Evidence that nitric oxide- and opioid-containing interneurons innervate vessels in the dorsal horn of the spinal cord of rats

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1. In the dorsal horn of the spinal cord, activation of small fibre nociceptive afferents leads to the release of nitric oxide and enkephalins by interneurons. In this work we encountered unexpected relationships among local spinal cord dorsal horn blood flow, specific forms of afferent input, nitric oxide and intrinsic opioids.

2. Selective rises in rat lumbar dorsal cord blood flow using laser Doppler flowmetry and microelectrode hydrogen clearance polarography were generated by ipsilateral, 'nociceptive' low (3 Hz) frequency stimulation of sciatic afferents. Inhibitors of nitric oxide synthase (NOS) prevented rises in flow during stimulation without influencing baseline flow. Ipsilateral hindpaw intradermal injection of capsaicin, a nociceptive activator, also generated large rises in flow sensitive to NOS inhibition.

3. During NOS blockade or morphine administration there were unexpected acute declines in the dorsal cord blood flow strictly confined to low frequency stimulation epochs. This acute vasoconstrictive effect was prevented by administration of an opioid receptor antagonist.

4. Using immunohistochemistry, terminals apparently innervating dorsal spinal cord blood vessels were labelled with antibodies against neuronal NOS and met-enkephalin.

5. We conclude that local nitric oxide and opioids, probably from interneurons, have competitive actions on dorsal horn microvessels once interneurons are activated during a nociceptive barrage. Collateral innervation of blood vessels may explain this property.

The concept that the metabolic activation of the central nervous system is closely coupled to changes in local blood flow is a classical tenet. Recently, nitric oxide (NO) has been suggested as an important link between local blood flow and cerebral activity (Dawson & Snyder, 1994; Akgoren *et al.* 1994). The dorsal horn of the spinal cord offers an ideal site to address the role of nitric oxide coupling because a proportion of its interneurons contain neuronal nitric oxide synthase (nNOS) (Valtschanoff *et al.* 1992; Zhang *et al.* 1993) and specific pathways within it can be activated by the type of afferent discharges it receives. Opioids might be expected to dampen the metabolic activity of nociceptive pathways. While there is no evidence that intrinsic opioids directly influence central nervous system blood flow, opioids can modulate blood flow in peripheral nerves and other tissues (Li & Duckles, 1991; Bartho *et al.* 1992; Zochodne & Ho, 1993; Schaafsma *et al.* 1997). These actions are probably mediated by opioid inhibition of smooth muscle adenylate cyclase activity (Sharma *et al.* 1975). An untested possibility is that specific neurotransmitters, rather than local metabolic demands couple changes in local perfusion to their release. This might occur by local diffusion of specific agents away from synapses to local neuroeffector junctions on blood vessels. An even more intriguing possibility is that interneurons might provide axon branches that collaterally innervate local vessels. In the peripheral nervous system, this arrangement between the parent nerve trunk and vasa nervorum exists in that local endoneurial branches exit to innervate epineurial vessels (Rechthand *et al.* 1986; Zochodne, 1993).

In this work, we studied changes in local spinal cord dorsal horn blood flow in response to afferent barrages. Two complementary techniques, laser Doppler flowmetry (LDF) addressing erythrocyte flux of surface vessels of the dorsal cord and microelectrode hydrogen clearance polarography addressing intrinsic dorsal horn grey matter blood flow, were used to study the role of local NO release on blood flow during a nociceptive afferent barrage. While examining the role of NO, we also encountered an unexpected but substantive role for direct opioid modulation of blood flow that raised interesting possibilities of how flow and neurotransmission may be coupled.

METHODS

Physiological preparation

Studies were carried out in male Sprague-Dawley rats ($n = 4$–6 per experiment) weighing 200–500 g. All experiments were carried out in accordance to the guidelines of the Canadian Council of Animal Care and the University of Calgary Animal Care Committee. Rats were anaesthetized with sodium pentobarbital (65 mg kg^{-1}; I.P.) supplemented (20 mg kg^{-1}) approximately every 2 h to maintain a relatively constant anaesthesia as judged by the level and stability of the mean arterial pressure. All measurements of local dorsal horn spinal cord blood were carried out in rats injected with the neuromuscular blocker tubocurare (1.5 mg kg^{-1}; I.P.) and artificially ventilated rats. The rat skull and pelvis were immobilized on a stereotactic frame and the spinal cord exposed through a multilevel low thoracic and lumbar laminectomy. Measurements of dorsal cord blood flow concentrated on the fifth lumbar (cord) level. A carotid catheter was used to measure mean arterial pressure, and to draw samples for arterial blood gases. Rats were killed at the end of the measurements by injections of a high dose of pentobarbital.

Dorsal horn spinal cord blood flow

Laser Doppler flowmetry (LDF) and hydrogen clearance microelectrode polarography (HC) were used to address dorsal cord blood flow. There were several purposes considered in using both techniques for different types of experiments: (i) the approach confirmed the most important findings of the work using two separate methods in different animals; (ii) LDF was particularly suited to multiple protocol testing with varying frequencies during the stimulation studies; (iii) HC provided quantitative information and had stricter localization to the dorsal horn grey matter (as confirmed by comparing our blood flow values with previous work using much larger hydrogen electrodes or other techniques, see Discussion); HC, however, was unsuitable for multiple protocols and limited to single interventions (three serial washout curves are considered to reflect optimal physiological stability using this method); (iv) HC provided quantitative dose–response data; and (v) LDF demonstrated opioid vasoconstriction in a dramatic and simple fashion, and was used to explore that property (see below).

