A spinal vasopressinergic mechanism mediates hyperosmolality-induced sympathoexcitation

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An elevation in plasma osmolality elicits a complex neurohumoral response, including an activation of the sympathetic nervous system and an increase in arterial pressure. Using a combination of *in vivo* and *in situ* rat preparations, we sought to investigate whether hypothalamic vasopressinergic spinally projecting neurones are activated during increases in plasma osmolality to elicit sympathoexcitation. Hypertonic saline (HS, I.V. bolus), which produced a physiological increase in plasma osmolality to 299 ± 1 mosmol (kg water)$^{-1}$, elicited an immediate increase in mean arterial pressure (MAP) (from 101 ± 1 to 121 ± 3 mmHg) *in vivo*. Pre-treatment with prazosin reversed the HS-induced pressor response to a hypotensive response (from 121 ± 3 to 68 ± 2 mmHg), indicating significant activation of the sympathetic nervous system. In an *in situ* arterially perfused decorticate rat preparation, hyperosmotic perfusate consisted of either 135 mM NaCl, or a non-NaCl osmolyte, mannitol (0.5%); both increased lumbar sympathetic nerve activity (LSNA) by 32 ± 5% (NaCl) and 21 ± 1% (mannitol), which was attenuated after precollicular transection (7 ± 3% and 1 ± 1%, respectively). Remaining experiments used the NaCl hyperosmotic stimulus. In separate preparations the hyperosmotic-induced sympathoexcitation (21 ± 2%) was also significantly attenuated after transection of the circumventricular organs (2 ± 1%). Either isoguvacine (a $GABA_A$ receptor agonist) or kynurenic acid (a non-selective ionotropic glutamate receptor antagonist) microinjected bilaterally into the paraventricular nucleus (PVN) attenuated the increase in LSNA induced by the hyperosmotic stimulus (control: 25 ± 2%; after isoguvacine: 7 ± 2%; after kynurenic: 8 ± 3%). Intrathecal injection of a V_{1a} receptor antagonist also reduced the increase in LSNA elicited by the hyperosmotic stimulus (control: 29 ± 6%; after blocker: 4 ± 1%). These results suggest that a physiological hyperosmotic stimulus produces sympathetically mediated hypertension in conscious rats. These data are substantiated by the *in situ* decorticate preparation in which sympathoexcitation was also evoked by comparable hyperosmotic stimulation. Our findings demonstrate the importance of vasopressin acting on spinal V_{1a} receptors for mediating sympathoexcitatory response to acute salt loading.

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Plasma osmolality is tightly controlled by homeostatic processes involving an integrative response of the cardiovascular, renal and neuro-endocrine systems (Share & Claybaugh, 1972). Experimentally, plasma osmolality can be increased by water deprivation (dehydration) or intravenous infusion of hypertonic saline (HS), which produce immediate neurohumoral responses including powerful activation of the sympathetic nervous system, an increase in arterial blood pressure and release of vasopressin from the posterior pituitary resulting in water retention (Hatzinikolaou *et al.* 1980, 1981; Garcia-Estan *et al.* 1989; Akins & Bealer, 1990; Weiss *et al.* 1996; Scrogin *et al.* 1999; Bealer, 2000; Stocker *et al.* 2005). This response is mediated, in part, through activation of the hypothalamic paraventricular nucleus (PVN) driven by specific forebrain areas in which central osmoreceptors are contained (Antunes-Rodrigues *et al.* 2004). These areas include the forebrain lamina terminalis (organ vasculosum lamina terminalis, OVLT, median preoptic area, MnPO, and subfornical organ, SFO), and on stimulation by plasma

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hyperosmolality provide a major source of excitatory afferent input to the PVN (Camacho & Phillips, 1981; Saper & Levisohn, 1983; Sawchenko & Swanson, 1983; McKinley *et al.* 1992; Johnson *et al.* 1996).

The PVN contains magnocellular and parvocellular divisions. In the former are found the vasopressin- and oxytocin-containing neurones that project exclusively to the posterior pituitary whereas the latter region contains the 'autonomic' neurones that excite the sympathetic nervous system (Swanson & Sawchenko, 1983). Parvocellular neurones project to premotor sympathoexcitatory neurones located either in the rostral ventrolateral medulla (RVLM) and/or the sympathetic preganglionic neurones of the intermediolateral cell column (IML) of the spinal cord. Previous studies have shown that the autonomic parvocellular neurones play a significant role in cardiovascular regulation, particularly in response to homeostatic disturbance including plasma salt loading (Kannan *et al.* 1989; Weiss *et al.* 1996; May *et al.* 2000; Chen & Toney, 2001; Toney *et al.* 2003). What is not clear is which descending PVN parvocellular pathway(s) (either via the RVLM and/or direct spinal) mediate(s) the sympathoexcitatory response to plasma hyperosmolality, and what is (are) the neurotransmitter substance(s) employed.

Previous studies have demonstrated vasopressinergic terminals within the spinal cord including both the superficial dorsal horn and IML (Buijs, 1978; Nilaver *et al.* 1980). Vasopressinergic mechanisms at the spinal level have been associated with both the increase in arterial pressure, induced when vasopressin was applied intrathecally, and following direct stimulation of the PVN (Malpas & Coote, 1994; Porter & Brody 1986*a*,*b*; Tan & Tsou, 1986). However, what physiological stimulus results in activating the PVN-spinal vasopressinergic pathway has not been revealed. In the present study, we hypothesized that hypothalamic vasopressinergic spinally projecting neurones are activated during hyperosmolality, to release vasopressin within the spinal cord that results in sympathoexcitation. To this end, we developed an *in situ* decorticate arterially perfused rat preparation containing hypothalamic structures. This preparation was based on those previously described (Paton, 1996; Pickering & Paton, 2006), and allowed very precise and repeatable changes in systemic osmolality without cumulative salt-loading effects. Here, we (1) demonstrate the integrity of the PVN in this *in situ* preparation; (2) compare hyperosmolality-induced sympathetic responses with those evoked in the conscious *in vivo* rat; and (3) show that the sympathoexcitatory response to physiological levels of salt loading is mediated, primarily, by the PVN, and depends on the integrity of V_{1a} receptors within the spinal cord.

Part of this study was presented to the Physiological Society in abstract form (Antunes *et al.* 2005).

Methods

All experimental procedures were approved by the University of Bristol Ethical Review Committee and were carried out under government licence in accordance with the Home Office Scientific Procedures Act (1986). *In vivo* implanted rats were killed by an overdose of sodium pentobarbitone given intravenously.

In vivo conscious rat studies

Adult male Wistar rats weighing 300–350 g were obtained from University of Bristol colony and kept at a constant temperature of 22°C and a relative humidity of 50–60% under a controlled light/dark cycle (12/12 h light/dark). Rats had access to normal rat chow and drinking water *ad libitum*.

Cardiovascular parameter recording. A radiotelemetry system (Data Sciences International, Arden Hills, MN, USA) was used for recording of arterial pressure as previously described (Waki *et al.* 2003, 2006). The system consists of three basic elements: (1) a transmitter for monitoring arterial pressure (TA11PA-C40); (2) a receiver (RP-1); and (3) an adapter (R11CPA) with an ambient pressure monitor (APR-1) to output analog signals of arterial pressure. The system is calibrated relative to atmospheric pressure. The pulsatile blood pressure signal was recorded and then stored on a computer via an analog input box (IP-810 A, GigaTex Co, Ltd, Miyagi, Japan) connected to an analog I/OPC card(AD 12–8(PM), CONTECCo Ltd, Japan) for digitization.

