

Structural and Functional Denervation of Human Detrusor after Spinal Cord Injury

Marcus J. Drake, Petter Hedlund, Ian W. Mills, Rachel McCoy, Gordon McMurray, Brian P. Gardner, Karl-Erik Andersson, and Alison F. Brading

Oxford Continence Group (MJD, IWM, RM, GM, AFB), University Department of Pharmacology, Oxford, and Urology Department (MJD), Churchill Hospital, Oxford, United Kingdom; and Department of Clinical Pharmacology (PH, K-EA), Lund University Hospital, Lund, Sweden; and National Spinal Injury Centre (BPG), Stoke Mandeville, Aylesbury, United Kingdom

SUMMARY: The bladder receives an extensive nerve supply that is predominantly cholinergic, but several putative transmitters are present, some of which are colocalized. Previous studies have shown increased levels of sensory nerves, reduced inhibitory transmitters, and structural and functional changes in the excitatory input in unstable bladder conditions. The present study compared the end-organ nerve supply to the bladder in spinal cord injury (SCI) with uninjured controls. Acetylcholinesterase histochemistry and double-label immunofluorescence were used to investigate neurotransmitter content, with confocal laser scanning microscopy to assess colocalization. Organ bath studies provided functional correlates for the structural changes in the excitatory innervation. Control samples had dense innervation of the detrusor containing a diverse range of transmitters. Hyperreflexic SCI samples showed patchy denervation, and areflexic SCI samples were diffusely denervated. Vasoactive intestinal polypeptide-, neuropeptide Y-, neuronal nitric oxide synthase-, and galanin-immunoreactive nerve fibers were reduced from frequent or moderately frequent to infrequent or very infrequent in SCI. Calcitonin gene-related peptide-immunoreactive fibers were infrequent in controls and SCI samples. Patterns of colocalization were unchanged, but significantly fewer fibers expressed more than one transmitter. The subepithelial plexus was markedly reduced and several of the smaller coarse nerve trunks showed no immunoreactivity to the transmitters assessed. There was no reduction in sensitivity to electrical field stimulation of intrinsic nerves in SCI, but the maximum force generated by each milligram of bladder tissue and the peak force as a proportion of the maximum carbachol contraction were significantly reduced and the responses were protracted. There was no significant functional atropine-resistant neuromuscular transmission in controls or SCI. The reported findings have clinical implications in the management of chronic SCI and development of new treatments. (*Lab Invest* 2000, 80:1491-1499).

Detrusor muscle receives an extensive nerve supply (Daniel et al, 1983), and there is a dense suburothelial plexus in the bladder (Gosling et al, 1983). Excitatory neuromuscular transmission in the normal human bladder is predominantly cholinergic (Brindley and Craggs, 1976; Chen et al, 1994; Kinder and Mundy, 1985; Sibley, 1984; Sjogren et al, 1982), but a wide range of putative transmitters is present. These include nitric oxide (NO) (Smet et al, 1994), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), and tachykinins (Smet et al, 1997). Several of these have been shown to immunolocalize within the same nerve fiber on double-labeling studies (Smet et al, 1997), and it is possible that they serve as modulators of neuromuscular trans-

mission and smooth muscle activity. Intramural ganglia are present in the lateral bladder wall and are heterogeneous in respect of VIP-, neuronal NO synthase- (n-NOS), neuropeptide Y (NPY)-, tyrosine hydroxylase (TH)-, and galanin (gal)-immunoreactivity (IR) (Smet et al, 1996). Pericellular varicosities, most often expressing CGRP- or VIP-IR, occur around ganglion cells (Smet et al, 1996). The arrangement suggests the possibility of local circuits regulating or reinforcing motor or sensory activity. Nevertheless, considerable uncertainty remains regarding the origin of terminal motor and sensory fibers and the precise function of the substances they contain.

Some studies into pathological causes of unstable bladder activity have looked for an increase in the number of sensory nerve fibers, hypothesizing that detrusor instability (DI) may result from abnormal afferent activity in the micturition reflex. Increased numbers of nerves containing acetylcholinesterase (AChE) (Moore et al, 1992), CGRP (Smet et al, 1997), and substance P (SP) (Smet et al, 1997) are present in the suburothelial region. However, there is no alteration in the overall suburothelial nerve density (Smet et al, 1997). Changes in detrusor innervation occur, with reduced densities of some transmitters in hyperreflexia (Kinder et al, 1985), idiopathic DI (Gu et al, 1983),

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Address reprint requests to: Dr. Marcus Drake, Department of Surgery, School of Surgical Sciences, The Medical School, University of Newcastle, Newcastle upon Tyne, NE2 4HH, United Kingdom. Fax: 44 191 222 8514; E-mail: m.j.drake@ncl.ac.uk

and bladder outflow obstruction (Chapple et al, 1992), and alterations in cholinergic nerve distribution in idiopathic DI (Charlton et al, 1999; Mills et al, 2000). The morphological studies have been supported by functional experiments. Partial denervation and cholinergic supersensitivity is evident in isolated perfused strips from people with unstable obstructed bladders (Harrison et al, 1987) or hyperreflexia due to spina bifida (German et al, 1995). In idiopathic DI there is reduced response to electrical field stimulation of intrinsic nerves (Mills et al, 2000) and increased spontaneous activity (Kinder and Mundy, 1987; Mills et al, 2000), but these are not associated with cholinergic supersensitivity (Mills et al, 2000). Ultrastructurally, elderly patients with idiopathic DI show detrusor denervation, seen as axon degeneration, associated with a specific muscle dysjunction pattern (Elbadawi et al, 1993).

