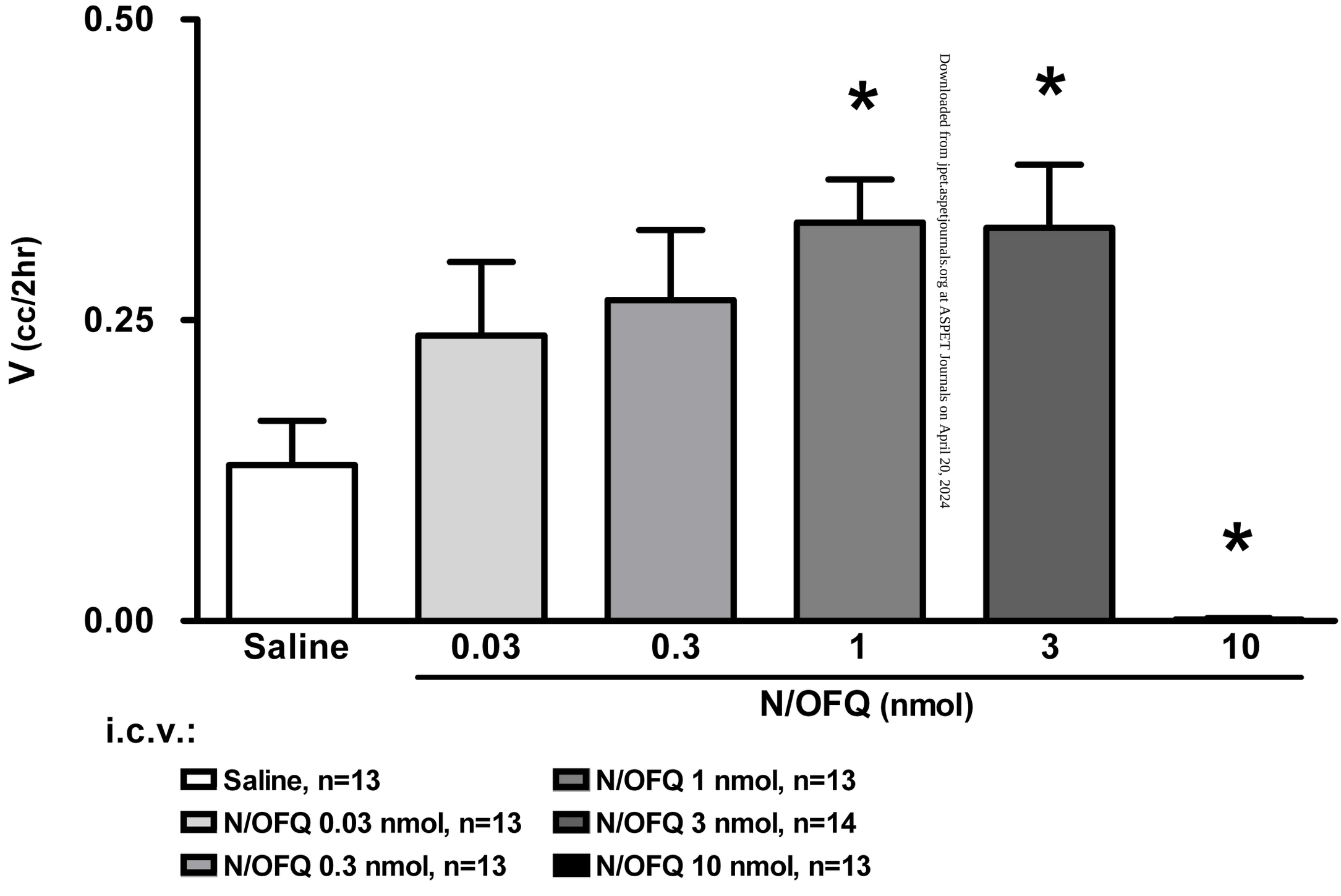

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Figure 1b

