Experimental Neurology 215 (2009) 264–270

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Reinnervation of transplanted vas deferens by cholinergic nerves normally supplying skeletal muscle

G. Vrbová, G. Burnstock *

Autonomic Neuroscience Centre, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK

ARTICLE INFO

Article history:
Received 13 May 2008
Revised 15 October 2008
Accepted 17 October 2008
Available online 31 October 2008

Keywords:
Acetylcholine
Denervation
Noradrenaline
Reinnervation
Soleus muscle
Vas deferens

ABSTRACT

The rat vas deferens was removed and either transplanted alongside the soleus muscle or into the bed of the soleus muscle that had previously been removed, and in this case the soleus nerve was connected to the transplant. The vas deferens reinnervated by the somatomotor nerve recovered the best. Contractions to transmural electrical stimulation could not be elicited from the denervated vas deferens, although noradrenaline and acetylcholine elicited contractions. The reinnervated vas deferens produced good contractile responses to transmural stimulation, and these were substantially reduced by a cholinergic muscarinic blocking agent, hyoscine, as compared to only a small reduction in the control vas deferens. Neostigmine potentiated the contraction of the transplanted vas deferens to a greater extent than that of the control. This indicated that a substantial component of the contractile response was produced by cholinergic fibres. Consistent with this was the finding that, while guanethidine blocked a greater proportion of the contraction in the control vas deferens, the contraction of the reinnervated transplant was less affected. Acetylcholine elicited a strong contraction in control vas deferens, but only a small response was obtained in the reinnervated transplant. However, the response to noradrenaline was greater in the transplant than in the control vas deferens. These results indicate that cholinergic nerves normally supplying skeletal muscle can reinnervate smooth muscle and that the alien somatomotor innervation altered the responsiveness of the smooth muscle of the vas deferens. Morphological studies confirm the shift from adrenergic to cholinergic fibres in the reinnervated vas deferens.

Introduction

The question as to whether peripheral target tissues can become reinnervated only by their own nerves, or whether they accept alien nerves, has been extensively studied using striated muscle. Langley and Anderson (1904), over a century ago, found that autonomic cholinergic nerves will establish functional connections with previously denervated skeletal muscle fibres. Subsequently, Flumerfelt et al. (1986) showed that the preganglionic parasympathetic neurons of the dorsal vagal nucleus were able to innervate motor end plates in the striated skeletal muscle of the tongue. Further studies have provided detailed information on the characteristics of nerves and the pattern of innervation of skeletal muscles reinnervated by alien nerves. It appears that the original physiological properties of the autonomic nerve endings are maintained in the new target (Landmesser, 1971; Bennett et al., 1973; Grinnell and Rheuben, 1979).

Other tissues, such as the nictitating membrane of the cat (Ceccarelli et al., 1972) or the sympathetic ganglion of the cat and rat, have also accepted innervation by alien nerves (Langley, 1898; De Castro, 1937; McLachlan, 1974; Östberg et al., 1976). As in skeletal muscle, in the sympathetic ganglion, the presynaptic terminals resembled those of the original nerve (Östberg et al., 1976). In the nictitating membrane however, Vera et al. (1957) reported that hypoglossal nerves reinnervating this preparation more closely resembled autonomic nerves, with extensively branching terminal varicose axons rather than those found at localized synaptic endplates on striated muscle fibres.

Regarding changes of the postsynaptic target tissue, Vera et al. (1957) found that contractions of the nictitating membrane elicited by stimulation of the hypoglossal nerve were unaffected by curare and blocked by atropine, indicating that the nature of the receptor molecule of the target tissue was not affected by the alien innervation. This held true for the frog sartorius muscle, where the alien innervation did not induce a change of the nicotinic acetylcholine (ACh) receptor molecule on the muscle fibre (Landmesser, 1971).

A series of recent experiments challenge the view that the nicotinic cholinergic nature of the receptors of the postsynaptic membrane of skeletal muscle is unaffected by the alien innervation. Brunelli and his colleagues connected glutamatergic nerves from the cortex to skeletal muscles and claimed that these nerves induced a glutamatergic neuromuscular junction where the postsynaptic receptors changed

Abbreviations: Acetylcholine, ACh; 6-hydroxydopamine, 6-OHDA.

* Corresponding author. Fax: +44 20 7830 2949.
E-mail address: g.burnstock@ucl.ac.uk (G. Burnstock).

0014-4886/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2008.10.011

from those responding to ACh to receptors responding to glutamate (Pizzi et al., 2006; Brunelli, 2007). However, these experiments are surprising and need to be further explored.

In other studies, alien innervation of various tissues was achieved not only by transposing nerves and leaving the target *in situ*, but also by moving the target to a new site. Grinnell and Rheuben (1979) transplanted the frog sartorius muscle and studied whether the trophic influence of the alien nerves on the maintenance of muscle mass was similar to that of the muscle's own nerve. In these experiments the alien nerves were shown to restrict ACh sensitivity from extrajunctional areas of the muscle fibres to the neuromuscular junction and reduce muscle atrophy.

In another series of experiments, the anterior eye chamber was used to study the potential of alien nerves to functionally reinnervate various target tissues, including heart muscle (Olson and Malmfors, 1970; Taylor et al., 1978; Unsicker et al., 1978). These experiments show that contacts between alien nerves and new target tissues are quite readily established and maintained, but did not analyse the question whether a chronic exposure of a target muscle to a new nerve input modified its physiological or pharmacological properties.