Spinal cord injury (SCI) has fundamental effects on bladder function, usually resulting in overactivity (hyperreflexia) in suprasacral lesions or inability to void (areflexia) in more distal lesions. Since the clinical manifestations of detrusor hyperreflexia are similar to those of idiopathic and obstructive DI, there may be common pathophysiology (Brading and Turner, 1994). Nevertheless, the different nomenclature for the clinical conditions has been proposed by the Standardization Committee of the International Continence Society (Abrams et al, 1986). SCI is of particular interest in that most people affected have achieved full maturity and normal bladder function before the injury, contrasting with the congenital neuropathic diseases that have been the subject of previous investigations into hyperreflexia (German et al, 1995; Kinder and Mundy, 1987; Saito et al, 1993). In the current study we aimed to investigate how SCI affects end-organ innervation in the bladder in terms of function, structure, and neurotransmitter expression and colocalization.

Results

Histochemistry, Immunofluorescence, and Confocal Microscopy

Detrusor Muscle. Controls showed very frequent AChE-positive nerves (Fig. 1) and very frequent fibers that are immunoreactive to the general nerve marker protein gene product (PGP) 9.5. These markers revealed slender nerve fibers and varicose terminals evenly distributed throughout the detrusor muscle. There were frequent VIP-immunoreactive varicose terminals and moderate numbers of fine varicose terminals with NPY-, n-NOS-, and gal-IR, infrequent CGRP-IR, and very infrequent TH- and SP-IR. TH-IR was more apparent in interfascicular planes. Hemoxygenase (HO)-1, HO-2, and somatostatin (SOM)-IR were absent. Colocalization was apparent between several transmitters (Fig. 2) and the associations are set out in Table 1.

SCI samples showed marked reduction in the number of nerve fibers in the detrusor using both AChE

histochemistry and PGP 9.5 immunofluorescence. The pattern of denervation was patchy in hyperreflexic SCI, in which some areas showed frequent nerve fibers adjacent to areas where nerve fibers were very infrequent (Fig. 1). Nerve loss was diffuse in areflexic SCI, down to very infrequent (absent in one specimen) (Fig. 1). VIP-, NPY-, n-NOS-, and gal-IR were reduced to infrequent or very infrequent. CGRP-IR was infrequent in controls and remained infrequent in SCI samples. TH- and SP-IR were almost absent from the muscle fascicles. There was no change in the patterns of colocalization assessed, but significantly fewer fi-

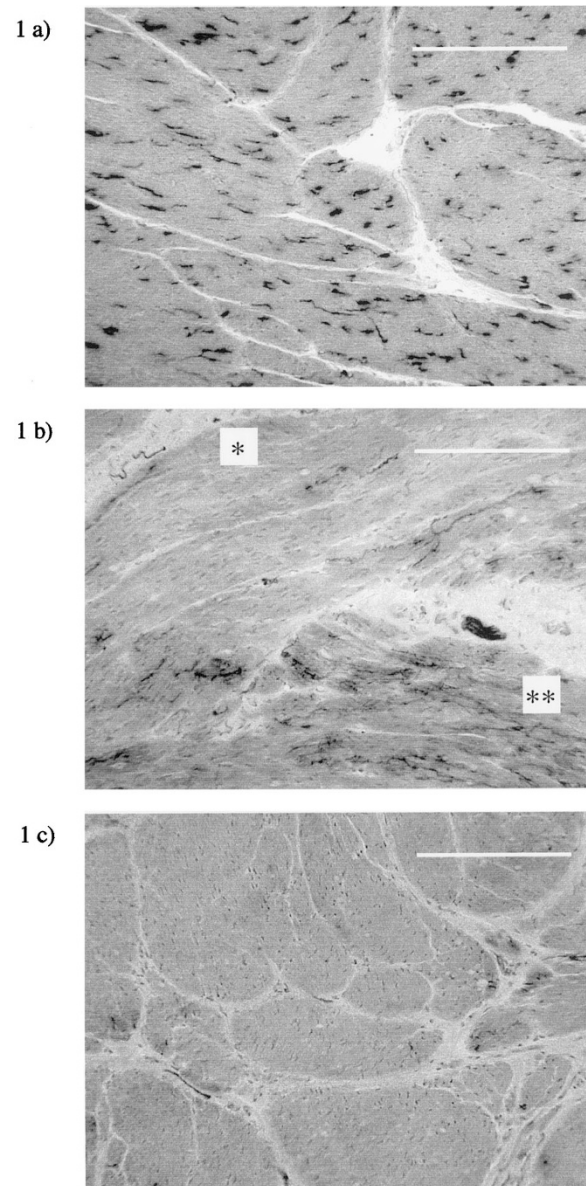


Figure 1.

