Are sympathetic preganglionic neurones (SPNs) directly inhibited by GABAergic neurones in the central region of the rat spinal cord?

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The activity of SPNs is mainly influenced by inputs that originate both supraspinally and at the level of the spinal cord. To date, the contribution of specific groups of local interneurones to SPN activity remains to be elucidated. We utilised *in situ* hybridisation and electrophysiological studies to determine whether there are local GABAergic neurones that can influence the activity of SPNs.

For *in situ* hybridisation, rats (150 g) were anaesthetised with Sagatal (60 mg kg^{-1} I.P.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Spinal cord sections (30 μm) were hybridised with digoxigenin-UTP-labelled GAD_{65} or GAD_{67} sense and antisense RNA probes using a modified version of the manufacturer's protocol (www.biochem.roche.com). mRNA for GAD_{65} and GAD_{67} was visualised in neurones in a number of regions in the spinal cord but one notable compact group of GAD mRNA positive neurones was located dorsal and lateral to the central canal at the edge of the grey matter.

For electrophysiology, thoracic spinal cord slices (250 μm) were cut from 10–14 day rats anaesthetised with urethane (2 g kg^{-1}, I.P., then humanely killed) and submerged in oxygenated ACSF at room temperature. Whole-cell recordings were obtained from SPNs identified electrophysiologically and histologically. Stimulating electrodes were placed in a position corresponding to the GABAergic neurones in the central region and in the lateral funiculus (lf) for comparison. Drugs were applied to the bathing medium.

Stimulation of the central region elicited IPSPs with short constant latencies in all SPNs tested ($n = 12$) that were maintained in the presence of the excitatory amino acid antagonist kynurenic acid (500 μM, sufficient to block lf mediated EPSPs). This suggested that the IPSPs were elicited by activating a monosynaptic pathway. Similar IPSPs could not be elicited by stimulating the white matter dorsal to the central region or by stimulation of the contralateral IML or lateral funiculus. Central region evoked IPSPs were always antagonised by application of the $GABA_A$ receptor antagonist bicuculline (5 μM; -5.4 ± 0.3 to -0.6 ± 0.1 mV, mean ± S.E.M. $n = 7$) and no further effect was observed on addition of strychnine. In contrast, IPSPs elicited by lf stimulation were antagonised in part by bicuculline (-5.5 ± 0.9 to 2.2 ± 0.8 mV) and were further blocked by strychnine (2 μM, 2.2 ± 0.8 to 0.1 ± 0.1 mV, $n = 6$).

These data show that SPNs are directly inhibited by stimulation in the central region of the spinal cord and this may be due to activation of a compact group of GABAergic neurones in this region. This theory is undergoing further investigation in the laboratory.

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All procedures accord with current UK legislation.

The potassium channel subunit Kv3.1b is expressed in both GABAergic and glutamatergic neurones in the nucleus tractus solitarii (NTS) of the adult rat

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Kv3.1b is a member of the *Shaw* family of potassium channels with rapid activation and deactivation kinetics, commonly expressed in fast-firing GABAergic neurones (Erisir *et al.* 1999). We have previously reported the presence of the Kv3.1b subunit in a subset of neurones in the NTS of the adult rat medulla oblongata (Deuchars & Atkinson, 2001). Here we examine the possibility that these Kv3.1b-expressing neurones are GABAergic.

Male rats (100–150 g) were anaesthetised with Sagatal (60 mg kg^{-1} I.P.), perfused transcardially with artificial cerebrospinal fluid in which NaCl was replaced with sucrose (217 mM) and the brain removed. Total RNA was isolated from medulla and reverse transcribed, followed by 35 cycles of PCR performed using specific oligonucleotide primers for glutamic acid decarboxylase ($GAD_{65/67}$), vesicular glutamate transporter 2 (VGLUT2) or Kv3.1. For *in situ* hybridisation rats were anaesthetised as above and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Coronal 30 μm sections of medulla were hybridised with digoxigenin-UTP-labelled GAD_{65}, GAD_{67} or VGLUT2 sense and antisense RNA probes using a modified version of the manufacturer's protocol (www.biochem.roche.com). Subsequently, sections were incubated in a polyclonal antibody that recognises the carboxy terminal (residues 567–585) of the Kv3.1b subunit (Alomone). Sections were then washed and placed in either donkey anti-rabbit conjugated Cy3 (1:1000, Jackson) or a biotinylated secondary anti-rabbit IgG (1:200, Vector), followed by streptavidin Alexa (1:1000, Molecular Probes). Sections were then prepared for light microscopic analysis.

RT-PCR reveals the presence of GAD_{65}, GAD_{67}, VGLUT2 and Kv3.1b transcripts in the rat medulla. Furthermore, *in situ* hybridisation shows mRNA for GAD_{65} and GAD_{67} in GABAergic neurones and mRNA for VGLUT2 in glutamatergic neurones throughout the medulla. Kv3.1b immunoreactivity (IR) was also observed throughout the medulla. In the NTS, Kv3.1b IR was present in neurones in the ventrolateral, interstitial, medial and dorsolateral subnuclei where the staining appeared to be membrane bound. A small proportion of NTS neurones positively labelled for GAD_{65}, GAD_{67} or VGLUT2 mRNAs were also immunoreactive for the Kv3.1b subunit. These results clearly demonstrate that the Kv3.1b potassium channel subunit is expressed in both GABAergic and glutamatergic neurones in the NTS of the adult rat medulla oblongata, which are likely to have fast firing phenotypes.