The present experiments aim to establish whether the smooth muscle of the vas deferens, which normally receives predominantly adrenergic innervation together with some cholinergic nerves, can become reinnervated by cholinergic somatomotor nerves when transplanted into a new site. In addition, the possibility that this innervation may change the nature of the ACh muscarinic receptors expressed by this tissue was also examined. The vas deferens of rat was removed and placed into the leg either to be left denervated or to be innervated by a somatomotor nerve. The changes in the pattern of innervation and sensitivity of the transplants to ACh and noradrenaline (NA) were then established.

Materials and methods

Surgery

All surgical experiments were carried out following Institutional guidelines for the performance of surgical procedures and following Home Office (UK) regulations covering the use of animals in Scientific Procedures. Adequate measures were taken to minimise pain or discomfort to the animals.

Forty-one male albino rats (Sprague–Dawley), weighing from 150 to 300 g were used. Under ether anaesthesia the right vas deferens was excised, and stripped of its connective tissue sheath. It was then transplanted to the lower leg of the rat in one of two ways. In twenty-seven rats, the soleus muscle was removed and the vas deferens firmly tied to the tendons which were left in place. The soleus nerve was dissected out, severed, and the proximal stump secured onto the surface of the transplanted vas deferens ('reinnervated transplants'). In the other series of 14 animals, the vas deferens was tied in parallel with the soleus muscle which was left undisturbed ('denervated transplants'). Four of the reinnervated transplants were injected with 6-hydroxydopamine (6-OHDA; 250 mg/kg, i.v.) 3–4 days prior to sacrifice to produce sympathectomy ('6-OHDA denervated').

The final experiments were carried out 3–4, 8, and 12 weeks following transplantation. The rats were killed by an overdose of ether and the control left vas deferens removed and placed in Krebs buffer. The transplanted vas deferens together with the soleus nerve was located within the leg, carefully dissected under a dissecting microscope, removed and placed into Krebs buffer.

In some cases, small amounts of tissue were taken from the end of both control and transplanted vas deferens and fixed for electron microscopic studies.

Experiments of the same type were grouped together regardless of the duration of recovery, since no significant changes occurred after the end of the third week.

Isolated organ studies

Both control (from 12 un-operated rats) and transplanted vas deferens were slit longitudinally. Those with the nerve attached to the vas deferens were opened along the side away from the nerve attachment. Both ends were tied ready for positioning in an organ bath. The control vas deferens was cut to approximately the same length as the transplants.

Both control and transplanted vas deferens were suspended in a 50 ml organ bath containing oxygenated (95% O_2/5% CO_2) Krebs buffer of the following composition (mM): NaCl, 133; KCl, 4.7; $NaHCO_3$, 16.4; $MgSO_4$, 0.6; NaH_2PO_4, 1.4; glucose, 7.7 and $CaCl_2$, 2.5; pH 7.3, maintained at 36°C. Tension was recorded on a Grass polygraph by means of Grass force-displacement transducers using rack and pinion mounting to allow time for adjustment of tension. Contractions were elicited by transmural stimulation of the intramural nerves of each vas deferens with two platinum ring electrodes placed around the tissue and connected to a Grass S8 stimulator. In some reinnervated transplants, the sutured nerve trunk, if present, was also stimulated through platinum ring electrodes independently of the intramural nerves. Denervated vas deferens were stimulated directly with 2 platinum ring electrodes. All muscles were stimulated at 10 min intervals. Stimuli of 0.2–2 ms duration and intensity to produce maximal contractions were used. Drugs were added to the bath in volumes of no more than 0.5 ml. All drug concentrations are expressed as the final dilution in the organ bath. Non-cumulative concentration–response curves were determined by successive injection in the bathing fluid of increasing concentrations of drugs. Responses were calculated as percentages of the maximal response. Arithmetic means of the individual EC_{50} values were used to determine shifts in the concentration–response curves. The Student's *t*-test was used for statistical analysis of the results. The effects of different drugs on the responses to transmural and sutured nerve trunk stimulation were observed by adding the drugs to the bath for 20 min.

On completion of the studies in the organ bath, the tissue was dried, weighed and divided into segments for fluorescence histochemistry and electron microscopy.

Materials

All drugs used in the experiments, ACh chloride, guanethidine sulphate, hyoscine hydrobromide, neostigmine methysulphate and NA bitartrate, were obtained from Sigma Chemical Co. (Poole, UK).

Fluorescence histochemistry

Sections of control and transplanted vas deferens were prepared for fluorescence histochemical demonstration of catecholamines with the Falck–Hillarp technique (see Falck and Owman, 1965), as modified by Evans et al. (1972). Briefly, contents of the vas deferens midway along its length were spread out on a slide in freshly oxygenated Krebs solution, and examined under a microscope. Histologic samples of these tissues were fixed in 10% formalin in normal saline, blocked in paraffin wax, and 5–10 μm sections were stained with haematoxylin and eosin, or iron haematoxylin and Masson trichrome stain for routine histologic observation.