Transmitter implant surgery. Each transmitter was implanted at least 5 days before any experimental protocol. Rats were anaesthetized with a mixture of ketamine (60 mg kg^{-1}) and medetomidine (250 mg kg^{-1}) injected intramuscularly. The level of anaesthesia was checked frequently by assessing limb withdrawal reflexes to noxious pinching. A midline laparotomy was made in the supine position, and the intestines were reflected to expose the abdominal aorta. The tip of the catheter (outside diameter 0.7 mm, thin-walled thermoplastic membrane) connected to transmitter (diameter 15 mm) was inserted into the abdominal aorta caudal to the root of the left renal artery and held in place with tissue adhesive (Vetbond, 3M). The body of the transmitter was sutured to the ventral wall of the abdominal cavity. After surgery, anaesthesia was reversed with a subcutaneous injection of atipamezole (1 mg kg^{-1}), a prophylactic antibiotic was injected intramuscularly (penicillin, 1000 U), and the rats were returned to their home cages for recovery (typically five days). The day before experiments rats were re-anaesthetized with halothane (5%) and the right femoral vein was cannulated with a catheter (PE-10

connected to PE-50 (Clay Adams, Parsippany, NJ, USA)), tunnelled subcutaneously, and exteriorized through the back of the neck for systemic drugs delivery.

Systemic drugs delivery. Rats initially received intravenous (I.V.) injections of isotonic saline (154 mM NaCl) in which the plasma osmolality was 289 ± 1 mosmol (kg water)$^{-1}$. On the following day the same group of rats ($n = 5$) received the same volume of hypertonic saline (HS, 3 M NaCl), which resulted in an increased plasma osmolality of 299 ± 1 mosmol (kg water)$^{-1}$ (peak response) as measured 90 s after injection. In a second group of rats, HS (I.V.) was injected after pre-treatment with prazosin (α_1-adrenoceptor antagonist, 1 mg kg^{-1} ml^{-1}, I.V.). The efficacy of this drug dose was challenged with phenylephrine (15 μg kg^{-1} ml^{-1}, I.V.), and found sufficient to block the evoked pressor response. In a third group, HS (I.V.) was injected after pretreatment with [β-mercapto-β,β-cyclopentamethylene-propionyl^1, *O*-me-Tyr^2,-Arg^8]-vasopressin (a V_{1a} receptor antagonist, 10 μg kg^{-1} ml^{-1}, I.V.). The efficacy of this antagonist was tested by intravenous injection of [Arg^8]-vasopressin acetate (AVP, 10 μg kg^{-1} ml^{-1}). To avoid the cumulative effects of salt loading, HS was administrated once only in each rat. Either HS or isotonic saline was administered at a volume of 0.14 ml 100 (g body weight)$^{-1}$ and lasted for 40 s.

Plasma osmolality measurements. Blood samples (200 μl) were collected via a venous catheter using heparinized syringes. After collection of each sample, an equal volume of isotonic saline was reintroduced (I.V.) in order to avoid changes to the circulating blood volume. For the first five minutes, blood samples were collected every minute after HS administration (NaCl 3 M, 0.14 ml kg^{-1}, I.V.). Subsequent blood samples were collected every 30, 90, 120, and 180 min post-HS injection. The plasma osmolality was measured using a freezing point depression osmometer (Camlab, Roebling Micro-osmometer, Cambridge, UK).

Data analyses. Telemetry data were acquired using Hey-Presto software (Waki *et al.* 2006) through an analog input box and I/O PC card (as described above) displayed on the computer screen and stored on the hard drive. The mean arterial pressure (MAP) was exported into Excel (Microsoft Corporation, USA) for further statistical evaluation. The MAP values were averaged over 180 min post-osmolality challenge. The first 5 min of recording immediately after injection of HS (I.V.) were averaged every 30 s, in order to obtain high temporal resolution of the changes in MAP elicited by HS and drugs. For the remaining time, MAP data was sampled every 15 min. Changes in MAP (mmHg) were statistically analysed by two-way ANOVA followed by Bonferroni *post hoc* test for multiple comparisons, and the differences were considered significant at $P < 0.05$. All values are expressed as the mean $\pm$ standard error of mean (S.E.M.), and n is the number of rats in each group.

Decorticate, unanaesthetized, arterially perfused *in situ* preparation of rat

Male Wistar rats weighing 70–90 g were anaesthetized deeply with halothane (5%), and the level of anaesthesia was assessed by a failure to respond to a noxious pinch of either a paw or the tail. The stomach, intestines and spleen were ligated and removed via midline laparotomy. The sternum was split and the ribcage retracted to allow access to the mediastinum. The pericardium was removed and the left phrenic nerve was isolated. The animal was submerged in cooled Ringer solution (see below) and the cerebral hemispheres exposed by removal of the parietal bones. The cerebral cortices, hippocampus and thalamic area were removed by gentle aspiration. The preoptic area and its adjacent septal nuclei and hypothalamic areas remained largely intact (see Fig. 7*A* and *B*). The preparation was skinned and transferred to the recording chamber. A double-lumen cannula was introduced into the ascending aorta via the left ventricle. The preparation was perfused at flow rates of 28 ± 2 ml min^{-1} using a roller pump (Watson Marlow 505S) with Ringer solution containing an oncotic agent (Ficoll 70, 1.25%; Sigma, St Louis, USA), gassed with carbogen (95% O_2 and 5% CO_2), warmed to 32°C and filtered using a nylon screen (pore size: 25 μm). After respiratory-related movements commenced, a neuromuscular blocker (vecuronium bromide, 40 μg ml^{-1}, Norcuron Organon Teknika) was added to the perfusate to stabilize mechanically the preparation. The second lumen of the cannula was used to monitor aortic perfusion pressure. Phrenic nerve activity (PNA) was recorded from its distal end using a glass suction monopolar electrode held in a 3-D micromanipulator. Rhythmic ramping PNA gave a continuous physiological index of preparation viability. The lumbar sympathetic chain (L2–L3) was visualized through a binocular microscope and recordings made from the distal cut end using a bipolar glass suction electrode. Signals were AC-amplified (Neurolog NL104) and band-pass filtered (8 Hz–3 kHz). Sympathetic nerve activity exhibited marked respiratory modulation, and was profoundly attenuated by an increase in perfusion pressure (arterial baroreceptor stimulation).

Brain transection. Brain transections were performed using a microblade coupled to a 3-D micromanipulator array. The blade was adjusted to the correct stereotaxic co-ordinates to allow precise transection at the precollicular level. Transections in the coronal plane were also made to remove all circumventicular organ (CVO) connections to the hypothalamus (see Fig. 7*A* and *B*).

Microinjection studies. The head of the preparation was fixed by ear bars and a nasal clamp. The angle of the head was positioned so that the dorsal surface of the brainstem was horizontal in every experiment; this aided accurate and consistent placement of micropipettes into the PVN. In pilot studies we mapped out the PVN based on co-ordinates relative to the superior colliculus (SC) (i.e. 2.7 mm rostral, 0.3 mm lateral to midline and 3.4 mm below the brain surface). A three-barrelled glass micropipette (external tip diameter 10–30 μm) was placed into the PVN using a 3-D micromanipulator. The volume microinjected (60 nl) was determined by viewing the movement of the meniscus through a binocular microscope fitted with a precalibrated eyepiece reticule. All microinjections were made bilaterally within a period of 60 s, and the osmotic stimulus tested 2, 15 and 30 min post-microinjection.

Intrathecal injections. A retractor attached to the pelvis was used to stretch the spinal cord to facilitate access. Two vertebral processes were removed at the L2–L3 level. A calibrated glass micropipette (Accu-Fill 90, Micropet; Clay Adams, Parsippany, NJ, USA) was positioned into the subarachnoid space using a 3-D micro manipulator and a volume of up to 0.5 μl injected. In order to check the approximate spread of drugs, a 2% solution of Pontamine Sky Blue was injected at the same site at the end of each experiment.

Osmotic stimulus. The *in situ* preparation was initially perfused with isosmotic Ringer solution containing (mM): NaCl 120, $NaHCO_3$ 24, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1.25, KH_2PO_4 1.25, dextrose 10; pH 7.3 after carbogenation. The osmolality, measured by a freezing point depression osmometer, was 291 ± 1 mosmol (kg water)$^{-1}$. Hyper- and hyposmotic ionic Ringer solution were prepared by adjusting the final concentration of NaCl. In addition, a non-NaCl osmolyte (mannitol) was used. Hyperosmotic Ringer solution contained either 135 mM NaCl or mannitol (0.5%), and resulted in osmolalities of 321 ± 1 and 320 ± 1 mosmol (kg water)$^{-1}$, respectively. The hyposmotic solution contained 105 mM resulting in 259 ± 1 mosmol (kg water)$^{-1}$. Initially the *in situ* preparation was perfused with isotonic Ringer solution. A second reservoir of either hyper- or hyposmotic Ringer solution was connected to the perfusion system via a three-way tap and circulated for 40 s. This exposure time was established in pilot experiments to produce a submaximal, reversible and highly reproducible increase in sympathetic nerve activity.

