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PHOTOINACTIVATION OF AN IDENTIFIED MOTONEURONE IN THE LOCUST *LOCUSTA MIGRATORIA*

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Summary

1. The common inhibitory motoneurone 1 (CI_1) in the mesothoracic ganglion of the locust was photoinactivated using a helium–cadmium laser or a mercury lamp as light source. Treated animals showed no signs of abnormal locomotory behaviour over periods of up to 40 days.

2. Photoinactivation of part of the neurone in the ganglion, i.e. the soma and the primary neurite, is sufficient to cause irreversible degeneration of all the peripheral extensions of the neurone. Three weeks after photoinactivation, all GABA immunoreactivity had disappeared from the axon branches of the photoinactivated neurone and from their terminals on one of the target muscles investigated, the anterior coxa rotator M92, and inhibitory postsynaptic potentials could no longer be elicited through stimulation. This was taken as proof of functional denervation of the muscle with regard to its inhibitory input. By this time, the axon of CI_1 in nerve N3C1, which supplies M92, had also disappeared.

3. Animals treated during the fourth or fifth instars showed a permanent loss of the photoinactivated mesothoracic CI_1 neurone after moulting into adulthood.

4. Denervation of M92 in the middle legs of instars and adults by axotomy of N3 always led to rapid functional reinnervation of the muscle. The first sign of reinnervation (excitatory neuromuscular activity upon mechanical stimulation of the tarsi) was detected electrophysiologically as early as 8 days after severing the motor nerve.

5. The eliminination of CI_1 by photoinactivation for a period of up to 40 days did not influence parameters of the target muscle, such as size, number of fibres and phenotypes of fibres defined histochemically according to their myofibrillar ATPase isoforms, irrespective of whether the operation was performed in instars or adults. Similarly, the short period of denervation following axotomy before reinnervation took place did not affect the fibre type composition of the muscle.

Key words: insect, nervous system, common inhibitor, degeneration, muscle fibre types, GABA, histochemistry, locust, *Locusta migratoria*.

Introduction

Photoinactivation has become a widely used technique for eliminating individual neurones or portions of neurones in invertebrates without damage to neighbouring cells. After filling a cell with a fluorescent dye, illumination with light of an appropriate wavelength will selectively inactivate only the illuminated portions (Miller and Selverston, 1979; Jacobs and Miller, 1985; Madison *et al.* 1988). When the illumination is confined to a portion of an axon of a dye-filled neurone, the soma induces profuse neurite outgrowth which, after several days, bridges the inactivated portion and forms new functional connections with the distal stump (Cohan *et al.* 1983). To kill a neurone, the dye-filled soma of the cell must be irradiated. This method of ‘fill and kill’ has been used to investigate the role of individual neurones in networks, e.g. the stomatogastric ganglion of crustaceans (see Selverston and Moulins, 1987), and in development (locust, Bentley and Caudy, 1983; Ball *et al.* 1985; leech, Jellies, 1990; Loer and Kristan, 1989).

Where this technique has been used to photoinactivate neuronal somata, the extent and rate of spread of damage to distal, non-illuminated parts of the cell have not been investigated. In the case of a motoneurone, one would like to know the degenerative effects on its primary neurite within the ganglion, on its axon exiting the central nervous system and on its terminals at the neuromuscular junctions.

Selective elimination of motoneurones is one way of studying the influence of neuronal activity on phenotype expression in target muscles. Photoinactivation of individual motoneurones should be a very useful technique for the selective and permanent denervation of muscles. The method employed so far for this purpose in insects has been to sever

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the nerve supplying a particular muscle (Usherwood, 1963; Rees and Usherwood, 1972*a*,*b*; Nüesch, 1985; Washio, 1989). This necessitates cutting all the axons in a given nerve; selective elimination of a single axon is rarely possible using this method. In addition, since the somata of the neurones remain functional, axotomized motoneurones in arthropods usually regenerate their axons rapidly and reinnervate their target muscles (crustaceans, Ely and Velez, 1982; Hunt and Velez, 1989; cockroach, Guthrie, 1967; Jacklet and Cohen, 1967; Pearson and Bradley, 1972; Washio, 1989). For the locust, however, little or no regeneration of motor axons and reinnervation of target muscles after axotomy has been reported (Usherwood, 1963; Edwards, 1969; Rees and Usherwood, 1972*b*; Lakes and Kalmring, 1991).

Skeletal muscles of arthropods are usually composed of several different muscle fibre types which can often be distinguished histochemically according to the activity of their different myofibrillar ATPase (mATPase) isoforms (for literature on insects, see Müller *et al.* 1992). The influence of neuronal activity on the expression and maintenance of different phenotypes of insect muscle fibres is unknown, but in mammalian skeletal muscles, the activity induced by motoneurones plays a decisive role in this context (Pette and Vrbová, 1992).