Electron microscopy

Tissue from reinnervated transplants was taken for electron microscopic examination in 13 rats, in some cases before and in other cases after organ bath experiments. Fixation and preparative procedures were according to the technique used by Heath et al. (1972). Briefly, following an initial 10 min fixation in buffered 1% OsO_4, the tissue was diced and placed in buffered 1% OsO_4 for 1 h. It was then

washed for 10 min in buffer and post-fixed in buffered 5% glutaraldehyde. Following another 10 min buffer wash, it was again placed in buffered 1% OsO_4 for 30 min. Fixatives were buffered at pH 7.4 with 0.2 M phosphate buffer. After a brief wash in distilled water, the tissue was block stained in a 2% aqueous solution of uranyl acetate for 30 min. It was then rapidly dehydrated in a graded series of alcohols, cleared in propylene oxide, infiltrated in a 50/50 solution of propylene oxide and Araldite and finally embedded in Araldite. Thin sections were cut on an ultramicrotome, stained with a saturated aqueous solution of uranyl acetate, followed by lead citrate, and subsequently examined with an electron microscope. Semi-thin sections (approximately 1 μm) were cut at the same time and stained with toluidine blue for light microscopy.

Results

Appearance of the transplant

The transplanted vas deferens was always distinguishable from the surrounding tissue by its lumen. Often it adhered to the adjacent muscle fascia and was surrounded by connective tissue. Numerous blood vessels were seen branching over the surface of the vas deferens. Compared to the control, the diameter of the transplanted vas deferens was reduced. When placed in the organ bath, it lengthened more than the control vas deferens when the same tension was applied to it. In 21 of the 27 reinnervated transplants, the soleus nerve was firmly attached to the vas deferens and in most cases a piece of nerve up to 10 mm long could be dissected for stimulation. In 6 experiments the nerve could not be found, and the vas deferens alone was dissected. The appearance of the vas deferens in the 4 animals that had been treated with the 6-OHDA was similar to that of the untreated ones.

Histological examination of the vas deferens showed that the reinnervated transplant retained the basic anatomical characteristics of the normal organ. Blood vessels penetrated into the transplanted vas deferens and supplied the outer regions of the transplant; however only a few blood vessels reached the muscles of the inner regions. Thus, while the outer longitudinal muscle was well maintained, the inner muscle region showed some necrosis. This necrosis was particularly marked in denervated transplants.

Table 1
A summary of the mean contractile response to transmural stimulation at 5 pulses/s at 10 V, 0.2 ms for 3 s (±S.E.M.) and the effect of various drugs on this response

Type of tissue	Tension developed (g)	Hyoscine (5×10^{-7} Mol) tension % of initial	Neostigmine (5×10^{-7} Mol) tension % of initial	Guanethidine (1×10^{-5} Mol) tension % of initial
Control Un-operated vas deferens	1.95±0.15 (12)	91.7±3.9 (10)	145±3.1[b] (2)	16.2±3.3[c]
Reinnervated transplants	0.09±0.02[a]	44.6±9.6 (9)	170±8.7[b] (2)	23.9±10.6[c] (9)

The effect of drugs is calculated as a percentage of the initial tension.
[a] Significant decrease compared to control vas $P<0.01$ (Student's t-test).
[b] Significant increase compared to control responses $P<0.01$ (Student's t-test).
[c] Significant decrease compared to control responses $P<0.01$ (Student's t-test).

Electrically elicited contractile responses and their sensitivity to cholinergic or adrenergic blocking agents

Examples of records of contractions of the normal vas deferens elicited by transmural stimulation (Fig. 1a) of the reinnervated transplant (Fig. 1b) and denervated transplant (Fig. 1c) are shown. Records of Fig. 1b also show responses of the reinnervated transplants to stimulation of the soleus nerve. In contrast to the strong contractile responses of the innervated vas deferens, the denervated transplanted vas deferens did not respond to electrical stimulation (see Fig. 1c). The means±S.E.M. values of the force developed by the vas deferens were calculated for each group separately and summarized in Table 1.

Of the 14 denervated transplant preparations, most did not respond to transmural electrical stimulation, but occasionally extremely weak contractions could be elicited (see Fig. 1c). In contrast, in 21 of the 27 reinnervated transplants that were seen to be connected to