Histological analysis. PVN injection sites were marked with 2% Pontamine Sky Blue (60 nl) contained in one barrel of a three-barrel micropipette. The brain was removed and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Coronal sections (45 μm thick) were cut using a cryostat (CM1900, Leica, Switzerland) and thaw-mounted on gelatin-subbed glass slides. The sections were counterstained with methyl green, and the injection sites documented on predrawn atlas sections from Paxinos & Watson (1996). Only data in which the microinjections were confirmed to be within the PVN were analysed in this study. The histology for the brain transection protocols followed the same procedure described above; however, these brains were cut in the sagittal plane for better visualization of the CVOs (Fig. 7).

Data analyses. All *in situ* rat data were acquired using a CED 1401 A–D interface (CED, Cambridge Electronic Design, Cambridge, UK) and a computer running Spike 2 software (CED) with custom-written scripts for data acquisition and on- and off-line analyses. Lumbar sympathetic nerve activity (LSNA) was displayed as a moving average (100 ms time constant) and measured as the area under the curve per second, both 40 s before and 40 s after an osmotic stimulus. To standardize the data across preparations, LSNA changes were expressed as a percentage of baseline. Baseline noise was assessed by application of lidocaine (2%) to the sympathetic chain at the end of the experiment. One-way ANOVA for repeated measures followed by Tukey's *post hoc* test was used, and differences were taken as significant at $P < 0.05$. All values are expressed as the mean $\pm$ standard error of mean (S.E.M.) and n is the number of preparations.

Drugs. The following drugs were either microinjected or given intrathecally: L-glutamate (20 mM), kynurenic acid (glutamatergic antagonist of ionotropic receptors, 100 mM), isoguvacine (selective $GABA_A$ agonist, 1 mM), β-mercapto-β,β-cyclopentamethylenepropionyl^1,*O*-me-Tyr^2,-Arg^8-vasopressin (V_{1a} receptor antagonist, 1 μg ml^{-1}), Phe^2,Ile^3,Orn^8-Vasopressin (V_1 receptor agonist, 1 μg ml^{-1}), AVP, pontamine Sky Blue dye (2%). All drugs were purchase from Sigma, UK, with the exception of V_1 agonist, which was purchased from Bachem, UK.

Results

In vivo studies

Intravenous injections of hyperosmotic saline (HS) increase arterial pressure in conscious rats ($n = 15$). HS injected intravenously, which produced an increase in plasma osmolality to 299 ± 1 mosmol (kg water)$^{-1}$ elicited an increase in MAP (120 ± 1 mmHg; $P < 0.05$; $n = 5$; Fig. 1) that reached a peak 90 s following the injection. An injection of the same volume of isotonic saline (I.V.) had no significant effect on either MAP (102 ± 2 mmHg;

$n = 5$) or plasma osmolality (289 ± 1 mosmol (kg water)$^{-1}$). Five minutes after HS injection, MAP started to return to baseline levels and was fully recovered 180 min later (Fig. 1 inset).

In a separate group, rats were pretreated with prazosin (I.V.). An injection of HS caused a biphasic response with an initial depressor response occurring over the first minute post-injection (68 ± 2 mmHg; $P < 0.001$; $n = 5$; Fig. 1) followed by a pressor effect (109 ± 3 mmHg) that peaked at 2 min and 30 s later. In a separate group of five rats that were pre-treated with the V_{1a} antagonist (I.V.), the injection of HS produced no significant change in MAP at any time point (Fig. 1). The resultant increases in the plasma osmolality were similar across all groups, and the average values are shown in Fig. 1.

The intravenous infusion rate of HS (0.14 ml.100 (g body weight)$^{-1}$ for 40 s) produced neither cardiac arrthymia nor a change in heart rate at any time point when compared to isotonic saline injections (data not shown). In a separate group of rats ($n = 3$), we verified that the pressor effect elicited by phenylephrine (ΔMAP: 54 ± 4 mmHg; 15 μg kg^{-1}, I.V.) was attenuated significantly by prazosin (1 mg kg^{-1} ml^{-1}; ΔMAP was 3 ± 1 mmHg). Moreover, the efficacy of the V_{1a} antagonist was confirmed by measuring the pressor response to I.V. injection of [Arg^8]-vasopressin acetate (AVP, 10 μg^{-1} ml^{-1}). The pressor responses elicited by AVP (ΔMAP: 57 ± 4 mmHg) were reduced significantly after administration of the V_{1a} antagonist (ΔMAP: 1 ± 0.5 mmHg).

In situ studies

The integrity of the hypothalamus is essential for the sympathoexcitation induced by different osmotically active solutes. To validate the integrity of the *in situ* decorticate preparation, we switched the isosmotic solution to a second reservoir containing hyperosmotic Ringer solution. We anticipated that this should elevate sympathetic nerve activity as seen in the *in vivo* rat. Indeed, the hyperosmotic perfusate (135 mM NaCl) induced sympathoexcitation ($32 \pm 5\%$; $n = 6$; Fig. 3). This effect was found to be reproducible eliciting consistent responses during repeated osmotic challenges. Next, we wanted to test whether the sympathetic response was mediated by hypothalamic structures. To this end, we challenged the preparation with a hyperosmotic stimulus (135 mM NaCl) before (intact hypothalamus, Fig. 2*A*) and after precollicular transection (no hypothalamus, Fig. 2*A*). Switching from the isosmotic to hyperosmotic perfusate increased the LSNA by $32 \pm 5\%$ (Fig. 3), which was attenuated significantly after the transection (i.e. $7 \pm 3\%$, $P < 0.01$; $n = 6$; Fig. 3). It is important to note that after precollicular transection the LSNA baseline did not change (Fig. 2*A*).

In a separate group of preparations ($n = 5$), the hyperosmotic stimulus was induced using 0.5% mannitol (320 ± 1 mosmol (kg water)$^{-1}$). Figure 2*C* shows tracings of integrated and raw LSNA where the hyperosmotic stimulus elicited sympathoexcitation ($21 \pm 1\%$, Fig. 3) in

