Hypothalamic paraventricular nucleus differentially supports lumbar and renal sympathetic outflow in water-deprived rats

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The present study sought to determine whether the hypothalamic paraventricular nucleus (PVN) contributes in a time-dependent manner to the differential patterning of lumbar and renal sympathetic nerve activity (SNA) in water-deprived rats. Mean arterial blood pressure (MAP) and both lumbar SNA (LSNA) and renal SNA (RSNA) were recorded simultaneously in control, 24 and 48 h water-deprived rats, and the PVN was inhibited bilaterally with microinjection of the $GABA_A$ agonist muscimol (100 pmol in 100 nl per side). Inhibition of the PVN significantly decreased RSNA in 48 h water-deprived rats but not in 24 h water-deprived or control rats (48 h, $-17 \pm 4\%$; 24 h, $-2 \pm 5\%$; control, $4 \pm 6\%$; $P < 0.05$). In addition, injection of muscimol significantly decreased LSNA in 48 and 24 h water-deprived rats but not in control rats (48 h, $-41 \pm 4\%$; 24 h, $-14 \pm 6\%$; control, $-3 \pm 2\%$; $P < 0.05$). Interestingly, the decrease in LSNA was significantly greater than the decrease in RSNA of 24 and 48 h water-deprived rats ($P < 0.05$). Inhibition of the PVN also significantly decreased MAP to a greater extent in 48 and 24 h water-deprived rats compared to control rats (48 h, -34 ± 5 mmHg; 24 h, -26 ± 4 mmHg; control, -15 ± 3 mmHg; $P < 0.05$). When 48 h water-deprived rats were acutely rehydrated by giving access to tap water 2 h before experiments, inhibition of the PVN with muscimol did not alter LSNA ($-12 \pm 8\%$) or RSNA ($7 \pm 4\%$) but did produce a small decrease in MAP (-15 ± 4 mmHg) that was not different from control rats. In a parallel set of experiments, acute rehydration of 48 h water-deprived rats significantly attenuated the increased Fos immunoreactivity in PVN neurones that project to the spinal cord or rostral ventrolateral medulla. Collectively, the present findings suggest that PVN autonomic neurones are synaptically influenced during water deprivation, and that these neurones differentially contribute to LSNA and RSNA in water-deprived rats.

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Neurones in the hypothalamic paraventricular nucleus (PVN) play a pivotal role in the regulation of sympathetic nerve activity (SNA) and arterial blood pressure (ABP). This is accomplished through both mono- and polysynaptic pathways to several autonomic regions of the central nervous system, including the dorsomedial medulla, rostral ventrolateral medulla (RVLM) and spinal intermediolateral cell column (IML) (Saper *et al.* 1976; Swanson & Kuypers, 1980; Strack *et al.* 1989; Shafton *et al.* 1998; Pyner & Coote, 2000; Cano *et al.* 2001, 2004; Stocker *et al.* 2004*a*). Electrical and chemical stimulation of the PVN has been reported to produce both increases and decreases in SNA and ABP (Porter & Brody, 1986; Katafuchi *et al.* 1988; Martin *et al.* 1991; Martin & Haywood, 1992, 1993; Deering & Coote, 2000; Kenney *et al.* 2001). Electrophysiological studies *in vivo* have demonstrated the PVN contains neurones projecting to the hindbrain and/or spinal cord that display sympathetic- and cardiac cycle-related discharge (Barman, 1990; Chen & Toney, 2003). In addition, the discharge of PVN parvocellular neurones is altered by a variety of inputs, including changes in ABP, blood volume, plasma osmolality (pOsm) and circulating angiotensin II (Ang II) levels (Ferguson, 1988; Lovick & Coote, 1988; Bains & Ferguson, 1995; Chen & Toney, 2000; Toney *et al.* 2003). These discharge responses probably have functional significance, since changes in SNA and/or ABP during increases in pOsm, angiotensin II levels and blood volume depend upon neurotransmission in the PVN (Gutman *et al.* 1988; Lovick *et al.* 1993; Haselton *et al.* 1994;

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Ferguson & Washburn, 1998; Chen & Toney, 2001; Yang & Coote, 2003). Thus, a variety of hormonal and visceral signals alter the excitability of PVN parvocellular neurones to influence SNA and ABP.

Water deprivation is a physiological challenge that decreases intravascular volume and increases pOsm and circulating Ang II levels (Stocker *et al.* 2002, 2004*b*; Brooks *et al.* 2004*b*) and therefore provides a model for investigation of the central neural circuitry and cellular mechanisms contributing to elevated SNA during conditions of neurohumoral activation. Recently, we demonstrated that PVN autonomic neurones are synaptically influenced during water deprivation (Stocker *et al.* 2004*a*), and these neurones support renal SNA (RSNA) and ABP in water-deprived rats (Stocker *et al.* 2004*b*). While the sympathoadrenal activation during water deprivation is reflected by increases in circulating catecholamines (Thornton & Proppe, 1988), tachycardia (Scrogin *et al.* 1999, 2002; Stocker *et al.* 2004*b*) and an exaggerated depressor response to ganglionic blockade (Stocker *et al.* 2004*b*), indirect comparisons of basal SNA suggest that lumbar SNA (LSNA) may be more elevated than RSNA in water-deprived rats (Scrogin *et al.* 1999, 2002). Thus, the neurohumoral activation during water deprivation may alter the excitability of specific neural pathways to produce differential changes in sympathetic outflow to various end organs.

The purpose of the present study was to determine whether the PVN contributes in a time-dependent manner to the differential patterning of sympathetic outflow during water deprivation. That is, would inhibition of the PVN produce differential effects between LSNA and RSNA as the duration of water deprivation and/or stimulus intensity (pOsm, blood volume, etc.) increased? In these experiments, LSNA and RSNA were recorded simultaneously in control, 24 and 48 h water-deprived rats and the PVN was inhibited bilaterally with microinjection of the $GABA_A$ agonist muscimol. Since rehydration rapidly reverses or attenuates homeostatic responses to water deprivation (i.e. vasopressin secretion; Thrasher *et al.* 1981, 1987; Appelgren *et al.* 1991; De Luca *et al.* 2002; Stricker *et al.* 2002; Ji *et al.* 2005), we sought to determine whether acute rehydration of water-deprived rats would attenuate: (1) the exaggerated depressor response to ganglionic blockade; (2) the changes in SNA that occur after inhibition of the PVN; and (3) the increased Fos immunoreactivity in PVN autonomic neurones.

Methods

Animals

Adult male Sprague–Dawley rats (Charles River Laboratories) weighing 250–375 g were housed in a temperature-controlled room (22–23°C) with a 14 h:10 h light:dark cycle (lights on at 7 am). Tap water and laboratory chow (Harlan Teklad LM-485, 0.3% NaCl) were available *ad libitum* except where noted. All experimental and surgical procedures conformed with the National Institutes of Health Guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Effect of bilateral microinjection of muscimol into the PVN on mean arterial pressure (MAP), RSNA and LSNA in control, 24 and 48 h water-deprived rats and rehydrated rats

Rats were deprived of water but not food for 24 or 48 h, whereas control rats had continuous access to both food and water. A fourth group of rats were deprived of water for 48 h but given access to tap water for 2 h prior to experiments. This group will be referred to as the 'rehydrated' group.

On the day of experiments, rats were anaesthetized with a mixture of α-chloralose (80 mg kg^{-1}) and urethane (800 mg kg^{-1}) (Sigma-Aldrich, St. Louis, MO, USA) given intraperitoneally. Catheters were implanted in the femoral artery and vein (PE-50 tubing) for recording ABP and administration of drugs, respectively. After tracheal cannulation, neuromuscular blockade was induced with gallamine triethiode (25 mg kg^{-1} h^{-1}, I.V.) and the lungs were artificially ventilated with oxygen-enriched room air. End-tidal partial pressure of CO_2 was maintained between 4.5 and 5.5% by adjusting ventilation rate (70–100 breaths min^{-1}) and/or tidal volume (2–3 ml). Body temperature was maintained at 37 ± 1°C with a water-circulating pad. An adequate depth of anaesthesia was assessed by absence of a withdrawal reflex (before neuromuscular blockade) or a pressor response to foot pinch. Supplemental doses of anaesthetic (10% of initial dose) were given as necessary.

