Somatic and visceral afferents to the 'vasodepressor region' of the caudal midline medulla in the rat

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Abstract

Previous research has found that the integrity of a restricted region of the caudal midline medulla (including caudal portions of nucleus raphé obscurus and nucleus raphé pallidus) was critical for vasodepression (hypotension, bradycardia, decreased cardiac contractility) evoked either by haemorrhage or deep pain. In this anatomical tracing study we found that the vasodepressor part of the caudal midline medulla (CMM) receives inputs arising from spinal cord, spinal trigeminal nucleus (SpV) and nucleus of the solitary tract (NTS). Specifically: (i) a spinal–CMM projection arises from neurons of the deep dorsal horn, medial ventral horn and lamina X at all spinal segmental levels, with approximately 60% of the projection originating from the upper cervical spinal cord (C1–C4); (ii) a SpV–CMM projection arises primarily from neurons at the transition between subnucleus caudalis and subnucleus interpolaris; (iii) a NTS–CMM projection arises primarily from neurons in ventrolateral and medial subnuclei. In combination, the specific spinal, SpV and NTS regions which project to the CMM receive the complete range of somatic and visceral afferents known to trigger vasodepression. The role(s) of each specific projection is discussed.

Introduction

Neurons of the caudal midline medulla (CMM), including the caudal portions of the nucleus raphé obscurus and nucleus raphé pallidus are known to influence sympathetic activity and to mediate changes in arterial pressure and heart rate (Gilbey *et al.*, 1981; Pilowsky *et al.*, 1986; Minson *et al.*, 1987; Yen & Hwang, 1989; Yen *et al.*, 1991; Morrison, 1993; D'Amico *et al.*, 1996; Faria *et al.*, 1996; Almada *et al.*, 1997). Recently, several laboratories reported that excitation of neurons in a discrete part of the CMM evoked a strong vasodepression response. Specifically in halothane and barbiturate anaesthetized rats, or barbiturate-anaesthetized rabbits, small microinjections (10–50 nL) of excitatory amino acids into the CMM, between the level of the obex to +1.5 mm above the obex, evoke sympathoinhibition, hypotension and bradycardia (Coleman & Dampney, 1995; Coleman & Dampney, 1998; Henderson *et al.*, 1998b; Heslop *et al.*, 2002; Verberne *et al.*, 1999). Further, it was found in both halothane and urethane anaesthetized rats, that this discrete CMM region is part of the neural network which mediates the hypotensive (phase II/decompensatory) response to progressive blood loss (Henderson *et al.*, 1998a, 2000, 2002; Heslop *et al.*, 2002). Specifically, microinjection of local anaesthetic (lignocaine) or a synaptic blocker (cobalt chloride) into the vasodepressor part of the CMM either blocks (lignocaine) or delays and attenuates (cobalt chloride) the hypotension and bradycardia triggered by a hypotensive haemorrhage (Henderson *et al.*, 1998a, 2000, 2002; Heslop *et al.*, 2002). Interestingly the same manipulations do not alter resting arterial pressure and heart rate; nor do they affect the hypotension and bradycardia evoked by excitation of 5-HT₃ cardiopulmonary vagal afferents or the bradycardia triggered by

baroreceptor loading (Coleman & Dampney, 1998; Henderson *et al.*, 2000). Such data suggest that the CMM 'vasodepressor' region plays no significant role in 'homeostatic' cardiovascular functions, although clearly its integrity is critical in evoking the decompensatory (phase II) response to progressive blood loss (for further discussion of this issue see Henderson *et al.*, 2000).

It has been thought that the decompensatory/phase II response to severe haemorrhage is triggered by signals arising in vagal (cardiac) afferents from right atrial and ventricular mechanoreceptors activated as a result of poor cardiac (diastolic) filling (Oberg & Thoren, 1970; Oberg & Thoren, 1972; Oberg & Thoren, 1973). However, it has been suggested also that nociceptive signals originating from ischaemic tissues may play an important role, i.e. if blood loss continues the combination of increased sympathetic (vasoconstrictor) drive to maintain arterial pressure (compensatory/phase I response) and falling cardiac output will produce severe ischaemia in many visceral and skeletal muscle beds (Evans & Ludbrook, 1991; Schadt & Ludbrook, 1991; Fitzpatrick *et al.*, 1993; Ludbrook, 1993; Evans *et al.*, 1994). With respect to the CMM and the role of nociceptive afferents triggering the decompensatory response, Johansson observed 40 years ago (Johansson, 1962) that electrolytic lesions of the midline medulla (including the CMM 'vasodepressor' region) blocked hypotension and bradycardia evoked by electrical stimulation of 'nociceptive' muscle afferents (i.e. A- δ and C-fibres in the sciatic nerve of the cat). The same midline medullary lesions, however, had no effect on the hypotension and bradycardia evoked by electrical stimulation of the vagus.