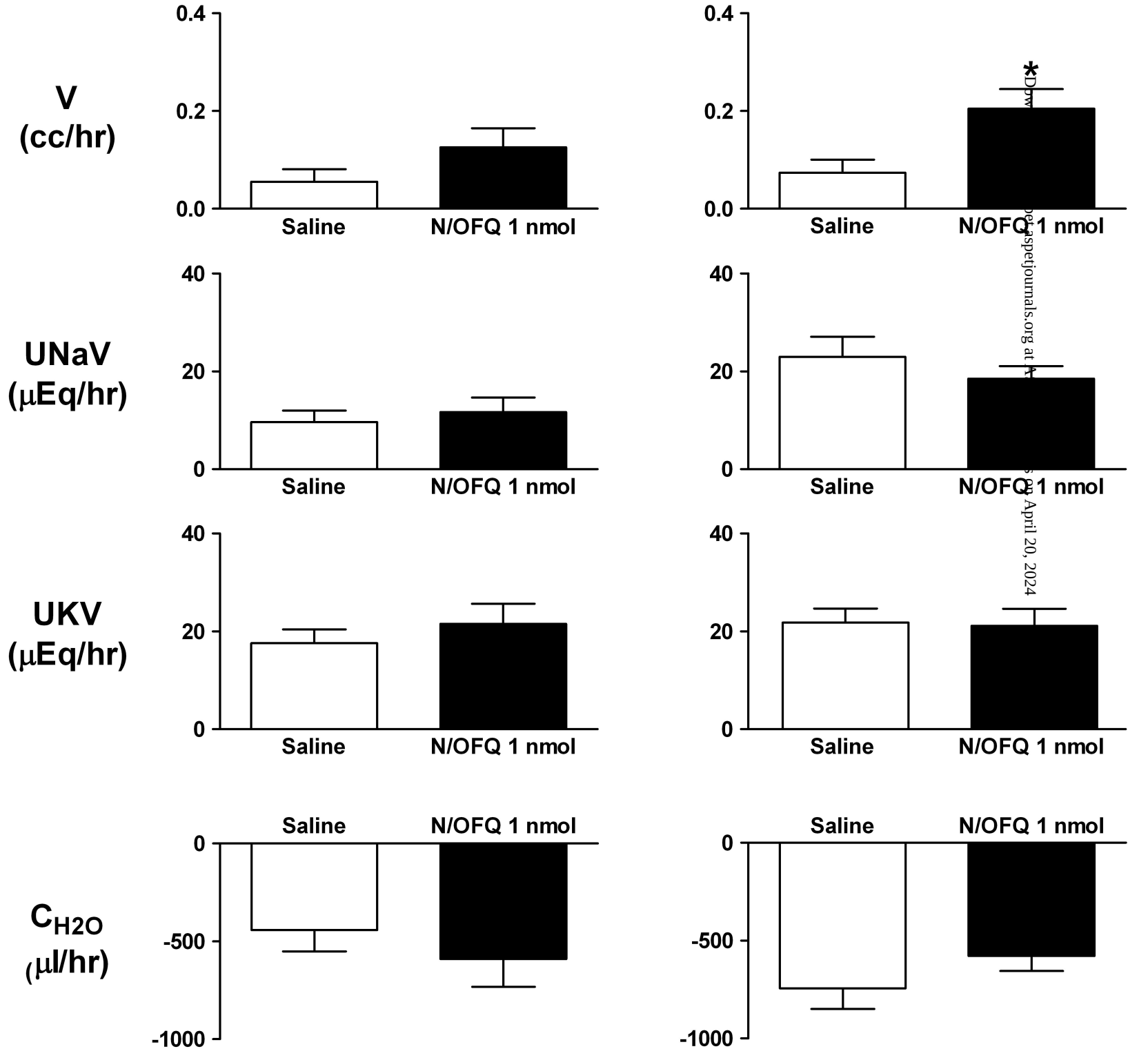

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Figure 2

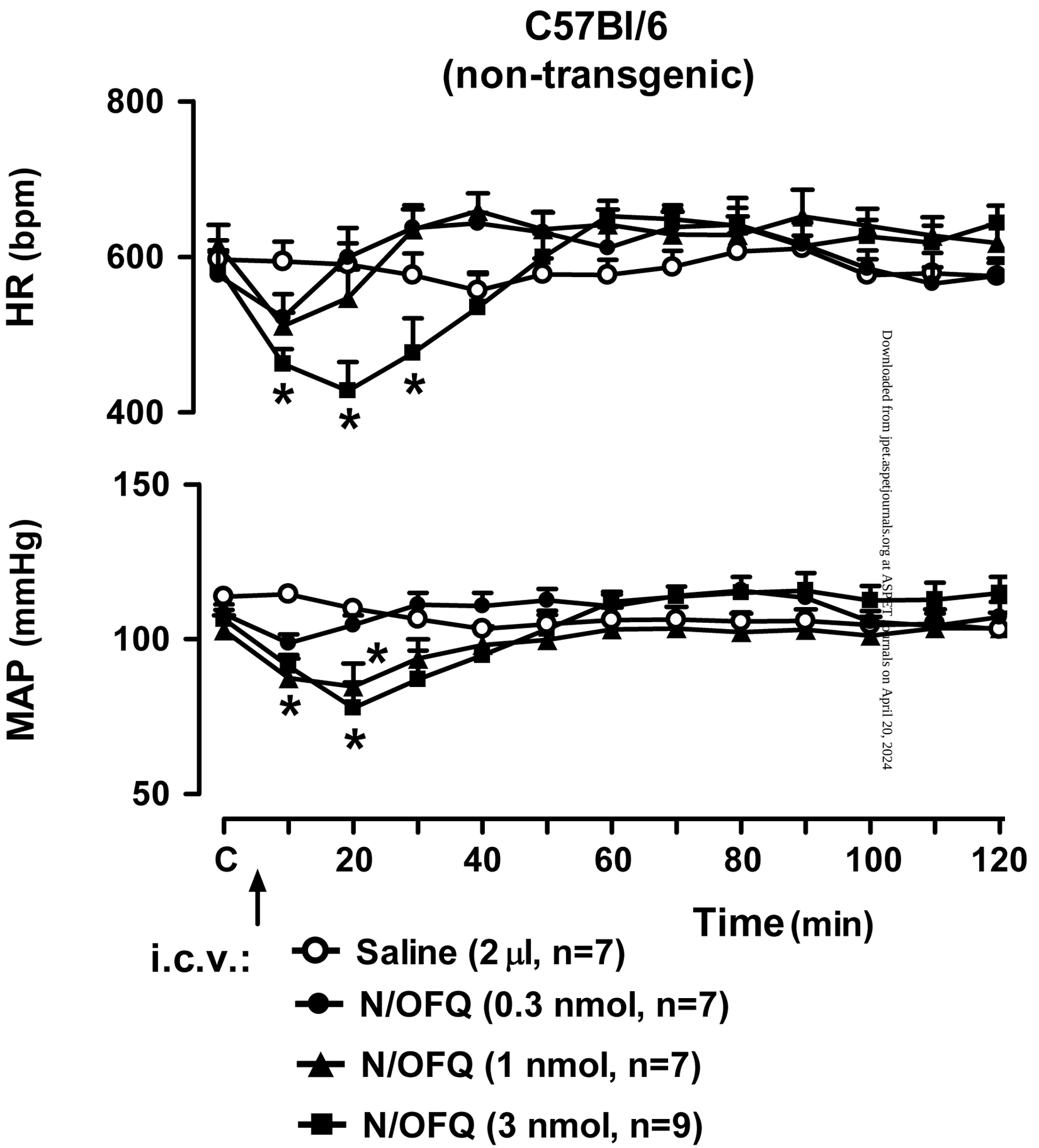