To record both LSNA and RSNA simultaneously, a left renal and a lumbar postganglionic sympathetic nerve were isolated though a retroperitoneal incision, and each was placed onto a separate stainless-steel electrode (0.125 mm o.d., A-M systems, Everett, WA, USA). Each nerve and electrode was covered with a silicon-based impression material (Super-Dent Light, Carlisle Laboratories, New York, NY, USA). Nerve signals were obtained using a high–impedance probe connected to an AC amplifier equipped with half-amplitude filters (bandpass, 30–3000 Hz) and a 60 Hz notch filter. Then the signal was amplified (10 000–20 000×), full-wave rectified and integrated (10 ms time constant) using a moving averager (MA-821RSP, Cwe Inc., Ardmore, PA, USA), and digitized at a frequency of 1000 Hz using a 1401plus analog-to-digital converter and Spike 2 software (Cambridge Electronic Design, Cambridge, UK). At the end of each experiment, background noise was determined

by the average value of integrated voltage over 5 min after a bolus injection of hexamethonium (30 mg kg^{-1}, I.V.) to block ganglionic transmission.

Microinjection of muscimol into the PVN was performed as previously described (Stocker *et al.* 2004*b*). Briefly, rats were placed into a stereotaxic head frame with the skull level between bregma and lambda. A small craniotomy was performed to remove bone overlying the cortex in order to allow a glass micropipette to be lowered into the PVN. Baseline variables were allowed to stabilize for at least 1 h before any microinjection was performed. Then, a blood sample (0.4 ml) was collected from the arterial line into microcentrifuge tubes containing heparin (6 i.u.) for determination of pOsm, haematocrit and plasma protein concentration. The blood sample was replaced with an equal volume of isotonic saline. Variables were allowed to stabilize for an additional 15 min before microinjections were performed. Then, muscimol (100 pmol dissolved in 100 nl isotonic saline; Sigma) was microinjected bilaterally into the PVN using a pneumatic picopump (WPI) connected to a single-barrelled glass micropipette (tip o.d. 30–50 μm) at the following stereotaxic coordinates: 1.6–2.0 mm caudal to bregma, 0.5–0.7 mm lateral to the mid-line and 7.8 mm ventral to the dura. Injections were made on one side over 30 s, the pipette removed and then lowered into the contralateral PVN. The two injections were approximately 2–3 min apart.

At the conclusion of microinjection experiments, animals were killed by injection of 4 M KCl (0.5 ml, I.V.) and 100 nl of 2% Chicago Sky Blue dissolved in isotonic saline was microinjected bilaterally into the PVN using the same coordinates and micropipettes as used for microinjections of muscimol. Brains were postfixed in 4% paraformaldehyde and then sectioned at 50 μm using a cryostat. The outermost dye distribution for each rat was mapped onto standard sections from the atlas of Paxinos & Watson (1998). Then sections from similar rostral–caudal levels were overlaid to obtain the largest distribution of dye for each group.

Effect of ganglionic blockade on MAP in control, 24 and 48 h water-deprived rats and rehydrated rats

To determine the contribution of the sympathetic nervous system to the maintenance of ABP, additional experiments were performed in a separate group of control, rehydrated and 24 and 48 h water-deprived rats. Rats were anaesthetized, neuromuscular blockade was induced, they were artificially ventilated and prepared for recording of ABP and SNA as described above. At least 1 h after all surgical procedures were completed, baseline ABP and SNA were recorded for a minimum of 15 min. Blood samples (0.4 ml) were collected from the arterial line as described above approximately 15 min before water-deprived and control rats were given a bolus injection of hexamethonium (30 mg kg^{-1}, I.V.). At the conclusion of these experiments, animals were killed by injection of 4 M KCl (0.5 ml, I.V.).

Effect of rehydration on Fos immunoreactivity in PVN autonomic neurones of water-deprived rats

In a parallel set of experiments, we examined the number of Fos immunoreactive nuclei in PVN autonomic neurones in control, 48 h water-deprived and rehydrated rats. Two weeks before experiments, rats were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, I.P.) and received a microinjection of 4% Fluorogold (FG, 50 nl; Fluorochrome, Denver, CO, USA) into the IML at T1–T3 as previously described (Stocker *et al.* 2004*a*). In addition, a second retrograde tracer, cholera toxin subunit B (CTB, 0.25% in isotonic saline, 20–30 nl; List Biological Laboratories, Campbell, CA, USA), was microinjected into the RVLM with the incisor bar positioned 11 mm below the interaural line. A small portion of the occipital bone was removed, and the area postrema visualized. A glass micropipette was angled 20 deg rostrally and lowered into the RVLM at the following coordinates in reference to the dorsal surface and caudal tip of area postrema: 1.8 mm lateral, 1.6–2.0 mm rostral and 2.8 mm ventral. Initially, the RVLM was located functionally by an increase in MAP $>$25 mmHg in response to microinjection of L-glutamate (2 nmol in 25 nl). Then the pipette was removed from the brain, emptied, filled with CTB and returned to the same position in the RVLM. All microinjections were performed over 1 min with glass micropipettes (o.d. 30–50 μm) connected to a pneumatic picopump (WPI). MAP was recorded through a cathether placed into the femoral artery and then removed after the microinjections. Once the overlying musculature and skin was sutured, each rat was given ampicillin (100 mg kg^{-1}, I.M.) and returned to its home cage.

Approximately 2 weeks after microinjection of the retrograde tracers, rats were randomly assigned to one of three groups: 48 h water deprivation, 48 h water deprivation plus 2 h access to tap water (rehydration) as described above, and control, which had continuous access to water and food. After 48 h water deprivation and/or rehydration, rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, I.P.), and blood (0.4 ml) was collected from the left ventricle into microcentrifuge tubes containing heparin (10 i.u.) using a 23 gauge needle. Samples were used for determination of haematocrit, pOsm and plasma protein levels as described below. Immediately after the blood sample was collected, rats were perfused transcardially with heparinized isotonic saline (30 i.u. ml^{-1}, 100 ml) followed by 4% paraformaldehyde (4°C, 300 ml) dissolved in 0.1 M phosphate-buffered saline (PBS). Brains were removed and postfixed in 4%

paraformaldehyde at 4°C for 1–4 days. The forebrain and hindbrain were sectioned at 30 μm using a sliding microtome. Sections were collected into five separate sets and stored in vials containing RNAse-free cryoprotectant at −25°C.

Fos and CTB immunocytochemistry was performed as previously described (Stocker *et al.* 2004*a*). Briefly, sections were incubated with a rabbit polyclonal anti-Fos antibody (1:10 000; Oncogene Research Products, San Diego, CA, USA) at 4°C for 72 h followed by biotinylated donkey antirabbit IgG for 2 h at room temperature (1:250). Tissue was reacted with an avidin–peroxidase conjugate (ABC Vectastain Kit, Vector Laboratories, Burlingame, CA, USA) in Tris buffer containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma), 2.5% nickel sulphate hexahydrate (Sigma) and 0.006% hydrogen peroxide. The reaction was terminated with several rinses in PBS. CTB was visualized by immunofluorescence. Briefly, sections were incubated in a goat anticholeragenoid antibody (1:3000; List Biological Laboratories, Campbell, CA, USA) for 48 h at 4°C followed by an overnight incubation in CY3 donkey antigoat IgG (1:250) at 4°C. CTB injection sites targeted at the RVLM were visualized by similar methods. All antibody incubations were performed in PBS containing 0.3% Triton X-100 and 1% donkey serum. Donkey serum and secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). All sections were mounted on glass slides, dehydrated in graded concentrations of alcohol, cleared in xylene and coverslipped with Cytoseal 60 (Fisher Scientific).