Detrusor muscle innervation. *a*, Control (44-year-old female cadaveric organ donor). Numerous black deposits of reaction product indicative of presumptive cholinergic nerves. *b*, Patchy denervation (33-year-old female hyperreflexic spinal cord injury [SCI]). Denervated fascicle (*) adjacent to a well-innervated fascicle (**). *c*, Diffuse denervation (48-year-old male areflexic SCI). Near-total detrusor denervation. A few nerve fibers are present in interfascicular planes. Acetylcholinesterase histochemistry. Scale bar = 100 μ m.

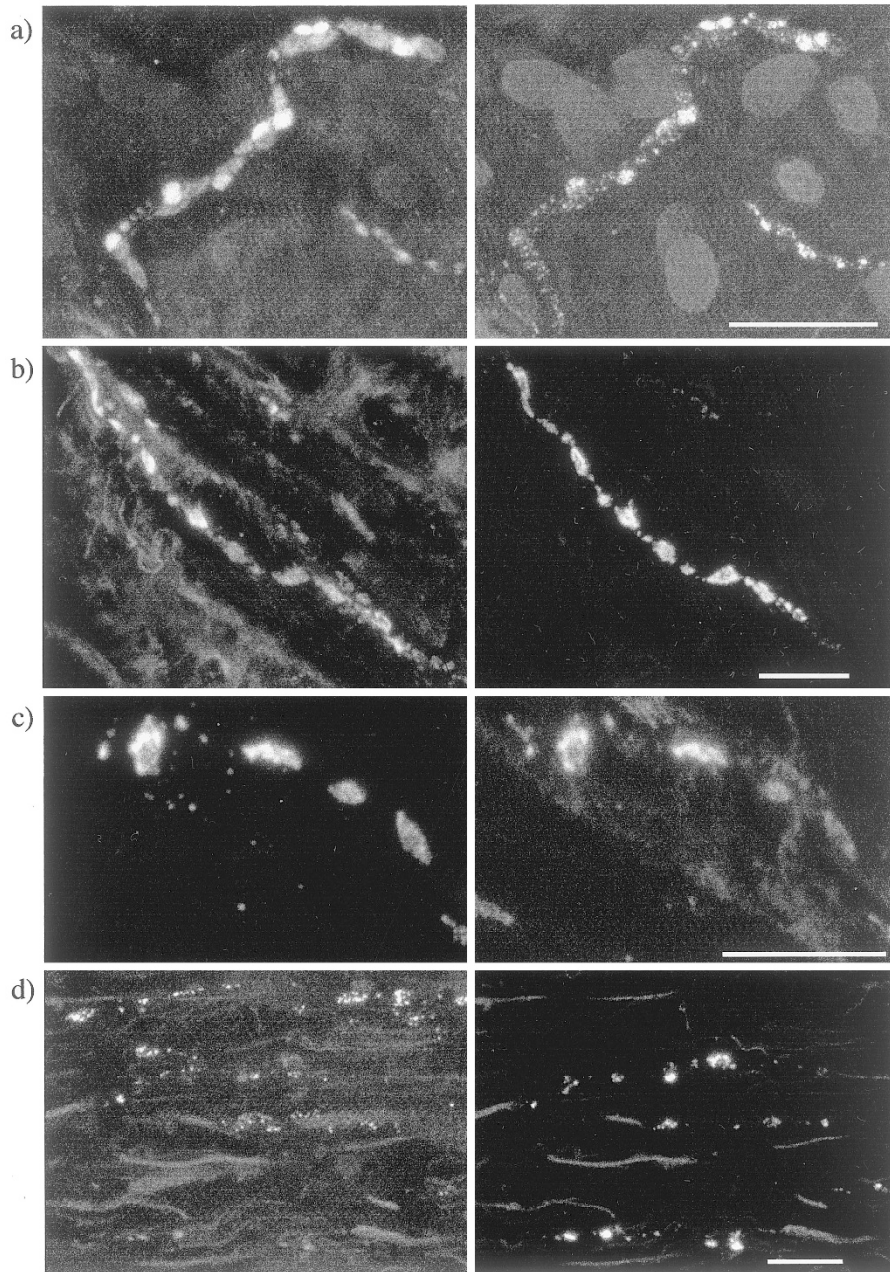


Figure 2.

Neurotransmitter colocalization. *a*, 33-year-old control; n-NOS (left) colocalized with vasoactive intestinal polypeptide (VIP) (right). *b*, 47-year-old areflexic SCI; n-NOS (left) colocalized with VIP (right). *c*, 33-year-old control; calcitonin gene-related peptide (CGRP) (left) colocalized with galanin (gal) (right). *d*, 47-year-old areflexic SCI; CGRP (left) close to but not colocalized with gal (right). Scale bar = 10 μm . Double-label immunofluorescence viewed by confocal microscopy.

bers expressed more than one transmitter ($p < 0.01$) (Table 1).