A previous neuroanatomical tracing study by Henderson *et al.* (1998b) found that the vasodepressor part of the CMM receives a significant projection from the ventrolateral column of the periaqueductal grey (vPAG). The vPAG itself receives direct afferents from spinal cord laminae in which primary afferents from deep tissues (muscles, joints, viscera) terminate (Keay *et al.*, 1997; Clement *et al.*,

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2000); and subnuclei of the nucleus of the solitary tract in which vagal and spinal afferents terminate (Herbert & Saper, 1992). Further, using immediate-early gene expression as a marker of neuronal activation, it has been found that vlPAG neurons are activated by noxious stimulation of muscles, joints or viscera (Keay & Bandler, 1993; Keay *et al.*, 1994; Clement *et al.*, 1996; Keay & Bandler, 1998; Keay *et al.*, 2002) or by a hypotensive haemorrhage (Keay *et al.*, 2002). Recently we, and others reported that neural blockade of the vlPAG (lignocaine) or injection into the vlPAG of opioid antagonists, delayed and attenuated the decompensatory response to haemorrhage (Cavun & Millington, 2001; Cavun *et al.*, 2001; Ip *et al.*, 2002). Such data suggest that haemorrhage-evoked hypotension and bradycardia may be triggered by a descending projection from the vlPAG to the CMM. However, the possibility that CMM neurons may be activated also by more direct somatic and visceral afferent pathways has yet to be evaluated. The aim of the present anatomical tracing study, using the retrograde transport of cholera-toxin subunit B, was to determine whether the CMM 'vasodepressor' region receives afferents of spinal, spinal trigeminal (SpV) or solitary tract nucleus (NTS) origins.

Methods

All experiments were carried out following the guidelines of the NHMRC 'Code of Practice for the Care and Use of Animals in Research in Australia' and with the approval from the University of Sydney Animal Care Ethics Committee.

Retrograde tracer injection was made in 20 male Sprague–Dawley rats (350–450 g). Each animal was anaesthetized with an (0.15 mL) intramuscular injection of ketamine (100 mg/kg), xylazine (4 mg/kg), and acepromazine (0.5 mg/kg). Each rat was placed (flat skull position) in a stereotaxic holder, and a small midline bone flap was removed exposing the caudal portion of the cerebellum and the dorsal surface of the medulla. A micropipette containing cholera toxin β -subunit (CTb) (List Biologicals, low salt), was lowered at 10° (caudo-rostral) angle onto the obex. The micropipette was then lowered +1.5 mm rostral, and 1.5–2.5 mm below the obex into the caudal midline medulla. CTb was deposited by iontophoresis using 2–15 μ A pulses (7 second duty cycle) over 15–30 min. The micropipette was then removed and the wound closed. Animals were then given 2 mL saline (i.p) and analgesics (buprenorphine, 0.075 mg/kg). Each rat was then placed in a recovery box (37–38 °C) for 3–6 h after which it was returned to its home-cage. During the next 2–4 days the mobility, activity level, eating, drinking and grooming of each rat was closely observed.

FIG. 1. Photomicrographs showing spinal cord neurons retrogradely labelled with cholera-toxin subunit B and visualized with Nickel-enhanced diaminobenzidine following injections into the vasodepressor region of the caudal midline medulla. The images show labelled neurons from the medial ventral horn (mVH), the lateral deep dorsal horn (IDDH) and lamina X. The scale bar in the bottom right of each image shows 20 μ m.

Ten days later, each animal was deeply anaesthetized (sodium pentobarbitone, 60 mg/kg) and transcardially perfused with heparinized saline (500 mL; 0.9%; pH 7.4; room temperature) followed by 4% paraformaldehyde in a borate buffer (500 mL; pH 9.4; 4 °C). The brain and spinal cord were removed and post fixed (1–2 h), before being placed in 30% sucrose (0.1 M phosphate buffer; pH 7.4; 4 °C) for a minimum of 2 days.

Retrograde label in spinal cord

For 13 brains, coronal sections of a medullary block, containing the CMM injection site, were cut at 60 μ m. For each medulla in which the injection site was within the vasodepressor part of the CMM (see below), the spinal cord was divided into eight spinal segmental regions: C1, C2–C4, C5–C8, T1–T5, T6–T10, T11–L2, L3–L6, and S1–S3, and coronal sections of each segmental region were cut at 40 μ m.

Retrograde label in SpV and NTS

For seven other brains the medulla was sectioned at 50 μ m and both the injection sites and the location of retrogradely labelled neurons within the medulla were examined.