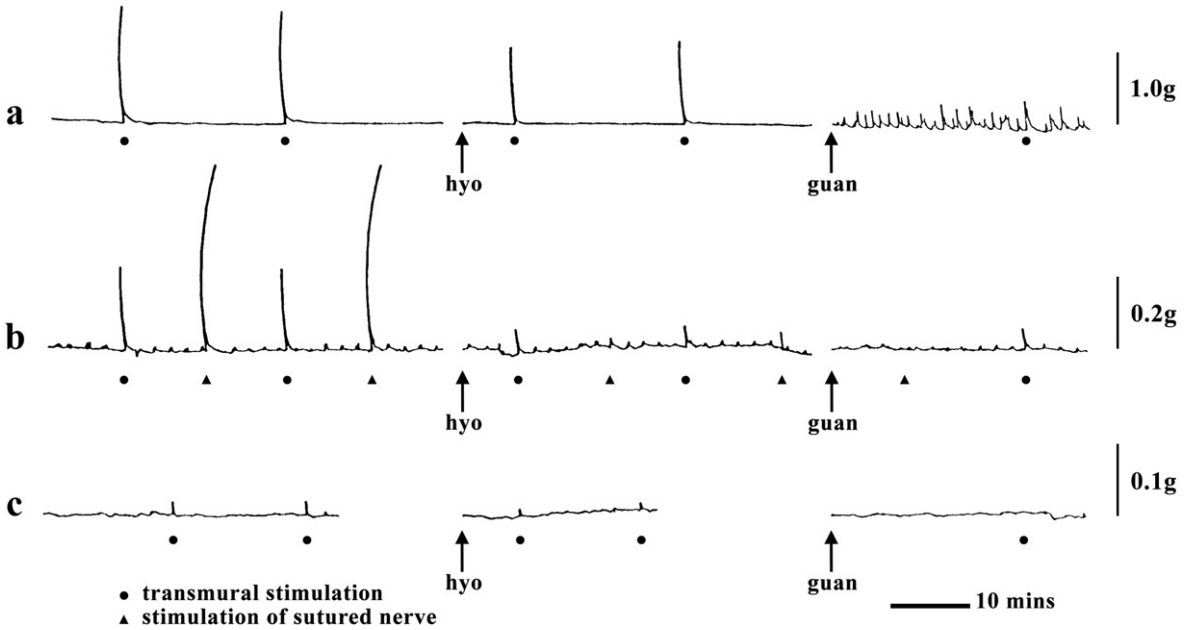

Fig. 1. Responses of isolated vas deferens to transmural electrical stimulation (●; 5 pulses/s, 0.2 ms pulse duration; 40 V for 3 s approximately every 10 min) and stimulation of sutured nerve on transplants (▲; 40 pulses/s, 0.4 ms pulse duration; 40 V for 3 s every 10 min). Hyoscine (hyo; 5×10^{-7} g/ml) and guanethidine (guano; 1×10^{-5} g/ml) were injected into the bath at arrows. (a) Control vas deferens. (b) Transplanted vas deferens ('reinnervated transplants')-sutured nerve response substantially reduced by hyoscine. (c) Denervated transplanted vas deferens. Note, this is an example where there were particularly dramatic changes.

the soleus nerve, transmural stimulation always elicited a contraction. The contractions produced by the reinnervated transplant were significantly weaker than those of the control vas deferens (see Figs. 1a and b and Table 1). Interestingly, in 2 preparations where contractions of the transplant were elicited by stimulation of the motor nerve, the contractions elicited by nerve stimulation were stronger than those produced by transmural stimulation (see Fig. 1b). The contractions of the transplanted vas deferens fatigued rapidly and it was necessary to have 10 min intervals between successive stimulations. Nevertheless, these results show that the smooth muscle of the vas deferens established functional connections with axons that previously innervated a striated muscle.

Whether the neuromuscular transmission between the nerves that reinnervated the transplanted vas deferens was cholinergic via muscarinic receptors was tested next. A standard dose (5×10^{-7} g/ml) of hyoscine (scopolamine), a competitive muscarinic M1 acetylcholine receptor antagonist, was added to the bath and the response to transmural or nerve stimulation was assessed. The records of the middle column of Fig. 1a illustrates that after the addition of hyoscine the strength of the contraction of the normal vas deferens was reduced by less than 10%, whereas the responses of the reinnervated transplants (Fig. 1b) were reduced by a much greater extent (50–80%). Table 1 summarises the results and shows that the control preparations maintained 91±3.9% of their original tension, while the reinnervated transplants could produce only 44±9.6% of their original force (see Table 1), indicating that the contribution of cholinergic innervation of the transplanted vas deferens was significantly greater than in the normal vas deferens.

Addition of the reversible cholinesterase inhibitor, neostigmine, potentiated the response of the transplant more than that of the control vas deferens (Table 1). This is consistent with the findings that show that the transplant derives a high proportion of its innervation from cholinergic fibres.

The contribution of adrenergic innervation was tested in experiments in which adrenergic transmission was blocked by guanethidine, an inhibitor of the Mg^{2+}ATPase dependent pump, which reduces the release of catecholamines. Fig. 1 shows records of contractions to electrical stimulation of control (a) reinnervated transplant (b) and denervated transplant (c) vas deferens before and after the addition of guanethidine to the bath (right side panels). The records illustrate that guanethidine reduced the contractile responses of the vas deferens. The results are summarised in Table 1, showing that guanethidine reduced the response of the control vas deferens to electrical stimulation to 16%±3.3 (n=9), and that of the reinnervated transplant vas deferens to 24±10.6 (n=9), which was significantly less ($P<0.01$, Student's t-test). Although the relative contribution of the cholinergic innervation was increased, these results taken together indicate that adrenergic nerve fibres still made a considerable contribution to the innervation of transplanted vas deferens.

The possibility that the response of the transplanted vas deferens altered its sensitivity to exogenously applied transmitters was tested next.

Responses of the transplanted vas deferens to NA and ACh

Three different preparations were compared: denervated transplants, reinnervated transplants and control vas deferens. The maximal responses to exogenously applied ACh and NA of the three groups of vas deferens are shown in Table 2. The response to ACh of the reinnervated transplant was much smaller then that of either the control vas deferens or the denervated transplanted vas deferens. This indicated that the distribution of ACh receptors, or the number of receptors, was altered in the reinnervated transplant. An alteration in acetylcholinesterase activity might also account for this.

Interestingly, the response to NA was similar in the control vas deferens and the reinnervated transplant, but the denervated transplant was significantly more sensitive to NA. This, together with the finding that guanethidine reduced the electrically elicited response of the reinnervated transplanted vas deferens, indicates that the reinnervated transplant, in addition to the cholinergic innervation, may have acquired some adrenergic innervation. Whether this was indeed the case was explored by visualising adrenergic innervation by fluorescent histochemistry.

Table 2
Maximum responses of control and transplanted vas deferens to exogenous noradrenaline or acetylcholine

Treatment	Control	Reinnervated transplant	Denervated transplant
Noradrenaline	66.6±9.2 (27)	88.0±4.9 (13)	122.3±16.2[a] (14)
Acetylcholine	31.7±5.7 (23)	1.8±0.4[b] (9)	30.5±5.6 (14)

The results are expressed as mean±S.E.M. maximum tension developed (g)/weight of muscle tissue.
Number of experiments is shown in parenthesis.
[a] Significant increase compared to control vas $P<0.01$ (Student's t-test).
[b] Significant decrease compared to control vas $P<0.01$ (Student's t-test).