Suburothelial Plexus. Controls showed an extensive suburothelial nerve plexus, with very frequent AChE-positive and PGP 9.5 immunoreactive fibers (Fig. 3), from which nerve fibers extended into the urothelium. Innervation was frequent for VIP-IR, moderate for NPY-, SP-, TH-, CGRP-, and n-NOS-IR, and infrequent for gal- and SOM-IR. HO-1-IR was absent. In SCI the subepithelial plexus was markedly reduced; in some areas it was absent, but changes in hyperreflexic cases were akin to those seen in fascicles in that some areas showed preservation of the plexus at a

reduced density (Fig. 3). Colocalization associations (Table 1) were the same as for controls, but significantly fewer fibers expressed more than one transmitter ($p < 0.05$) (Table 1).

Other Regions. Coarse nerve trunks were seen in interfascicular planes in the detrusor, taking up a characteristic "wavy" conformation. Large nerve trunks in controls showed VIP-, CGRP-, NPY-, TH-, and SP-IR. Nearly all of the smaller trunks contained VIP-IR, but they often lacked one or more of the other transmitters. In SCI, VIP-IR remained the commonest finding, but some smaller trunks were devoid of VIP-IR, with several showing no IR to the transmitters

Table 1. Colocalization of transmitters in nerve fibers in controls and SCI as assessed with confocal laser scanning microscopy

Transmitter	Nerve site (frequency) ^a	Proportion colocalizing second transmitter (control/SCI) ^b						
		VIP	NPY	CGRP	n-NOS	TH	Gal	SP
VIP	Muscle (F/I)	—	4/3	0/0	2/1	NA	NA	0/0
	Suburo. ^c (F/I)	—	4/4	0/0	2/1	NA	NA	0/0
NPY	Muscle (M/I)	4/2	—	1/0	NA	3/1	NA	NA
	Suburo. (M/VI)	4/3	—	2/2	NA	1/1	NA	NA
CGRP	Muscle (I/I)	0/0	2/0	—	NA	NA	3/1	2/1
	Suburo. (M/I)	0/0	2/1	—	NA	NA	3/1	2/1
n-NOS	Muscle (M/VI)	3/2	NA	NA	—	CA/0	NA	NA
	Suburo. (M/I)	3/2	NA	NA	—	0/0	NA	NA
TH	Muscle (VI/A)	NA	4/1	NA	CA/0	—	NA	NA
	Suburo. (M/VI)	NA	4/1	NA	0/0	—	NA	NA
Gal	Muscle (M/VI)	NA	NA	2/1	NA	NA	—	NA
	Suburo. (I/VI)	NA	NA	2/1	NA	NA	—	NA
SP	Muscle (VI/A)	0/0	NA	5/5	NA	NA	NA	—
	Suburo. (M/I)	0/0	NA	5/5	NA	NA	—	—

^a A = Absent; VI = Very infrequent; I = Infrequent; M = Moderate; F = Frequent; VF = Very frequent; (control/SCI).

^b 0 = No colocalization present; 1 = 1–20%; 2 = 21–40%; 3 = 41–60%; 4 = 61–80%; 5 = 81–100%; CA = close association observed, but transmitters not colocalized; NA = not assessed.

^c Suburo = suburothelial plexus.

assessed. Intramural ganglia were very infrequent in the dome of the bladder for both controls and SCI. Fine nerve fibers were observed around 86% of arteries in controls stained for PGP 9.5. They showed frequent TH-, NPY-, and VIP-IR, with moderate or infrequent SP- and CGRP-IR. In SCI, perivascular fibers were seen for 47% of arteries. The same transmitters were present as for controls, but with a proportionate reduction in the frequency of the nerves showing IR.

Functional Studies. The numbers of strips studied were: controls 127 (mean weight 3.7 mg), hyperreflexic SCI 108 (3.4 mg), and areflexic SCI 92 (3.5 mg). Electrical field stimulation (EFS) caused a rapid rise in isometric tension that was frequency-dependent in the range 1 to 50 Hz (Fig. 4). The time course of EFS responses in the SCI strips was significantly altered. The time to peak response for controls (expressed as mean \pm SEM) was 5.8 ± 0.4 seconds, compared with 12.8 ± 0.9 seconds for hyperreflexic SCI and 11.2 ± 0.5 seconds for areflexic SCI ($p < 0.01$). The 50% to 50% contraction time for controls was 14.4 ± 0.7 seconds, compared with 26.9 ± 2.5 seconds for hyperreflexic SCI and 22.1 ± 2.1 seconds for areflexic SCI ($p < 0.01$). There was no reduction in the sensitivity to EFS in SCI (Fig. 5a). However, the maximum force generated by each milligram of bladder tissue was significantly reduced (Fig. 5b) at 0.61 ± 0.06 g/mg for hyperreflexic cases and 0.43 ± 0.07 g/mg for areflexic, compared with 1.37 ± 0.09 g/mg in controls ($p < 0.01$). The peak EFS response as a proportion of the maximum carbachol contraction (Fig. 5c) was $72.1 \pm 3.6\%$ for controls, $62.3 \pm 2.8\%$ for hyperreflexic SCI, and $51.2 \pm 2.5\%$ for areflexic SCI ($p < 0.01$). In the control strips, $13.6 \pm 4.1\%$ of the EFS response at 30 Hz was atropine resistant, compared with $11.7 \pm 3.1\%$ and $11.1 \pm 3.3\%$ for hyperreflexic and areflexic

SCI, respectively ($p = 0.23$). Additional perfusion with tetrodotoxin further reduced the responses for control strips to $10.1 \pm 2.9\%$, for hyperreflexic SCI strips to $8.9 \pm 2.4\%$, and for areflexic strips to $8.7 \pm 3.2\%$ ($p = 0.69$).