All sections were washed, free-floating, in 50% ethanol followed by 3% hydrogen peroxide in 50% ethanol. Sections were rinsed in 0.1 M phosphate buffer (PB) solution (pH 7.4) for 30 min, followed by blocking in normal horse serum (NHS; 10% in PB; 30 min). Sections were incubated overnight in goat anticholeraenoid (List Biologicals; 1 : 15 000–1 : 30 000 in phosphate buffered horse serum (PBH; 0.1% bovine serum albumin, 2% NHS in 0.1 M PB, pH 7.4; room temperature). Sections were washed in PB and incubated for 2 h in biotinylated

FIG. 2. (A) Camera-lucida reconstruction (left) and photomicrograph (right) of a coronal section of the medulla illustrating the centre of the CTb injection into the vasodepressor region of the CMM from animal 24. The injection site was visualized with nickel-enhanced diaminobenzidine (see Methods). (B–D) Camera-lucida reconstructions of the injection sites of the 15 animals from which data are reported. Note that the injections in rats 27 and 40 (row D) show no spread into the inferior olivary nuclei located below.

FIG. 3. Left-hand column, standard coronal sections of each spinal segmental region analysed: (A) C1; (B) C2–C4; (C) C5–C8; (D) T1–T5; (E) T6–T10; (F) T11–L2; (G) L3–L6 and (H) S1–S3 (see next page). The retrogradely labelled neurons from 10 (40 μ m) sections are shown on each section (black dots) for each spinal segmental region (data from rat 24). Right-hand column, histograms illustrating the laminar locations of retrogradely labelled neurons within each spinal segmental region. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons within that spinal segmental region for all animals analysed. The laminar regions are shown in the adjacent schematic diagram of a coronal section of the cord.

antigoat IgG (Vector; 1 : 200 in PBH). Sections were washed again for 30 min in PB and incubated in Extr-Avidin Peroxidase (Sigma; 1 : 1000 in PBH; 2.5 h). Following a final wash in PB, the bound antibody was visualized with diaminobenzadine (DAB) as the chromagen using the glucose oxidase technique (see (Keay *et al.*, 1997) for details).

Sections were then mounted onto gelatinized slides, dried overnight, dehydrated through graded alcohols and coverslipped with DPX mounting medium (Ajax Chemicals).

Sections were observed under the light microscope and injection sites were reconstructed using a camera-lucida attachment. Cells were

FIG. 3. continued

considered to be retrogradely labelled if the CTb-immunoreactivity (IR) could be clearly differentiated from background staining at $\times 100$, and the soma contained a granular brown reaction product visible at $\times 200$ (see Fig. 1). To plot the location of retrogradely labelled neurons in the spinal cord, representative ('standard') sections at each of the 8 spinal segmental regions (C1, C2–C4, C5–C8, T1–T5, T6–T10, T11–L2, L3–L6, S1–S3) were drawn using the camera-lucida attachment. For numerical analysis the CTb-IR cells on 10 randomly selected

sections, at each segmental level, were plotted onto the 'standard sections'. The mean number of CTb-IR (labelled) cells per section ($40\mu\text{m}$) was then calculated for each spinal segmental region.

For each spinal segmental region the boundaries of the superficial dorsal horn (SDH), lateral deep dorsal horn (IDDH), medial deep dorsal horn (mDDH), lamina X, medial ventral horn (mVH), lateral ventral horn (IVH), and lateral spinal nucleus (LSN) were delineated using cytoarchitectonic criteria based on Nissl stained spinal cord

FIG. 4. (A) Graph illustrating the distribution of retrogradely labelled neurons within each of the eight spinal segmental regions analysed. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in each animal. (B) Histogram illustrating the distribution of retrogradely labelled neurons within each lamina region. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in each animal. The lamina regions analysed are shown in the schematic diagram of a coronal section of the cord (above right). (C) Graphs illustrating the distribution of retrogradely labelled neurons within: (i) the medial ventral horn; (ii) the lateral deep dorsal horn; (iii) the medial deep dorsal horn; and (iv) lamina X; of the eight spinal segmental regions analysed. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in each lamina region. The lamina region analysed is shown in the schematic diagram of a coronal section of the cord above each graph.

sections. These boundaries followed the schema proposed by Molander and Grant (Molander & Grant, 1995). The SDH included laminae I, II, III, and lateral part of lamina IV. The deep dorsal horn (lamina V) was subdivided into two regions, a lateral (IDDH) and medial (mDDH) sector. The lateral deep dorsal horn contained only the 'reticular' cells of lamina V. The medial deep dorsal horn contained neurons of lamina V, medial lamina IV and lamina VI of the C4–T8, and L3–L6 segments. The ventral horn was divided into medial and lateral sectors. The medial ventral horn (mVH) contained primarily neurons of lamina VII, with some lamina VIII neurons. The lateral ventral horn (lVH) contained the remaining lamina VIII neurons and the motor neurons of lamina IX.