Fluorescent histochemistry

Fluorescence histochemistry confirmed the well-documented presence of a dense network of fluorescent adrenergic fibres in the control vas deferens (Fig. 2a). Examination of the transplanted vas deferens on the third post-operative day showed that adrenergic denervation resulting from stripping the vas deferens prior to transplantation was complete; only a few fluorescent fibres were present in the transplant (Fig. 2e). In the transplanted and reinnervated vas deferens some fluorescent fibres were visible in the periphery 7 days after transplantation, and after 3 to 6 weeks there was a small increase in the density of fluorescent fibres (Fig. 2b). Thus these transplants became reinnervated by adrenergic as well as cholinergic fibres. This was not the case for the denervated vas deferens, where innervation remained sparse (Fig. 2d). The innervation of the transplanted vas deferens appeared to be patchy, both when viewed in cross and longitudinal sections. It seemed that a single fibre or group of fibres entered the smooth muscle of the transplant, branched repeatedly and formed a localized plexus. In several cases, innervation was confined to regions of the transplant close to its adhesion to the surrounding tissue while the opposite, unattached surface of the transplant was completely free of fluorescent fibres. The 'patches' showed many varicosities, suggesting junctional relationships with the reinnervated smooth muscle, but the density of any one plexus patch never reached more than approximately 20% of normal vas deferens innervation and in many preparations there were very few patches of reinnervation, the majority of the transplant being completely devoid of adrenergic fibres. Treatment with 6-OHDA effectively depleted the ground plexus and perivascular adrenergic fibres of control and transplanted vas deferens (results not shown).

The adrenergic innervation of the reinnervated transplant may have reached the vas deferens via the motor nerve. Sections of control motor nerves and motor nerves that innervated the transplant revealed some adrenergic fibres running within the nerve trunk (Fig. 2c) (see also Dahlström, 1965; Dahlström and Häggendal, 1966). There was some enhancement of fluorescence in the adrenergic fibres in motor nerves of 6-OHDA-treated rats indicating preterminal accumulation of catecholamines.

The central necrotic region of the transplanted vas deferens surrounding the lumen rarely contained any fluorescent fibres, but in most preparations a halo of small, dull, golden autofluorescent particles indicated the presence of lysosomes, a characteristic sign of necrotic tissue (Fig. 2b). Fluorescent mast cells were seen occasionally in control tissue, generally at the periphery, while in the vas deferens many were observed throughout the entire section with a greater

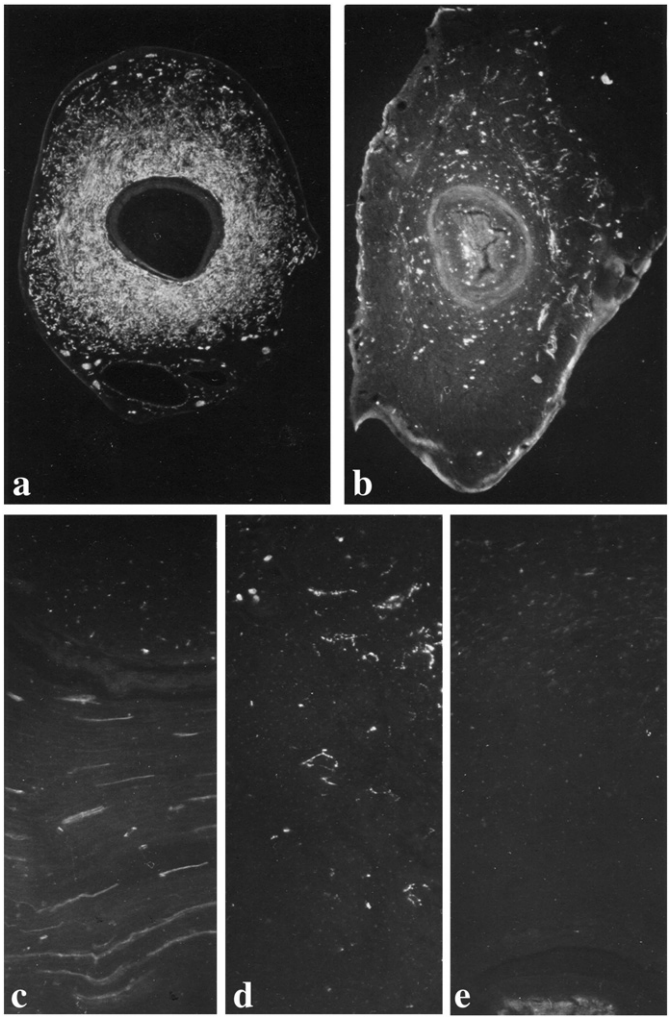

Fig. 2. Fluorescence histochemistry. (a) Control vas deferens showing a dense network of fluorescent adrenergic nerve fibres. (b) Vas deferens removed 6 weeks after 'soleus removed, nerve sutured' transplantation. The fluorescent adrenergic fibres are localized in the upper right portion of the vas deferens. A large number of autoflourescent particles and mast cells are visible in the inner 'necrotic' regions of the muscle coat. Note that the transplant still retains its basic anatomical structure. (c) Section taken through the sciatic nerve trunk illustrating the presence of a small proportion of fluorescent adrenergic nerve fibres. (d) Vas deferens removed 12 weeks after control transplantation. There are very few fluorescent fibres visible in the muscle indicating a poor reinnervation by adrenergic nerves. (e) Vas deferens removed 3 days after 'soleus removed, nerve-sutured' transplantation. Note the absence of fluorescent nerve fibres, indicating a complete adrenergic denervation. Magnification for a and b is ×100; c – e is ×400.

concentration within regions of necrosis. The distribution of mast cells was variable.