Fos-positive neurones in the PVN were examined under light microscopy. The PVN was sampled at three rostral–caudal levels as previously described (Stocker *et al.* 2004*a*). Level 1 was the most rostral and consisted of the dorsal parvocellular, medial parvocellular and ventrally located posterior magnocellular subnuclei. Level 2 displayed a prominent posterior magnocellular region and both dorsal and ventrolateral parvocellular divisions. Level 3 was the most caudal and consisted of the medial and lateral parvocellular divisions. Based upon previous anatomical studies (Swanson & Kuypers, 1980; Stocker *et al.* 2004*a*), these levels of the PVN have efferent projections to the RVLM and IML. Digital images from the PVN were collected using an Olympus IX50 microscope connected to a Spot camera (Spot RT Slider, Diagnostic Instruments, Inc., Sterling Heights, MI, USA) using Spot imaging software (version 3.24). The number of Fos-positive and retrogradely labelled neurones was quantified by an individual blind to the experimental groups. For purposes of visualization, FG- and CTB-labelled neurones were pseudocoloured green and red, respectively, and images were digitally overlaid. Accordingly, double-labelled neurones (FG + CTB) appear yellow in the merged images.

Determination of hematocrit, pOsm and plasma protein

Hematocrit was determined from duplicate capillary tubes measured with a Lancer microhaematocrit tube reader (St Louis, MO, USA). Blood samples were centrifuged (10 000*g*, 30 s), and pOsm was determined from duplicate plasma samples analysed using a vapour pressure osmometer (model no. 5520, Wescor Inc., Logan, UT, USA). Plasma protein concentration was determined by protein refractometry (Refractometer Veterinary ATC, VWR International, Suwanee, CA, USA).

Data analysis

All values of SNA and MAP were measured as a 60 s average of each variable. Changes in integrated SNA were calculated by subtracting background noise taken from a 5 min average after hexamethonium injection. Then these values were expressed as a percentage of the baseline value (equal to 100%) that was calculated from values averaged at −5, −2.5 and −1 min before the microinjection. The effects of bilateral microinjection of muscimol into the PVN on SNA and MAP were analysed by a one-way ANOVA (Systat 10.2, Systat Software, Inc., Richmond, CA, USA). When significant F values were obtained, independent t tests with layered Bonferroni corrections were performed to compare differences between groups. Data from ganglionic blockade experiments and pOsm, haematocrit and plasma protein concentrations were analysed similarly. Linear regression analysis was performed using SigmaPlot 2000 (SPSS, Inc., Chicago, IL, USA).

The number of Fos-positive nuclei in each subdivision of the PVN was compared between water-deprived, rehydrated and control rats using a two-way ANOVA (group × subdivision) followed by a layered Bonferonni analysis. The number of retrogradely labelled or retrogradely labelled plus Fos-positive neurones was analysed similarly. When data were expressed as a percentage, groups were compared by a Mann–Whitney U test.

A P value less than 0.05 was considered statistically significant for all comparisons.

Results

Effect of bilateral microinjection of muscimol into the PVN on MAP, RSNA and LSNA in control, 24 and 48 h water-deprived rats

Two major goals of the present study were to determine: (1) whether inhibition of the PVN produced differential changes in RSNA and LSNA, and (2) whether the magnitude of these effects depended on the duration of water deprivation. As expected (Stocker *et al.* 2004*b*),

microinjection of muscimol into the PVN of a control rat did not alter RSNA or LSNA but did produce a small decrease in ABP (Fig. 1). In marked contrast, injection of muscimol into the PVN of a 48 h water-deprived rat decreased both RSNA and LSNA, and this was associated with a prompt decrease in ABP (Fig. 2). In 24 h water-deprived rats, LSNA and ABP but not RSNA decreased after injection of muscimol into the PVN (trace not shown).

Figure 3 summarizes the group responses for control, 24 and 48 h water-deprived rats. Microinjection of muscimol significantly decreased MAP in all groups; however, the decrease in 24 and 48 h water-deprived rats was significantly greater than that of control rats (Fig. 3).

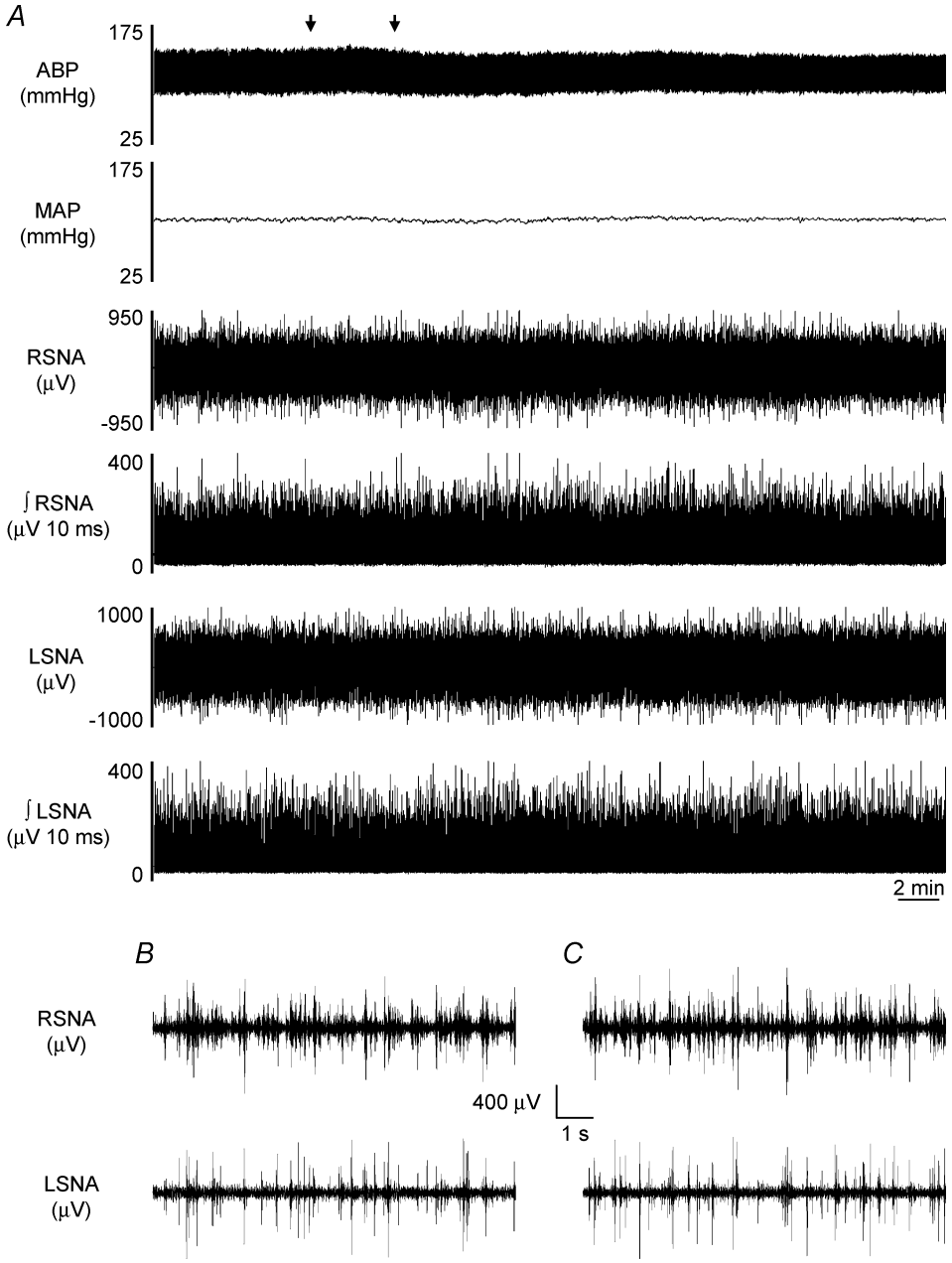

Figure 1. ABP, MAP, RSNA, ∫RSNA, LSNA and ∫LSNA in a control rat receiving bilateral microinjection of the $GABA_A$ agonist muscimol (100 pmol in 100 nl per side) into the PVN

A, microinjection of muscimol into the PVN of a control rat did not significantly alter LSNA or RSNA but did produce a small decrease in MAP. Arrows indicate microinjection of muscimol into the PVN. *B* and *C* show specimen data from 10 s before and after, respectively, microinjection of muscimol into the PVN. Summary data are presented in Fig. 3.

Baseline MAP did not differ between groups (Table 1). In addition, inhibition of the PVN with muscimol significantly decreased RSNA in 48 h water-deprived rats but not in control or 24 h water-deprived rats (Fig. 3). By contrast, injection of muscimol significantly decreased LSNA in both 24 and 48 h water-deprived rats compared to that of control rats; however, the decrease was significantly greater in 48 compared to 24 h water-deprived rats (Fig. 3). When the changes in RSNA and LSNA were compared within groups, LSNA decreased significantly more than RSNA in both 24 and 48 h water-deprived rats.