We have investigated the long-term effects of photoinactivation of the soma of an identified motoneurone in the locust with regard to the spread of degeneration within the neurone from the ganglion to its peripheral target muscle. We have also compared this method of denervation with axotomy. It must be borne in mind that, in contrast to mammalian muscles, most insect muscle fibres are innervated polyneuronally and photoinactivation of a single identified motoneurone leads to only partial denervation of the fibres. We have also investigated the effects of this method of denervation on selected muscle parameters such as muscle size, number of fibres and, in particular, distribution of different histochemically defined fibre types (Müller *et al.* 1992). For this pilot study, we have chosen the common inhibitory neurone 1 (CI_1) in the mesothoracic ganglion of the locust for several reasons. (1) Its soma can be quickly identified by its typical contralateral position in the ganglion (Watson *et al.* 1985) and by its characteristic electrical responses to acoustical stimuli and to stimulation of mechanoreceptors on the leg (Schmidt and Rathmayer, 1993). (2) The peripheral axon branches of CI_1 can be identified in electrophysiological *en passant* recordings from leg nerves, by immunocytochemical staining for γ-aminobutyric acid (GABA) and in ultrastructural sections of the peripheral axon branches. The spread of degeneration into the distal axon portion after photoinactivation of central parts of the neurone can be followed in several nerve branches containing collaterals of CI_1. (3) CI_1 activity affects the mechanical performance of leg muscles by selectively inhibiting the slow-contracting fibres and thus speeding up fast contractions (Pearson and Bergman, 1969; Ballantyne and Rathmayer, 1981; Wolf, 1990). (4) In the muscle studied, the anterior coxal rotator muscle of the middle leg (M92, according to Snodgrass 1929), CI_1 innervates the slow-contracting fibres (type I) and only one population of fast-contracting fibres (type IIa, according to Müller *et al.* 1992). Thus, different populations of muscle fibres are available for examining possible postsynaptic effects due to elimination of one presynaptic input. (5) Permanent removal of the inhibitory input by injection of pronase into the axon results in changes in the transmission properties of the excitatory axons to abdominal extensor muscles in the rock lobster (Parnas *et al.* 1982).

We show that, in the locust, it is possible to eliminate a single motoneurone permanently by photoinactivating its soma as early as larval stage 4. Following the operation, the animals survive through several moults, including that into adulthood (up to 40–60 days). In all cases, photoinactivation of central portions of the neurone led to progressive axon degeneration and, finally, permanent loss of all peripheral axonal branches. However, the absence of synaptic input from the common inhibitor CI_1 did not influence the histochemically defined phenotypes of muscle fibres.

Some of the results have been reported previously in abstract form (Bässler and Rathmayer, 1992; Bässler *et al.* 1993).

Materials and methods

Animals

Male and female *Locusta migratoria* not older than 1 week after the imaginal moult or 1–2 days after the moults to the fourth or fifth instar were taken from a crowded breeding colony at the University of Konstanz (room temperature 24 °C, 100 W light bulb in the cages). No differences were found with respect to gender. Operated animals were maintained in separate colonies.

Photoinactivation

Animals were tethered ventral side up on a Plasticine platform. A small piece of sternal cuticle was excised above the mesothoracic ganglion. The ganglion was exposed by displacing and partly removing overlying tracheal sacs, fatty tissue and salivary glands. The ganglion was stabilised by a small glass hook beneath it. Saline was used as economically as possible, especially in instars to avoid dilution of hormones present. The soma of CI_1 was impaled with glass microelectrodes and identified by the electrical activity, e.g. the shape of the action potentials and the response to mechanical stimulation of the middle leg (Schmidt and Rathmayer, 1993). Conventional glass microelectrodes were used. They contained a 5 % solution of Lucifer Yellow CH in distilled water and had a tip resistance of 80–120 MΩ. The motoneurone was filled with dye by injection of hyperpolarising current (approximately 10 nA for 5–30 min).

To kill the dye-filled cell, the whole ganglion was illuminated with a beam of blue light emitted from a helium–cadmium laser (Omnichrome 456SM, 442 nm, beam diameter 1.2 mm, power density 10 000 mW cm^{-2}) for 5–15 min or from a medium-pressure mercury arc lamp

(Büschges *et al.* 1989: HQI-T150, beam 5 mm×2 mm, power density 330 mW cm^{-2}) for 1–60 min. The accompanying changes in the membrane potential could be recorded with the intracellular electrode in the soma of CI_1.