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Figure 3

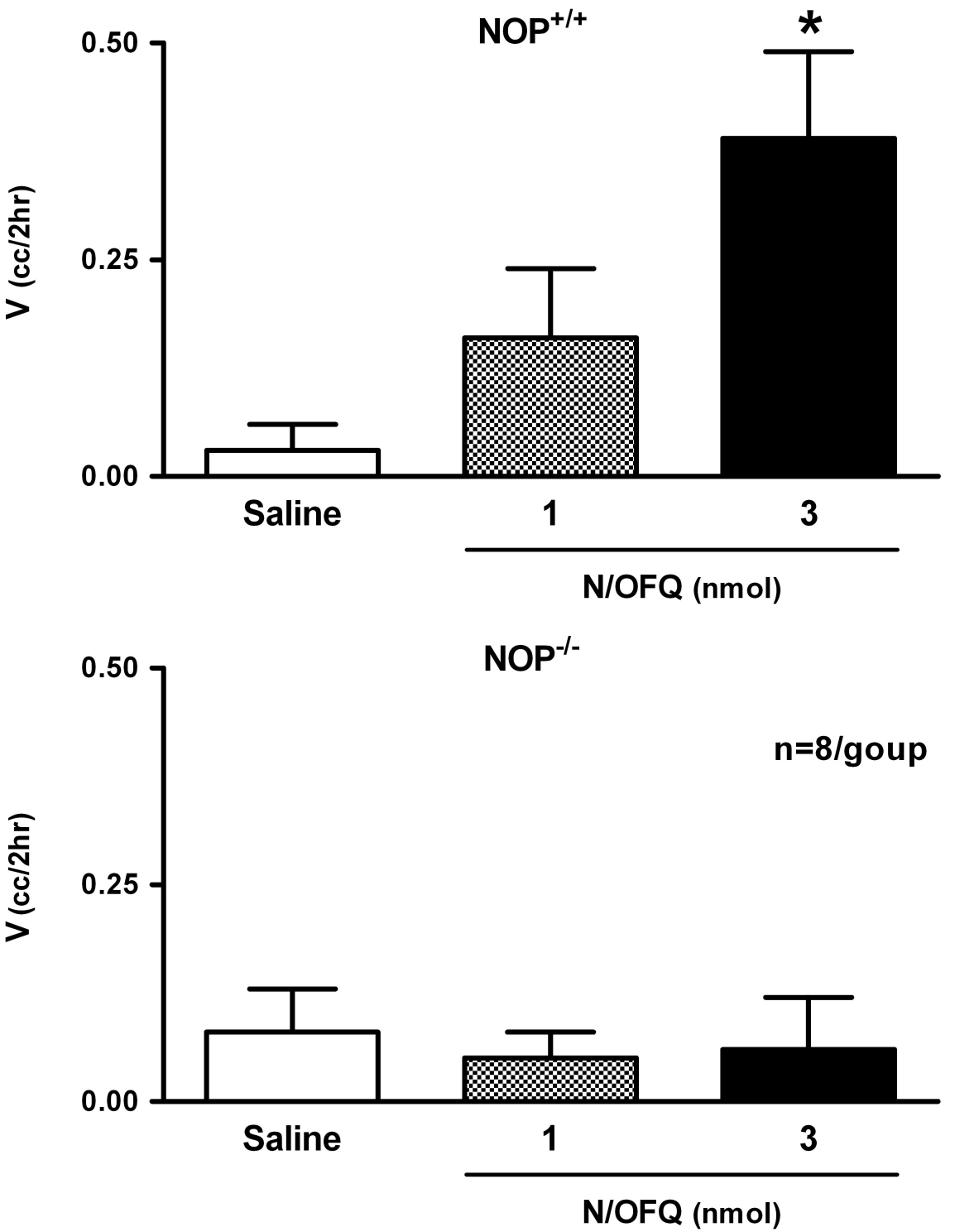