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Distension of the main pulmonary artery stimulates vagal afferent activity in anaesthetized dogs

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Previously we have reported that distension of the main pulmonary artery by pressures in excess of 30 mmHg leads to an increase in systemic vascular resistance (McMahon *et al.* 2000). These pressures are higher than those normally observed in the pulmonary circulation, indicating that physiological pressures may not elicit this response. However, this failure to observe a pressor response at pressures below 30 mmHg might also reflect the effects of general anaesthesia on autonomic reflex mechanisms. The aim of the present study, therefore, was to determine whether or not vagal afferent fibres respond to physiological changes in pressure in this reflexogenic region.

Dogs were anaesthetized with α-chloralose (100 mg kg^{-1} I.V.) and artificially ventilated. A cardiopulmonary bypass was established that received blood from the right atrium and inferior vena cava. A pouch consisting of the entire extrapulmonary parts of the pulmonary arteries and the trunk was created and this was independently perfused with venous blood from a pressurized reservoir. The cephalic and subdiaphragmatic circulations were perfused with oxygenated blood. Afferent activity was recorded from single units or small multi-units in slips dissected from the left or right cervical vagi. The animals were humanely killed at the end of each experiment.

An increase in pulmonary pouch pressure from 5.0 ± 2.0 to 46.0 ± 4.5 mmHg elicited an increase in activity from 12.0 ± 4.1 to 40.3. ± 7.6 impulses s^{-1} (means ± S.E.M., $P < 0.05$, paired t test, $n = 9$). At a mean pulmonary pressure of 20.5 ± 1.0 mmHg, activity was significantly increased by 7.0 ± 2.5 impulses s^{-1} ($P < 0.05$), representing 25 % of the overall response. In additional experiments, the chest cavity was resealed and ventilation was simulated by application of a pulsatile negative pressure (approximately 8 mmHg) to the thoracic cavity at a frequency of 0.3 Hz. The mean pulmonary arterial pressure corresponding to 50 % of the overall response was lower during simulated ventilation than in its absence (21.7 ± 2.2 *versus* 16.8 ± 2.4 mmHg, $P < 0.05$, $n = 7$). We conclude that afferent activity from baroreceptors located in the extrapulmonary pulmonary arteries does increase to distension of this region. This response is enhanced by changes in intrathoracic pressure during ventilation. It is likely, therefore, that the vagal afferents attached to these receptors contribute to normal circulatory control.

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Changes in sympathetic nerve activity recorded from skeletal muscle arteries of the anaesthetised rat during graded levels of systemic hypoxia

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We recently demonstrated the ability to record sympathetic nerve activity from the surface of arterial vessels of 80–120 μm within skeletal muscle; this activity has characteristic baroreceptor modulation (Hudson *et al.* 2002). We have now investigated the changes in muscle sympathetic nerve activity (MSNA) produced by graded levels of systemic hypoxia.

Experiments were performed on eight spontaneously breathing male Wistar rats (256–322 g) anaesthetised with Saffan (7–12 mg kg^{-1} h^{-1}, I.V.). The animal was killed with an anaesthetic overdose at the end of the experiment. MSNA was recorded directly from the surface of arterial blood vessels of the right spinotrapezius muscle using the focal recording technique (Hudson *et al.* 2002). Hypoxic gas mixtures (12, 10 and 8 % O_2 in N_2) given consecutively via the tracheal cannula for 2 min each over a 6 min period evoked graded falls in mean arterial blood pressure (MABP) graded increases in heart rate (HR) and respiratory rate (Marshall & Metcalfe, 1988). Concomitantly there were graded increases in MSNA.

In four animals which experienced the complete protocol of 12, 10 and 8 % O_2, MABP fell from a baseline of 118 ± 10 to 99 ± 12, 81 ± 6 and 67 ± 8 mmHg (means ± S.E.M.), respectively, while HR increased from 391 ± 28 to 401 ± 23, 416 ± 16 and 419 ± 8 beats min^{-1} and respiratory rate increased from 88 ± 7 to 110 ± 7, 115 ± 9 and 113 ± 9 breaths min^{-1}. Concomitantly, MSNA (2–3 units in each recordings) increased by 81 ± 15, 125 ± 11 and 193 ± 9 % during 12, 10 and 8 % O_2. Cross-correlation histogram analysis showed MSNA to have strong cardiac- (6.5–7 Hz) and respiratory- (1.5–1.9 Hz) related rhythms at rest and during each period of hypoxia.

These results indicate that graded systemic hypoxia evokes graded increases in MSNA as a consequence of peripheral chemoreceptor stimulation and/or baroreceptor unloading, even though there is an accompanying graded vasodilatation in skeletal muscle (Marshall & Metcalfe, 1988).

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Projections from the central nucleus of amygdala to brainstem neurones that are activated by alteration of blood pressure

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The central nucleus of amygdala (CeA) is a forebrain nucleus involved in the integration of cardiovascular functions during stress and anxiety (Iwata *et al.* 1987). Anatomical and physiological studies indicate that the CeA sends projections to several brainstem nuclei, including the nucleus of the solitary tract (NTS). We have recently shown that axons projecting from the CeA to the NTS contain γ-aminobutyric acid (GABA) and the vesicular GABA transporter (Saha *et al.* 2000). Some CeA terminals in the NTS were also immunopositive for somatostatin, and apposed dendrites displaying immunoreactivity for the sst2A receptor subunit (Saha *et al.* 2002).