After photoinactivation, a few crystals of phenylthiourea/antibiotic mixture (phenylthiourea:penicillin:streptomycin 2:1:1) were applied to the wound to prevent infection, agglutination and melanisation of the haemolymph (Schneidermann, 1967). The wound was sealed with Parafilm and insect wax (beeswax/violin resin mixture 1:2).

Survival rate after the operation was high in young adult animals (80 %), but lower in fourth or fifth instars.

Neuromuscular recordings

The general procedures for neuromuscular recordings from M92 were adapted from Müller *et al.* (1992). The neck connectives were severed. The animals were immobilised dorsal side up on a Plasticine platform. To gain access to muscle M92, the wings were removed and the animal was opened by a cut along the dorsal midline. The gut, parts of the meso–metathoracic suture and the posterior rotator of the coxa attached to it were removed, together with the tracheal sacs. For extracellular nerve recordings, a pair of hook electrodes (stainless-steel pins, diameter 50 μm) was placed on nerve N3C1 (the motor nerve supplying M92; for nomenclature, see Snodgrass, 1929) in bipolar configuration and insulated with a drop of Vaseline. For selective stimulation of CI_1, a pair of hook electrodes was attached to nerve N3A3 or N4D4. These nerves supply other muscles and contain an axon branch of CI_1 (Hale and Burrows, 1985). Voltage pulses applied to the bipolar electrode activated CI_1 antidromically. Spontaneous and stimulus-evoked inhibitory and excitatory junction potentials were recorded intracellularly from muscle fibres of M92. The glass microelectrodes for this purpose were filled with 1 mol l^{-1} KCl and had a tip resistance of 40–70 MΩ.

Recordings were digitised (PCM-601ESD, Sony) and stored on video tape (Blaupunkt, RTV-740 HIFI) for later display on a chart recorder (Gould ES 1000) or digital oscilloscope (Gould digital storage type 1425) and *x,y* plotter (Gould colorwriter 6120).

GABA immunocytochemistry

Antisera against conjugates of γ-aminobutyric acid (GABA) coupled covalently to keyhole limpet haemocyanin (KLH, Calbiochem-Behring, La Jolla, CA) raised in rabbits were used to stain axon branches of inhibitory motoneurones in peripheral nerves and in muscles.

Immunocytochemistry was performed on whole mounts or paraffin sections using the indirect peroxidase–antiperoxidase (PAP) technique of Sternberger (1979), modified by Homberg *et al.* (1987).

After dissection, ganglia were fixed overnight (4 °C) in 5 % glutaraldehyde, 1 % acetic acid, 75 % picric acid. Whole mounts were washed six times for 1 h or overnight in 0.1 mol l^{-1} Tris/HCl, 0.3 mol l^{-1} NaCl (pH 7.4) containing 0.1 % Triton X-100. To reduce non-specific staining, ganglia were incubated for 30 min in 1 mol l^{-1} 2-aminoethanol (in phosphate buffer) and subsequently in 0.1 mol l^{-1} Tris/HCl, 0.3 mol l^{-1} NaCl (pH 7.4) containing 0.5 % Triton X-100 and 2 % normal goat serum (NGS, Gibco, Chagrin Falls, Ohio) for 3 h. Antisera in dilutions ranging from 1:1000 to 1:5000 and containing 1 % NGS were applied to the ganglia for 52 h. An overnight incubation in goat anti-rabbit IgG (R5506, Sigma) at a dilution of 1:40 was followed by incubation in rabbit PAP (Dakopatts) diluted 1:300, again overnight. Ganglia were pretreated for 1 h with a solution of 3,3′-diaminobenzidine (DAB) (0.03 %) in 0.1 mol l^{-1} phosphate buffer, pH 7.4. The reaction was triggered by H_2O_2 (0.015 %). Finally, ganglia were dehydrated in alcohols and cleared in methyl salicylate for microscopic inspection.

To apply this technique to paraffin sections, fixed ganglia were dehydrated in ethanol, infiltrated with toluene and embedded in Paraplast Plus (Sherwood Medical Co.). Sagittal sections (14 μm) were cut with a rotary microtome. The sections were dried on microscope slides at 50 °C. Deparaffinized and rehydrated sections were processed by the PAP technique described above for whole mounts, except that antisera were applied to sections overnight, and goat anti-rabbit IgG and rabbit PAP were applied for 1 h. The pretreatment with DAB was omitted.