Water deprivation also produced time-dependent increases in pOsm, haematocrit and plasma protein concentration (Table 1). That is, 24 and 48 h

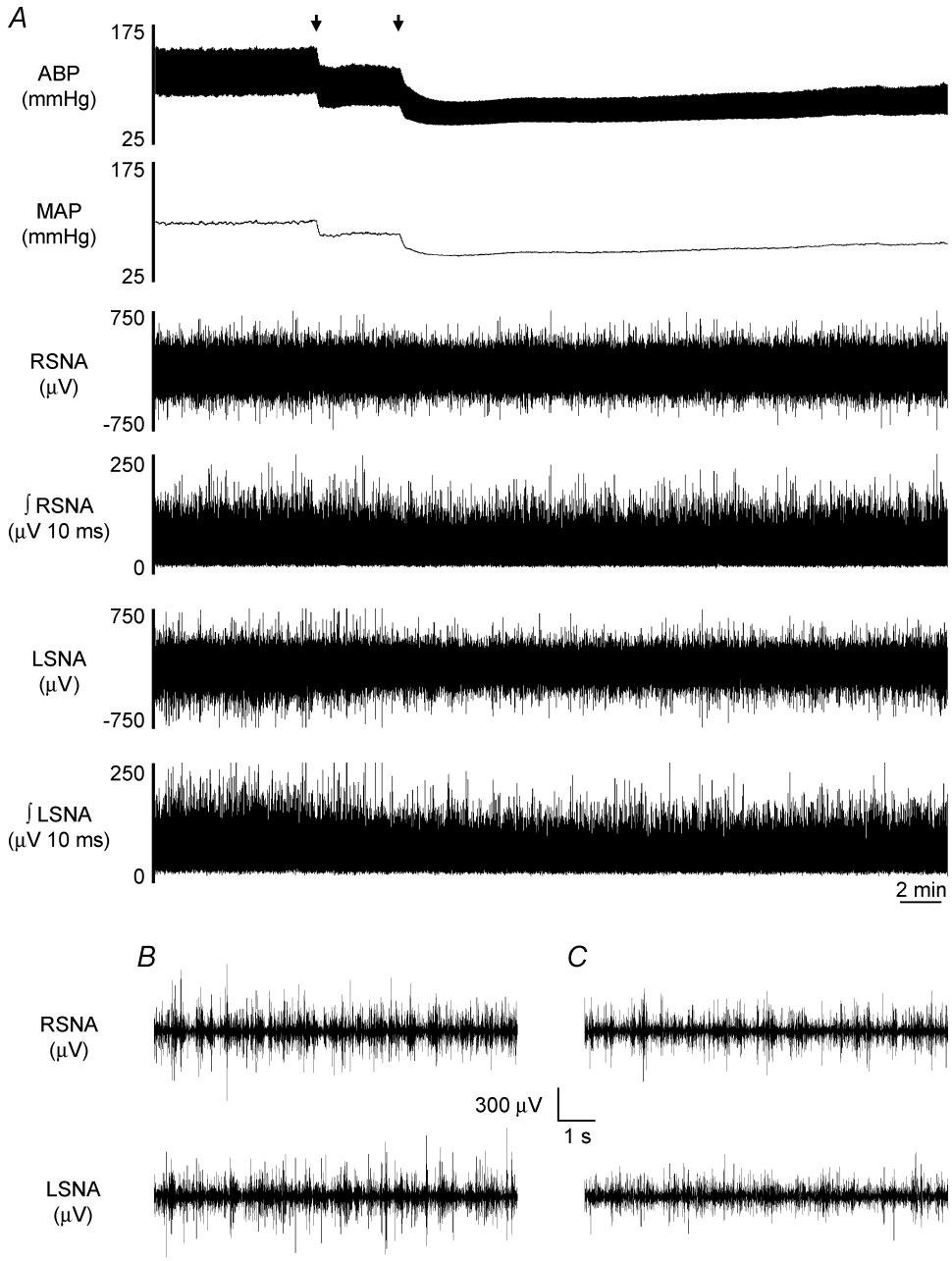

Figure 2. ABP, MAP, RSNA, ∫RSNA, LSNA and ∫LSNA in a 48 h water-deprived rat receiving bilateral microinjection of the $GABA_A$ agonist muscimol (100 pmol in 100 nl per side) into the PVN
A, in marked contrast to a control rat (see Fig. 1), microinjection of muscimol into the PVN of a 48 h water-deprived rat produced a reduction in MAP, RSNA and LSNA from baseline values. Arrows indicate microinjection of muscimol into the PVN. *B* and *C* show specimen data from 10 s before and after, respectively, microinjection of muscimol into the PVN. Summary data are presented in Fig. 3.

Table 1. Baseline MAP, pOsm, haematocrit and plasma protein concentrations of control, 24 and 48 h water-deprived rats and rehydrated rats that received bilateral microinjection of muscimol into the PVN or ganglionic blockade with hexamethonium (30 mg kg^{-1}, I.V.)

Group	*n*	Baseline MAP (mmHg)	pOsm (mosmol l^{-1})	Haematocrit (%)	Plasma protein (g dl^{-1})
Microinjection of muscimol into the PVN					
Control	6	105 ± 2	290 ± 2	49 ± 1	5.3 ± 0.1
24 h water deprivation	7	107 ± 3	303 ± 1*	53 ± 1*	6.2 ± 0.1*
48 h water deprivation	7	101 ± 4	311 ± 1*†‡	58 ± 1*†	7.0 ± 0.1*†‡
Rehydration	9	111 ± 3	293 ± 2	55 ± 1*	5.4 ± 0.1
Ganglionic blockade					
Control	8	97 ± 3	291 ± 2	50 ± 1	5.1 ± 0.1
24 h water deprivation	8	101 ± 4	300 ± 2*	55 ± 1*	6.4 ± 0.1*
48 h water deprivation	8	103 ± 2	312 ± 2*†‡	60 ± 1*†	7.0 ± 0.1*†‡
Rehydration	8	96 ± 6	292 ± 1	55 ± 1*	5.4 ± 0.1

Values are means ± S.E.M. *Significant difference from control rats ($P < 0.05$), †significant difference between 24 and 48 h water-deprived rats ($P < 0.01$), ‡ significant difference between 48 h water-deprived and rehydrated rats ($P < 0.01$).

water-deprived rats had significantly elevated pOsm, haematocrit and plasma protein concentrations compared to control rats, and these values were significantly higher in 48 than in 24 h water-deprived rats. Moreover, a linear regression analysis across control, 24 and 48 h water-deprived rats revealed that the peak changes in MAP, RSNA and LSNA after microinjection of muscimol into the PVN correlated with haematocrit, plasma protein concentration and pOsm (Fig. 4).

Effect of bilateral microinjection of muscimol into the PVN on MAP, RSNA and LSNA in rehydrated rats

To determine whether acute rehydration would attenuate the inhibitory effect of muscimol injection into the PVN on SNA and ABP following water deprivation, a separate group of rats were water deprived for 48 h and given access to tap water 2 h before experiments. During this time, rehydrated rats ingested 30.4 ± 1.4 ml of water, which resulted in pOsm and plasma protein concentrations that were significantly lower than those of 48 h water-deprived rats but were not different from those values of control rats (Table 1). Bilateral microinjection of muscimol into the PVN of rehydrated rats did not alter LSNA or RSNA but did produce a small decrease in ABP (Fig. 5). Figure 6 summarizes the group responses. Acute rehydration significantly attenuated the decrease in MAP, RSNA and LSNA compared to those responses of 48 h water-deprived rats (Fig. 6). In fact, peak changes in MAP, RSNA and LSNA of rehydrated rats were not different from those of control rats (Fig. 6).

All microinjection sites in the PVN were similar to those published previously by our laboratory (Stocker *et al.* 2004*b*). The spread of injected dye impacted the dorsal, ventrolateral and lateral parvocellular and magnocellular subnuclei throughout the rostral–caudal extent of the PVN without rupturing the wall of the third ventricle. Dye did not consistently spread to a nucleus outside the PVN.