To determine the location of retrogradely labelled neurons in both the NTS and spinal trigeminal nucleus (SpV), seven sections (spaced ~500 µm apart) from the C1 spinal segment through to the level 1.5 mm rostral to the obex were selected. Each section was drawn and the labelled cells plotted onto these sections using the camera-lucida attachment. The NTS was subdivided in accordance with the schema of Herbert and Saper (Herbert *et al.*, 1990; Herbert & Saper, 1992). The SpV regions were subdivided on the basis of a number of recent studies (Hathaway *et al.*, 1995; Jacquin *et al.*, 1983; Jacquin *et al.*, 1986; Imbe *et al.*, 1999; Zhou *et al.*, 1999; Imbe *et al.*, 2001).

Results

The data of 15 rats in which the CTb injections were restricted to the vasodepressor part of the CMM were analysed in detail. For eight rats (numbers 19–26) retrograde label in the spinal cord was analysed. For 5 rats (29, 31, 33, 34 and 43) retrograde label in the medulla (NTS, SpV) was evaluated, and in the remaining two rats (27 and 40) retrograde label in both spinal cord and medulla was analysed. Camera-lucida reconstructions of each of the centres of the injection sites are shown in Fig. 2. The patterns of retrograde label in both spinal cord and medulla for two animals in which there was no appreciable spread of CTb into the underlying inferior olives (injections 27 and 40) were compared with those of the other 13 animals.

Spinal cord

CTb-IR neurons were distributed bilaterally, at each of the spinal segmental regions investigated (see Fig. 3). However, there were striking differences in the numbers of labelled cells in each of the

FIG. 5. Graph illustrating the distribution of retrogradely labelled neurons within the spinal nucleus of the trigeminal (SpV) at each of the eight medullary sections (500 µm apart) analysed. The rostro-caudal location of the sections are defined according to distance (mm) from obex. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in the SpV of each animal.

8 spinal segmental regions (Figs 3 and 4A, Table 1). Approximately 60% of the labelled cells were found within the upper cervical spinal segments (C1–C4), of which at least 40% were contained solely within the C1 segment. A similar distribution of retrograde label was found for the two CMM injection sites without involvement of the inferior olive (Table 1). Each of the 7 remaining spinal segmental regions (C5 and below) contained between 4 and 10% (mean values) of the total number of CTb-IR neurons.

At each spinal segmental region labelled neurons were located only within specific laminae (see Figs 3 and 4B). The SDH was notable by a near total absence of CTb-IR neurons. The greatest number of retrogradely labelled spinal neurons were found within the medial half of the ventral horn (mVH = $44 \pm 2\%$). A further 42% of all labelled neurons were located within the deep dorsal horn, $23 \pm 1\%$ laterally (IDDH); $19 \pm 2\%$ medially (mDDH). However, it is important to note that the number of labelled neurons within the mDDH was reduced by half for the two rats in which the CMM injection sites did not infringe upon the inferior olives. Lamina X, surrounding the central canal contained 10% of all labelled neurons. Smaller numbers of

TABLE 1. Numbers of retrogradely labelled (CTb-IR) neurons in 10 randomly selected coronal sections at each spinal cord segmental region

Animal	Numbers of retrogradely labelled (CTb-IR) neurons (also as percentages of all labelled cells found in each region)								Total
	C1	C2–C4	C5–C8	T1–T5	T6–T10	T11–L2	L3–L6	S1–S3	
19	178 (32.9%)	81 (14.9%)	44 (8.1%)	36 (6.7%)	37 (6.8%)	49 (9.1%)	86 (15.9%)	30 (5.5%)	541
20	–	36	6	9	7	5	21	3	–
21	287 (53.9%)	82 (15.4%)	34 (6.4%)	24 (4.5%)	16 (3.0%)	29 (5.5%)	42 (7.9%)	18 (3.4%)	532
22	140 (42.0%)	50 (15.0%)	21 (6.3%)	16 (4.8%)	18 (5.4%)	34 (10.2%)	36 (10.8%)	18 (5.4%)	333
23	113 (47.9%)	40 (16.9%)	18 (7.6%)	11 (4.7%)	8 (3.4%)	20 (8.5%)	19 (8.1%)	7 (3.0%)	236
24	210 (50.8%)	60 (14.5%)	41 (9.9%)	17 (4.1%)	12 (2.9%)	23 (5.6%)	40 (9.7%)	10 (2.4%)	413
25	–	82	46	21	33	42	47	31	–
26	100 (41.1%)	48 (19.8%)	14 (5.8%)	11 (4.5%)	10 (4.1%)	29 (11.9%)	24 (9.9%)	7 (2.9%)	243
27	141 (42.3%)	63 (18.9%)	22 (6.6%)	20 (6.0%)	25 (7.5%)	26 (7.8%)	25 (7.5%)	11 (3.3%)	333
40	298 (44.3%)	104 (15.5%)	75 (11.1%)	49 (7.3%)	30 (4.5%)	55 (8.2%)	46 (6.8%)	16 (2.4%)	673