Here we have used the expression of the immediate early gene *c-fos* to identify NTS neurones that are activated following infusion of either a hypertensive agent, phenylephrine (PE) or a hypotensive agent, sodium nitroprusside (NP). Adult rats were anaesthetized with pentobarbitone (40 mg kg^{-1}, I.P.) and infused for 1 h with either PE (1 mg ml^{-1} in saline, $n = 8$) or NP (1 mg ml^{-1} in saline, $n = 6$), at a rate sufficient to maintain mean arterial pressure at least 25 % above or below the resting level, respectively. Control rats ($n = 4$) were infused with saline for the same length of time. Axons originating from the CeA were identified in the same animals by microinjection of biotin dextran amine (BDA, 0.2 μl of 10 %) in the CeA 12–15 days earlier, under the same conditions of anaesthesia. At the end of the infusion period, the rats were humanely killed by perfusion with aldehyde fixative, and vibratome sections of the brainstem were processed for Fos-protein immunohistochemistry. PE-induced hypertension resulted in *c-fos* expression in neurones mainly restricted to the dorsomedial subnucleus of the NTS. On the other hand, NP-induced hypotension produced more widespread *c-fos* expression in the NTS which was most concentrated in the medial and ventral subnuclei. Following microinjection of BDA into the CeA, anterogradely labelled varicose fibres were observed in close apposition with Fos-immunoreactive neurones in the dorsomedial NTS.

The results suggest that the CeA may directly influence the activity of NTS neurones that are involved in regulation of blood pressure in response to stressful conditions. Further electron microscopic studies are needed to confirm that the terminals derived from the CeA axons form synapses with the activated neurones in the NTS.

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The contribution of neuropeptide Y to sympathetically evoked vasoconstriction of rat tail artery *in vitro*

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An early study that used neuropeptide Y (NPY) as an agonist suggested that NPY contributes to sympathetically evoked responses in rat tail artery *in vitro* (Nield, 1987). A recent study using the specific NPY Y_1 antagonist, BIBP3226, suggested that neurally released NPY does not contribute to sympathetically evoked responses in this vessel (Duckles *et al.* 1997). Current studies in rat tail artery from this laboratory showed that small numbers of impulses evoke a contraction that relies more on ATP, and with increased numbers of impulses relies more on noradrenaline (Bradley Johnson, unpublished observations), but it is not known whether NPY affects responses to these stimuli preferentially. Here we examine whether sympathetically released NPY contributes to neurally evoked responses in rat tail artery, and whether any contribution of NPY is influenced by the number of sympathetic impulses.

Tail arteries were excised from humanely killed male Sprague-Dawley rats (250–350 g). Isometric contractile responses to electrical field stimulation were measured in response to several batteries of different impulse numbers (all at the same frequency of 20 Hz, 1 ms pulses, supramaximal) in endothelium-denuded vessels. Experiments were conducted in the presence of NPY and the selective NPY Y_1 antagonist, BIBP 3226. Electrically evoked responses were abolished in the presence of tetrodotoxin (1 μM) or guanethidine (10 μM).

NPY had a direct agonist action and potentiated sympathetically mediated responses in a dose-related manner (10^{-9} to 10^{-6} M). NPY (75 nM) potentiated responses to very short trains (5 impulses) delivered every minute (4/5 experiments) to 169 ± 30 % of control (mean ± S.E.M.). BIBP 3226 (75 nM) decreased this response to 36 ± 5 % of control ($n = 5/5$). In the presence of NPY, the response to a couplet (2 impulses) was significantly potentiated by 77 ± 13 % ($P < 0.05$, ANOVA and SNK; $n = 12$). The response to a short train (20 impulses) was significantly potentiated, but only to 28 ± 4 % greater than control ($P < 0.05$). In the presence of BIBP 3226, responses to the couplet (53 ± 10 %) and short train (12 ± 4 %) were significantly reduced ($P < 0.05$ in each case). In the presence of NPY or BIBP 3226 the couplet was affected significantly more than the train ($P < 0.05$ in each case, Student's paired *t* test).

This study confirms a role for endogenously released NPY in sympathetically evoked responses in rat tail artery. As it preferentially affects the responses to a couplet, our data raises the possibility that NPY preferentially affects the actions of sympathetically released ATP in this tissue.

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TRH potentiates excitatory inputs from the raphe pallidus to rat hypoglossal motoneurones, *in vitro*

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Thyrotropin-releasing hormone (TRH) is co-localised with 5-hydroxytryptamine (5-HT) and glutamate in raphe pallidus projections to rat hypoglossal motoneurones (Kachidan *et al.* 1991; Nicholas *et al.* 1992), with this peptide having been shown to have an excitatory action on these latter neurones (Bayliss *et al.* 1992). Since we have previously demonstrated that 5-HT inhibits glutamatergic inputs from the raphe pallidus to hypoglossal motoneurones via an action at presynaptic terminals (Bouryi & Lewis, 2001), the aim of the present study was to determine whether TRH could also modulate glutamate release via an action at this site.

Male Wistar rats (12–14 days) were terminally anaesthetised with sodium pentobarbitone (120 mg kg^{-1}, I.P.) and 300 μm coronal slices of the medulla prepared (Lewis, 1994). Whole-cell voltage-clamp recordings were made from identified hypoglossal motoneurones. Synaptic pathways from the raphe pallidus were activated utilising a stimulating electrode placed within this region, with pharmacological agents being applied in the superfusate as required.

Recordings were made from 21 hypoglossal motoneurones located within the ventral region of the nucleus. TRH (10 μM) evoked a membrane depolarisation of 6.53 ± 4.7 mV (mean ± S.D., $P < 0.001$, Student's paired t test, $n = 15$), an increase in cell input resistance of 34.0 ± 33 % ($P < 0.001$, $n = 15$) and an increase in spontaneous excitatory postsynaptic currents (EPSCs) in the majority of neurones. TRH increased the amplitude of EPSCs (120 ± 16 %, $n = 7$) evoked by stimulation within the raphe pallidus whilst decreasing the paired pulse ratio from 1.22 ± 0.13 to 1.14 ± 0.09 ($P < 0.05$, $n = 4$). In seven neurones, TRH also evoked spikelets. The spontaneous EPSCs, but not the spikelets, were abolished by 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulphonamide disodium (NBQX, 10 μM). The amplitudes of the spikelets were unaffected by membrane voltage in the recorded cell.