Electron microscopy

Although tissue taken in the region of attachment of the sutured nerve from preparations used in organ bath studies (nerve-sutured transplants) was badly preserved due to delayed fixation, it nevertheless provided useful information about reinnervation. No nerve fibres were seen in the inner necrotic areas of transplant, but in the outer areas, the innervation appeared to be distributed in a similar arrangement as that in the normal vas deferens sympathetic ground plexus. Single and small bundles of nerve fibres were seen close to muscle cells, although close contacts between terminal varicose fibres and muscles were seen less frequently. These contacts were associated with collagenous connective tissue to a larger extent than in normal vas deferens (Fig. 3a).

External to the muscle layer of the reinnervated transplanted vas deferens, large nerve bundles were seen surrounded by collagen and perineurium. These bundles often contained myelinated fibres (size 2 μm) (Figs. 3a and b), and often up to six fibres were seen in one bundle. Myelinated axons were seen occasionally in nerve bundles which had penetrated the muscle coat (Fig. 3b), but deep within the muscle no myelinated fibres were seen, even in large bundles. The large bundles were not always accompanied by perineurium, and most of their fibres were filled with neurofilaments and neurotubules. Blood vessels penetrated the muscle and were accompanied by small groups of nerve fibres (Fig. 3b). Nerve fibres sometimes ran parallel to and between muscle cells (Fig. 3) accompanied by Schwann cells. Single axon profiles with an incomplete Schwann cell investment and filled with mainly clear vesicles were occasionally seen close to muscle cells, but rarely as close as 20 nm, which is common in the normal vas deferens. Axons contained mitochondria and occasionally profiles contained many membrane-bound vesicles (Fig. 3b). Most profiles contained predominantly clear vesicles together with some large granular vesicles (Fig. 3a). Small granular vesicles were found in some axons.

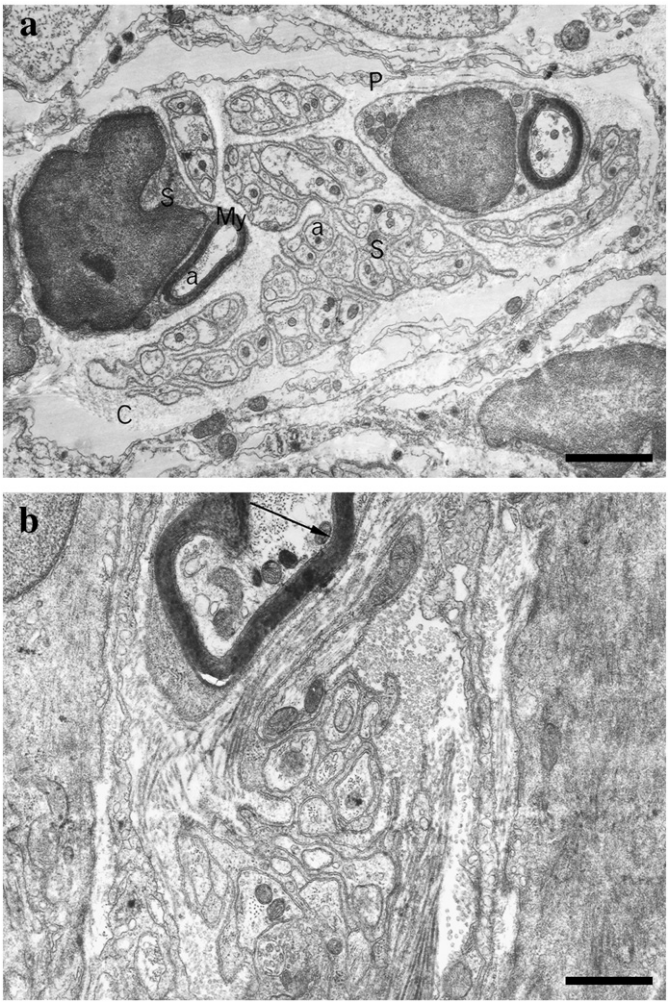

Fig. 3. (a) 'Reinnervated' transplant at 8 weeks. Large nerve bundle at the edge of the vas deferens, surrounded by perineurium (P), supported by collagen (C). Note myelinated axons (My) and non-myelinated axons (a) embedded in Schwann cells (S). Osmium tetroxide fixation. (b) Transplant 3 weeks. Nerve bundle with myelinated and non-myelinated axons, between two muscle cells. The myelinated axon is cut obliquely and is about 2 μm in width at the arrow. Osmium tetroxide fixation. Calibration bar in a = 4 μm and in b = 2 μm.