The LDF signal records a product of erythrocyte velocity and mass or erythrocyte flux. We employed a 1.0 mm tipped fibre optic probe containing the afferent and efferent fibres (separated by 250 μm) positioned with a micromanipulator so as to just touch the dorsal surface of the spinal cord at 60–80 deg and bathed in mineral oil maintained at 37 °C with a heating lamp, that was kept on until immediately before the signal reading. The probe was connected to a perfusion monitor (Perimed PF3). Each flux value was taken as the mean from 10 individual but closely adjacent areas in the region of interest. The sensing sphere of the LDF probe has been estimated at 1 mm^3. Erythrocyte (RBC) flux values were in arbitrary units. Results and methodology for the technique in peripheral nerve and spinal cord have been published (Zochodne *et al.* 1995, 1999; Windhorst *et al.* 1997).

HC allows direct quantitative measurements of intrinsic local grey matter blood flow. The 3–5 μm tipped platinum microelectrode, linearly sensitive to hydrogen, was inserted approximately 1 mm into the dorsal horn of the spinal cord at approximately L5 using a micromanipulator. We verified that the electrode tip was correctly placed in the dorsal horn histologically. We routinely recorded two to three washout curves in physiologically stable preparations (mean arterial pressure > 100 mmHg) with blood gases maintained at $P_{O_2} > 80$ Torr and P_{CO_2} 35–45 Torr. Results using our microelectrode HC technique for measurements of local flow in peripheral nerve and dorsal root ganglia have been published (Zochodne & Ho, 1991; Zochodne *et al.* 1995, 1999). Resistance measurements were calculated as mean arterial pressure/flow.

Protocols

Stimulation frequency studies. LDF recordings were made at baseline then during ipsilateral sciatic nerve stimulation at 3, 10 and 100 Hz (15 V, 0.1 ms duration stimuli) in varying sequences. Each epoch lasted approximately 10 min and was separated by 20 min. Hydrogen clearance curves were recorded during ipsilateral or contralateral 3 Hz sciatic stimulation.

Pharmacological studies. Hydrogen clearance was measured after systemic (intra-arterial) injection of 0.10, 1.0 or 10 mg kg^{-1} L-NAME (N^G-nitro-L-arginine-methyl ester), 10 mg kg^{-1} D-NAME (N^G-nitro-D-arginine-methyl ester), 10 mg kg^{-1} 7-NI (7-nitroindazole), or 10 mg kg^{-1} L-NNA (N^G-nitro-L-arginine), before and during 3 Hz ipsilateral sciatic stimulation. L-NAME and L-NNA are broad spectrum NOS (nitric oxide synthase) inhibitors whereas 7-NI is thought to have relative selectivity for the neuronal isoform of NOS. D-NAME is the inactive enantiomer of L-NAME. LDF was measured before, during and after 3 Hz ipsilateral sciatic stimulation with 10 mg kg^{-1} L-NAME without or with sympathetic or opioid receptor blockade or with morphine (0.2 mg kg^{-1}) in the absence of L-NAME. 'Supramaximal' sympathetic blockade used 50 mg kg^{-1} guanethidine hydrochloride I.P. for each of 3 days before the measurements or 0.06 mg kg^{-1} phentolamine I.P. at the time of the measurements. Opioid blockade was carried out using 0.05, 0.10 or 0.20 mg kg^{-1} naloxone I.P. at the time of the measurements. Further LDF measurements were made before and after ipsilateral paw intradermal injection of 0.2% capsaicin (in 10% Tween 80 and 10% ethanol; injectate 0.10 ml) or with carrier alone without or with 10 mg kg^{-1} L-NAME given as above.

Immunohistochemistry

A separate group of rats, which had not been used in the blood flow studies, were killed with an overdose of pentobarbital (I.P) and samples of spinal cord taken and fixed in modified Zamboni's fixative (2% paraformaldehyde, 0.5% picric acid and 0.1 M phosphate buffer) overnight at 5 °C. Tissues were then washed in phosphate-buffered saline (PBS), then dimethyl sulfoxide, and again with PBS. They were covered with PBS and 20% sucrose, left at 5 °C overnight and then embedded in optimum cutting temperature (OCT) compound (Miles), frozen and sectioned at 20 μm. Sections were placed onto poly-D-lysine-coated slides and then incubated for 48 h at 4 °C with one of the following antibodies: (i) mouse monoclonal anti-met-enkephalin antibody (1:150; Chemicon International, Temecula, CA, USA); (ii) rabbit polyclonal anti-factor VIII (FVIII) antibody (1:200; Sigma, St Louis, MO, USA); (iii) mouse monoclonal anti-neuronal nitric oxide synthase (nNOS) antibody (1:500; Bio/Can, Mississauga, Ontario, Canada). Slides were then washed with PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody (1:50; Sigma) or CY3 conjugated sheep anti-mouse immunoglobulin G antibody (1:100; Sigma) for 1 h at room temperature. After further PBS washing, coverslips were mounted with bicarbonate-buffered glycerol (pH 8.6) and viewed with a fluorescence microscope (Zeiss Axioplan) and digital camera (Zeiss Axiovision). To confirm labelling of vessels, additional double labelling experiments were carried out by combining the antibody to FVIII (labelling vessels) with that labelling met-enkephalin or nNOS. Appropriate filtering was used to eliminate 'bleed-through' artifact from double labelling. Control experiments were carried out with the primary antibody eliminated (negative control) with each of the single labelling experiments and