Discussion

The major findings in the reported study relate to detrusor and suburothelial denervation in SCI. Hyperreflexic cases were distinguished by the patchiness of the denervation, particularly in the detrusor and to a lesser extent in the suburothelial plexus. Areflexic cases showed diffuse, severe nerve loss. The reduction was global for all tested transmitters and appeared to be a loss of both the slender fibers and varicose terminals rather than merely loss of the IR. Detrusor denervation occurs in humans with congenital neuropathic hyperreflexia (Charlton et al, 1999; German et al, 1995), idiopathic DI (Charlton et al, 1999; Mills et al, 2000), and obstructive DI (Harrison et al, 1987; Speakman et al, 1987). In some, the denervation has been reported as patchy, akin to the current findings (Charlton et al, 1999; Mills et al, 2000). In hyperreflexic SCI, the apparent density in areas retaining innervation was reduced compared with age-matched controls. This suggests that the denervation in hyperreflexic cases may be a combination of two processes, one leading to a patchy change, on which is superimposed a diffuse denervation. The diffuse nerve loss in areflexic SCI cases is similar to a severe version of the nerve loss seen with ageing in normal humans (Gilpin et al, 1986). We postulate, therefore, that SCI leads to a general acceleration of the ageing process, either directly, indirectly due to obstruction from dyssynergic sphincter activity, or as a consequence of the increased functional demands placed

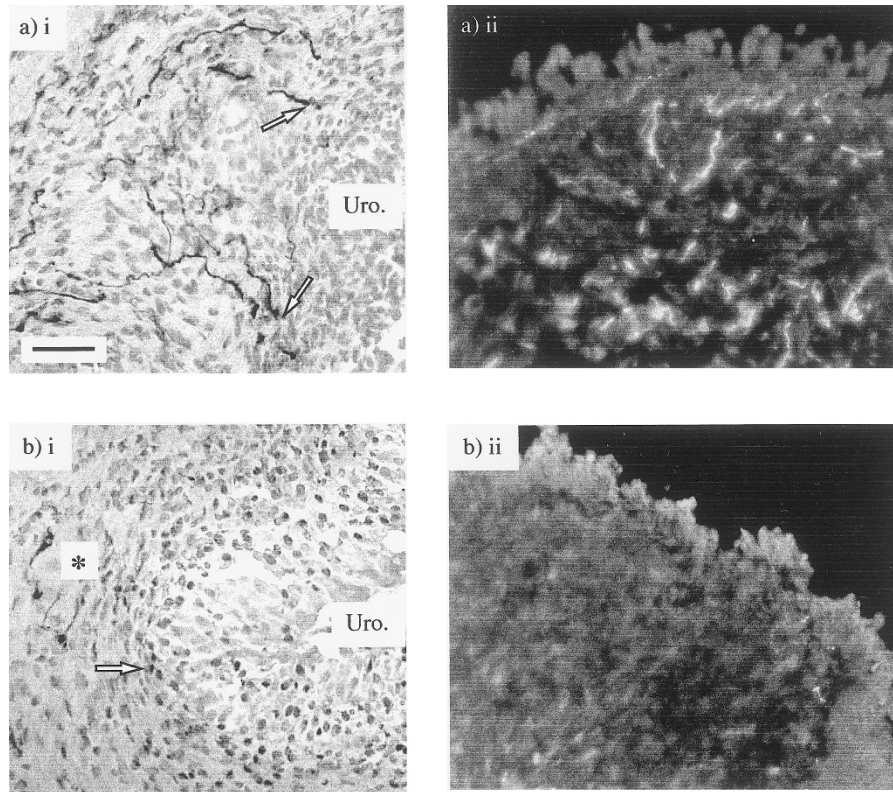


Figure 3.

Suburothelial plexus. *a (i)*, Control (44-year-old male cadaveric organ donor). Suburothelial nerve fibers (*arrowhead*) were seen approaching the urothelial basement membrane. Acetylcholinesterase histochemistry. *a (ii)*, Section from the same control specimen. Protein gene product (PGP) 9.5 immunofluorescence. *b (i)*, SCI (33-year-old male hyperreflexic). Marked suburothelial nerve loss, with only sporadic fibers still seen (*). *Arrowhead* indicates region of urothelial basement membrane. Acetylcholinesterase histochemistry. *b (ii)*, Section from the same SCI specimen showing an area of total denervation. PGP 9.5 immunofluorescence. Uro. = urothelium; scale bar = 10 μ m (applies to all).

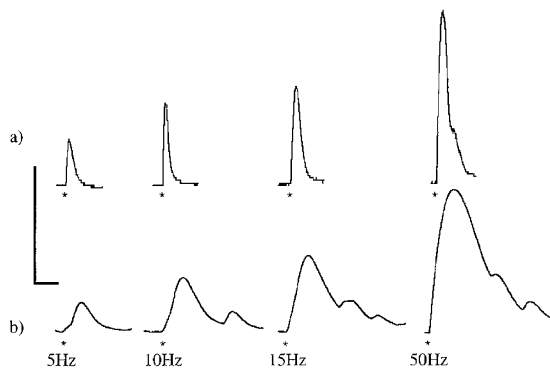


Figure 4.