FIG. 6. Left-hand column, standard coronal sections of each of the eight coronal sections of medulla in which SpV label was analysed (+1.5 mm to –3.0 mm relative to obex). Sections A–C correspond to the subnucleus interpolaris (SpVi), sections C and D correspond to the 'transitional region' SpVi/SpVc, and sections E–G (see next pages) correspond to the subnucleus caudalis (SpVc). The retrogradely labelled neurons from one side of a single section (50 µm) are shown as black dots (data from rat 43). Right-hand column, histograms illustrating: (i) the dorso-ventral, and (ii) the medio-lateral locations of retrogradely labelled neurons at each of the medullary levels. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons within that medullary rostrocaudal level for all animals analysed. The dorso-ventral and medio-lateral regions are shown schematically in the figure.

FIG. 6. continued

TABLE 2. Numbers of retrogradely labelled (CTb-IR) neurons in the dorsal, intermediate and ventral aspects of the SpV

Animal	Numbers of retrogradely labelled (CTb-IR) neurons (also as percentages of all labelled cells found in each region)			Total
	Dorsal (ophthalmic)	Intermediate (maxillary)	Ventral (mandibular)	
29	275 (30.3%)	291 (32.0%)	343 (37.7%)	909
31	210 (40.8%)	156 (30.3%)	149 (28.9%)	515
33	138 (31.2%)	88 (19.9%)	216 (48.9%)	442
34	198 (39.1%)	172 (33.9%)	137 (27.0%)	507
43	92 (26.7%)	76 (22.0%)	177 (51.3%)	345
27	69 (33.8%)	38 (18.6%)	97 (47.5%)	204
40	69 (30.1%)	48 (21.0%)	193 (55.3%)	310

neurons were found in the lateral portion of the ventral horn and the lateral spinal nucleus (<5%).

Analysis of the distribution of labelled neurons within each laminar region revealed that the greatest numbers of labelled cells in the mVH, DDH and lamina X were located in the C1–C4 region (Fig. 4C). However, it was interesting to note that for lamina X in particular, the thoraco-lumbar junction (T11–L2) and the lumbar enlargement (L3–L6) were locations of the next greatest number of labelled cells (Fig. 4C). That is, whilst lamina X contributed only around 10% of the total number of CMM projecting neurons (Fig. 4B), 40% of the labelled lamina X neurons were found at upper cervical spinal cord (UCC) segments and 30% were located between T11 and L6 (Fig. 4C). Finally, as illustrated in Fig. 3, for each spinal segmental region analysed (with the exception of the sacral segmental region), the mVH

contained always the largest number of labelled cells (41–53%). In the sacral cord, however, the IDDH contained the greatest number of labelled cells.

Spinal trigeminal nucleus (SpV)

CTb-IR neurons were distributed bilaterally within the SpV, with ~70% of all SpV labelled neurons found at the transitional region between the subnucleus caudalis (laminar) and the subnucleus interpolaris (alaminar) (see Fig. 5). Strikingly, whilst the C1 spinal segment was characterized by a very large number of labelled cells, it immediately rostral extension, SpV subnucleus caudalis, was almost devoid of CTb-IR cells (compare Figs 6 and 3). The pattern of SpV label was the same for CMM injections with and without olivary involvement (Table 2).

For the purposes of analysis, the SpV was divided dorsoventrally into the three regions which correspond to the terminal fields of the ophthalmic, maxillary and mandibular divisions of the trigeminal nerve (Jacquin *et al.*, 1986; Jacquin *et al.*, 1983). There were only slight differences in the proportions of labelled neurons found within each of these regions ($33 \pm 2\%$ dorsal; $25 \pm 2\%$ intermediate; $42 \pm 4\%$ ventral) (Fig. 7A, Table 2). However, there was a suggestion that: (i) the majority of CTb-IR neurons within the ventral division were caudal to those in the dorsal and intermediate divisions (Fig. 7A); and (ii) there were more labelled neurons medially ($57 \pm 2\%$) than laterally ($43 \pm 2\%$) (Fig. 7B).

Nucleus of the solitary tract (NTS)

CTb-IR neurons were located bilaterally within the nucleus of the NTS (Fig. 8), with 75% of all labelled NTS neurons found between the obex

Fig. 7. (A) Histogram illustrating the distribution of retrogradely labelled neurons within the spinal nucleus of the trigeminal (SpV): (i) within the dorsal ('maxillary'), intermediate ('mandibular') and ventral ('ophthalmic') regions, and (ii) within its medial and lateral aspects. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in the SpV of each animal. (B) Graph illustrating the rostro-caudal distribution (defined according to the distance from obex) of labelled neurons within the dorsal ('maxillary'), intermediate ('mandibular') and ventral ('ophthalmic') regions at each of the eight medullary sections analysed. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in the SpV of each animal. (C) Graph illustrating the rostro-caudal distribution (defined according to the distance from obex) of labelled neurons within the medial and lateral SpV at each of the eight medullary sections analysed. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in the SpV of each animal.