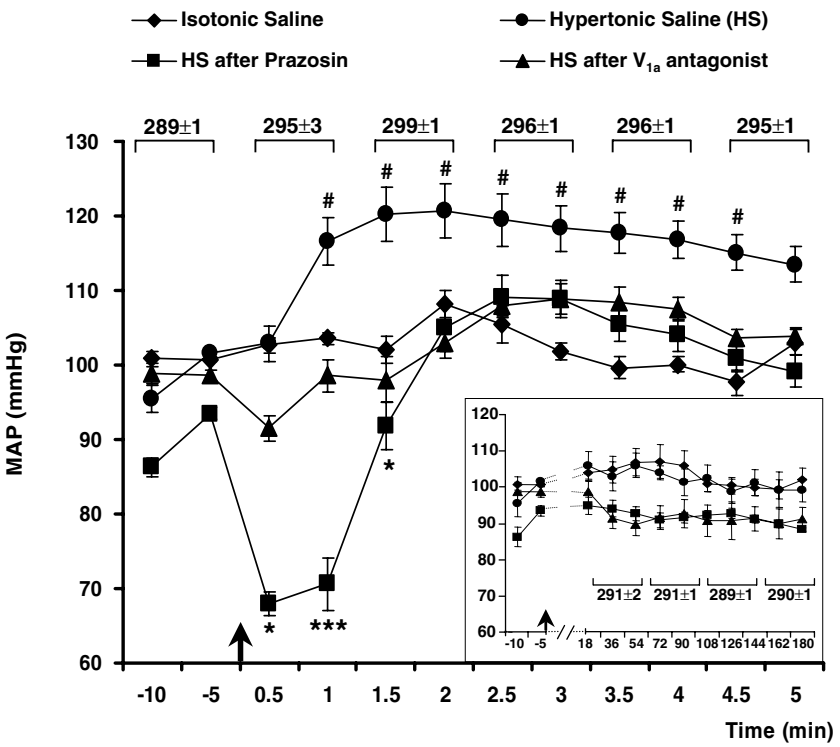
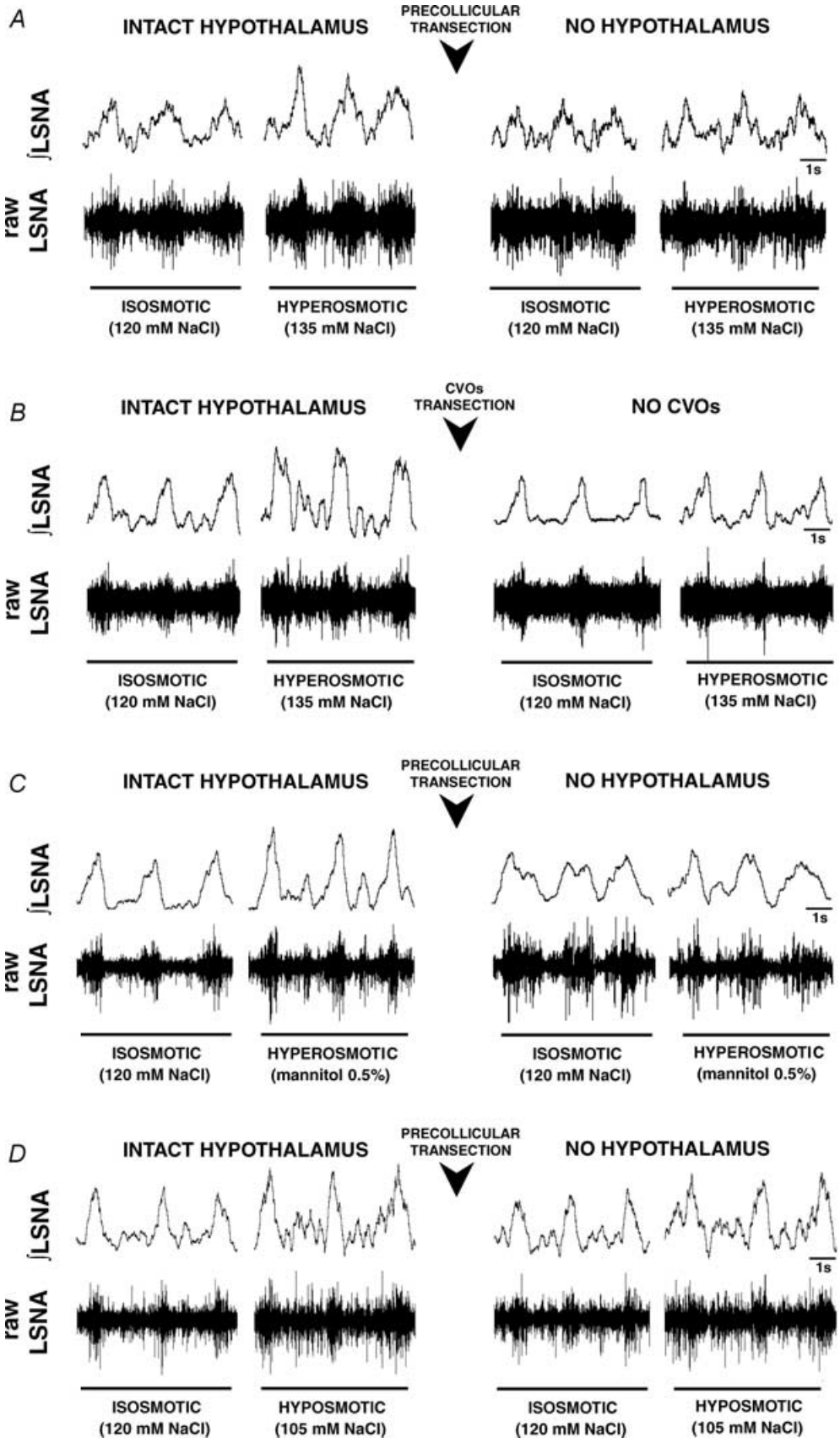


Figure 1. Intravenous injections of hyperosmotic saline (HS) increase arterial pressure in conscious rats
HS (3 M, I.V.) produced an increase in plasma osmolality (values show are per min for 5 min) and in the MAP, which reached a significant peak at 90 s (299 ± 1 mosmol (kg water)$^{-1}$). Prazosin reversed the pressor response evoked by HS into a depressor effect at 30 and 60 s ($n = 5$). HS injection failed to produce an increase in the MAP after administration of a V_{1a} receptor antagonist. Isotonic saline injected intravenously produced a negligible effect on the MAP ($n = 5$). Inset graph, the pressor response evoked by HS recovered after 180 min and plasma osmolality values are show every 40 min. Arrows show the time when HS or isotonic saline were injected. #$P < 0.05$ compared to isotonic saline injection. *$P < 0.05$ and ***$P < 0.001$ compared to HS injection over the same time course. Error bars show S.E.M.



a preparation with an intact hypothalamus. Following precollicular transaction, this response was almost abolished ($1 \pm 1\%$, $P < 0.001$; Fig. 3).

The integrity of the CVOs is essential for the hyperosmolality evoked sympathoexcitation. In order to verify central detection of the hyperosmotic stimulus, we performed a selective ablation of the CVOs sparing the paraventricular nucleus (see Fig. 7*B*). Figure 2*B* shows recordings of the integrated and raw LSNA from a group of preparations ($n = 4$) during isosmotic (120 mM NaCl) and hyperosmotic conditions (135 mM NaCl), before and after transection of the CVOs. The increase in the LSNA by $21 \pm 2\%$; $n = 4$) was significantly reduced ($2 \pm 1\%$; $P < 0.001$; Fig. 3) after transection of the CVOs. Importantly, this lesion did not change basal LSNA.

Hyposmotic-induced sympathoexcitation is not mediated via the hypothalamus. For comparison, we also challenged the preparation with a hyposmotic stimulus (105 mM NaCl; Fig. 2*D*) before and after precollicular transection. As with the hyperosmotic stimulus, the hyposmotic perfusate increased LSNA ($20 \pm 3\%$, n 6; Fig. 3). However, in stark contrast to hyperosmotic perfusate, the sympathoexcitation persisted after removing the hypothalamus (i.e. $21 \pm 3\%$, $n = 6$; Fig. 3).

Because the changes in sympathetic activity to a hyperosmotic stimulus (135 mM NaCl) were robust and repeatable it was only this variable that we studied further.

The PVN plays a significant role in mediating the hyperosmotic-induced sympathoexcitation. To further validate the preparation and to demonstrate a role for the PVN in mediating the hyperosmolality-evoked sympathoexcitatory responses, we performed a number of microinjection experiments using two different protocols: (i) a reversible inactivation of the PVN with bilateral microinjections of isoguvacine (a $GABA_A$ receptor agonist); (ii) blockade of excitatory amino acid ionotropic receptors with kynurenic acid (a non-selective ionotropic glutamatergic receptor antagonist). Following isoguvacine, baseline LSNA was unperturbed but the hyperosmotic-induced increase in LSNA ($25 \pm 2\%$) was attenuated significantly ($7 \pm 2\%$; $P < 0.01$; n 5; Fig. 4*A*), but recovered to a level not different from control 15 min after the microinjection (i.e. $23 \pm 2\%$). Similarly, the hyperosmotically induced sympathoexcitation ($25 \pm 2\%$; Fig. 4*B*) was attenuated significantly at 2 min ($8 \pm 3\%$; $P < 0.01$) and 15 min ($13 \pm 4\%$; $P < 0.05$) after kynurenic acid microinjections into the PVN in a separate group of preparations ($n = 5$). By 30 min, the response had recovered fully (i.e. $27 \pm 3\%$). As seen with isoguvacine, bilateral microinjection of kynurenic acid failed to alter basal LSNA.