Example electrical field stimulation (EFS) traces. *a*, Control (42-year-old female, strip weight 2.1 mg). *b*, SCI (44-year-old male hyperreflexic, strip weight 3.9 mg). Protracted, multiphasic response and reduced force generation per milligram of tissue. Scale: vertical bar, 1 mg isometric tension; horizontal bar, 15 seconds. * indicates the point of stimulation.

on the injured individual (Whiteneck et al, 1993). Despite denervation of a large proportion of the muscle in hyperreflexic SCI specimens, the ability to achieve detrusor contraction in vivo is maintained. In normal bladder, muscle innervation is particularly dense (Daniel et al, 1983) and may be surplus to the minimum required for bladder emptying, potentially providing an innervation reserve. Alternatively, changes in detrusor

properties, such as increased smooth muscle coupling, may compensate and thereby facilitate widespread contraction after partial denervation.

The etiology of the nerve loss is not apparent, but all parts of the dome of the bladder were affected in the current study. The range of transmitters seen in the coarse nerve trunks was reduced, which suggests a proximal process, perhaps at the level of the pelvic ganglia. In view of the persisting immunolocalization of PGP 9.5 by coarse nerve trunks in SCI samples, a local process selectively affecting terminal parts of nerve fibers is also possible. There is evidence that transmural ischemia may explain denervation in idiopathic DI (Mills, 1999).

Failure to observe HO-1 and 2 in these experiments, and low levels of other transmitters, may not be a true reflection of their representation in the dome of the bladder. Nonvisualization could also occur if the transmitter is labile in vivo or suffers rapid depletion post mortem, a comment that applies to immunolocalization studies in general. HO-2 produces carbon monoxide (CO), a putative transmitter found in the human urethra (Ho et al, 1999). HO-1 is inducible in response to oxidative stress. HO-2 undergoes axonal transport so that the local concentration in axons may be insufficient to allow immunolocalization (Vollerthun et al, 1995). Notwithstanding, functional studies show a

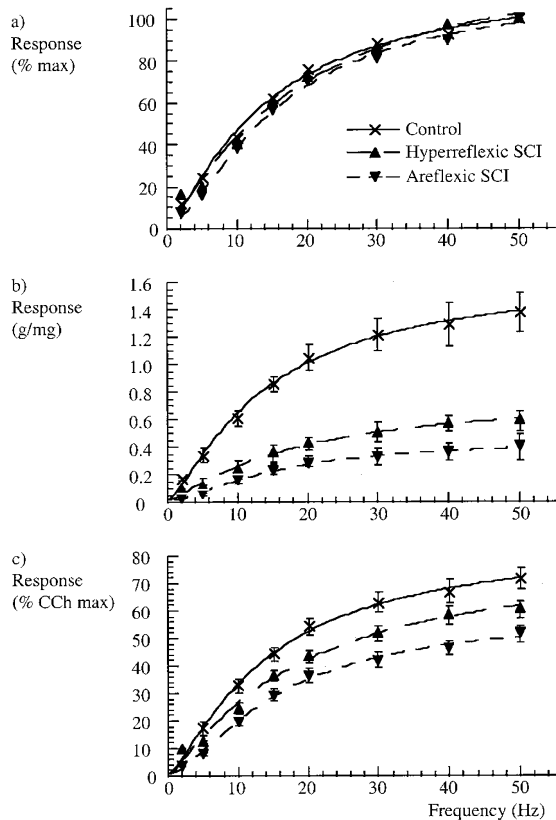


Figure 5.

Frequency-response curves of control and SCI detrusor strips to electrical field stimulation. Points are means \pm SEM. *a*, Contractile response of control ($n = 127$), hyperreflexic SCI ($n = 108$), and areflexic SCI ($n = 92$) strips expressed as a percentage of the maximal response to EFS. *b*, Contractile response of strips expressed in grams per milligram of tissue. *c*, Contractile response of strips expressed as the percentage of the maximal response to carbachol (CCh).

lack of effect of CO on human intravesical ureter (Iselin et al, 1997), and because the status of CO as a neurotransmitter remains uncertain, lack of HO enzymes in the human bladder might be expected.

Colocalization of neurotransmitters has been reported in the human bladder on the basis of coincident profiles on immunolocalization studies. At least three distinct populations of nerves are present, showing IR to VIP alone, CGRP alone, and CGRP with SP or neurokinin A (Smet et al, 1997). Postnatally, intramural ganglion cells colocalize NOS and NPY, SP and CGRP, or NPY and VIP (Dixon et al, 1998). The functional significance of transmitter colocalization is not known. These substances may have a trophic function or modulate muscle excitability or gene expression. Consequently, transmitter depletion and reduced colocalization may play a role in the reduced compliance and structural derangement of the bladder wall in SCI. The current results show similar colocalization patterns for the detrusor and the suburothelial region, except that fewer NPY-IR fibers contained TH-IR suburothelially, whereas the same proportion of TH-positive fibers contained NPY-IR in both regions. This implies two populations of NPY-IR nerves, those

with or without TH-IR, the latter occurring more commonly in the suburothelial plexus.