A: Rostral NTS

B: Intermediate NTS

FIG. 8. Top panel, left-hand column, standard coronal sections of rostral NTS (+1.5 to +1.0 mm rostral to obex), intermediate NTS (+0.5 mm to obex) and caudal NTS (−0.5 to −1.5 mm from obex; see part C on next page). The retrogradely labelled neurons from both sides of a single section (50 μ m) are shown as black dots (data from rat 43). Right-hand column, histograms illustrating the subnuclear distributions of retrogradely labelled neurons in the NTS. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons within that medullary rostrocaudal level for all animals analysed. The NTS subnuclei are shown schematically along with each histogram. Note that label in dorsal column nuclei has been removed for clarity.

and the region +0.5 mm (intermediate NTS) (Fig 9A and C). Labelled neurons were found predominantly within the ventrolateral and dorsal parasolitary NTS subnuclei (Figs 8, and 9B and C, and Table 3). However, it is important to note that many fewer labelled neurons were found within the dorsal parasolitary NTS subnucleus for the two CMM injections which did not infringe upon the inferior olive (Table 3: 11.5 and 28%, without inferior olive involvement vs. 25 to 53% with olivary involvement).

Conversely the numbers of labelled neurons within the ventrolateral NTS subnucleus was greater in animals in which the CMM injections sites did not infringe upon the inferior olives (39 and 55% without olivary involvement vs. 20–46%, with olivary involvement). There was little difference in the mean numbers of labelled cells in the medial NTS (22.4% without olivary involvement vs. 17.7%, with olivary involvement). The rostral and commissural NTS subnuclei

contained the fewest numbers of CTb-IR cells for all CMM injections (i.e. with or without olivary involvement; $5 \pm 1\%$ and $4 \pm 0.5\%$, respectively).

Discussion

The aim of the present study was to determine, using the method of the retrograde transport of CTb, whether the vasodepressor region of the CMM received spinal, SpV or NTS inputs. The results of our experiments revealed that the vasodepressor part of the CMM receives inputs from: (i) spinal neurons of the deep dorsal horn, medial ventral horn and lamina X at all rostro-caudal levels of the spinal cord (however, $\sim 60\%$ of this total spinal input to the CMM arises from upper cervical spinal segments C1–C4); (ii) SpV neurons, primarily at the interface of subnucleus caudalis and subnucleus interpolaris; and (iii) neurons

C: Caudal NTS

FIG. 8. continued

within the intermediate region of the NTS, specifically from the ventrolateral, medial and dorsal parasolitary subnuclei.

Technical considerations

The problem of uptake and transport by damaged fibres of passage is always a concern whatever the choice of tracer or method of injection. We attempted to minimize both the spread of tracer and damage to fibres of passage by iontophoretic application of CTb using micropipettes with relatively small tip diameters ($<30\mu\text{m}$). The 'vasodepressor' part of the CMM is a relatively circumscribed region; from obex to approximately $+1.5\text{mm}$ rostral to obex, encompassing the caudal nucleus raphé obscurus and raphé pallidus, bounded dorsally by the hypoglossal nucleus, ventrolaterally by the inferior olives and medial reticular formation (Henderson *et al.*, 1998b, 2000, 2002), and only injections in which the core of the CTb deposit was located within these boundaries were selected for analysis. Particular attention was paid to comparing CMM injections with and without inferior olivary nucleus involvement.

Location of CMM-projecting spinal neurons

The distribution of CMM-projecting spinal neurons reported here is in broad agreement with general observations that spinal projections to medial medullary structures arise primarily from the deep dorsal horn

(laminae IV/V), the medial aspects of the intermediate and ventral grey (laminae VI–VIII) and lamina X (Menetrey *et al.*, 1983; Nogueira *et al.*, 2000). In this study, however, retrograde tracer injections were restricted to the vasodepressor part of the CMM.