Table 1. Quantitative dorsal horn grey matter blood flow studies using hydrogen clearance polargraphy

Intervention (n)	Flow * (ml (100 g)$^{-1}$ min^{-1})	Resistance † (mmHg ml^{-1} (100 g)$^{-1}$ min^{-1})	Mean arterial pressure ‡ (mmHg)
None (6)	63.9 ± 6.3	2.13 ± 0.22	129 ± 7
3 Hz stim (6)	127.1 ± 14.5	1.41 ± 0.14	147 ± 4
3 Hz + L-NAME (10 mg kg^{-1}) (10)	50.4 ± 4.3	3.23 ± 0.29	151 ± 3
3 Hz + D-NAME (10 mg kg^{-1}) (5)	116.4 ± 10.3	1.37 ± 0.14	153 ± 3
L-NAME alone (10 mg kg^{-1}) (5)	60.3 ± 6.8	2.70 ± 0.31	153 ± 4
3 Hz + 7-NI (10 mg kg^{-1}) (6)	51.0 ± 8.8	3.66 ± 0.54	131 ± 13
3 Hz + L-NNA (10 mg kg^{-1}) (5)	71.4 ± 8.0	2.59 ± 0.43	168 ± 2

* ANOVA $P < 0.001$: None *vs.* 3 Hz, 3 Hz + D-NAME, $P < 0.05$; 3 Hz *vs.* 3 Hz + L-NAME, 3 Hz + 7 NI, 3 Hz + L-NNA, $P < 0.05$. † ANOVA $P < 0.001$: None *vs.* 3 Hz, 3 Hz + D-NAME, $P < 0.05$; 3 Hz *vs.* 3 Hz + L-NAME, 3 Hz + 7-NI, 3 Hz + L-NNA, $P < 0.05$. ‡ ANOVA $P < 0.002$: None *vs.* 3 Hz, $P < 0.05$; none *vs.* 3 Hz + L-NAME, L-NAME alone, 3 Hz + D-NAME, 3 Hz + L-NNA, $P < 0.05$

by labelling neurons (positive control) in the spinal cord (with met-enkephalin or nNOS antibodies, respectively) and brain (nNOS).

Statistical analysis

Values were calculated as means ± S.E.M. Comparisons among experimental groups were made using one way standard or repeated measurements analysis of variance (ANOVA) and post-ANOVA unpaired or paired Student's two-tailed t tests.

RESULTS

Blood flow studies

At low rates of sciatic nerve stimulation (3 Hz), local rises in dorsal spinal cord blood flow approximated 25% by LDF, whereas rises of intrinsic grey matter flow measured by hydrogen clearance were greater, approximately double with stimulation. While 3 Hz

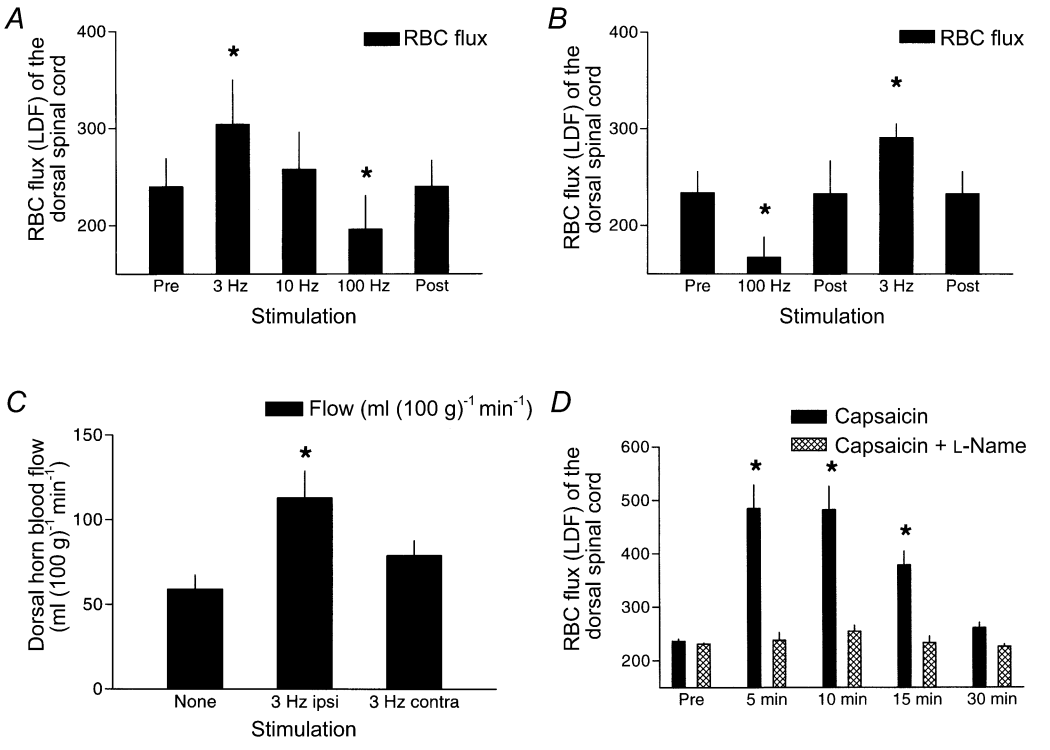

Figure 1. The influence of afferent barrages on dorsal spinal cord blood flow measured by laser Doppler flowmetry (*A*, *B* and *D*) or microelectrode hydrogen clearance polarography (*C*)

Ipsilateral, but not contralateral simulation increased blood flow, independent of the order of the stimulation train (*A*, *B* and *C*). The hydrogen clearance studies provide selective dorsal horn grey matter blood flow values (*C*). Capsaicin stimulation evoked a much larger rise in RBC (erythrocyte) flux (*D*). * Significantly different from pre (*A*, *B* and *D*) or none (*C*). (See statistical description in Tables 1 and 2.)