Effect of ganglionic blockade on MAP in control, 24 and 48 h water-deprived and rehydrated rats

To determine the contribution of the sympathetic nervous system to the maintenance of ABP, separate groups of control, 24 and 48 h water-deprived rats and

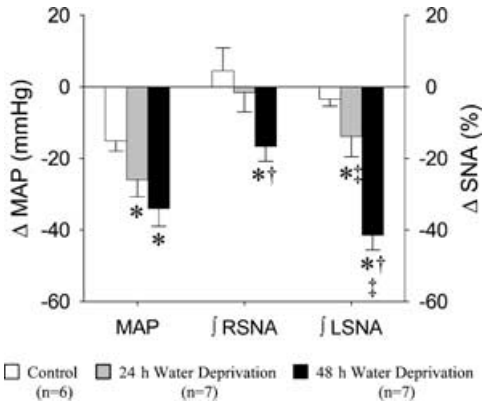

Figure 3. Peak decreases in MAP, ∫RSNA and ∫LSNA of control, 24 and 48 h water-deprived rats after bilateral microinjection of muscimol into the PVN

Microinjection of muscimol significantly decreased MAP in all groups; however, the decrease in MAP was significantly greater in 24 and 48 h water-deprived rats compared to control rats. Injection of muscimol significantly decreased RSNA in 48 h water-deprived rats but not in control or 24 h water-deprived rats. The same treatment decreased LSNA in both 24 and 48 h water-deprived rats, but the decrease in LSNA was significantly greater in 48 h water-deprived rats. When LSNA and RSNA responses were compared within groups, inhibition of the PVN decreased LSNA significantly more than RSNA in both 24 and 48 h water-deprived rats. *Significant difference from control rats ($P < 0.05$), †significant difference between 24 and 48 h water-deprived rats ($P < 0.05$), ‡ significant difference from RSNA within the same group ($P < 0.05$). Values are means ± S.E.M.

rehydrated rats were administered the ganglionic blocker hexamethonium. As expected (Stocker *et al.* 2004*b*), hexamethonium significantly decreased MAP in all groups, and these responses were time-dependent (Fig. 7). That is, the drop in MAP was greater as the duration of water deprivation increased. When 48 h water-deprived rats were given access to water, these rehydrated rats ingested 29.4 ± 1.3 ml and no longer displayed an exaggerated fall in MAP in response to hexamethonium. In fact, the fall in MAP of rehydrated rats after hexamethonium was not different from that of control rats (Fig. 7). Baseline MAP was not different among groups (Table 1). Again, water deprivation increased pOsm, haematocrit and plasma protein concentrations in a time-dependent manner, and acute rehydration restored pOsm and plasma protein concentrations (Table 1).

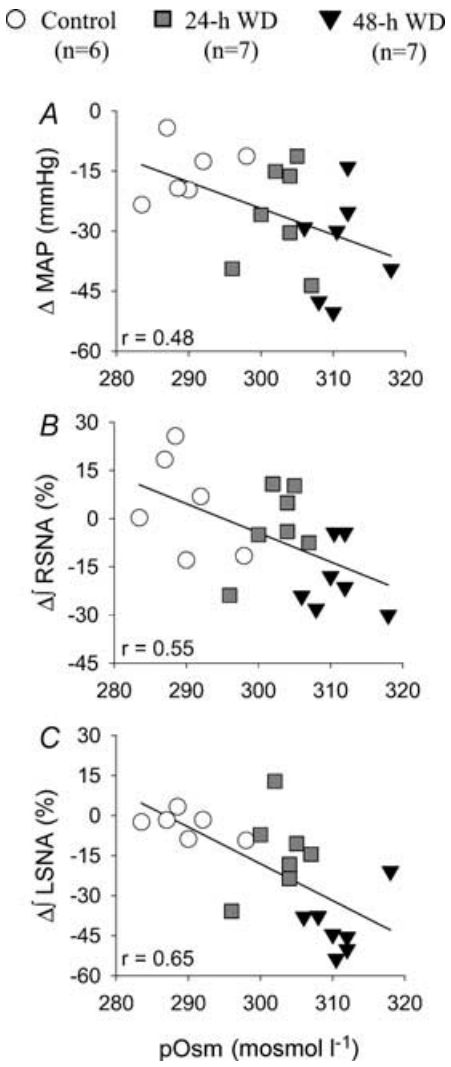

Figure 4. Scatter plots of peak changes in MAP, ∫RSNA and ∫LSNA plotted as a function of pOsm in control, 24 and 48 h water-deprived (WD) rats

A linear regression analysis revealed that the peak changes in MAP (*A*), RSNA (*B*) and LSNA (*C*) significantly correlated with pOsm (all values of $r > 0.48$, $P < 0.05$). In addition, these peak changes also correlated with haematocrit and plasma protein concentrations (plots not shown, all values of $r > 0.44$; $P < 0.05$). Integrated SNA values were expressed as a percentage of baseline (see Methods).

Effect of rehydration on Fos immunoreactivity in PVN autonomic neurones of water-deprived rats

In a parallel set of experiments, we determined the effect of acute rehydration on Fos immunoreactivity in spinally-projecting and RVLM-projecting PVN neurones of 48 h water-deprived rats. As expected (Stocker *et al.* 2004*a*), 48 h water-deprived rats had a significantly greater number of Fos-positive nuclei in the dorsal parvocellular region at level 1, both dorsal and ventrolateral parvocellular regions at level 2, and the lateral parvocellular region at level 3 (Fig. 8). When 48 h water-deprived rats were given access to water, rats ingested 33.0 ± 1.7 ml and had a significantly reduced number of Fos-immunoreactive nuclei throughout the parvocellular divisions of the PVN (Fig. 8). In fact, the number of Fos-positive nuclei in rehydrated rats was not different from those values in control rats at every level and subdivision of the PVN. Again, the ingestion of water restored pOsm, haematocrit and plasma protein concentrations, and values were not different from those of control rats (data not shown).

Since rehydration of 48 h water-deprived rats significantly reduced the number of Fos-positive nuclei in autonomic regions of the PVN, we determined whether the decrease in Fos immunoreactivity occurred in PVN neurones with projections to the spinal cord and/or RVLM. In agreement with our previous findings (Stocker *et al.* 2004*a*), 48 h water deprivation significantly increased the number of spinally-projecting and RVLM-projecting neurones that were Fos positive, particularly in the ventrolateral and lateral parvocellular divisions of the ipsilateral PVN (Fig. 9). Acute rehydration of 48 h water-deprived rats significantly reduced the number of Fos-positive nuclei among both spinally-projecting and RVLM-projecting PVN neurones (Fig. 9). In fact, the effect of 48 h water deprivation on Fos immunoreactivity was abolished or significantly attenuated in both spinally-projecting and RVLM-projecting neurones in every subdivision and level of the PVN. Although a greater percentage of spinally projecting PVN neurones were Fos positive in 48 h water-deprived *versus* control rats (control, $0.8 \pm 0.8\%$; water deprived, $8.0 \pm 2.3\%$; $P < 0.05$), this effect was eliminated when 48 h water-deprived rats were rehydrated (rehydrated, $1.3 \pm 0.4\%$). Similarly, a greater percentage of RVLM-projecting PVN neurones were Fos positive in 48 h water-deprived rats compared to control rats (control, $2.2 \pm 0.7\%$; water deprived, $18.7 \pm 2.7\%$; $P < 0.01$), and rehydration again attenuated

this response (rehydrated, $7.8 \pm 1.7\%$). With regard to PVN neurones that project to both the spinal cord and RVLM, a significantly greater percentage of these neurones were Fos positive in 48 h water-deprived rats compared to control or rehydrated rats (control, $1.0 \pm 0.7\%$; water deprived, $4.7 \pm 1.5\%$; rehydrated, $1.5 \pm 1.1\%$; $P < 0.05$). The absolute numbers of PVN neurones retrogradely labelled from the spinal cord and/or RVLM and injection site targeted at the RVLM were not different across groups (data not shown) and were similar to those reported previously (Stocker *et al.* 2004*a*).