As summarized in Figs 2 and 3, the UCC contained by far the greatest number of CMM-projecting neurons, with $\sim 40\%$ of all neurons labelled in the spinal cord located within a single segment, C1. Those UCC laminae containing the greatest numbers of CMM-projecting neurons (mDDH, IDDH and mVH) correspond to regions which receive primary afferents of deep neck muscles, many of which are driven by nociceptive signals (Abrahams *et al.*, 1979, 1981, 1984a, b, 1989; Mysicka & Zenker, 1981; Ammann *et al.*, 1983; Pfaller & Arvidsson, 1988; Chudler *et al.*, 1991; Prihoda *et al.*, 1991; Yezierski & Broton, 1991; Yezierski & Mendez, 1991) and propriospinal afferents arising from neurons of the SDH and LSN at thoracic and lumbar spinal levels (Craig *et al.*, 1992; Matsushita & Ikeda, 1973; Matsushita *et al.*, 1979; Giesler & Elde, 1985; Leah *et al.*, 1988; Gonzalo & Rotinen, 1990; Pubols & Haring, 1995; Matsushita, 1998). This suggests that: (i) nociceptive signals arising from neck muscles; and (ii) signals arising from thoracic and abdominal viscera, or somatic structures of the hindlimb (including nociceptive/thermal inputs relayed from SDH and LSN which ascend to the UCC via propriospinal pathways) are likely sources of spinal afferent drive onto the CMM

FIG. 9. (A) Graph illustrating the distribution of retrogradely labelled neurons within the nucleus of the solitary tract (NTS) at each of the seven medullary sections (500 μ m apart) analysed. The rostro-caudal location of the sections are defined according to distance (mm) from obex. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in the NTS of each animal. (B) Histogram illustrating the distribution of retrogradely labelled neurons within subnuclei of NTS; vl, ventrolateral nucleus; med, medial nucleus; com, commissural nucleus; dPS, dorsal parasolitary nucleus; rostral, rostral nuclei. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in the NTS of each animal. (C) Graph illustrating the rostro-caudal distribution (defined according to the distance from obex) of labelled neurons within the ventrolateral nucleus, medial nucleus, commissural nucleus, dorsal parasolitary nucleus and rostral nuclei at each of the seven medullary sections analysed. The data are expressed as a percentage ($\pm$ SEM) of the total number of neurons labelled in the NTS of each animal.

'vasodepressor' region (Andrew & Craig, 2001; Craig *et al.*, 1994; Craig, 1996; Han *et al.*, 1998; Craig & Andrew, 2002).

Other than the UCC, lamina X at the level of thoraco-lumbar junction (T11–T12 and L3–L6) stood out as an additional region of interest (see Fig. 3). At these spinal segmental regions lamina X is a major site of termination of renal (T10–L2) (Knuepfer *et al.*, 1988; Simon & Schramm, 1984; Wyss & Donovan, 1984; Weiss & Chowdhury, 1998) and pelvic visceral (T10–L6) afferents (Birdier *et al.*, 1991; Morgan *et al.*, 1981; Kuo *et al.*, 1983; Nadelhaft & Booth, 1984; McKenna & Nadelhaft, 1986; Morgan *et al.*, 1986a, b). As $\sim$ 30% of lamina X neurons projecting to the CMM originate at the level of the thoraco-lumbar junction, these results indicate a possible, reasonably direct route via which renal and pelvic visceral signals could modulate the activity of CMM 'vasodepressor' neurons (see below, discussion of afferent signals triggering decompensation).

Location of CMM-projecting SpV neurons

The SpV transition region (i.e. junction of subnuclei caudalis and interpolaris) contains terminals of all three branches of the trigeminal nerve. As revealed by both single unit recording and immediate early gene expression (*c-fos*) studies, neurons within the SpV transition region are profoundly activated by either acute or chronic noxious

stimulation of superficial or deep orofacial structures (e.g. cornea, temporomandibular joint, tongue, vibrissae pad) (Bereiter *et al.*, 1994; Hathaway *et al.*, 1995; Meng & Bereiter, 1996; Imbe *et al.*, 1999; Zhou *et al.*, 1999; Imbe *et al.*, 2001). Our observation that the SpV transition region contained $\sim$ 70% of CMM-projecting SpV neurons (see Fig. 7 and Table 2) suggests that pain of orofacial origin may strongly influence the activity of CMM 'vasodepressor' neurons. In this regard it is well documented that noxious stimulation of the orofacial region can evoke a marked vasodepression (the so-called trigeminal depressor reflexes). In newborn animals such stimulation can produce a 'vaso-vagal' episode akin to the hypotension and bradycardia characteristic of the decompensatory response to haemorrhage. Furthermore, it has been reported that lesion or microinjection of GABA agonists (muscimol) into the CMM blocks the hypotension (but not the bradycardia) evoked by electrical stimulation of either the Vth nerve or spinal tract of V (Clement & McCall, 1989; McCall & Harris, 1987).