Table 2. Erythrocyte flux of the dorsal horn of the spinal cord using laser Doppler flowmetry

Intervention (n)		RBC flux (a.u.)	Resistance (mmHg a.u.$^{-1}$)	MAP (mmHg)
Stimulation studies				
Prior to stimulation (8)		238 ± 20 *	0.42 ± 0.04 †	94 ± 4 ‡
3 Hz stimulation (8)		299 ± 30	0.41 ± 0.04	114 ± 5
10 Hz stimulation (5)		258 ± 30	0.50 ± 0.06	115 ± 3
100 Hz stimulation (8)		185 ± 24	0.73 ± 0.12	114 ± 6
Post stimulation (8)		238 ± 19	0.47 ± 0.04	106 ± 3
Pharmacological studies: L-NAME, sympathetic blockade				
Prior to intervention (4)		263 ± 10	0.34 ± 0.01	89 ± 4
L-NAME (10 mg kg^{-1}) (4)		274 ± 25	0.58 ± 0.05	151 ± 5
L-NAME + 3 Hz stimulation (4)		0	n.a.	154 ± 3
Post L-NAME + 3 Hz (4)		276 ± 12	0.48 ± 0.13	131 ± 4
Guanethidine pretreatment (2)		237 ± 44	0.35 ± 0.06	80
Guanethidine + L-NAME (2)		292 ± 40	0.46 + 0.13	130 ± 20
Guanethidine + L-NAME + 3 Hz (2)		0	n.a.	128 ± 8
Post (2)		248 ± 45	0.49 ± 0.08	118 ± 3
Phentotamine pretreatment (3)		208 ± 2	0.39 ± 0.01	82 ± 1
Phentotamine + L-NAME (3)		228 ± 4	0.55 ± 0.01	125 ± 4
Phentotamine + L-NAME + 3 Hz (3)		0	n.a.	123 ± 3
Pharmacological studies: opioids				
Prior to naloxone (10)		216 ± 11	0.40 ± 0.03	83 ± 3
Naloxone (0.05 mg kg^{-1}) + L-NAME (3)		222 ± 5	0.66 ± 0.03	147 ± 6
+ 3 Hz		19 ± 4	8.7 ± 2.2	133 ± 7
Post		206 ± 4	0.57 ± 0.03	117 ± 5
Naloxone (0.10 mg kg^{-1}) + L-NAME (3)		220 ± 3	0.65 ± 0.01	143 ± 1
+ 3 Hz		67 ± 17	2.8 ± 1.0	137 ± 3
Post		198 ± 6	0.57 ± 0.03	113 ± 7
Naloxone (0.20 mg kg^{-1}) + L-NAME		298 ± 28	0.44 ± 0.06	125 ± 9
+ 3 Hz		224 ± 70	0.45 ± 0.03 (3)	134 ± 5
Post		238 ± 33	0.59 ± 0.13	125 ± 3
Morphine (0.2 mg kg^{-1}) (3)		205 ± 2	0.46 ± 0.03	95 ± 6
+ 3 Hz		0	n.a.	93 ± 3
Post		198 ± 6	0.47 ± 0.02	93 ± 7
Pharmacological studies: capsaicin				
Capsaicin 0.2% (4)	predose	236 ± 4 §	0.40 ± 0.04 ‖	95 ± 12
	postdose	484 ± 44	0.25 ± 0.03	119 ± 6
Capsaicin carrier (3)	predose	253 ± 5	0.52 ± 0.13	98 ± 24
	postdose	243 ± 5	0.43 ± 0.11	103 ± 23

* Repeated measures ANOVA $P < 0.0001$: prior *vs.* 3 Hz, 100 Hz, $P < 0.01$. † Repeated measures ANOVA $P = 0.0002$: prior *vs.* 100 Hz, $P < 0.05$; post *vs.* 3 Hz, 100 Hz, $P < 0.05$. ‡ Repeated measures ANOVA $P < 0.0001$: prior *vs.* 3 Hz, 100 Hz, 10 Hz, $P < 0.01$. § Capsaicin postdose *vs.* predose, post carrier, $P < 0.001$. ‖ Capsaicin postdose *vs.* predose, $P = 0.006$. a.u., arbitrary units; n.a., not applicable.

stimulation was associated with an expected rise in mean arterial pressure, microvascular resistance declined indicating that rises in dorsal cord blood flow were not passive (Table 1). Using LDF, in the initial stimulation protocol, there was a rise in mean arterial pressure and resistance was similar to the prestimulation baseline result but lower than the subsequent post-stimulation value (Table 2). A subsequent, separate repeat experiment using 3 Hz stimulation alone confirmed both a rise in LDF erythrocyte flux and a drop in resistance during 3 Hz stimulation compared to pre-stimulation (additional data not shown). At 10 Hz, there was no significant change in LDF erythrocyte flux but 100 Hz stimulation was associated with a paradoxical fall in erythrocyte flux and rise in resistance despite rises in mean arterial pressure. Stimulation of the contralateral sciatic nerve evoked only a borderline rise in dorsal cord blood flow (hydrogen clearance), and a fall in resistance despite a robust rise in mean arterial pressure. Injection of intradermal capsaicin into the rat paw generated large rises in dorsal cord blood flow erythrocyte flux (approximately doubling) and a profound fall in resistance lasting 30 min. The carrier had no such effect. Results are illustrated in Fig. 1*A–D* and Table 2.