Discussion

The extent and nature of reinnervation of the transplanted vas deferens was variable. The smooth muscle of the vas deferens is normally innervated mainly by adrenergic fibres (Burnstock and Costa, 1975). However, the present results show that in some of the reinnervated transplants, the contribution of the cholinergic nerves exceeded that of the adrenergic nerves. The nerve fibres from the sutured motor nerve trunk entered the vas deferens, so that electrical stimulation of the nerve trunk resulted in a strong contraction. This contraction was largely abolished by hyoscine, showing that much of the muscle was innervated by cholinergic nerves. The finding that the response of the vas deferens was reduced by a muscarinic blocking agent suggests that the alien innervation did not alter the nature of the receptors in the vas deferens, although some involvement of nicotinic receptors, while unlikely, cannot definitively be ruled out. Only the transplants that were connected to a motor nerve were reinnervated, whereas the denervated transplants had no cholinergic innervation and only a very sparse adrenergic reinnervation. Although we cannot exclude the possibility that the target influenced the nature of the transmitter released, we consider it unlikely since the response of the nerve stimulation was blocked by a substance that blocks cholinergic transmission (hyoscine) and also since changes in the nature of the transmitter released are induced mainly neonatally or during embryonic development.

The peripheral nerve used in the present experiments to reinnervate the vas deferens was a mixed nerve and thus had a sensory, as well as motor, component. However, in view of the finding that the contraction elicited through the nerve was significantly reduced by hyoscine and potentiated by neostigmine, it is unlikely that sensory fibres significantly contributed to the reinnervation of the smooth muscle. It could be argued that the cholinergic vasodilator fibres may have reinnervated the vas deferens, had it not been known that in rats there are few cholinergic vasodilator fibres (Uvnäs, 1966).

The somatomotor nerve fibres reinnervating the smooth muscle of the vas deferens formed plexuses, so that the pattern of innervation resembled that of the original tissue. This is similar to results obtained by Vera et al. (1957) on the nictitating membrane.

Electron microscopy in the present study showed that myelinated motor fibres entered the smooth muscle of the soleus nerve-sutured transplanted vas deferens. The arrangement of the reinnervating nerves, once they had penetrated into the smooth muscle, appeared to be similar to a normal autonomic ground plexus, with varicosities in close apposition to smooth muscle (see Burnstock, 2008) rather than skeletal motor endplate synaptic terminals. The density of innervation, however, was quite low.

An interesting finding of this study is that the motor nerve altered the sensitivity of the smooth muscle to ACh. Whereas the transplant that remained denervated was as sensitive to ACh as the control vas deferens, the transplant reinnervated by a motor nerve became remarkably insensitive to ACh. Luco and Vera (1964) also found a decrease in sensitivity to ACh of the nictitating membrane after reinnervation by a somatomotor nerve. However, the nictitating membrane does not normally receive any cholinergic innervation, although the presence of muscarinic receptors in the nictitating membrane of the cat has been suggested (Mantione et al., 1983). It is interesting that the somatomotor nerve is much more efficient in desensitizing the vas deferens to ACh than its own autonomic cholinergic nerve. This may be due to the larger amounts of ACh released from somatomotor nerves as compared to autonomic cholinergic ones (Grinnell and Rheuben, 1979).

The findings that, upon somatomotor nerve stimulation, strong contractions of the smooth muscle of the vas deferens were elicited, together with the results showing a low sensitivity to diffusely applied ACh, indicate that a redistribution of ACh receptors or a change in receptor numbers may have taken place as a result of the alien innervation. However, since nerve elicited contractions were blocked by the muscarinic ACh receptor (AChR) blocker hyoscine our results show that the nature of the receptor on the smooth muscle membrane did not change. In a series of recent studies Brunelli (2007) claims that the receptors on skeletal muscle fibres can be changed from cholinergic to glutamatergic when a skeletal muscle is reinnervated by a nerve that releases glutamate. This interesting result is at odds with results obtained by Langley and many others (see Grinnell and Rheuben, 1979) who failed to show that a skeletal muscle fibre could change its type of receptor when reinnervated by a nerve other than cholinergic. Our present results that the AChR of the reinnervated transplanted vas deferens remained muscarinic are consistent with the work showing that alien innervation is unable to change the expression of the nature of the receptor on the membrane.

The increased sensitivity to NA was found in all transplants and was greatest in denervated transplants. There appeared to be a good correlation of reinnervation and degree of hypersensitivity, in that those preparations that had only a few fluorescent nerve fibres showed a greater degree of hypersensitivity than those that were demonstrably well reinnervated by adrenergic fibres. Thus the loss of uptake of NA in the absence or decrease in the numbers of adrenergic fibres seems to be responsible for the hypersensitivity to NA of the denervated or partially reinnervated transplants. This is consistent with the proposal that denervation hypersensitivity to NA is due to lack of uptake of the transmitter (Birmingham, 1970), while denervation hypersensitivity to ACh is due to the diffuse distribution of AChRs on muscle fibres. It is therefore not surprising that the responsiveness to NA can change independently to that of ACh.

These results indicate that the motor nerve endings continue to release ACh, which brought about the changes in responsiveness of the smooth muscle of the vas deferens. This appears to be different from results obtained on neonatal sympathetic neurons, where the target had some influence over the transmitter choice (Schotzinger and Landis, 1988; Stevens and Landis, 1990).

References

Bennett, M.R., McLachlan, E.M., Taylor, R.S., 1973. The formation of synapses in mammalian striated muscle innervated with autonomic preganglionic nerves. J. Physiol. (Lond.) 233, 501–517.

Birmingham, A.T., 1970. Sympathetic denervation of the smooth muscle of the vas deferens. J. Physiol. (Lond.) 206, 645–661.

Brunelli, G.A., 2007. Experimental neurotisation of the brachial plexus by means of nerve grafting from the corticospinal tract of the proximal spinal cord at the T3–T4 level. J. Hand Surg. Eur. 32, 620–625.