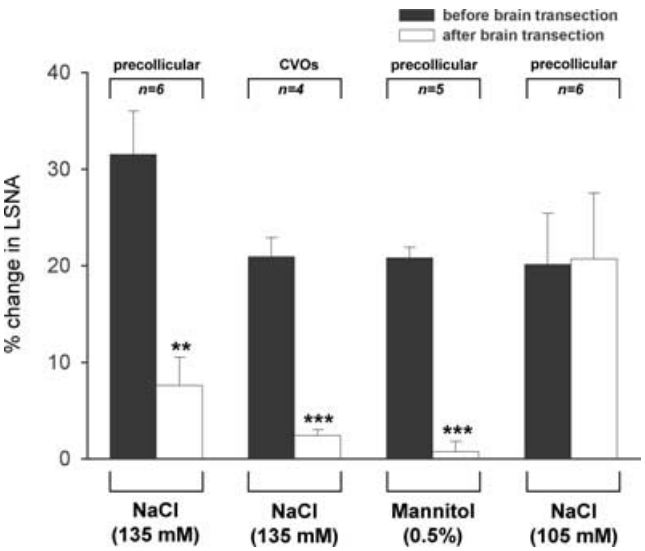


Figure 3. LSNA before and after brain transection (precollicular or CVOs) induced by hyperosmotic (135 mM NaCl or mannitol 0.5%) or hyposmotic (105 mM NaCl) stimuli in the *in situ* rat preparation

n is the number of preparations utilized in every protocol. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to before brain transection. Error bars are S.E.M.

Hyperosmotic perfusate microinjected directly into the PVN does not affect the LSNA. We tested whether PVN neurones exposed directly to the same osmotic stimulus (hyperosmotic Ringer solution, 135 mM NaCl) would be able to produce a sympathoexcitatory response. At sites within the PVN that produced a sympathoexcitatory response to application of L-glutamate ($38 \pm 4\%$, $n = 3$, Fig. 5), microinjections of hyperosmotic Ringer solution (320 mosmol (kg water)$^{-1}$), 30 min after the L-glutamate injection produced a negligible response ($4 \pm 1\%$; n.s.). When injected in the reverse order (i.e. hyperosmotic

Figure 2. Lumbar sympathetic nerve activity (LSNA) changes during three distinct osmotic conditions (isosmotic, hyperosmotic and hyposmotic) before and after precollicular transection and selective ablation of the CVOs

Representative traces of changes in raw and integrated ($\int$) LSNA during isosmotic (120 mM NaCl) and hyperosmotic stimuli (135 mM NaCl) before (intact hypothalamus) and after precollicular brain transection (no hypothalamus, *A*) or CVOs transection (*B*). Recordings in *C* show the sympathoexcitatory effect of mannitol (0.5%) before and after precollicular transection. A hyposmotic stimulus (105 mM NaCl, *D*) also produced sympathoexcitation, but this was not affected by precollicular brain transection. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to before brain transection.

Ringer solution before L-glutamate), the same effect was seen but in the reverse order (i.e. hyperosmotic Ringer solution, $2 \pm 1\%$; L-glutamate, $33 \pm 5\%$; $n = 3$; Fig. 5). These studies are consistent with those previously reported *in vivo* (Larsen & Mikkelsen, 1995; Stocker & Toney, 2005), suggesting that in the *in situ* preparation the detection of the hyperosmotic perfusate to produce sympathoexcitation resides outside the PVN.

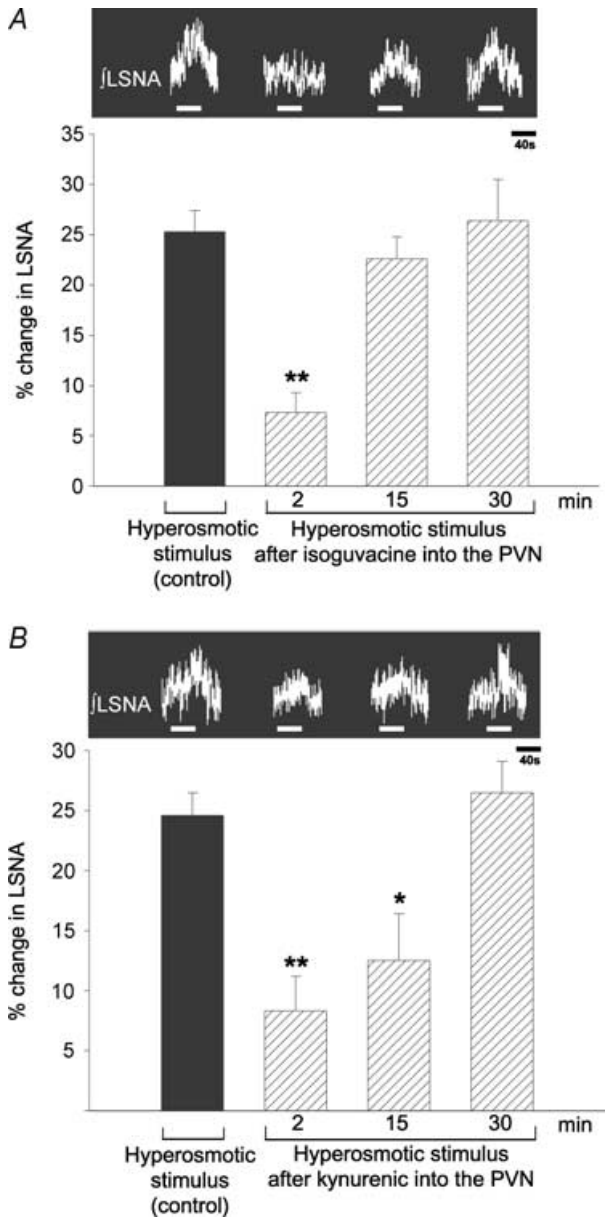


Figure 4. Reversible inactivation of the PVN and blockade of excitatory amino acid (EAA) receptors within the PVN reduce hyperosmotic-induced sympathoexcitation *in situ*
A, isoguvacine (1 mM; 60 nl) microinjected bilaterally into the PVN attenuated the increase in the LSNA elicited by the hyperosmotic stimulus. This effect reversed 15 and 30 min later. $^{**}P < 0.01$ compared to control, 15 and 30 min; ($n = 5$). *B*, blockade of EAA receptors with kynurenic acid (100 mM, 60 nl) microinjected bilaterally into the PVN attenuated the increase in LSNA elicited by a hyperosmotic stimulus; total recovery was at 30 min $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to control and 30 min, respectively; ($n = 5$). Hatched bars: hyperosmotic stimulus (40 s) after microinjections into the PVN. Error bars are S.E.M.

Vasopressin (V_{1a} receptors) at the level of spinal preganglionic sympathetic neurones is involved in the hyperosmotically mediated sympathoexcitation. To establish an effective blocking concentration of the V_{1a} receptor antagonist, we studied the response of intrathecal injections of a V_1 receptor agonist, Phe^2,Ile^3,Orn^8-vasopressin, on LSNA before and after intrathecal injections of a V_{1a} receptor antagonist ($n = 3$). As seen in Fig. 6*A*, intrathecal injection of Phe^2,Ile^3,Orn^8-vasopressin ($1\ \mu g\ ml^{-1}$) evoked an increase in LSNA ($18 \pm 1\%$), a response not dissimilar to that evoked by the hyperosmotic perfusate (see above). However, after applying the V_{1a} receptor antagonist ($1\ \mu g\ ml^{-1}$), the response to the agonist was attenuated dramatically ($6 \pm 2\%$; $P < 0.05$). Thirty minutes after the V_{1a} antagonist was applied, a second intrathecal injection of the V_1 agonist produced an increase in LSNA that was similar to the control response (i.e. $19 \pm 2\%$). Thus, this determined the concentration of the V_{1a} receptor antagonist to use during hyperosmolality-induced sympathoexcitation.