The broad spectrum NOS inhibitor L-NAME had no influence on baseline dorsal cord blood flow irrespective of whether it was assessed by LDF or hydrogen clearance. L-NAME was associated with a rise in mean arterial pressure. L-NAME completely blocked stimulation-driven rises in dorsal cord blood flow (both LDF and hydrogen clearance measurements) in a dose-dependent fashion. L-NAME also completely blocked capsaicin-driven rises in dorsal cord blood flow (LDF) (Fig. 1*D*). D-NAME, the inactive enantiomer of L-NAME, did not influence the rises in dorsal cord blood flow (hydrogen clearance) with 3 Hz stimulation. L-NNA and 7-NI both blocked rises in dorsal cord blood flow (hydrogen clearance) with 3 Hz stimulation. None of D-NAME, L-NNA or 7-NI influenced baseline dorsal cord blood flow. Results are illustrated in Figs 2*A* and *B*.

We observed an unexpected and severe reduction of erythrocyte flux with 3 Hz stimulation during NOS blockade with L-NAME at 10 mg kg^{-1}. Immediate LDF recordings following termination of the stimuli indicated that there was a very gradual recovery of perfusion that returned to baseline values by 10–15 min after

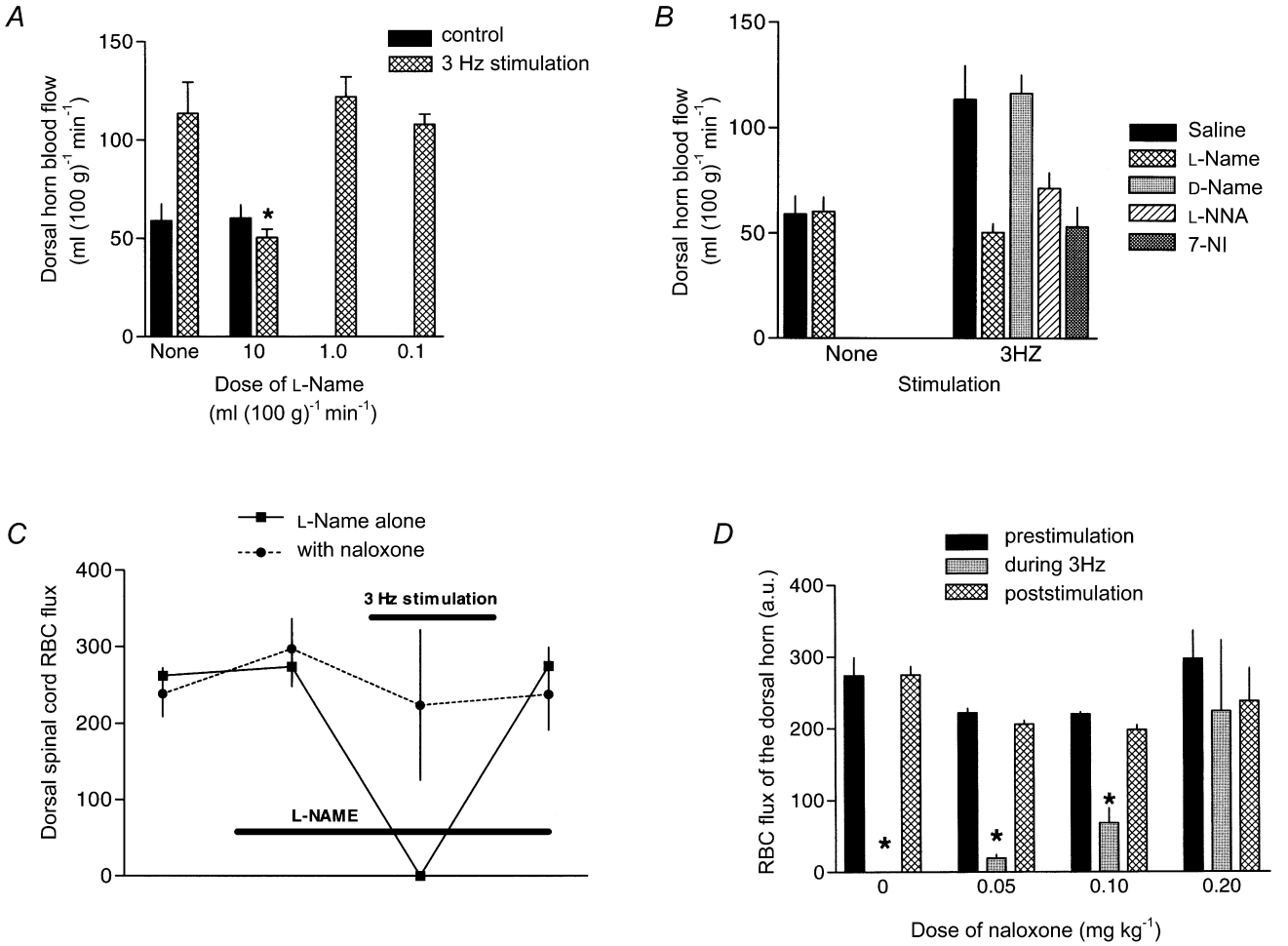

Figure 2. The influence of NOS (nitric oxide synthase) inhibitors on simulation-driven rises in dorsal horn grey matter blood flow measured by microelectrode hydrogen clearance polarography (*A* and *B*) and with 3 Hz stimulation (*C*)