These data demonstrate that TRH has multiple actions on hypoglossal motoneurones including a postsynaptic excitatory action, potentiation of excitatory glutamatergic inputs from the raphe pallidus via an action at presynaptic terminals and the synchronisation of activity in adjacent neurones following the opening of gap junctions.

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Activation of adenosine $A2_A$ receptors in the intermediolateral cell column (IML) enhances inhibitory but not excitatory synaptic transmission

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Adenosine is an important neuromodulator in the CNS. Its actions are mediated via G-protein-coupled receptors classified as A1, $A2_A$, $A2_B$ and A3. When administered intrathecally, agonists at the A1R (Koh *et al.* 1996) and $A2_A$R (Koh *et al.* 2000) decrease blood pressure and heart rate. Deuchars *et al.* (2001) determined the presence and function of A1Rs in the IML but since the neuronal circuitry mediating the $A2_A$R effect is unclear we studied the distribution and role of $A2_A$Rs in the spinal cord.

For immunohistochemical studies, rats (100–150 g) were injected intraperitoneally with 0.1 ml 1 % Fluorogold and 7 days later were humanely killed by Sagatal (60 mg kg^{-1} I.P.) and perfused transcardially with 4 % paraformaldehyde/0.1–0.5 % glutaraldehyde. Thoracic spinal cord slices (50 μm) were cut and incubated in anti-$A2_A$R antibody (Santa Cruz, Biotechnology) followed by a Cy3 conjugated secondary antibody. $A2_A$R immunoreactivity was observed throughout the spinal cord. Intense punctate staining was observed in a compact region of the IML. These punctate structures, suggestive of terminals, apposed Fluorogold labelled sympathetic preganglionic neurones (SPNs). For electro-physiology, thoracic spinal cord slices (250 μm) from terminally urethane-anaesthetised (2 g kg^{-1} I.P.) 10- to 12-day-old rats were submerged in ACSF equilibrated with 95 % O_2 and 5 % CO_2 at 20°C. Whole-cell recordings were made from SPNs and interneurones in the IML, identified by electrophysiology and histology. Bath application of the $A2_A$R agonist CGS-21680 (1 μM) had no significant effect (Student's paired t test) on EPSPs evoked by stimulation of the lateral funiculus but significantly enhanced the amplitude of bicuculline and strychnine sensitive IPSPs (control 5.1 ± 0.6 mV, CGS-21680 7.2 ± 0.8 mV, mean ± S.E.M., $n = 19$, $P \leqslant 0.005$). This increase was blocked by the selective $A2_A$R antagonist, ZM-241385 (1 μM, $n = 6$). Application of CGS-21680 changed the paired pulse ratio ($n = 6$) of evoked IPSPs, but had no effect on input resistance suggesting a presynaptic site of action.

This study indicates that $A2_A$Rs are found in the IML where their activation selectively increases inhibitory transmitter release from terminals presynaptic to IML neurones. These results suggest $A2_A$Rs may play a vital role in the control of sympathetic outflow and provide evidence for a way in which activation of inhibitory A1Rs and/or excitatory $A2_A$Rs causes similar cardiovascular responses.

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Cardiac vagal afferent fibres projecting to nucleus tractus solitarii and cervical spinal dorsal horn in the rat

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Vagal afferent fibres from the heart may play a role in reflex control of the circulation in hypertension and heart failure (Hainsworth, 1991) and in conveying nociceptive information associated with ischaemic heart disease (Foreman, 1999). In this study, the central distribution of afferent fibres from the heart was investigated by injecting cholera toxin B-subunit (CTb, 0.02 ml of 1 % aqueous solution) into the pericardium in halothane (95 % in O_2) anaesthetised, artificially ventilated adult (250–300 g, $n = 10$) Wistar rats. After a 10-day recovery period, the rats were humanely killed by perfusion with aldehyde fixatives under halothane anaesthesia, and vibratome sections of the medulla and spinal cord were labelled with mouse, goat or rabbit antibodies to CTb for fluorescence immunohistochemistry. CTb labelled axons formed *en passant* and terminal varicosities bilaterally in the nucleus tractus solitarii (NTS), and at caudal levels formed a band in the dorsal part of the commissural subnucleus, in the dorsomedial subnucleus and around the tractus solitarius. At area postrema level, they were prominent in the dorsomedial and interstitial NTS subnuclei, but were sparsely distributed in the medial subnucleus. More rostrally, they maintained the same position, but were markedly reduced in number. Examination in coronal, parasagittal and horizontal planes revealed labelled axons entering the commissural NTS bilaterally, and at progressively more caudal levels these could be followed in bundles laterally to the central canal, from where they radiated along the interface of the dorsal horn with the overlying white matter. Beaded fibres and varicosities were seen surrounding neuronal perikarya in the superficial cervical dorsal horn. The vagal origin of these fibres was confirmed by their elimination on the ipsilateral side in unilaterally vagotomised rats, and by no reduction in their numbers after removal of the sympathetic stellate ganglion. Both these procedures were performed under halothane anaesthesia immediately before injection of CTb. Dual labelling with specific antibodies to vesicular glutamate transporters VGLUT1 and VGLUT2 (Varoqui *et al.* 2002) suggested that subpopulations of the vagal fibres express VGLUT1 and are glutamatergic in nature.

Our observations are consistent with vagal afferent fibres from the heart having predominantly excitatory central projections terminating in areas of the medulla oblongata and cervical spinal cord that are concerned with both cardiovascular and nociceptive reflex control.