Location of CMM-projecting NTS neurons

The major findings were that CMM-projecting NTS neurons were located predominantly in: (i) the ventrolateral subnucleus which receive vagal, but little direct spinal input, and (ii) the medial

TABLE 3. Numbers of retrogradely labelled (CTb-IR) neurons in each of the subnuclei of the solitary tract

Numbers of retrogradely labelled (CTb-IR) neurons (also as percentages of all labelled cells found in each region)						
Animal	Commissural	Medial	Ventrolateral	Parasolitary	Rostral	Total
29	18 (3.8%)	86 (18.4%)	16 (24.8%)	235 (50.2%)	13 (2.8%)	368
31	25 (6.6%)	72 (18.9%)	77 (20.2%)	203 (53.3%)	4 (2.8%)	381
33	17 (3.4%)	94 (18.5%)	233 (46.0%)	150 (29.6%)	13 (2.6%)	507
34	18 (2.8%)	73 (11.3%)	185 (28.5%)	322 (49.7%)	50 (7.7%)	648
43	10 (4.4%)	49 (21.6%)	100 (44.1%)	57 (25.1%)	11 (4.8%)	227
27	11 (4.3%)	66 (25.9%)	99 (38.8%)	72 (28.2%)	7 (2.7%)	255
40	13 (3.7%)	66 (21.6%)	193 (55.3%)	40 (11.5%)	37 (10.6%)	349

subnucleus, which in addition to vagal input, receives significant spinal input from laminae I, IV/V and X (Menetrey & Basbaum, 1987). The projection to the CMM arising from the dorsal parasolitary subnucleus appears to target predominantly the inferior olive (see Table 3), specifically the medial accessory nucleus (Barmack *et al.*, 1998).

As discussed in the Introduction, earlier findings indicate that cardiovascular reflexes triggered by activation of high pressure baroreceptor afferents or 5-HT₃ sensitive cardio-pulmonary vagal afferents, or hypotension and bradycardia evoked by electrical stimulation of the vagus, are not altered by either lesion or neural blockade of the CMM vasodepressor region (Johansson, 1962; Coleman & Dampney, 1998; Henderson *et al.*, 2000). Thus it may seem surprising that vagal-recipient NTS subnuclei have strong projections to the CMM. In this regard, it is important to remember that the CMM 'vasodepressor' region includes populations of neurons known to play important roles in gastrointestinal functions and respiratory regulation (Garrick *et al.*, 1994; Holtman *et al.*, 1986a, b; Lalley, 1986a, b; McCown *et al.*, 1986; Millhorn, 1986; Yen & Hwang, 1989; Morin *et al.*, 1990a, b; Yang *et al.*, 1990; Garrick *et al.*, 1992; Yang *et al.*, 1992; Tache *et al.*, 1993; Kaneko *et al.*, 1995; Taché *et al.*, 1995). In particular, anatomical and electrophysiological evidence indicate that vagal afferent fibres carrying signals from lung stretch receptors terminate and influence the activity of ventrolateral NTS neurons (including those of the dorsal respiratory group) (Bellingham & Lipski, 1992; Kalia & Sullivan, 1982). Such signals are an important afferent drive likely relayed via the NTS–CMM projection identified here.

Afferents triggering phase II/decompensatory response

As stated in the Introduction the integrity of the CMM 'vasodepressor region' is critical for the expression of the decompensatory/phase II response to progressive blood loss (Henderson *et al.*, 2000; Henderson *et al.*, 2002; Heslop *et al.*, 2002), as well as the hypotension and bradycardia triggered by pain of deep origin (muscle) (Johansson, 1962). The aim of this study was to establish if the CMM vasodepressor region received direct spinal, SpV, and/or NTS projections, likely to relay somatic and visceral signals initiating decompensation. Our anatomical findings indicate that the vasodepressor part of the CMM receives afferents from spinal, SpV and NTS regions each of which receives somatic and visceral afferents, which are likely candidates to trigger components of the phase II/decompensatory response to haemorrhage. Specifically our data suggest the following: (i) vagal afferents (non-5HT₃-sensitive) to CMM-projecting NTS neurons are still candidates to trigger the bradycardia, decrease in cardiac contractility and renal sympathoinhibition which are characteristic of a phase II/decompensatory response to haemorrhage; (ii) nociceptive spinal afferents to CMM projecting neurons are candidates to drive the vasodepressor response characteristic of pain of deep origin (e.g. muscle), as well as the phase II/decompensatory response to haemorrhage; and (iii) nociceptive afferents to CMM-projecting SpV ('transition region') neurons are candidates to drive the hypotensive component of trigeminal depressor reflexes

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Abbreviations

CMM, caudal midline medulla; CTb, cholera toxin β -subunit; DAB, diaminobenzidine; IR, immunoreactivity; IDDH, lateral deep dorsal horn;

LSN, lateral spinal nucleus; IVH, lateral ventral horn; mDDH, medial deep dorsal horn; mVH, medial ventral horn; NTS, nucleus of the solitary tract; PB, phosphate buffer; PBH, phosphate buffered horse serum; SpV, spinal trigeminal nucleus; SDH, superficial dorsal horn; UCC, upper cervical spinal cord; vlPAG, ventrolateral column of the periaqueductal grey.

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