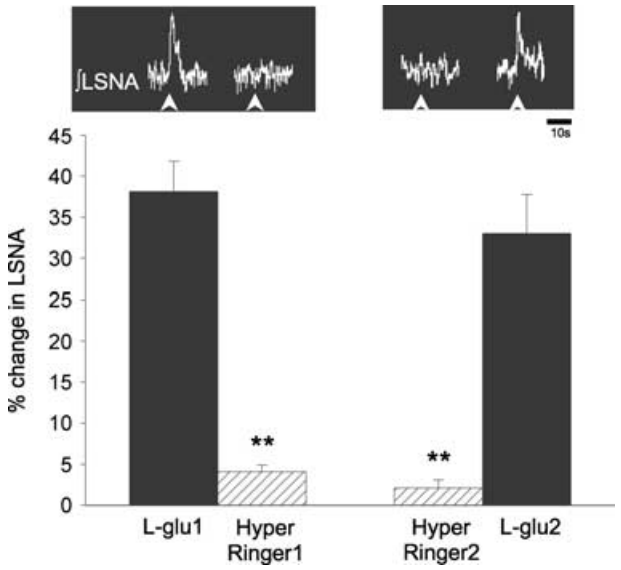


Figure 5. Effect of hyperosmotic perfusate microinjected directly into the PVN on the lumbar sympathetic nerve activity *in situ*
L-Glutamate (L-glu, 20 mM) or hyperosmotic Ringer solution (Hyper Ringer solution, 320 mosmol (kg water)$^{-1}$) was microinjected unilaterally into the PVN. Despite switching the order in which the different injections were made, hyperosmotic injections were ineffective. In each case there was a 30-min interval between each microinjection. $^{**}P < 0.01$ compared to L-glu1 and L-glu2; ($n = 3$). Arrowheads show the time when L-glutamate or hyperosmotic Ringer solution were microinjected into the PVN. Error bars are S.E.M.

Figure 6*B* shows that the hyperosmotically induced increase in LSNA ($29 \pm 6\%$, $n = 5$) was attenuated significantly at both 2 min ($4 \pm 1\%$; $P < 0.001$) and 15 min (13 ± 3; $P < 0.05$) post-intrathecal injection of the V_{1a} receptor antagonist ($1\ \mu g\ ml^{-1}$). Thirty minutes later thehyperosmotically induced increase in LSNA had recovered to control levels ($25 \pm 3\%$; Fig. 6*B*). In addition, the injection of V_{1a} antagonist intrathecally did not affect basal LSNA.

Brain transection and PVN microinjection sites. Careful histological analysis revealed that the transections effectively dissociated the CVO from the preparation while keeping the PVN intact (Fig. 7*A* and *B*), while those made at the pre-collicular level effectively removed all brainstem and spinal inputs from the hypothalamus. As demonstrated by the coronal (Fig. 7*C*) and schematic diagrams (Fig. 7*D*), all of the microinjections sites (isoguvacine, kynurenic acid, L-glutamate and hyperosmotic Ringer solution) that provided the data presented here were found within the PVN. In a group of three rats, microinjection sites (isoguvacine and kynurenic acid) were found to be outside the PVN. In these animals the HS-induced sympathoexcitation was not affected, concluding that the effects of isoguvacine or kynurenic acid are highly site-specific and confined to the PVN.

Discussion

The haemodynamic and autonomic responses to osmotic challenges were studied in two different experimental models: an *in vivo* conscious rat and an *in situ* decorticate arterially perfused rat preparation. In addition to demonstrating the viability of the CVOs and PVN in our perfused *in situ* decorticate rat model, our main finding indicates an involvement of a vasopressinergic mechanism within the spinal cord, for mediating hyperosmolality-induced sympathoexcitation.

HS in conscious rats elicited an immediate increase in MAP (Fig. 1) that completely recovered 180 min later. This pressor response was expected since it is well-established that hypertonic saline injected intravenously produces a pressor effect (Garcia-Estan *et al.* 1989; Akins & Bealer, 1990; Bealer, 2000). Studies performed by Garcia-Estan *et al.* (1989) have shown that the hypertensive response was due to a sympathetically mediated vasoconstriction. Our results are in agreement with this, since the pretreatment with prazosin blunted the pressor effect elicited by HS (I.V.). After prazosin, we revealed an initial hypotensive response that could be due to a direct effect of HS-induced vasodilatation on some vascular beds (Steenbergen & Bohlen, 1993; Pedrino *et al.* 2005), which is normally masked by the concomitant sympathetically mediated vasoconstriction. Since the administration of isotonic

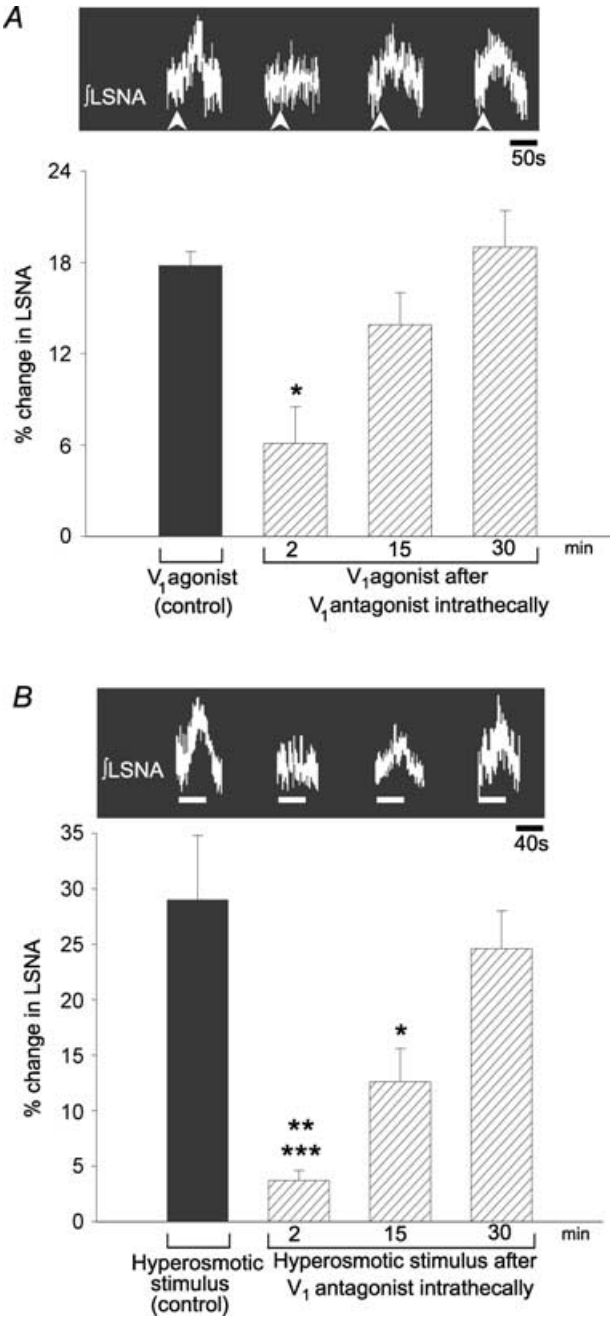


Figure 6. Intrathecal injection of a V_{1a} receptor blocker reduces the hyperosmotically induced sympathoexcitation *in situ*
A, the efficacy of a V_{1a} receptor antagonist ($1\ \mu g\ ml^{-1}$) was tested by assessing whether it attenuated the increases in LSNA elicited by V_{1a} receptor agonist ($1\ \mu g\ ml^{-1}$) via intrathecal injections. $^{*}P < 0.05$ compared to control; ($n = 3$). Arrowheads show the time when V_{1a} agonist was microinjected into the PVN. *B*, intrathecal injection of the V_{1a} receptor antagonist ($1\ \mu g\ ml^{-1}$) attenuated the increases in the LSNA elicited by hyperosmotic stimulus (control) at 2 and 15 min. The response recovered 30 min later. $^{***}P < 0.001$ and $^{*}P < 0.05$ compared to control; $^{**}P < 0.01$, compared to 30 min; ($n = 5$). Error bars are S.E.M.