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Functional role of nitric oxide in spinal sympathetic networks activated by PVN stimulation in anaesthetised rats

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There are contrasting reports on the action of nitric oxide (NO) in spinal regulation of sympathetic neurone activity. *In vivo* experiments on rabbits showed NO donors given intrathecally facilitated sympathetic activity to the kidney (Hakim *et al.* 1995), whereas *in vitro* experiments on slices of rat spinal cord showed that NO potentiated glycine inhibitory currents in immature sympathetic preganglionic neurones (Wu & Dun, 1996). The latter study indicated that the NO effect followed synaptic excitation of the neurones. Therefore in rats anaesthetised with urethane, chloralose mixture (650 mg kg^{-1}, 50 mg kg^{-1}) I.V. we determined whether NO acted to enhance or depress synaptic excitation of spinal sympathetic neurones involved in the pathway from the paraventricular nucleus (PVN) of the hypothalamus to renal sympathetic nerve. Recordings were made of renal sympathetic nerve activity (RSNA) and arterial blood pressure. For activation of neurones in PVN a glass micropipette containing D,L-homocysteic acid (DLH) was placed stereotaxically, and sites marked with pontamine sky blue for later histological identification. Changes in the efficacy of spinal circuits were induced by intrathecal (I.T.) application of drugs via a catheter inserted into the subarachnoid space via the foramen magnum so that its tip lay at T_{10}. Drugs were administered in a volume of 10 μl washed in with 20 μl of artificial CSF. Statistical analysis was performed using a Student's two-tailed, paired *t* test. Rats were killed by overdose of urethane anaesthetic at the end of experiments. I.T. application of the NO precursor L-arginine (100 nM) significantly reduced RSNA by 12 ± 3 % ($P \leqslant 0.05$). An increase of RSNA of 69 ± 14 % produced by I.T. glutamate (0.2 M) was enhanced to 94 ± 15 % by preceding the same dose with I.T. L-NMMA (200 nM) ($P \leqslant 0.05$). Similarly, an increase in RSNA of 83 ± 15 % produced by microinjection of DLH (100 nl, 0.2 M) into PVN was potentiated by 126 ± 21 % ($P \leqslant 0.05$) when elicited following I.T. L-NMMA (200 nM). I.T. L-NMMA alone had no significant effect on baseline RSNA activity. I.T. application of the glycine receptor antagonist strychnine (3 mM) prior to PVN stimulation also enhanced the PVN-RSNA response. I.T. strychnine alone had no significant effect on baseline RSNA. The potentiation of PVN-RSNA responses by strychnine was not further increased by prior I.T. L-NMMA, indicating that the effects of block of glycine receptors and NO synthesis are not additive. These results suggest that NO acts as a retrograde inhibitory messenger following PVN synaptic activation of spinal sympathetic neurones and that glycine interneurones may be involved in this action.

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Light and electron microscopic localisation of $P2X_4$ receptor subunit immunoreactivity in the dorsal vagal complex of the rat

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Ionotropic ATP-gated receptors comprise three (or multiples of three) of the seven subunits cloned so far, $P2X_{1-7}$. $P2X_4$ receptor subunits ($P2X_4RS$) are expressed throughout the CNS. In the nucleus tractus solitarii (NTS) an antibody against an extracellular portion of the $P2X_4RS$ revealed sparsely distributed $P2X_4RS$-positive neurones, mainly in the intermediate portion of the commissural NTS, and along the postremal border (Yao *et al.* 2001). Here we compare this pattern with that obtained using an antibody against a peptide sequence corresponding to an intracellular portion of the subunit (C-terminus, amino acids 370–388, Alomone Labs), as well as examine the localisation ultrastructurally.

Male Wistar rats (150–200 g) were humanely killed by anaesthesia with sodium pentobarbital (Sagatal, 60 mg kg^{-1} I.P.) and transcardial perfusion with fixative containing 0.1 M phosphate buffer, 4 % paraformaldehyde and 0–0.5 % glutaraldehyde. Transverse sections (50 μm) of medulla oblongata were cut on a vibrating microtome and incubated in rabbit anti-$P2X_4RS$ (1:500) for 12–24 h. Sections were washed and placed in either donkey anti-rabbit IgG conjugated to Cy3 (1:1000, Jackson) or to a biotinylated anti-rabbit IgG (1:500; Jackson Immunoresearch) followed by peroxidase conjugated extravadin (1:1500; Sigma), visualised with diaminobenzidine. Sections were subsequently prepared for fluoroscence or light and electron microscopy.

Using fluorescence microscopy neuronal somata in the dorsal vagal nucleus contained moderate levels of $P2X_4RS$ immunoreactivity, while somata in the NTS were very lightly labelled. A dense band of immunoreactivity was observed in the sub-postremal NTS. The area postrema was densely labelled. In the NTS conventional light microscopy revealed fine punctate staining of the neuropil. In addition, some stained elements appeared to be glial in nature, although glial somata were not evident. Electron microscopy revealed that immunoreactivity was present both pre- and postynaptically in neurones. Post-synaptically the peroxidase reaction product could be located at the synaptic specialisation. However, presynaptically the reaction product was rarely located at the synaptic grid. In addition, glial cell processes surrounding presynaptic terminals were also labelled.

These data indicate diverse subcellular localisation of the $P2X_4RS$ in the NTS. We are currently investigating the origin and chemical nature of the presynaptic terminals bearing the $P2X_4$ receptors, as well as those that are adjacent to $P2X_4RS$ containing glial processes.

Yao, S.T. *et al.* (2001). *Neuroscience* **108**, 673–685.

All procedures accord with current UK legislation.

Effects of sympathetic impulse numbers on contributions of noradrenaline and ATP to vasoconstriction in rat tail and femoral arteries

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In vitro studies reported that responses to sympathetic vasoconstrictor stimulation with single or a few electrical impulses are mainly mediated by ATP (Bao, 1993). When impulse numbers increase to $\geqslant 20$, noradrenaline (NA) provides the main contribution. However, *in vivo*, contributions of NA dominated responses of tail and hindlimb vasculature to stimulation with both couplets and short trains (2 and 20 impulses at 20 Hz; Johnson *et al.* 2001). Here we studied how the number of impulses delivered alters contributions of NA and ATP to responses evoked in tail and femoral arteries, and whether responses gained *in vitro* are the same as those gained *in vivo*.