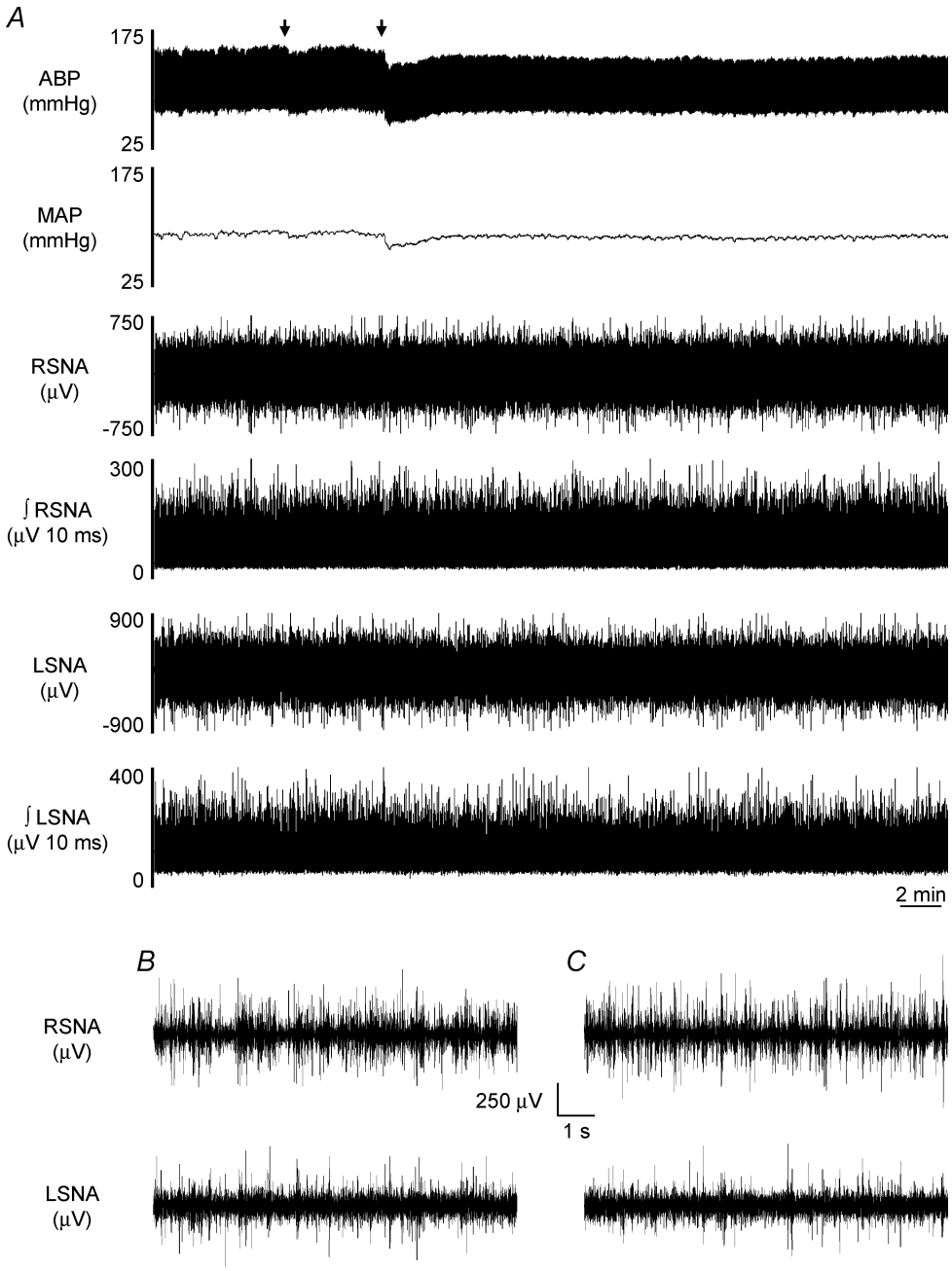

Figure 5. ABP, MAP, RSNA, ∫RSNA, LSNA and ∫LSNA in a rehydrated rat receiving bilateral microinjection of the $GABA_A$ agonist muscimol (100 pmol in 100 nl per side) into the PVN
A, in marked contrast to a 48 h water-deprived rat, microinjection of muscimol into the PVN of a rehydrated rat did not alter LSNA or RSNA but did produce a small decrease in MAP. Arrows indicate microinjection of muscimol into the PVN. *B* and *C* show specimen data from 10 s before and after, respectively, microinjection of muscimol into the PVN. Summary data are presented in Fig. 6.

Discussion

Previous studies have demonstrated that PVN autonomic neurones are synaptically influenced during water deprivation and contribute to RSNA and the maintenance of ABP (Stocker *et al.* 2004*a*,*b*). Since indirect measurements of basal SNA suggest that water deprivation increases LSNA more than RSNA (Scrogin *et al.* 1999, 2002), we sought to determine whether the PVN contributes to the differential patterning of LSNA and RSNA as the duration of water deprivation or stimulus intensity (pOsm, blood volume, etc.) increases. The present study provides several new key observations: (1) in addition to decreasing RSNA, inhibition of the PVN in water-deprived rats decreases LSNA and the magnitude of this effect was greater than that of RSNA, (2) the contribution of the PVN to LSNA and RSNA was time-dependent, the magnitude of the responses increased as the duration of water deprivation and/or stimulus intensity increased, and (3) acute rehydration attenuated the contribution of the PVN to LSNA and RSNA as well as the increased Fos immunoreactivity in PVN neurones with projections to the spinal cord and RVLM.

Water deprivation produces time-dependent changes in intravascular volume, pOsm and plasma Ang II and vasopressin levels (Fejes-Tâoth *et al.* 1985; Stocker *et al.* 2002, 2004*a*; Brooks *et al.* 2004). Therefore, we hypothesized that as the duration of water deprivation and/or stimulus intensity (pOsm, blood volume, etc.) increased, this would be associated with time-dependent increases in SNA. Indeed, the present findings demonstrate that the contribution of sympathetic outflow to the maintenance of ABP was time dependent in water-deprived rats, since the magnitude of the depressor response to ganglionic blockade increased as the time of water deprivation increased. If these time-dependent increases in SNA in water-deprived rats depend on the tonic activity of PVN neurones, we reasoned that inhibition of the PVN should decrease SNA and this response should depend on the duration of water deprivation. In fact, bilateral microinjection of the $GABA_A$ receptor agonist muscimol produced time-dependent decreases in LSNA, since the magnitude significantly increased as the time of water deprivation increased. Although RSNA in control and 24 h water-deprived rats did not change after inhibition of the PVN, it did significantly decrease in 48 h water-deprived rats as reported previously (Stocker *et al.* 2004*b*). Interestingly, LSNA decreased significantly more than RSNA in both 24 and 48 h water-deprived rats. Similar observations have been reported in a rat model of hypertension also associated with an elevated SNA, since inhibition of the PVN produced a greater decrease in LSNA of spontaneously hypertensive rats compared to control

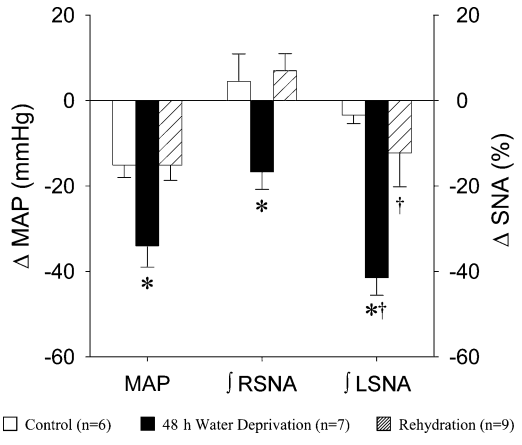

Figure 6. Peak decreases in MAP, ∫RSNA and ∫LSNA of control, 48 h water-deprived rats and rehydrated rats receiving bilateral microinjection of muscimol into the PVN
Acute rehydration of 48 h water-deprived rats prevented the significant decrease in MAP and eliminated the inhibition of RSNA and LSNA produced by microinjection of muscimol into the PVN. Indeed, the level of MAP and changes in RSNA and LSNA of rehydrated rats after microinjection of muscimol were not different from those values of control rats. The results of control and 48 h water-deprived rats from Fig. 3 are presented here for purposes of comparision. *Significant difference from control and rehydrated rats ($P < 0.05$), †significant difference between LSNA and RSNA within the same group ($P < 0.05$). Values are means ± S.E.M.