A and *B*, rises in blood flow were blocked by L-NAME at 10 mg kg^{-1} but not by lower doses and were blocked by L-NNA and 7-NI (both 10 mg kg^{-1}) but not D-NAME. L-NAME and L-NNA are broad spectrum NOS inhibitors whereas 7-NI has relative specificity for the neuronal NOS isoform. D-NAME is the inactive enantiomer of L-NAME. * Blood flow value significantly lower than with no treatment, than with D-NAME or than with saline (ANOVA $P < 0.05$). In *C*, stimulation at 3 Hz during L-NAME (10 mg kg^{-1}) NOS blockade results in a vasoconstrictive response, limited to the stimulation epoch and reversed by naloxone (0.20 mg kg^{-1}). The naloxone reversal was dose dependent. In *D*, RBC (erythrocyte) flux is indicated before, during and after 3 Hz stimulation. During stimulation RBC (erythrocyte) flux dropped to negligible values (vasoconstriction) but this was blocked in a dose-related fashion by naloxone, an opioid antagonist. * RBC flux significantly lower than prestimulation value. (See statistical description in Tables 1 and 2.)

discontinuing the stimulus train. Two approaches to sympathetic blockade had no influence on this vasoconstrictive response to afferent stimulation: pretreatment with guanethidine or administration of phentolamine despite appropriate sympatholysis as indicated by reductions in baseline mean arterial pressure. In contrast, there was a dose-related reversal of the vasoconstrictive response with naloxone. Morphine administration alone was associated with a slight fall in mean arterial pressure and baseline erythrocyte flux. In

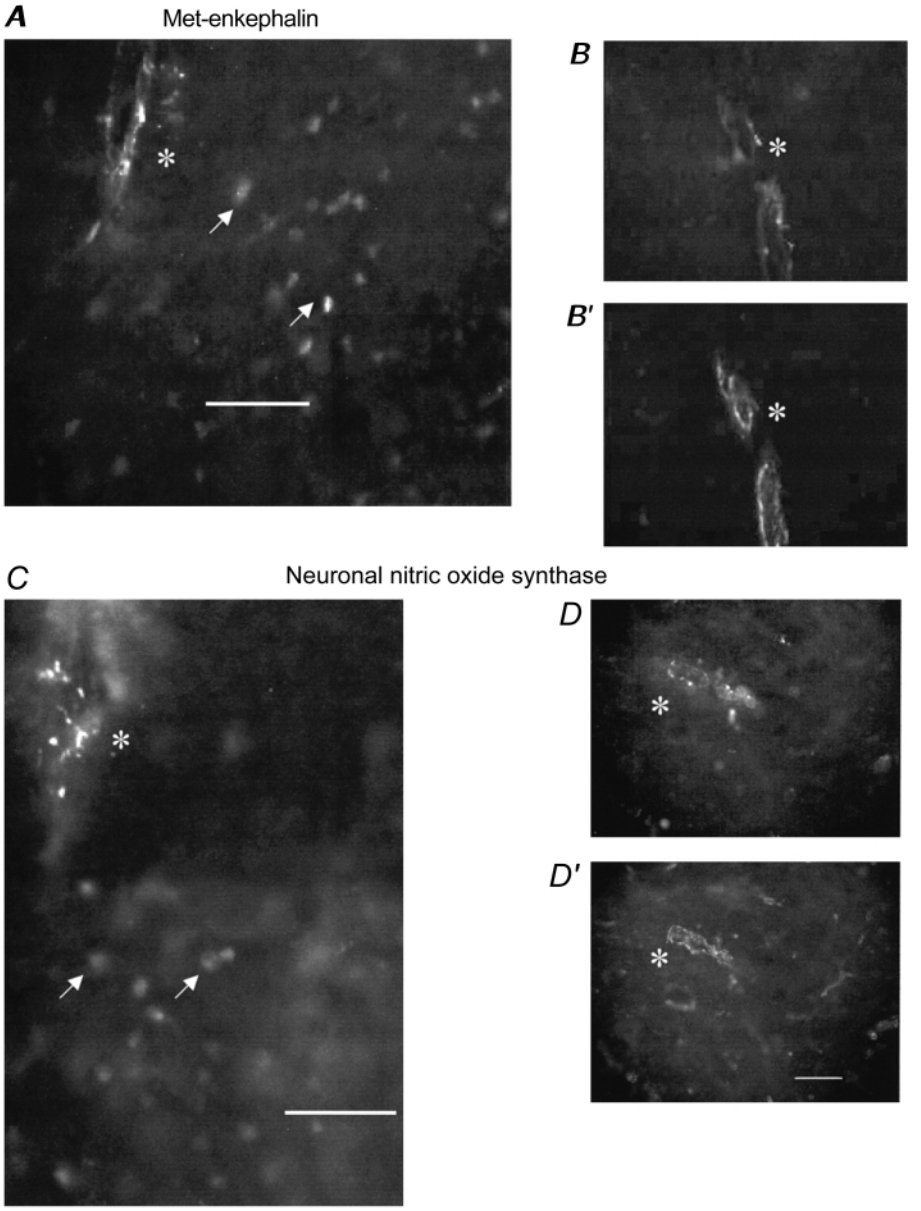

Figure 3. Immunohistochemical labelling of sections of the lumbar spinal cord dorsal horn of the rat