Male Sprague-Dawley rats (250–350 g) were killed humanely. Isometric contractile responses of excised tail and femoral arteries to electrical field stimulation were compared. Batteries of different impulse numbers (1−100 impulses at 20 Hz, 1 ms pulses, supramaximal) were applied to endothelium-denuded vessels in the presence of antagonists for NA or ATP (phentolamine, 2×10^{-6} M and suramin, 10^{-4} M).

Electrical stimuli evoked responses in tail artery ($n = 12$) around 16 times greater than in femoral artery ($n = 7$; significant from $\geqslant 4$ impulses, $P < 0.001$, ANOVA and SNK). In both arteries, absolute responses to stimuli were markedly reduced by phentolamine (significant at all impulse numbers in tail, $P < 0.001$). Relative responses to two impulses were reduced by 82 ± 4 % (mean ± S.E.M.) and 87 ± 13 %, respectively, whilst responses to 20 impulses were reduced by 93 ± 2 and 85 ± 7 %, respectively. When suramin was given first, absolute responses were reduced (in tail at impulse numbers $\geqslant 6$, $P < 0.001$ for each; in femoral artery at 100 impulses, $P < 0.001$). Relative responses to two impulses were reduced by 82 ± 6 and 75 ± 18 % in tail and femoral arteries, respectively, but responses to 20 impulses were reduced by only 31 ± 11 and 58 ± 27 %, respectively. Relative decreases with suramin in response to two impulses were significantly different from decreases in response to 20 impulses in tail arteries ($P < 0.001$). Responses remaining after the first antagonist were usually abolished after subsequent addition of the second.

We confirm that in tail artery, ATP contributes more to responses to few impulses and NA contributes more to greater numbers. We extend these observations to femoral artery, which shows a greater reliance on ATP at higher numbers of impulses than tail artery. This is in contrast to *in vivo* studies in tail and hindlimb vascular beds, confirming that conclusions from *in vitro* studies must be applied to the *in vivo* situation with caution.

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Population of neurones within dorsal and dorsomedial regions of the nucleus of the solitary tract that are sensitive to 4-AP and TEA

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The Kv3 subfamily of Kv channels consists of four genes (Kv3.1–3.4) and each Kv3 gene encodes multiple products by alternative splicing. Kv3.1 and Kv3.2 channels have been located within distinct populations of central neurones, with these channels endowing repetitive firing properties on neurones (for review, see Rudy *et al.* 1999). We have previously shown the existence of Kv3.1b subunits of these channels in specific neurones within the nucleus of the solitary tract by immunoreactivity (NTS, Deuchars & Atkinson, 2001). The aim of the present study was to examine the role of the Kv3.1 channels in determining the firing properties of NTS neurones. Male Wistar rats (15–21 days) were terminally anaesthetized with sodium pentobarbitone (120 mg kg^{-1}, I.P.) and humanely killed by decapitation. 300 μm coronal slices of the medulla oblongata were prepared. Whole-cell patch-clamp recordings were made from 50 NTS neurones from dorsal and dorsomedial regions of this nucleus and their firing properties were examined pre- and post-application of 4-AP (30 μM) and TEA (0.5 mM). At these specific concentrations if similar effects are observed then it is likely to be due to action on the Kv3.1 subunit (Coetzee *et al.* 1999). In 38 neurones 4-AP increased the action potential (AP) duration from 4.5 ± 1.5 to 10.3 ± 3.7 ms (mean ± S.D., $P < 0.05$, Student's paired *t* test), decreased the AHP amplitude from 17.4 ± 3.2 to 10.4 ± 2.9 mV ($P < 0.05$) and the steady-state firing frequency from 12.7 ± 0.9 to 8.7 ± 1.8 Hz ($P < 0.05$). TEA was tested in 25 of these 38 and seen to increase the AP duration from 5.1 ± 1.3 to 12.5 ± 4.2 ms ($P < 0.05$), and decrease the AHP amplitude from 18.6 ± 2.3 to 14.1 ± 4.1 mV ($P < 0.05$) and the steady-state firing frequency from 13.4 ± 1.3 to 7.5 ± 1.4 Hz ($P < 0.05$). Twelve neurones were insensitive to the application of the pharmacological agents. Two neurones were sensitive to TEA and insensitive to 4-AP. These data indicate that a subset of NTS neurones are sensitive to low concentrations of 4-AP and TEA, and are suggestive of a role for Kv3.1 channels in the neuronal output of specific neurones within dorsal and dorsomedial regions of the NTS. Ongoing studies are looking at the phenotype of these neurones and the circuits they are involved in.

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Enhancement of the baroreceptor-heart rate reflex by nitric oxide in the nucleus ambiguus of anaesthetised rats