Electron microscopy

For electron microscopy, specimens were dissected, prefixed overnight in 3 % glutaraldehyde and 3 % formaldehyde in buffer, postfixed for 1 h with 2 % osmium tetroxide, stained overnight (4 °C) in 0.5 % uranyl acetate (in 70 % ethanol), dehydrated in alcohol and propylene oxide, and embedded in Spurr's epoxy resin. Thin cross sections of the nerves were examined in a Zeiss EM 900 electron microscope.

Histochemistry

The coxa rotator muscle was isolated from decapitated animals. For preparation of the muscle, the ventral part of the mesothoracic cuticle and the coxa of the middle leg were removed. After removal of connective tissue and tracheae, the muscle was stretched to resting length.

The preparation was dried with cellulose tissue, embedded in Tissue Tec medium (Miles, Elkhart, USA) and shock-frozen in liquid nitrogen. Preparations were mounted with Tissue Tec on the cold chuck of a cryotome (Frigocut 2000, Reichert and Jung) at −25 °C. Serial cross sections (21 μm) were made and placed on dry, glycerine–albumin-coated slides. They were quickly thawed and air-dried on a hotplate at 40 °C for at least 30 min. After staining (see below), sections were dehydrated, cleared in xylene and mounted in Entellan (Merck). All assays were carried out at room temperature.

Staining for mATPase activity was performed according to Padykula and Hermann (1955), with the modification that pH 8.4 was used for the reaction (Müller *et al.* 1992).

To detect fibre-specific isoforms of mATPase on the basis of their different pH stabilities (Brooke and Kaiser, 1970; Guth and Samaha, 1970), muscle sections were pre-incubated for

10 min at pH 10.2 before transfer to the reaction medium at pH 9.4 (Müller *et al.* 1992).

The fibre composition of the sectioned muscles was determined by counting the total number of fibres and the number of fibres with high mATPase activity at pH 8.4 and at pH 10.2. The number of fibres which stained at both pH values was inferred indirectly by subtracting the total number of fibres from the sum of stained fibres obtained at pH 8.4 and after treatment of the sections at pH 10.2. The total muscle area was also measured (Pamela-system at ATARI-ST). The statistical significance of the data was calculated using the Mann–Whitney test (*U*-test for non-parametric data).

Results

Photoinactivation of CI_1

The common inhibitory neurone 1 (CI_1) in the mesothoracic ganglion of the locust is situated ventrally near the midline (Fig. 1A). After filling CI_1 with Lucifer Yellow through an intracellular electrode, only the soma and primary neurite show fluorescence upon illumination of the whole ganglion (Fig. 1B). Only those portions of the neurone where dye is present and that are reached by the light should be affected by photoinactivation. To determine the penetration depth of the light into the ganglionic tissue *in vivo*, we employed a photoconversion technique (Maranto, 1982) that converts DAB into a brown insoluble deposit. Using blue light emitted from a He–Cd laser, this insoluble deposit was always visible in the soma and the primary neurite of CI_1 and in its dendrites located deeper in the dorsal lateral neuropile (*N*=3, data not shown). This suggests that, during laser illumination, not only the soma and the primary neurite but also the deeper dendritic branches of the neurone in the neuropile are reached by the light and are thus photoinactivated, although no fluorescence can be seen in the dendritic branches in *in vivo* preparations.

In simultaneous intracellular recordings from a dye-filled CI_1 soma and from a target muscle fibre of M92, a 1:1 correlation of action potentials and inhibitory postsynaptic potentials (IPSPs) was seen (Fig. 2A). At the onset of laser illumination, the neurone depolarised from a membrane potential of approximately −50 mV to −30 mV. This depolarisation elicited a discharge of action potentials at increasing frequency (Fig. 1C). After 1 min, action potentials were no longer detectable and the cell remained depolarised. In all preparations examined (*N*=170), the action potential discharge lasted for 0.5–5 min. In some cases, the membrane potential later returned to more negative values. However, this was never accompanied by a reappearance of electrical activity in the soma.

Up to a frequency of about 60 Hz, each action potential of CI_1 elicited a hyperpolarising IPSP in the muscle fibre (Fig. 2C–F). At higher frequencies, individual IPSPs could no longer be distinguished (Fig. 2G–I) because the membrane of the muscle fibre was usually hyperpolarised by the summated IPSPs to their reversal potential. When the discharge of CI_1 stopped, the membrane potential of the muscle fibres returned to resting values (Fig. 2K). Although spontaneous excitatory postsynaptic potentials (EPSPs) were occasionally recorded