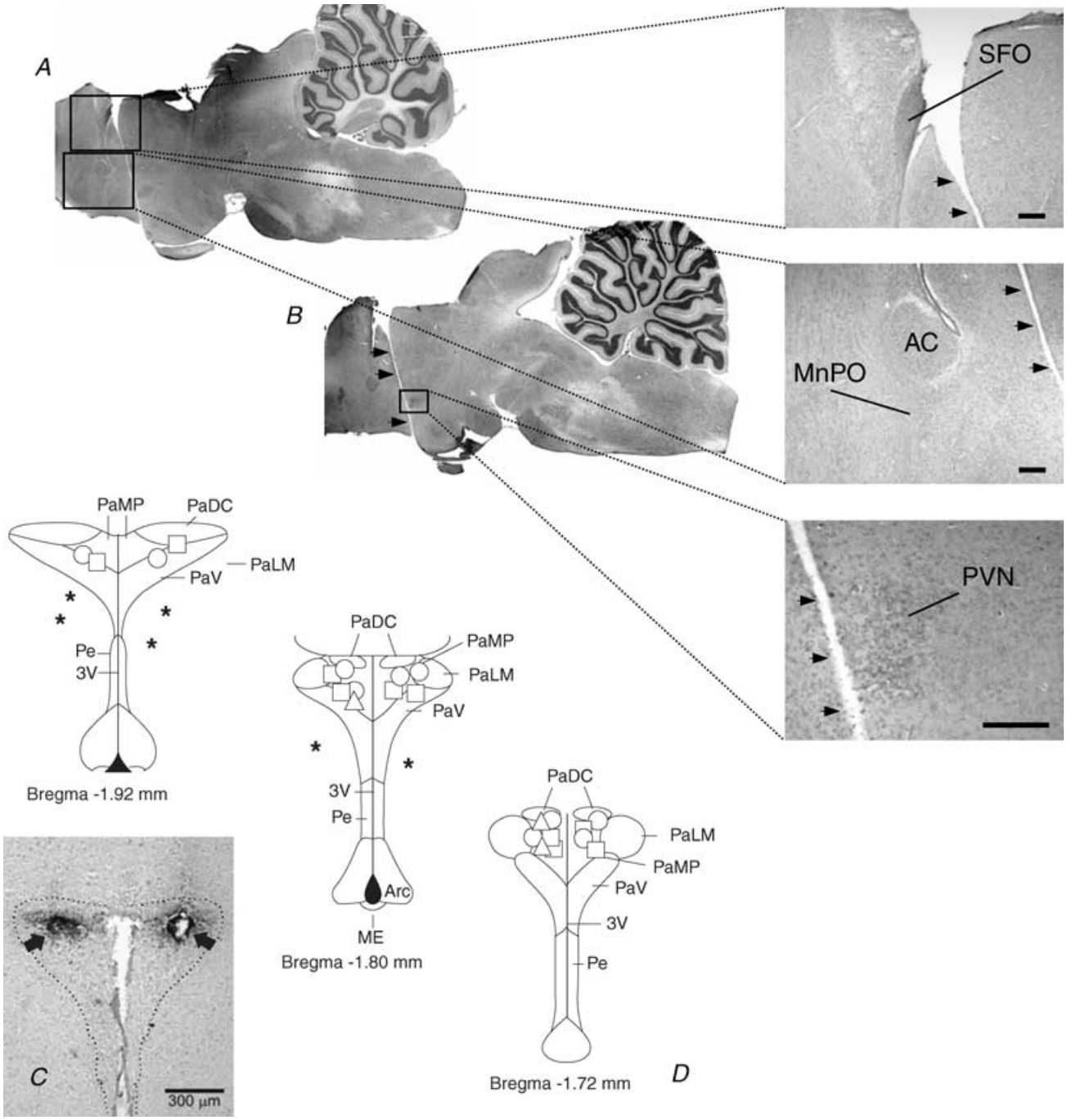


Figure 7
A, photomicrographs of a sagittal brain section, taken 0.1 mm lateral to the midline from a decorticated *in situ* preparation showing the intact lamina terminalis. The magnified areas depict the SFO and MnPO at higher magnification, respectively. The arrowheads indicate the level of the transection to disconnect the CVOs from the PVN. *B*, photomicrographs of a sagittal section, taken from the same brain as shown in *A*, 0.3 mm lateral to the midline. The magnified area shows that the transection (indicated by arrowheads) was made rostral to the PVN. The arrowheads indicate the level of transection. Scale bars: 200 μm. *C*, representative photomicrograph of the site of a bilateral microinjection into the PVN. The arrows mark the position of the glass micropipette tip. *D*, schematic diagram of coronal sections of the hypothalamus at the level of PVN modified from the atlas of Paxinos & Watson (1996), showing the centre of bilateral microinjections of isoguvacine (○), kynurenic acid (□) or unilateral microinjections of L-glutamate and hyperosmotic Ringer solution (△) into the PVN. Asterisks (*) represent microinjection sites of isoguvacine and kynurenic acid outside of the PVN, in a group of three rats that were ineffective in altering HS-induced sympathoexcitatory responses. Abbreviations: AC, anterior commissure; SFO,

saline had no effect on basal arterial pressure, we can rule out that the increase in blood pressure was not secondary to volume expansion.

Since peripheral administration of HS increases circulating levels of vasopressin, it has been postulated that it may be responsible for the rise in arterial pressure (Landgraf *et al.* 1988; Russ *et al.* 1992). Therefore, we sought to investigate the role of vasopressin on the hyperosmolality-induced pressor responses in conscious rats. Our results have shown that injection of HS produced no significant change in MAP at any time point after pre-treatment with a V_{1a} receptor antagonist (Fig. 1). Therefore, we suggest that the initial increase in MAP with acute salt loading is due to a vasoconstrictor effect of both the sympathetic nervous system (α_1-adrenoceptor and V_{1a} receptor), whereas the prolonged elevations of MAP following hyperosmolality are mediated by a V_{1a} receptor-sensitive mechanism only.

Taking the results from our *in vivo* experiments, we hypothesized that the integrity of hypothalamic vasopressinergic spinally projecting neurones was essential for the sympathoexcitation. This was assessed in a *in situ* decorticate arterially perfused rat preparation that left intact the lamina terminalis areas comprising the median preoptic nucleus (MnPO), subfornical organ (SFO) and organum vasculosum of the lamina termalis (OVLT), as well as the adjacent septal nuclei and hypothalamic areas (Fig. 7*A*). This preparation avoided the need for anaesthesia, allowed accurate control of the circulating osmolality, and exhibited robust, reproducible sympathetic nerve activity responses using physiologically relevant adjustments in osmolality. We suggest that the absence of anaesthesia helped preserve the sensitivity of the system to osmotic changes that were well within physiological limits. By using separate perfusate we also avoided the compounding problem of cumulative salt loading effects during repeated hyperosmotic challenges as can be the case *in vivo*.

The hypothalamus plays a critical role in homeostatic adjustments related to fluid balance disturbance (Antunes-Rodrigues *et al.* 2004). Therefore, we sought to test the importance of hypothalamic structures (particularly the PVN) in mediating the osmolality-induced sympathoexcitation. When the *in situ* preparation was challenged with an acute hyperosmotic stimulus (135 mM NaCl) of 321 ± 1 mosmol (kg water)$^{-1}$, for 40 s, we observed an increase in the LSNA before, but not after, removal of the hypothalamus (precollicular transection). Interestingly, we did observe a small residual increase in the LSNA following HS after the precollicular transection ($7 \pm 3\%$). This may be a direct effect of excess sodium chloride on the brainstem or spinal cord neurones (see Isawa *et al.* 2000). We also determined that the preparation was sensitive to another, non-NaCl osmolyte, namely mannitol. Our data show that a hyperosmotic stimulus using mannitol elicited an increase in sympathetic nerve activity, which was practically eliminated after precollicular transection.

Several studies have demonstrated the presence of osmosensitive receptors in neurones of the lamina terminalis, which contains the CVOs (for review see Bourque *et al.* 1994; Bourque & Oliet, 1997). These brain areas lack a complete blood–brain barrier, and they are intrinsically sensitive to changes in fluid osmolality. As the precollicular transection removes the entire hypothalamus we could not predict exactly which forebrain area could drive the hyperosmotic-induced sympathoexcitation. Hence, we performed a selective ablation of the CVOs, while leaving the PVN intact (Fig. 7*A* and *B*). After severing connections from the CVOs, the increase in LSNA elicited by hyperosmotic stimulus was almost abolished indicating that the integrity of CVOs and their neuronal connections to the PVN are essential for osmolality-driven sympathoexcitation. Indeed, the absence of peripheral osmoreceptors in our preparation, such as those located in mesenteric and hepatic portal vasculature (Vallet & Baertschi, 1980; Baertschi & Vallet, 1981; Hosomi & Morita, 1996), further support a role for central osmoreceptors in mediating the sympathoexcitation to salt loading.