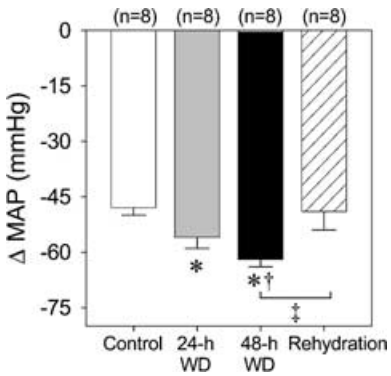

Figure 7. Peak decreases in MAP of control, 24 and 48 h water-deprived (WD) rats and rehydrated rats after ganglionic blockade with hexamethonium (30 mg kg^{-1}, I.V.)
Ganglionic blockade with hexamethonium produced a significantly greater decrease in the MAP of 24 and 48 h water-deprived rats compared to control rats. Moreover, the change in MAP was significantly greater in 48 *versus* 24 h water-deprived rats. The decrease in MAP of rehydrated rats was not different from that of control rats but was significantly less than that of 48 h water-deprived rats. Baseline MAP was not different between control (97 ± 3 mmHg), 24 h water-deprived (101 ± 4 mmHg), 48 h water-deprived (103 ± 2 mmHg) and rehydrated rats (102 ± 5 mmHg). *Significant difference from control rats ($P < 0.05$), †significant difference between 24 and 48 h water-deprived rats ($P < 0.05$), ‡significant difference between 48 h water-deprived and rehydrated rats ($P < 0.05$). Values are means – S.E.M.

Wystar-Kyoto rats (Allen, 2002), whereas the decrease in RSNA was not different between strains (Akine *et al.* 2003). Collectively, the present findings suggest that the PVN differentially supports LSNA and RSNA in water-deprived rats, and this contribution is time-dependent. Whether this differential contribution of the PVN results from activation of separate neuronal populations of PVN autonomic neurones linked to lumbar *versus* renal sympathetic nerves remains to be determined. Functionally, this differential contribution of the PVN to LSNA *versus* RSNA may permit the maintenance of ABP while simultaneously preserving the autoregulatory capacity of the kidney under conditions of reduced intravascular volume and elevated pOsm. However, future studies need to investigate this possibility.

Previous studies have demonstrated that acute rehydration of water-deprived rats reduces Fos immunoreactivity in magnocellular neurones of the PVN and supraoptic nucleus and inhibits plasma vasopressin and oxytocin secretion (Thrasher *et al.* 1981, 1987; Appelgren *et al.* 1991; De Luca *et al.* 2002; Stricker *et al.* 2002; Ji *et al.* 2005). In the present study, acute rehydration of 48 h water-deprived rats was accompanied by: (1) an attenuated fall in ABP after ganglionic blockade (i.e. reduced SNA support of ABP); (2) a decreased contribution of the PVN to both LSNA and RSNA; and (3) an attenuated increase in Fos immunoreactivity of PVN autonomic neurones. Although stimulation of oropharyngeal and hepatic osmoreceptors by the ingested water could underlie these responses, rats drank most of the water in the initial 30 min, which would allow more than 90 min for water to leave the gastrointestinal tract and enter the circulation. Since pOsm and plasma protein concentrations of rehydrated rats were not different from those of control rats, a more likely explanation for the observed effects is that rehydration eliminated the

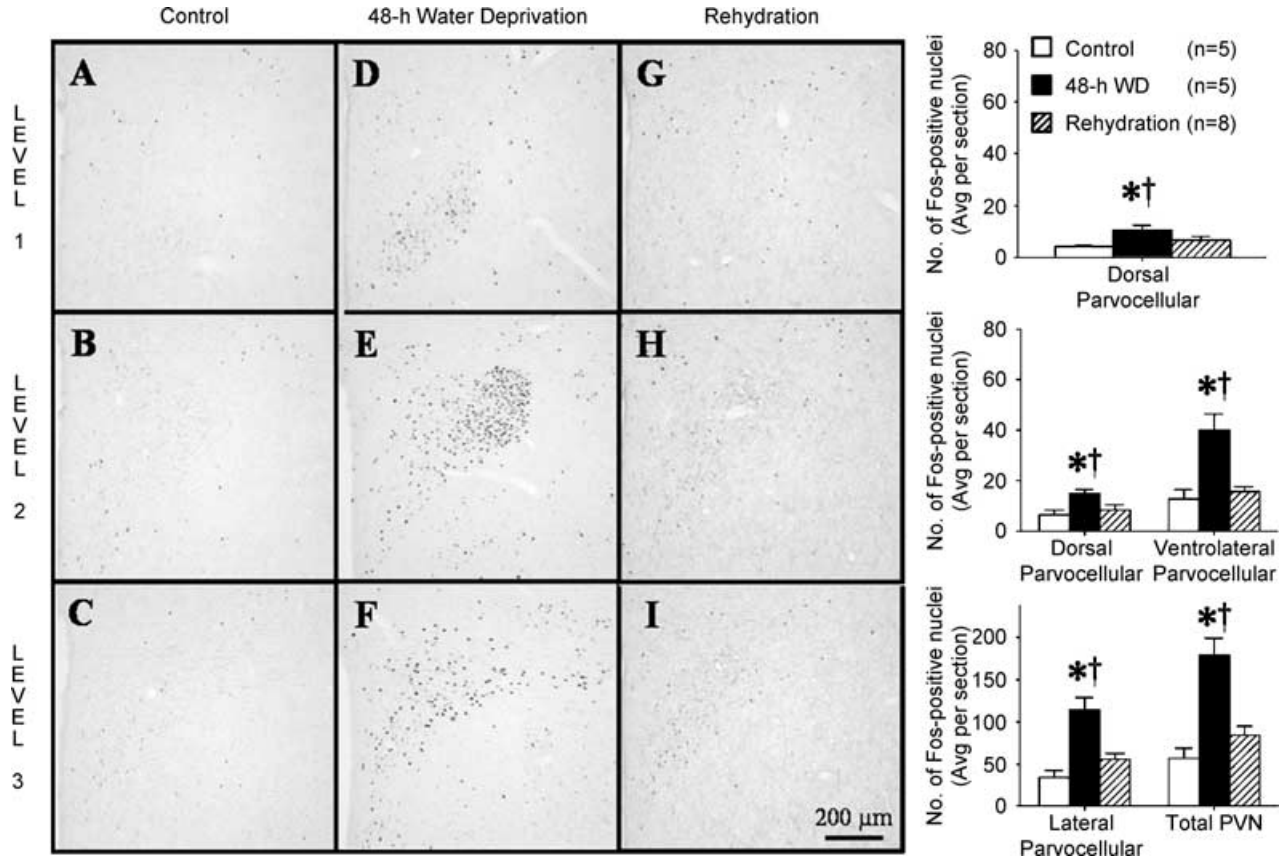

Figure 8. Fos immunoreactivity in control (*A*, *B* and *C*), 48 h water-deprived (*D*, *E* and *F*) and rehydrated rats (*G*, *H* and *I*) at three different rostral–caudal levels of the PVN

Summary counts of Fos immunoreactive nuclei at each level in the dorsal, ventrolateral and lateral parvocellular divisions of the PVN in all three groups are located on the right-hand side. The number of Fos immunoreactive nuclei was significantly higher in 48 h water-deprived rats compared to control rats at every level of the PVN. In addition, the number of Fos immunoreactive nuclei in rehydrated rats was significantly less than in 48 h water-deprived rats and similar to those of control rats. *Significant difference from control rats ($P < 0.05$), †significant difference from rehydrated rats ($P < 0.05$). Values are means ± S.E.M. Abbreviations: WD, water deprivation.

underlying stimulus by restoring pOsm and/or blood volume.