The reduced peak force generated by bladder strips in acquired spinal cord dysfunction due to SCI contrasts with increased contractility of strips in congenital neurogenic bladder dysfunction (Saito et al, 1993). The study group in the latter investigation was younger than the controls (mean 12.4 years as against 44.8 years), making direct comparison difficult. The reduction in the current study could in part be due to connective tissue infiltration decreasing the amount of muscle present in each strip, but the greater reduction in response to EFS as compared with carbachol stimulation implies partial functional denervation. The protracted time course of the EFS response suggests that the total force generated may be similar to controls. This might indicate abnormal spread of excitation in response to nerve activity, which is likely to be myogenic. No significant atropine-resistant neuromuscular transmission was present and in essence all the residual contraction was also resistant to tetrodotoxin, indicating that it was due to direct muscle stimulation rather than nerve stimulation and neurotransmitter release. Atropine virtually eliminates nerve-induced contraction in normal human bladder (Kinder and Mundy, 1985; Sjogren et al, 1982), but noncholinergic nonadrenergic (NANC) neuromuscular communication is present in several other species. Atropine-resistance in humans has been reported in some pathological conditions (Saito et al, 1993; Sjogren et al, 1982), but such findings could actually have been due to direct muscle stimulation. NANC neurotransmission may be more prevalent with low-frequency EFS (Brading and Williams, 1990; Luheshi and Zar, 1990), with the cholinergic component predominating at higher frequencies. Although a relatively high frequency (30 Hz) was used for investigating atropine resistance, it could be assumed to achieve maximal release of any NANC neurotransmitter, such that muscarinic blockade should reveal any NANC-mediated contraction. This is seen in strips from animals with a large NANC component, such as the rat and the guinea pig (Brading and Williams, 1990). Consequently, it is unlikely that any major NANC-mediated contraction was missed.

The etiology of unstable detrusor activity remains to be explained. Unstable contractions may be due to abnormal muscle properties ("the myogenic hypothesis") (Brading and Turner, 1994). Alternatively, the contractions may follow abnormal activation of the micturition reflex ("neurogenic hypothesis") (de Groat, 1997). The presence of subepithelial denervation, although not excluding the possibility, suggests that hyperreflexic contractions in SCI may not be due to exaggerated afferent activity. Likewise, abnormally protracted contraction in response to intrinsic nerve stimulation supports a myogenic etiology.

The reported findings are of clinical importance in the context of current bladder management deficiencies. All methods currently available are potentially associated with considerable morbidity, and there is increasing recognition of the need for novel ap-

proaches. In light of the extensive structural and functional changes demonstrated, the etiology of which remains to be determined, new approaches should only be developed with due consideration to the possibility of declining effectiveness with time should denervation prove progressive.

Materials and Methods

Study Subjects

Informed consent and local ethical committee approval were obtained. Eighteen people with SCI and recent urodynamic evaluation were studied; 11 had detrusor hyperreflexia (7 male, 4 female, mean age 41.4 years) and 7 had areflexia (4 male, 3 female, mean age 44.4 years). Samples were obtained at elective open operation. Control samples (10 female, 7 male, mean age 48.7 years) were obtained from 11 cadaveric organ donors and from macroscopically normal areas of the bladder of 6 carcinoma patients undergoing cystectomy (2 had previously received mitomycin intravesical chemotherapy). Urodynamic investigation was not possible for controls, but none had any history of lower urinary tract symptoms other than hematuria.

Morphological Studies

Tissue Handling. Full thickness bladder samples were taken from the bladder dome and placed immediately in ice cold Krebs solution. Samples for immunofluorescence studies were prefixed in a freshly prepared solution of paraformaldehyde (4% in phosphate buffered saline [PBS], pH 7.4, 4° C) for 4 hours, then rinsed and cryoprotected with sucrose (15% in PBS, 3 × 12 hours, 4° C). Fixation was carried out within 12 hours to avoid neurotransmitter depletion (Gu et al, 1985). Samples for histochemistry were not prefixed. Samples were frozen in OCT compound (Tissue-Tek; BDH Laboratory Supplies, Poole, United Kingdom) by immersion in isopentane cooled with liquid nitrogen and stored at -70° C.

Single- and Double-Label Immunofluorescence. Cryostat sections, 8 μm thick, were thaw-mounted onto glass slides, air dried, and incubated for 2 hours in PBS containing Triton X-100 (0.2%) and bovine serum albumin (0.1%). Primary antisera were diluted in the same solution and applied for 24 hours at room temperature, after which excess antibody was removed by washing in PBS (3 × 10 minutes). The second primary antibody for double-labeling studies was then applied for a further 24 hours. Appropriate secondary antibodies (diluted in PBS) were applied for 90 minutes each at room temperature, then washed (PBS, 3 × 10 minutes). Texas Red (TR)-conjugated (dilution 1:160) and/or fluorescein isothiocyanate (FITC)-conjugated (dilution 1:80) affinity purified antibodies raised against the source animals of the primary antibodies were used.