We suppose that the hyperosmolality-induced sympathoexcitation involves excitation of PVN neurones by central osmoreceptors (SFO, OVLT and MnPO); the latter are well known to detect changes in osmolality (see: Sawchenko & Swanson, 1983; McKinley *et al.* 1992; Bains & Ferguson, 1995; Weiss *et al.* 1996; Toney *et al.* 2003). This is supported by the absence of sympathoexcitation after: (i) selective CVO lesions; (ii) hyperpolarization of the PVN with isoguvacine; and (iii) blockade of ionotropic glutamatergic transmission in the PVN; the latter emphasizing the importance of glutamate receptors. Moreover, our data do not support the presence of osmoreceptors in the PVN (at least up to 321 ± 1 mosmol (kg water)$^{-1}$), since hyperosmotic perfusate injected into the PVN had a negligible effect on LSNA (Fig. 5). Finally, we found that the integrity of the

subfornical organ; MnPO, median preoptic nucleus; PVN, paraventricular hypothalamic nucleus; PaDC, paraventricular hypothalamic nucleus, dorsal cap; PaLM, paraventricular hypothalamic nucleus, lateral magnocellular part; PaMP, paraventricular hypothalamic nucleus, medial parvicellular part; PaV, paraventricular hypothalamic nucleus, ventral part; Pe, periventricular hypothalamic nucleus; ME, median eminence; Arc, arcuate hypothalamic nucleus; 3V, third ventricle.

PVN is essential for mediating hyperosmolality-induced sympathoexcitation

Interestingly, an acute hyposmotic stimulus (259 ± 1 mosmol) also produced an increase in LSNA that persisted after precollicular transection and is therefore not hypothalamic dependent. Autonomic adjustments to hyposmotic stimulus are controversial. Brooks *et al.* (2005) showed that intracarotid hypotonic fluid infusion in dehydrated rats evoked a decrease in LSNA. In contrast, Brown *et al.* (2005) found that water (providing a hyposmotic stimulus), but not saline drinking in human subjects, increased sympathetic activity. In light of our results, we suggest that hyposmotic perfusate may act directly on the brainstem/spinal cord to elicit spontaneous firing of neurones due to an imbalance of sodium chloride (Isawa *et al.* 2000). This remains to be tested.

It is well established that an osmotic challenge elicits a sympathoexcitation, and parvocellular neurones from the PVN play a pivotal role in mediating this response (Swanson & Kuypers, 1980; Kannan *et al.* 1989; Badoer, 1996; Toney *et al.* 2003; Stocker *et al.* 2004*a*). Indeed, the PVN contains parvocellular neurones with projections to the intermediolateral cell column of the spinal cord and rostroventrolateral medulla, two major areas regulating sympathetic nerve activity (Cechetto & Saper, 1988; Swanson & Sawchenko, 1983; Coote, 1995; Stern, 2004; Stocker *et al.* 2004*b*). While we have demonstrated a role for glutamatergic transmission in the PVN for the hyperosmolality-driven sympathoexcitation, we recognize the importance of circulating (DiBona & Jones, 2001) and CVO-released angiotensin II (ANGII) in the PVN (Li & Ferguson, 1993), as well as its interactions with GABA (Chen & Toney, 2001; Unger *et al.* 1983). Indeed, $GABA_A$ (Decavel & Van den Pol, 1990; Haywood *et al.* 2001) and ANGII-AT_1 receptors (Tsutsumi & Saavedra, 1991) are both expressed in autonomic regions of the PVN. As ANGII excites spinally projecting PVN neurons by attenuating GABAergic synaptic inputs presynaptically (Li *et al.* 2003), we acknowledge a possible contributing role for ANGII in producing the sympathoexcitation to hyperosmolality in this study.

With regard to PVN efferent pathways, we cannot rule out the glutamatergic projections to the RVLM-spinal projections. Brooks *et al.* (2004) have reported that antagonism of glutamate receptors in the RVLM reduced the increase in LSNA produced by acute hyperosmolality in water-deprived rats, suggesting a role for the RVLM. This contrasts to our study showing a dominant role for the PVN-spinal neurones. The difference might be explained by the presence of anaesthesia in the sudy of Brooks *et al.* (2004) (2004). Further, Stocker *et al.* (2004*b*) demonstrated that parvocellular PVN neurones projecting to the RVLM and spinal cord were activated (c-Fos immunoreactivity) by water deprivation. Thus, we accept that PVN neurones projecting to the RVLM may also contribute to the hyperosmolality-induced sympathoexcitation (see Stocker *et al.* 2006).

Projections from the PVN to the brainstem and spinal cord were demonstrated (Saper *et al.* 1976; Shafton *et al.* 1998; Pyner & Coote, 1999, 2000), giving an anatomical correlate for the long-known influence of the hypothalamus on the autonomic nervous system (Swanson & Sawchenko, 1983). Fibres immunoreactive for vasopressin make up an abundant terminal network in the spinal cord, both in the superficial dorsal horn and in the intermediolateral cell column (Buijs, 1978; Nilaver *et al.* 1980), and spinal vasopressin has been associated with sympathoexcitation (Porter & Brody 1986*a*,*b*; Malpas & Coote, 1994). Since blockade of vasopressin receptors (subtype V_{1a}) in the spinal cord at the lower thoracic and upper lumbar level attenuated the sympathoexcitation induced by hyperosmotic stimuli, we suggest that it is the vasopressinergic hypothalamic-spinally projecting neurones that mediate the hyperosmolality-induced sympathoexcitation on the preganglionic sympathetic neurones. One possible caveat of the present study is that while we demonstrated that the V_{1a} receptor antagonist used blocked the sympathoexcitation to spinally applied vasopressin, we did not show specificity of action in terms of whether other non-vasopressinergic excitatory responses (reflex or centrally evoked) were affected as a consequence of non-specific drug action. However, in the absence of any change in the ongoing activity (both tonic and respiratory modulated), we are confident that the antagonist was primarily blocking V_{1a} receptors.

Hallbeck & Blomqvist, (1999) have shown that over 40% of the spinally projecting neurones in the PVN express vasopressin mRNA, and demonstrated that these neurones are localized to specific parts of the PVN. Moreover, studies performed by Motawei *et al.* (1999) have demonstrated that terminals containing vasopressin-like immunoreactivity are similarly distributed to PVN-descending axons, and in addition terminate near to and are closely associated with preganglionic sympathetic neurones. Malpas & Coote (1994) have demonstrated that V_{1a} antagonist administered intrathecally in anaesthetized rats was able to block the increases in the renal sympathetic activity in response to electrical stimulation of the PVN, or to intrathecal vasopressin injection, ensuring the important role of vasopressin in the PVN-spinal pathway. Thus, based on the fact that the main source of vasopressin in the spinal cord arises from parvocellular neurones of the PVN (Sawchenko & Swanson, 1982; Lang *et al.* 1983; Cechetto & Saper, 1988), and that sympathetic preganglionic neurones express vasopressin V_{1a} receptors (Sermasi *et al.* 1998), which are directly excited by vasopressin (Ma & Dun, 1985; Sermasi & Coote, 1994), we suggest that PVN vasopressinergic spinally projecting neurones could mediate the hyperosmolality-induced sympathoexcitation reported herein.

In summary, the *in situ* preparation is a promising model for understanding hypothalamic, brainstem and spinal cord mechanisms at the systems and cellular levels in the homeostatic regulation of blood pressure and volume. We have demonstrated that this preparation exhibits appropriate autonomic responses to repetitive hyperosmotic stimuli within a physiological osmolality range ($\pm$ 30 mosmol (kg water)$^{-1}$ from isotonic 291 $\pm$ 1 mosmol (kg water)$^{-1}$), and that this response is dependent upon the integrity of the PVN with involvement of glutamatergic neurotransmission. In conclusion, we state that vasopressin plays a significant role at the level of the spinal cord for relaying the hyperosmolality-induced sympathoexcitation.

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