Burnstock, G., 2008. Non-synaptic transmission at autonomic neuroeffector junctions. Neurochem. Int. 52, 14–25.

Burnstock, G., Costa, M., 1975. Adrenergic Neurones: Their Organisation, Function and Development in the Peripheral Nervous System. Chapman and Hall, London, p. 225.

Ceccarelli, B., Clementi, F., Mantegazza, P., 1972. Adrenergic re-innervation of smooth muscle of nictitating membrane by preganglionic sympathetic fibres. J. Physiol. (Lond.) 220, 211–227.

Dahlström, A., 1965. Observations on the accumulation of noradrenaline in the proximal and distal parts of peripheral adrenergic nerves after compression. J. Anat. 99, 677–689.

Dahlström, A., Häggendal, J., 1966. Studies on the transport and life-span of amine storage granules in a peripheral adrenergic neuron system. Acta Physiol. Scand. 67, 278–288.

De Castro, F., 1937. Sur la régénération fonctionelle dans le sympathique (anastomoses croisées avec des nerfs de type iso –et hétéromorphes). Une référence spéciale sur la constitution des synapses. Trav. Lab. Rech. Biol. Univ. Madrid 31, 271–345.

Evans, B., Gannon, B.J., Heath, J.W., Burnstock, G., 1972. Long-lasting damage to the male genital organs and their adrenergic innervation in rats following chronic treatment with the antihypertensive drug guanethidine. Fertil. Steril. 23, 657–667.

Falck, B., Owman, C., 1965. A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. Acta Univ. Lund. (Sect. 11) 7, 1–23.

Flumerfelt, B.A., Kiernan, J.A., Krcek, J.P., Sholdice, J., 1986. Reinnervation of skeletal muscle in the tongue by preganglionic parasympathetic vagal neurons. J. Anat. 146, 117–130.

Grinnell, A.D., Rheuben, M.B., 1979. The physiology, pharmacology and trophic effectiveness of synapses formed by autonomic preganglionic nerves on frog skeletal muscle. J. Physiol. (Lond.) 259, 219–241.

Heath, J.W., Evans, B.K., Gannon, B.J., Burnstock, G., James, V.B., 1972. Degeneration of adrenergic neurons following guanethidine treatment: an ultrastructural study. Virchows Arch. Abt. B. Zellpath 11, 182–197.

Landmesser, L., 1971. Contractile and electrical responses of vagus-innervated frog sartorius muscles. J. Physiol. (Lond.) 213, 707–725.
Langley, J.N., 1898. On the union of cranial autonomic (visceral) fibres with-the nerve cells of the superior cervical ganglion. J. Physiol. (Lond.) 23, 240–270.
Langley, J.N., Anderson, H.K., 1904. The union of different kinds of nerve fibres. J. Physiol. (Lond.) 31, 365–391.
Luco, J.V., Vera, C., 1964. Sensitivity to acetylcholine of the nictitating membrane reinnervated by cholinergic fibers. Acta Physiol. Lat. Am. 14, 289–294.
Mantione, C.R., de Groat, W.C., Fisher, A., Hanin, I., 1983. Selective inhibition of peripheral cholinergic transmission in the cat produced by AF64A. J. Pharmacol. Exp. Ther. 225, 616–622.
McLachlan, E., 1974. The formation of synapses in mammalian sympathetic ganglia reinnervated with preganglionic or somatic nerves. J. Physiol. (Lond.) 237, 217–242.
Olson, L., Malmfors, T., 1970. Growth characteristics of adrenergic nerves in the adult rat. Fluorescence histochemical and 3H noradrenaline uptake studies using tissues transplanted to the anterior chamber of the eye. Acta. Physiol. Scand. (Suppl.) 348, 1.
Östberg, A.-J.C., Raisman, G., Field, P.M., Iversen, L.L., Zigmond, R.E., 1976. A quantitative comparison of the formation of synapses in the rat superior cervical sympathetic ganglion by its own and by foreign nerve fibers. Brain Res. 107, 445–470.
Pizzi, M., Brunelli, G., Barlati, S., Spano, P., 2006. Glutamatergic innervation of rat skeletal muscle by supraspinal neurons: a new paradigm in spinal cord injury repair. Curr. Opin. Neurobiol. 16, 323–328.
Schotzinger, R.J., Landis, S.C., 1988. Cholinergic phenotype developed by noradrenergic sympathetic neurons after innervation of a novel cholinergic target *in vivo*. Nature 335, 637–639.
Stevens, L.M., Landis, S.C., 1990. Target influence on transmitter choice by sympathetic neurons developing in the anterior chamber of the eye. Dev. Biol. 137, 109–124.
Taylor, D., Seiger, A., Freedman, R., Olson, L., Hoffer, B., 1978. Electrophysiological analysis of reinnervation of transplants in the anterior chamber of the eye by the autonomic ground plexus of the iris. Proc. Natl. Acad. Sci. U. S. A 75, 1009–1012.
Unsicker, K., Tschechne, B., Tschechne, D., 1978. Formation of cholinergic synapses on adrenal chromaffin cells in anterior eye chamber transplants. Brain Res. 152, 334–340.
Uvnäs, B., 1966. Cholinergic vasodilator nerves. Fed. Proc. Fed. Am. Soc. Exp. Biol. 25, 1618–1622.
Vera, C.L., Vial, J.D., Luco, J.V., 1957. Reinnervation of nictitating membrane of cat by cholinergic fibers. J. Neurophysiol. 20, 365–373.