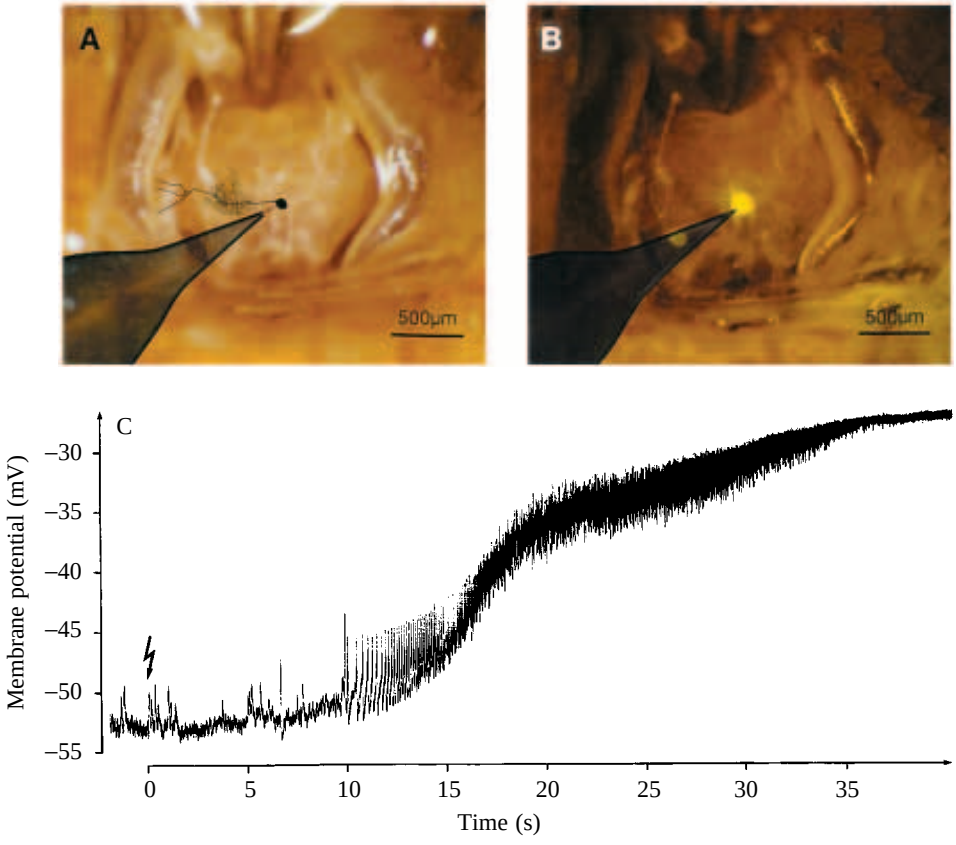

Fig. 1. (A) Ventral view of the dissection which exposes the mesothoracic ganglion of a locust after removing a piece of the ventral cuticle and the tracheal sacs. CI_1 was filled intracellularly with Lucifer Yellow *via* a glass microelectrode. For clarity, a *camera lucida* drawing of CI_1, showing its ventral soma and primary neurite, the dorsal dendritic branches and the three branches leaving the ganglion to the left through nerves N3, N4 and N5, is superimposed on the photograph. (B) Same preparation as in A but during illumination with blue light emitted from a laser beam. In the centre of the ganglion, the soma and the primary neurite of CI_1 show fluorescence (other yellow structures are tracheae). (C) Intracellular recording from the soma of a dye-filled CI_1 during illumination. The arrow indicates the onset of laser illumination.

(Fig. 2G–L), no further IPSPs occurred even if the membrane potential of the neurone had repolarised. Therefore, in experiments in which we studied the long-term effects of elimination of CI_1, laser illumination was turned off after the cessation of the high-frequency burst discharge of the neurone.

The time needed for photoinactivation and cessation of action potential discharge of CI_1 was prolonged (at least 15–60 min) when a medium-pressure mercury arc lamp was used for illumination instead of the He–Cd laser (Büschges *et al.* 1989). The penetration depth of this blue light is obviously restricted. This was confirmed by the photoconversion technique, where only the soma and the primary neurite but not the more dorsal dendritic branches showed brown staining (N=3).

Despite the operation and the long time required for identifying CI_1 through recording, subsequent dye-filling and final illumination, the survival rate of the treated animals was surprisingly high. Of the young adults, 80 % survived for periods of 3–4 weeks. During this time, abnormalities in locomotion were not obvious in animals observed in the colony or in animals walking on a treadmill (H. Wolf, unpublished observation).

Long-term effects of photoinactivation of CI_1 on its peripheral axon branches

CI_1 divides within the mesothoracic ganglion into three major branches, which exit the ganglion through nerves N3, N4 and N5. Within N3, further branching of CI_1 into nerves N3A, N3B and N3C occurs. Details of the branching pattern are described in Watson *et al.* (1985).