NOP+/+
*
0.50
0.25
0.00
V (cc/2hr)
Saline
1
3
N/OFQ (nmol)
NOP-/-
n=8/goup
0.50
0.25
0.00
V (cc/2hr)
Saline
1
3
N/OFQ (nmol)

Figure 4a

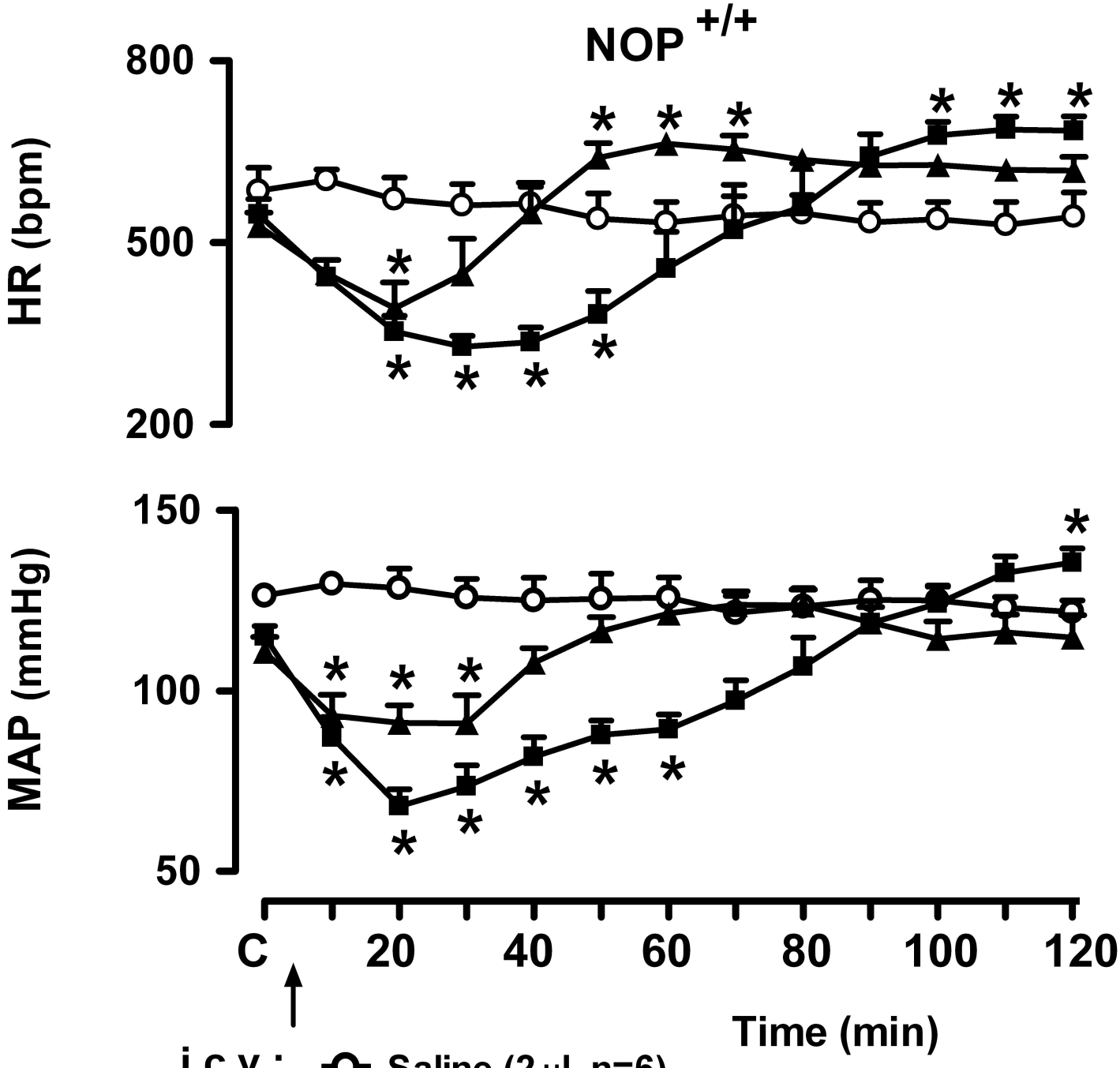

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