In *A* and *B* vascular-like profiles are labelled with an antibody directed against met-enkephalin, a peptide arising from interneurons. In *A*, there is also labelling of apparent interneurons (arrows) and the field is just at the junction of superficial dorsal horn (containing neurons) and white matter (containing the innervated vascular profile). Note the discrete, punctate immunoreactivity associated with the vessel (asterisk). In *B′*, the same section as *B* is labelled with an antibody to factor VIII confirming that the met-enkephalin-associated profiles are intrinsic spinal cord blood vessels. In *C* and *D*, vascular-like profiles (asterisk) are labelled with an antibody directed against neuronal nitric oxide synthase (nNOS). As with met-enkephalin, labelling is discrete and punctate. In *D′*, the same section as *D* is labelled as above with an antibody directed against factor VIII confirming that the profile is a blood vessel. In *C* there is also apparent labelling of interneurons (arrows) and the field is at the junction of the superficial dorsal horn and white matter. Note that *B*, *B′*, *D* and *D′* are taken from deeper layers of the dorsal horn where neuronal labelling is less evident. Bar = 50 μm.

the absence of L-NAME, morphine alone was associated with a 3 Hz stimulation-associated vasoconstrictive response identical to that observed with L-NAME, that recovered to baseline following stimulation. Results are illustrated in Fig. 2*C* and Table 2.

Immunohistochemistry

Blood vessels both within the grey matter of the lumbar spinal cord and penetrating vessels from the dorsolateral surface of the cord arising from extrinsic parent vessels labelled on their endothelial surface with an antibody to factor VIII. While the smallest factor VIII labelled vessels, presumed to be capillaries, did not colabel with the antibody to nNOS, larger feeding vessels could be identified with small, intense, linear and punctate probably adventitial (from their location) nNOS profiles (Fig. 3*C* and *D*). Less intense and adjacent rounded nNOS-labelled profiles that did not colabel with factor VIII probably represented interneurons. It was not possible to verify that individual nNOS neurons extended innervating branches to these vessels in the present work. The nNOS-associated vessels probably represented arterioles as judged by their wall thickness and internal elastic lamina, but some venular innervation could not be excluded. Using the same approach, similar sized arterioles (and probably some venules) colabelled with antibodies to factor VIII and met-enkephalin (Fig. 3*A* and *B*). Met-enkephalin also labelled apparent adjacent interneuron profiles not colabelled with factor VIII. The studies did not permit assessment of whether enkephalin and nNOS terminals innervated the same vessels. A frequent finding was that of nNOS or met-enkephalin innervating vessels near the grey–white matter junctions in the dorsolateral cord.

DISCUSSION

Our findings indicate an unexpected relationship between local dorsal spinal cord blood flow and specific activation of afferent pathways. Only afferent stimulation at strengths recruiting small myelinated and unmyelinated 'nociceptive' afferents succeeded in influencing dorsal cord blood flow. Intradermal capsaicin, a potent activator of nociceptive pathways, mimicked and exceeded the stimulation-driven rises in dorsal cord blood flow. The rises in dorsal cord blood flow with stimulation were mediated by nitric oxide but were accompanied by powerful opioid activation, with a direct and opposite action on blood flow that was only apparent during NOS blockade or morphine supplementation. The immunohistochemical studies indicated apparent innervation of cord vessels by terminals containing neuronal nitric oxide synthase and met-enkephalin. Together, the physiological and labelling studies suggested direct NO and opioid modulation of spinal cord blood flow.