As mentioned above, water deprivation increases pOsm and circulating Ang II levels and decreases intravascular volume (Stocker *et al.* 2002, 2004*b*; Brooks *et al.* 2004*b*). In this regard, electrophysiological studies *in vivo* have demonstrated that each of the aforementioned stimuli influence the discharge of PVN autonomic neurones (Ferguson, 1988; Lovick & Coote, 1988; Bains & Ferguson, 1995; Chen & Toney, 2000; Toney *et al.* 2003). Therefore, one or a combination of these signals is likely to mediate the greater dependence of ABP on SNA, the increased contribution of the PVN to LSNA and RSNA, and the increased Fos immunoreactivity in PVN autonomic neurones. However, Scrogin *et al.* (1999) reported that restoration of pOsm and blood volume normalized the elevated LSNA in 48 h water-deprived rats, whereas restoration of blood volume, by itself, had no effect. Consistent with the possibility of pOsm increasing SNA in water-deprived rats, intravenous infusion of hypertonic NaCl increases LSNA in anaesthetized rats (Brooks *et al.* 2004*b*) and in unanaesthetized, baroreceptor-denervated rats (Weiss *et al.* 1996), whereas intracarotid injection of hypertonic NaCl has been reported to increase RSNA (Chen & Toney, 2001). Interestingly, Brooks *et al.* (2004*a*) have reported that blockade of excitatory amino acid receptors in the RVLM decreases ABP in water-deprived rats, and this response was attenuated following intravenous infusion of 5% dextrose in water but not isotonic saline (Brooks *et al.* 2004*b*). These observations suggest that increased osmolality, and not reduced intravascular volume, contributes to the greater dependence of ABP on excitatory amino acid transmission in the RVLM. Further investigation is required to explore whether the elevated pOsm mediates the contribution of the PVN to LSNA and RSNA alone or in combination with reduced intravascular volume.

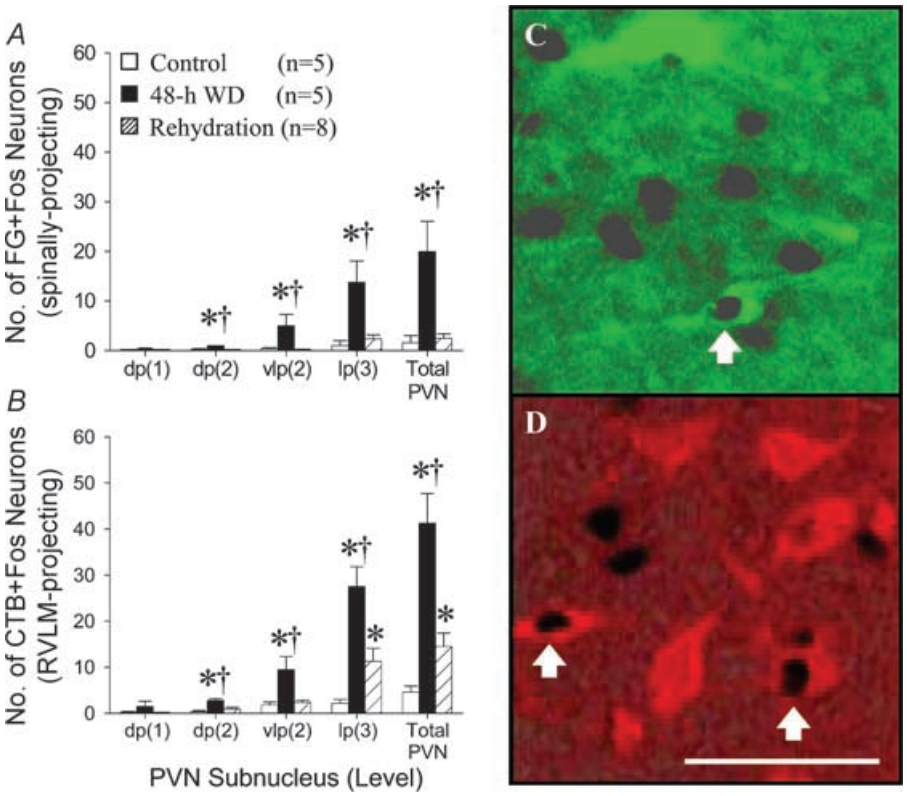

Figure 9. Fos immunoreactivity in spinally-projecting and RVLM-projecting PVN neurones of control, 48 h water-deprived rats and rehydrated rats

A and *B*, a significantly greater number of spinally-projecting and RVLM-projecting PVN neurones were Fos immunoreactive in 48 h water-deprived rats compared to control rats in the dorsal (level 2), ventrolateral (level 2), and lateral (level 3) parvocellular divisions. Moreover, rehydration significantly attenuated the increased Fos immunoreactivity in both spinally-projecting and RVLM-projecting PVN neurones. Examples of Fos-positive nuclei in spinally-projecting (*C*) and RVLM-projecting PVN neurones (*D*). White arrow denotes double-labelled neurone. Calibration bar is 25 μm. *Signficant difference from control rats ($P < 0.05$), †significant difference from rehydrated rats ($P < 0.05$). Values are means + S.E.M. Abbreviations: dp, dorsal parvocellular; vlp, ventrolateral parvocellular; lp, lateral parvocellular.

Several observations raise the possibility that the contribution of the PVN to LSNA and RSNA in water-deprived rats could involve activation of a neural pathway to the RVLM. First, neurotransmission in both the PVN and RVLM contribute to the maintenance of ABP in water-deprived rats (Brooks *et al.* 2004*a,b*; Stocker *et al.* 2004*b*). Second, water deprivation increases Fos immunoreactivity in PVN neurones that project to the RVLM and in RVLM neurones that project to the spinal cord (Stocker *et al.* 2004*a*). Recent evidence suggests that this putative PVN to RVLM pathway activated during water deprivation could be glutamatergic. For example, the increase in RVLM neuronal discharge evoked by PVN stimulation is blocked by iontophoretic application of the excitatory amino acid receptor antagonist kynurenic acid (Yang *et al.* 2001). As already noted above, Brooks *et al.* (2004*a*) have reported that tonic activation of excitatory amino acid receptors in the RVLM supports ABP in water-deprived rats. It should be emphasized, however, that there is no currently available data identifying the neurochemical phenotype of PVN neurones that project to the RVLM. Thus, definitive evidence for activation of a glutamatergic pathway from the PVN to RVLM during water deprivation is absent. To the extent that PVN inputs to the RVLM contribute significantly to the regulation of SNA during water deprivation, it might be expected that interruption of neurotransmission at either site would produce similar changes in LSNA and RSNA. However, since functional studies of the RVLM in water-deprived rats to date have not recorded SNA (Brooks *et al.* 2004*b*), this possibility cannot be assessed at the present time. If a glutamatergic pathway from the PVN to the RVLM does participate in the regulation of SNA in water-deprived rats, these observations must be reconciled with data indicating that increases in ABP and SNA produced by acute $GABA_A$ receptor blockade in the PVN are attenuated by blockade of Ang II type 1, but not excitatory amino acid receptors in the RVLM (Tagawa & Dampney, 1999).

Since the PVN contains a number of classical neurotransmitters (i.e. GABA and glutamate) and numerous peptides (Decavel & Van den Pol, 1990; Ferguson & Washburn, 1998; Hallbeck & Blomqvist, 1999; de Wardener, 2001; Hallbeck *et al.* 2001; Herman *et al.* 2003; Stern, 2004), one or a combination of these could mediate time-dependent responses in sympathetic outflow during water deprivation. Although PVN neurones are consistently labelled following injection of the transneuronal tracer pseudorabies virus into a number of sympathetically innervated organs, the topographical organization and neurochemical phenotype of labelled neurones may depend on the target organ (Strack *et al.* 1989; Sved *et al.* 2001). While this possibility remains to be fully explored, these observations are consistent with the notion that PVN neurones may differentially control sympathetic outflow to various end organs. Moreover, functional studies provide evidence supportive of regionally specific control of SNA by the PVN. For example, excitation of PVN neurones with D,L-homocysteic acid in anaesthetized rabbits has been reported to increase ABP, splanchnic, adrenal and cardiac SNA, but to decrease RSNA (Deering & Coote, 2000). In addition, local injections of noradrenaline, glutamate and GABA produce differential activation of PVN neurones (Cole & Sawchenko, 2002). Taken together, these observations raise the possibility that differential control of LSNA and RSNA by the PVN during water deprivation, as shown in the present study, could result from specific neurochemical inputs that target distinct populations of PVN neurones. Our findings also indicate that studies designed to evaluate the contribution of a specific brain region to the control of SNA may not reveal the full extent or complexity of regulation when sympathetic outflow is indexed by nerve activity to a single end organ.

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