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Nitric oxide (NO) produced within the central nervous system (CNS) blunts the baroreflex control of heart rate. Evidence suggests that this action could be occurring within the nucleus tractus soiltarii (Paton *et al.* 2001) where the enzyme NO synthase (NOS) has been localised immunohistochemically. However, NOS is also abundant in the nucleus ambiguus (NA) where vagal neurones supplying the heart are situated (Maqbool *et al.* 1995). There is only one report indicating that NO within the NA may play a role in the vagal control of heart rate and this suggests that cardiac vagal neurones are excited (Ruggeri *et al.* 2000). No studies have examined how NO in the NA may influence the baroreceptor heart rate reflex. In the present study in fifteen Wistar rats anaesthetised with urethane (650 mg kg^{-1} I.V.) we sought to determine the influence of NO on vagal tone and the baroreceptor reflex. Using stereotaxic co-ordinates the heart rate response to microinjection (50 nl) of the NO donor sodium nitroprusside (SNP, 1 mM) and the NO synthase (NOS) inhibitor, N^G-nitro-L-arginine (L-NNA, 3 mM) into functionally identified cardioinhibitory sites in the NA was determined. To estimate baroreflex sensitivity (BRS), RR interval and mean arterial pressure (MAP) changes were calculated following a bolus injection of phenylephrine hydrochloride (1–2 μg kg^{-1} I.V.). Data (means ± S.E.M.) were subjected to Student's paired *t* test ($P < 0.05$). Rats were humanely killed with an overdose of urethane at the end of the experiment. Unilateral microinjection of SNP in the NA induced a significant decrease in heart rate (−68 ± 14 b.p.m.; $P < 0.001$), with no significant change in MAP (2 ± 2 mmHg, $n = 12$ sites). Bilateral NOS inhibition produced a non-significant change in heart rate (+2 ± 1 b.p.m.), with no significant change in MAP (−2 ± 1 mmHg, $n = 22$ sites). However, bilateral NOS inhibition resulted in a significant attenuation in the slope of the regression line between RR interval and MAP compared with control (0.595 ± 0.095 ms $mmHg^{-1}$ at control; 0.136 ± 0.046 ms $mmHg^{-1}$ following L-NNA; $P < 0.001$, $n = 11$ sites).

The results confirm that NO within the NA of anaesthetised rats excites cardiac vagal neurones. There appears to be no tonic NO modulation of cardiac vagal motoneurone activity regulating heart rate, as blockade of NO synthesis does not alter resting heart rate. NO at synapses within the NA facilitates the baroreflex control of heart rate.

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Light and electron microscopic localisation of the angiotensin type 1 receptor (AT1R) in regions of spinal cord of the rat that influence autonomic outflow

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Intrathecal administration or microinjection of angiotensin II (angII) in the rat increases arterial pressure and heart rate via an increase in sympathetic nerve activity (Lewis & Coote, 1993). The AT1R appears to mediate such cardiovascular responses to angII since they are blocked by intrathecal losartan (Park & Henry, 1997). Consistent with a possible direct effect on sympathetic preganglionic neurones (SPNs), angiotensin II-like immunoreactivity was detected in synaptic contacts with neurones in the thoracic lateral horn (Galabov, 1992). To determine the site of the angII actions we examined the localisation of the AT1R in the spinal cord.

Male Wistar rats (100–150 g) were intraperitoneally injected with fluorogold (0.1 ml, 1 % in NaCl; Fluorochrome Inc.) and allowed to recover for 5 days prior to being humanely killed by anaesthesia with Sagatal (60 mg kg^{-1}, I.P.) and transcardial perfusion with fixative containing 4 % paraformaldehyde/ 0–0.2 % glutaraldehyde in 0.1 M phosphate buffer. Transverse sections (50 μm) of thoracic spinal cord were cut on a vibrating microtome (Leica) and incubated in an affinity-purified antibody to the AT1 receptor (1:400, RDI). Sections were then washed and place in either donkey anti-rabbit IgG conjugated to Cy3 (1:1000, Jackson Immunochemicals) or to a biotinylated anti-rabbit IgG followed by peroxidase conjugated extravidin (Sigma), visualised with diaminobenzidine. Sections were then prepared for fluorescence or light and electron microscopic examination as appropriate.

In the intermediolateral cell column (IML) a subset of fluorogold SPNs contained immunoreactivity for the AT1R associated with their somatic membrane. In addition, punctate-like labelling was also apparent within this region. In lamina X, in the central autonomic area, AT1 receptor labelling was also occasionally detected in neurones dorsal to the central canal. The ependymal cells surrounding the central canal were strongly immunoreactive. Ultrastructural analysis revealed that in the IML AT1 receptor labelling was evident in neuronal somata where it was associated with the cytoplasm, and rough endoplasmic reticulum as well as the somatic membrane. Reaction product was also detected in myelinated axons and presynaptic terminals. In addition, immunoreactive glial cell processes were also apparent. The localisation to a subset of SPNs suggest that angII can act to directly influence sympathetic outflow. However, indirect actions are also possible via angII acting on presynaptic terminals or glial cells.

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Changes in the noradrenergic innervation of lumbosacral spinal cord following systemically administered DSP-4 have no effect on micturition pattern in the male rat

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Aged (24 month) male rats exhibit different *in vivo* micturition patterns to those of young adult (3 month) animals. In young rats, micturition episodes are smaller and more frequent during the night than during the day. In aged rats the pattern seen during the day continues at night and the volume of urine released over 24 h is greatly increased. These changes could be due to previously observed degeneration of long descending projections to spinal cord regions involved in the control of micturition (Santer *et al.* 2002). Since a proportion of these descending inputs are monoaminergic we used the neurotoxin DSP-4 (*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine) to deplete noradrenergic projections from the locus coeruleus in young rats to see whether this would result in the emergence of the aged pattern of micturition. Prior to DSP-4 injections the rats were placed in a metabolic cage for 24 h and urine samples collected on an electronic balance connected to a PC. Rats were then injected with Zimelidine (10 mg kg^{-1} I.P.) to protect 5-HT-containing neurones before the administration of DSP-4 (50 mg kg^{-1}) either intraperitoneally ($n = 4$) or subcutaneously ($n = 4$). After a period of 7–10 days the micturition patterns of the rats were re-assessed on at least two separate occasions. Rats were then humanely killed by terminal anaesthesia ('Euthatal', sodium pentobarbitone, 200 mg kg^{-1}) and perfused with 4 % paraformaldehyde. The effect of the DSP-4 on the distribution of catecholamines in the lumbosacral spinal cord was assessed using immunocytochemistry for tyrosine hydroxylase (TH, antibody TZ 1010, Affiniti Research Products Ltd). DSP-4 administration caused a marked reduction in TH immunopositive axons in both dorsal and ventral regions of the lumbosacral spinal cord, including regions containing motorneurones innervating the pelvic floor musculature. Despite this, statistical comparisons (Student's paired *t* test) of micturition data before and after DSP-4 treatment revealed no significant change. The maintenance of *in vivo* micturition characteristics in post-DSP-4 lesion rats may, however, reflect the fact that labelling within lumbosacral autonomic regions was generally preserved. Since lumbosacral monoaminergic labelling in autonomic regions in aged rats is known to decline, these findings suggest that DSP-4 lesions are not a good model for studying normal patterns of micturition during ageing.