Our measurements of dorsal horn grey matter blood flow at baseline are comparable to reported values in rats and other animals using a variety of techniques: ^{14}C-iodoantipyrine autoradiography (Cawthon *et al.* 1980; Zivin & Waud, 1983; Hickey *et al.* 1986), ^{14}C-butanol distribution (Sakamoto *et al.* 1988; Kinoshita & Monafo, 1993), trapping of labelled microspheres (Marcus *et al.* 1977; Hickey *et al.* 1986; Wallace & Tator, 1986), the fast clearance component of ^{133}xenon clearance (Griffiths, 1973), and hydrogen clearance (Scremin & Decima, 1983; Hayashi *et al.* 1983; de la Torre & Goldsmith, 1988; Guha *et al.* 1989; Rubinstein & Arbit, 1990). In the literature there is significant variability in the reporting of 'normal' spinal cord blood flow values in various animal species with higher (> 80 ml (100 g)$^{-1}$ (min)$^{-1}$) results in some laboratories obtained using ^{14}C-iodoantipyrine autoradiography (Crosby, 1985; Holtz *et al.* 1988, 1989; Kristensen *et al.* 1993) or trapping of labelled microspheres (Nystrom & Norlen, 1983; Hitchon *et al.* 1996). In other laboratories, in contrast, low values have been reported using the trapping of labelled microspheres (Marcus *et al.* 1977; Linsberg *et al.* 1989; Hoy *et al.* 1994; Sandor *et al.* 1994; Hitchon *et al.* 1996), ^{133}xenon clearance (Ducker & Perot, 1971; Griffiths, 1973, 1979), and hydrogen clearance (Griffiths *et al.* 1975; Kobrine *et al.* 1975, 1978; Senter *et al.* 1978; Dohi *et al.* 1984; Hansebout *et al.* 1988). Some of this variation may be because the microsphere method measures a composite flow value from grey and white matter. Indeed, some of the hydrogen clearance measurements have probably been selective for white matter, explaining the lower values recorded (Senter *et al.* 1978; Young *et al.* 1981; Chehrazi *et al.* 1989). Our hydrogen clearance microelectrodes were considerably smaller than those used in earlier spinal cord blood flow measurements (400 μm compared to our 3–5 μm microelectrode tip) or in more recent measurements with smaller microelectrodes (75 μm diameters; Seki & Maeda, 1993). While LDF does not yield absolute values of spinal cord blood flow, several studies have verified its close relationship (including the epidural approach we used) to values made using other techniques (Shimoji *et al*, 1987; Linsberg *et al.* 1989; Hitchon *et al.* 1996). In the present study, hydrogen clearance and LDF studies were complementary and allowed us to verify several critical findings in closely related vascular beds of the dorsal spinal cord.

There have been few studies of the effect of afferent stimuli on spinal cord blood flow. Koltzenburg *et al.* (1990) noted a rise in blood flow after 1–10 Hz stimulation (but not 50 Hz) using single-site LDF recordings but concluded that rises in mean arterial pressure completely explained these rises. Kobrine *et al.* (1978) recorded ipsilateral rises in spinal cord blood flow with 1 Hz stimulation. Takahashi *et al.* (1988) noted rises with stimulation but these were at high stimulation rates (50–100 Hz) and high intensity (50–100 V). Hitchon *et al.* (1996) noted a decline in baseline spinal cord blood flow with NOS inhibition that we did not observe.

Our findings contrast with our expectation that dorsal cord blood flow would simply be responsive to metabolic activation, irrespective of the afferent barrage it experiences. The relationship between nitric oxide-mediated rises in dorsal cord blood flow and a 'nociceptive' afferent barrage verifies previous suggestions that nitric oxide may help to couple neuronal activity with local blood flow (Dawson & Snyder, 1994). Nitric oxide may mediate spreading depression but not hypercarbic hyperaemia (Fabricius *et al.* 1996). In our studies, it is likely that nitric oxide was generated from NOS-containing interneurons since nNOS is expressed in a very small percentage of L4 and L5 normal dorsal root ganglia neurons (Vizzard *et al.* 1995). Nitric oxide is released in the dorsal horn of the spinal cord during peripheral nociceptive activation, for example chronic arthritis (Wu *et al.* 1998*b*). There are rises in NO_2, a nitric oxide metabolite, in the dorsal horn of rats treated with paw capsaicin (Wu *et al.* 1998*a*). Direct application of nitric oxide donors to the intrathecal space promotes nociceptive behavioural responses (Inoue *et al.* 1997) and intrathecal NOS antagonists provide analgesia in pain models (Meller *et al.* 1992, 1994; Haley *et al.* 1992; Goettl & Larson, 1996; Roche *et al.* 1996). Ambient generation of nitric oxide in the dorsal horn of the spinal cord has been detected using *in vivo* electrochemical monitoring (Rivot *et al.* 1997). It may have been of interest to determine whether chronic elimination of nociceptive afferents would prevent the changes we observed. Such approaches, however, such as high dose neonatal or adult capsaicin could complicate cord circuitry by superimposing an injury response (Wall *et al.* 1982).

Stimulation-related local nitric oxide release accompanied activation of a powerful vasoconstrictive action, apparently opioid mediated. This vasoconstriction was 'unmasked' during NOS blockade but was also seen when morphine was administered without blocking NOS. While intrinsic opioid release may be anti-nociceptive, it acted directly on microvessels in an active and dramatic fashion, not simply blocking nitric oxide-driven hyperaemia. Like nitric oxide, the opioid actions were strictly stimulation-associated and neither the opioid agonist morphine, nor the antagonist naloxone had any substantial influence on baseline blood flow. Similarly, after stimulation, blood flow recovered, despite the continued presence of circulating L-NAME or morphine. A simple diffuse blockade of metabolic activity in the dorsal horn by opioids would not account for our findings. The opioid actions we uncovered during NOS blockade may have been a consequence of high intrinsic opioid concentrations in the perineuronal and perivascular extracellular space during epochs of intense local activity. This mechanism, however, might have difficulty explaining the 'on–off' vasoconstrictive property we observed. In view of the labelling studies it is more likely that nNOS- and opioid-containing interneurons have direct collateral branches that innervate local microvessels. Further work, examining ultrastructural labelling and tracing of interneuron projections would be of considerable interest in verifying this supposition.

Paradoxically, it may be that direct effects of opioids on local blood flow explain previous findings that opioid antagonists are of potential benefit in spinal cord injury, where ischaemia is an important mechanism of damage. A final intriguing possibility is that opioid actions help to suppress certain types of neural activity by controlling the blood supply available to support it. Further work is required to distinguish what population of intrinsic opioids may be responsible for their modulation of blood flow.

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