Intracellular recordings from muscle fibres of M92 of control animals usually show spontaneous EPSPs (Fig. 2). IPSPs are seen when CI_1 is activated antidromically by stimulating nerve N3A3 (Fig. 3A, arrowheads). Recruiting CI_1 selectively by orthodromic stimulation of nerve N3C1, which innervates M92, is difficult because this nerve also contains the two excitatory axons for this muscle. In animals tested 1 week after photoinactivation of CI_1, IPSPs could never be elicited by this antidromic stimulation (Fig. 3B, arrowheads). However, spontaneous EPSPs were still regularly present in these animals. This indicates that, with regard to the muscle studied, the entire photoinactivation procedure affected only the CI_1 motoneurone, leaving the excitatory motoneurones to the muscle intact.

The spread of degeneration and loss of function of CI_1 from the ganglion into the peripheral branches of the neurone were studied indirectly by immunocytochemical staining with specific antibodies for the CI_1 transmitter, GABA. Each mesothoracic hemiganglion contains the somata of three CI neurones ($CI_{1–3}$). The axons of CI_2 and CI_3 exit the ganglion *via* nerve N5, the axon branches of CI_1 *via* nerves N3, N4 and N5 (Fig. 3C; see also Hale and Burrows, 1985). In immunocytochemical studies with GABA antibodies, the axon branches of CI neurones are easy to detect.

In control animals as well as on the side contralateral to the photoinactivated CI_1, the three axon branches of CI_1 and the axons of CI_2 and CI_3 stained clearly with the antibody (Fig. 3C). On the photoinactivated side, the axons of the CI_2 and CI_3 neurones were present, but the axons of CI_1 were missing (Fig. 3D). The loss of GABA immunoreactivity in different branches of CI_1 appeared at various times after

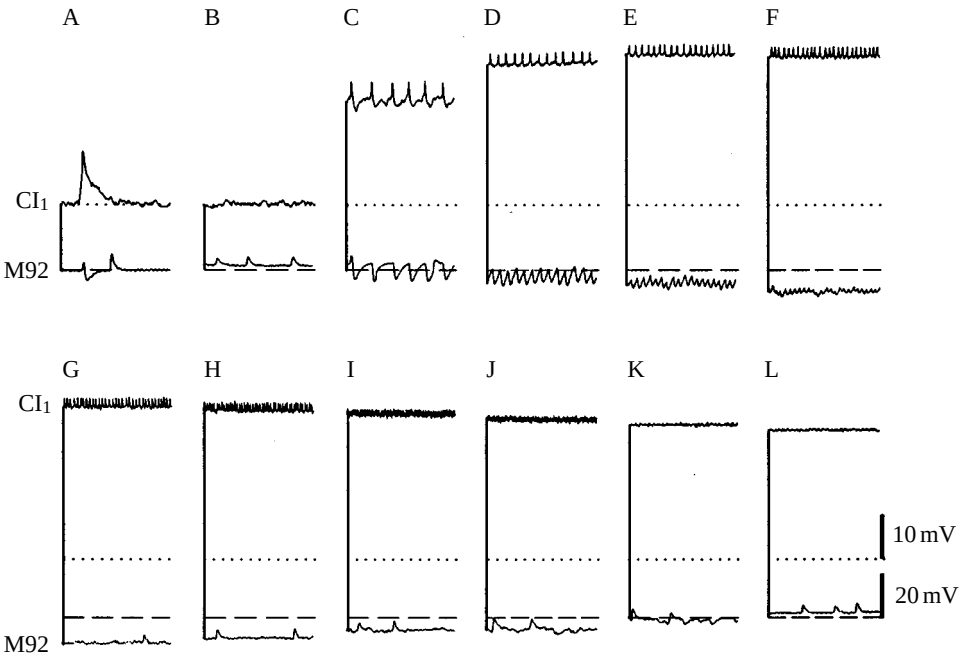

Fig. 2. Simultaneous intracellular recordings from the soma of CI_1 (upper traces) and a double-innervated slow-contracting type I fibre of M92 (lower traces) during laser illumination of the dye-filled CI_1. Each trace shows a 500 ms sequence from a total recording time of 75 s after the onset of illumination. The dotted lines indicate the resting membrane potential of CI_1 before illumination, the dashed lines the resting potential of the muscle fibre. (A) Before illumination. Two spontaneous EPSPs and one IPSP, elicited by a spontaneous action potential in CI_1. (B) 1 s after the onset of illumination. (C) After 13 s. Depolarisation of CI_1 has begun, accompanied by discharge. (D,E,F) 19, 25 and 31 s, respectively, after onset of illumination. High-frequency discharge of CI_1 elicits high-frequency IPSPs. (G–J) After 37, 49, 55 and 61 s, respectively. High-frequency discharge of CI_1 hyperpolarises the muscle fibre to the reversal potential of IPSPs. (K) After 67 s. (L) After 73 s. CI_1 remains depolarised without activity; no IPSPs are seen in the muscle fibre, but spontaneous EPSPs are visible. The membrane potential of the muscle fibre returns to the control value (K) and a slightly depolarised level (L).