Primary antibodies were as follows. From rabbit, PGP 9.5 (working dilution 1:1000; UltraClone, Wellow, Isle of Wight, United Kingdom), NPY (1:400; Peninsula

Laboratories, Belmont, California), gal (1:1500; Peninsula) and HO- 1 and 2 (1:500; StressGen, Victoria, BC, Canada). From guinea pig, VIP (1:640; Euro-Diagnostica, Malmö, Sweden), SP (1:1000; Euro-Diagnostica). From mouse, CGRP (1:500; Euro-Diagnostica), TH (1:1000; Incstar, Stillwater, Minnesota). From rat, SOM (1:500; Euro-Diagnostica). From sheep, n-NOS (1:1500; Euro-Diagnostica). The secondary antibodies used were: swine anti-rabbit (FITC, 1:80; Dako A/S, Glostrup, Denmark), donkey anti-rabbit (TR, 1:160; Jackson ImmunoResearch, West Grove, Pennsylvania), goat anti-guinea pig (FITC, 1:80; Sigma Chemical, St. Louis, Missouri), swine anti-guinea pig (TR, 1:160; Dako), donkey anti-mouse (TR, 1:160; Jackson), donkey anti-rat (TR, 1:160; Jackson), and donkey anti-sheep (FITC, 1:80; Sigma).

Antiserum specificity control experiments showed no IR in sections incubated in the absence of primary antibody, or in antibody preadsorbed with excess (100 μg/ml) of the respective antigen. As cross reactions to antigens with similar amino acid sequences could not be excluded completely, the structures demonstrated are referred to as showing antigen-IR. Nerve fiber frequency (very frequent, frequent, moderate, infrequent, very infrequent, absent) was assessed by two independent observers as compared with PGP 9.5-immunolocalized nerve frequency, which was used in controls to determine "very frequent." In double-label immunofluorescence studies, "close apposition" means the two fluorophores had very similar positions but with visible structural differences. "Coinciding profiles" refers to identical structural details. "Colocalization" means the two antigens were present in the same nerve fiber on confocal microscopy.

Confocal Laser Scanning Microscopy. Sections were viewed with a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) with a ×100 oil immersion plan apochromat lens. Sequential scanning was performed using excitation wavelengths of 488 and 668 nm from a Krypton-Argon laser. Immunofluorescence was selected with a 522 ± 30 band pass filter for FITC and a 605 ± 30 nm band pass filter for Texas Red.

AChE Histochemistry. The Hedreen and Bacon modification (Hedreen et al, 1985) of the Karnovsky-Roots stain (Karnovsky and Roots, 1964) was used.

Functional Studies

Isometric Tension Measurement. Specimens were transferred to Krebs solution at 4° C immediately after retrieval. Detrusor strips (1 × 1 × 5 mm) were micro-surgically dissected and 5/0 silk ligatures applied to each end. They were mounted at an initial tension of 1 g in 0.2 ml superfusion organ baths (Brading and Sibley, 1983) and perfused at a flow rate of 1 ml per minute with carboxygenated Krebs solution (O₂ 97%, CO₂ 3%, pH 7.4) at 37° C (± 0.1). One end was attached to a force transducer (Pioden UF1; Harvard Apparatus, Kent, United Kingdom); isometric tension was recorded with an Apple Macintosh PowerBook 1400c computer using Chart software and MacLab 8

hardware (Ad Instruments Pty, New South Wales, Australia).

Experimental Procedure. After 90 minutes at equilibration, the maximum contractile response of each strip was ascertained with a 10-second application of carbachol (10^{-4} M). Forty minutes later, frequency-response curves to EFS were constructed by stimulating with a Grass S48 electrical stimulator (Grass Instruments, Quincy, Massachusetts) via recessed platinum ring electrodes. Parameters were set to maximize nerve response, with minimum direct muscle stimulation (5-second pulse trains, 0.05-msec pulse width, 50 V amplitude, and varying frequencies from 1 to 50 Hz) (Sibley, 1984). Graduated increases in the period between stimulations, from 5 minutes at low frequencies (1 to 2.5 Hz) to 30 minutes for high frequencies (20 to 50 Hz), were applied to avoid desensitization. Atropine resistance was investigated by continuous exposure of the strips to atropine (10^{-6} M) for at least 30 minutes before and during repeated stimulation at 30 Hz. Strips were then exposed to tetrodotoxin (5×10^{-7} M) with atropine (10^{-6} M) to assess the nerve-mediated contribution to any residual contraction at 30 Hz. Finally, strips were blotted on tissue paper and weighed.

Drugs and Solutions

Carbamylcholine chloride (carbachol), atropine sulfate, and tetrodotoxin (Sigma Chemical, Poole, United Kingdom) were used. Drugs were dissolved in deionized water and diluted in Krebs solution (NaCl 119 mM, KCl 4.6 mM, CaCl_2 1.5 mM, MgCl_2 1.2 mM, NaHCO_3 15 mM, NaH_2PO_4 1.2 mM, and glucose 5.5 mM). Isopentane for tissue freezing was obtained from BDH, United Kingdom.

Statistics

Results are expressed as mean \pm SEM. Analysis of variance was used for comparison of parametric data. The Wilcoxon signed ranks test was used for comparison of paired nonparametric data. Statistical significance was inferred at $p < 0.05$.

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