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$GABA_A$ receptor subunit expression in the rat nucleus tractus solitarii

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The synaptic action of the inhibitory transmitter γ-aminobutyric acid (GABA) in the nucleus tractus solitarii (NTS) of the medulla is mediated by both $GABA_A$ and $GABA_B$ receptors (Kubo & Kihara, 1987; Suzuki *et al.* 1993). $GABA_A$ receptors can be composed of five structurally and pharmacologically different classes of subunit proteins, which exist in at least 16 major isoforms: $\alpha_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, ϵ, θ and π (see Barnard *et al.* 1998). Recently, we have detected the expression of mRNAs encoding six $GABA_A$ receptor subunits (α_1, α_2, α_3, β_2, β_3, γ_2) in the rat NTS by polymerase chain reaction (PCR), and confirmed the cellular localisation of the receptor proteins using specific antibodies (Saha *et al.* 2001). Both PCR and immunohistochemistry showed that the $GABA_A$ α_5 subunit is absent in the NTS. Here we have investigated the presence of further subunits of the $GABA_A$ receptor in rat NTS.

Adult Wistar rats were humanely killed by decapitation or perfusion under deep anaesthesia (halothane, 5% in O_2). Total RNA was isolated from punches of freshly dissected, unfixed NTS tissue and reverse transcribed to first strand cDNA. The cDNA was amplified by PCR using primers specific for the $GABA_A$ subunits α_4, α_6, β_1, γ_1, γ_3, δ, ϵ, θ and π. The presence of some of these subunits (β_1, γ_3, δ) was indicated by PCR, and this was confirmed by immunohistochemistry on sections from perfused tissue using antibodies specific for the individual subunits. These gave a labelling pattern in the NTS consisting of moderate to strongly labelled cell bodies interspersed with immunolabelled punctata in the neuropil. The differential patterns of immunoreactivity in neuronal somata and dendrites of NTS neurones were generally in agreement with the PCR results, confirming that mRNA expression is correlated with receptor protein synthesis. The α_6, γ_1, ϵ, θ and π subunits were detected at low expression levels by PCR, but immunolocalization in the NTS could not be confirmed. The results suggest that neurones in the NTS can express $GABA_A$ receptors comprising various combination of α, β, γ and δ subunits, with regional differences in expression within the nucleus that might be related to specific autonomic functions.

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Right atrial receptors inhibit renal sympathetic nerve activity via GABA interneurones in the PVN of rats

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Stimulation of right atrial receptors in the rat reflexly reduces sympathetic nerve activity to the kidney (RSNA, Pyner *et al.* 2002). However, it is unclear whether the inhibitory effect on renal sympathetic vasomotor tone is mediated at the paraventricular nucleus (PVN) of the hypothalamus or at the spinal cord. Evidence has shown that endogenous nitric oxide (NO) within the PVN can inhibit RSNA (Zhang *et al.* 1997), an action mediated via GABA interneurones in PVN (Zhang & Patel, 1998). The present experiments were designed to test whether the volume reflex reduction in RSNA involves PVN or a spinal site or both. Thirteen Wistar rats, weighing 312.4 ± 5.7 g, were anaesthetised with urethane and chloralose and recordings were made of arterial blood pressure, heart rate and RSNA. A small inflatable balloon was inserted down the right superior vena cava to stretch the vein-atrial junction. A double-barrelled glass micropipette was placed stereotaxically in PVN, for microinjections of drugs. An intrathecal catheter was placed via the cisterna magna with its tip at T10. Drugs were applied intrathecally in a volume of 10 ml. Statistical analysis was performed using a Mann-Whitney *U* test, following a two-way repeated measure ANOVA. Results are expressed as means ± S.E.M. Rats were killed by overdose of urethane at the end of experiments. Atrial receptors stimulated by balloon inflation reduced RSNA by 28 ± 3.7% without changing blood pressure. Microinjection of the $GABA_A$ receptor antagonist bicuculline (0.05 mM, 100 nl) into PVN increased RSNA by 63 ± 13% and this was little changed when combined with balloon inflation (RSNA increased by 75 ± 16%). Microinjection of NOS inhibitors L-NAME (0.1 mM, 100 nl) or L-NMMA (0.2 mM, 100 nl) into PVN elicited increases in RSNA of 36 ± 8 and 54 ± 10%, respectively. These increases were prevented by balloon inflation, RSNA activity being 8 ± 8 and 2 ± 2%, respectively, compared with baseline control ($P \leqslant 0.01$). To rule out the possibility that the atrial reflex inhibition was in part dependent on PVN spinal projections shown to inhibit RSNA via spinal D1 receptor, a dopamine D1 receptor antagonist SCH23390 (0.05 mM) was intrathecally applied to the spinal cord. The effect of balloon inflation on RSNA was not significantly reduced. Furthermore intrathecal application of GABA receptor antagonist bicuculline (0.03 mM) had no significant effect on the reflex inhibition of RSNA. It was concluded that atrial receptor activation causes an inhibition of RSNA at the PVN and this effect is mediated by GABA.

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