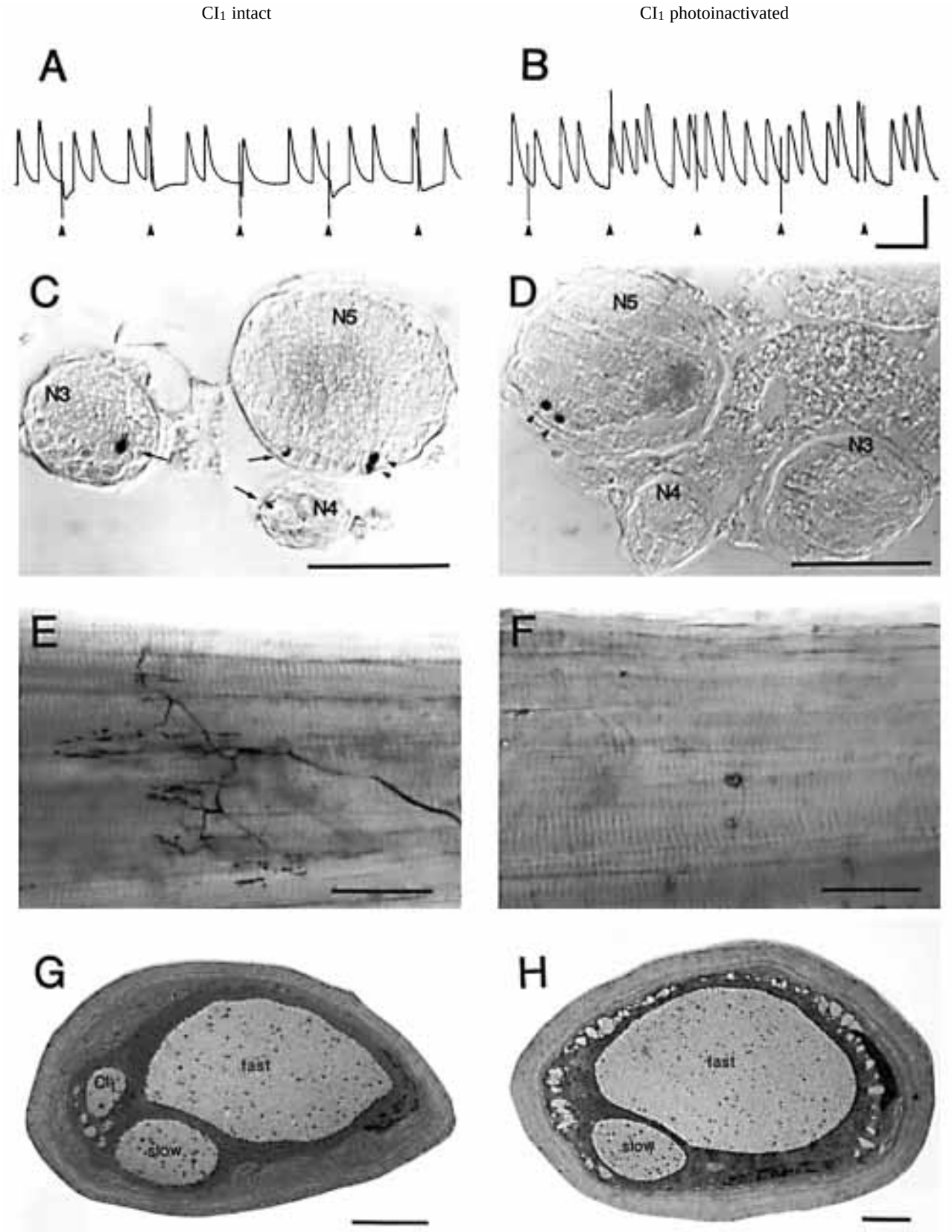
CI1 intact
CI1 photoinactivated
A
B
C
N5
N3
N4
D
N5
N3
N4
E
F
G
fast
CI1
slow
H
fast
slow