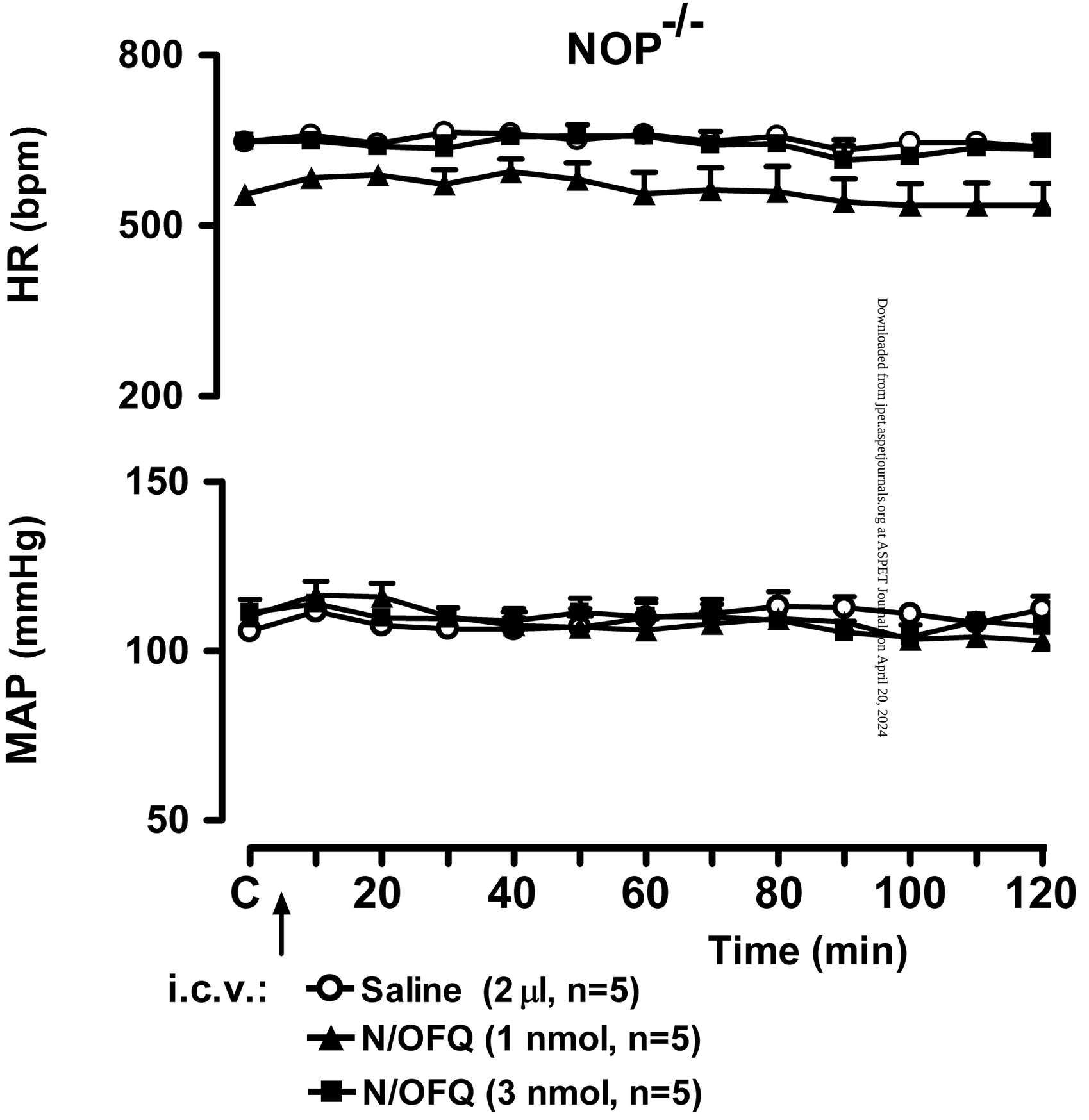
Figure 4b



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