Fig. 3

Fig. 3. (A,B) Intracellular recordings from a double-innervated slow-contracting type I fibre of M92. Scale bars, 200 ms, 10 mV. Antidromic electrical stimulation of CI_1 through N3A3 (arrowheads: stimulation artefacts). (A) Control animal. Spontaneous EPSPs and IPSPs evoked by antidromic stimulation. Membrane potential −32 mV. (B) 18 days after photoinactivation of CI_1. Only spontaneous EPSPs are present. Membrane potential −30 mV. (C,D) GABA immunoreactivity in cross sections of mesothoracic nerves N3, N4 and N5 on the operated and the contralateral intact side showing the axon branches of CI_1 (arrows), CI_2 and CI_3 (arrowheads). Scale bars, 100 µm. (C) Control side, CI_1 is present. (D) 21 days after photoinactivation of CI_1. CI_1 is absent on the photoinactivated side. (E,F) GABA immunoreactivity of M92. Scale bars, 50 µm. (E) Control side, terminals of CI_1 are visible. (F) 17 days after photoinactivation of CI_1. The fibres lack immunoreactive terminals. (G,H) Electron micrographs of cross sections through nerve N3C1 innervating M92. Scale bars, 5 µm. (G) Control side, three axon profiles are obvious. (H) 21 days after photoinactivation. The axon profile of CI_1 is absent; fast, axon of the fast excitatory motoneurone; slow, axon of the slow excitatory motoneurone.

photoinactivation (Table 1). There was also variability between preparations. However, by 20 days at the latest, GABA immunoreactivity had disappeared in all peripheral branches of the photoinactivated neurone in all preparations (*N*=21).

Fine peripheral axon branches and synaptic terminals (in the form of varicosities) of CI_1 were also stained by the GABA antibody in whole mounts or in cross sections of muscles (Fig. 3E). After photoinactivation, these structures gradually lost their GABA immunoreactivity. After 18 days, all GABA immunoreactivity on fibres of M92 had disappeared (Fig. 3F; Table 1).

The absence of synaptic potentials and the loss of GABA immunoreactivity provide good indirect evidence for the loss of function of the peripheral axon branches and the neuromuscular junctions of CI_1 after photoinactivation of the soma. To obtain further proof, the motor nerve N3C1, which innervates M92, was sectioned for ultrastructural examination. This nerve contains three large-diameter motor axons as well as 5–6 small unidentified axons. The three motor axons differ in diameter and can be identified from their size as originating from the fast and slow excitatory and the CI_1 motoneurones (Müller *et al.* 1992).

In about 75 % of the preparations, the axon of CI_1 was still present in this nerve 10–11 days after photoinactivation. After 16 days, however, it was no longer detectable (Fig. 3H; Table 1) although, in two of these preparations, some GABA immunoreactivity was still present in more distal parts of N3, indicating that degeneration had not yet spread that far.

Photoinactivation of CI_1 in instars

The previous findings show that it takes about 3 weeks for photoinactivation of the soma to affect all parts of a motoneurone. Since this time constitutes almost the entire life span of an adult locust, we used instars to prolong the time available for studying possible post-operational changes on the target muscle after photoinactivation. We were able to eliminate the mesothoracic CI_1 in fourth and fifth instars (*N*=65). Almost 50 % of the operated instars survived and moulted into adulthood. They did not differ in appearance or behaviour from control animals.

Elimination of CI_1 by photoinactivation in juveniles was tested 3 weeks after the final moult (up to 6 weeks after the operation). Electrical activity in the form of IPSPs in M92 or of spontaneous discharge of CI_1 in extracellular recordings from N3C1 was present on the contralateral side, but never on the side of the photoinactivated CI_1. In addition, GABA immunoreactivity was absent from the axon branches of CI_1 and from the terminals on muscle fibres on the side of inactivation. In electron micrographs of the ipsilateral N3C1, the axon profile of the CI_1 branch was not present. There was no ingrowth of GABA-immunoreactive axons into N3C1, e.g. from CI_2 or CI_3. This proves that CI_1 can be

Table 1. *Summary of all experiments employing photoinactivation of CI_1*

Time after inactivation (days)	GABA immunoreactivity							Electron microscopy		
	N3				M92			N3C1		
	N	A	B	C	*N*	Positive	Negative	*N*	CI_1 present	CI_1 absent
10–11	8	7	1		7	4	3	4	3	1
12–13	12	6	3	3	8	4	4	4	2	2
14–15	8	3	4	1	4	2	2	2	1	1
16–17	5	1	1	3	2	1	1	5		5
18–19	6	1	2	3	3		3	2		2
≥20	21			21	3		3	4		4

States of GABA immunoreactivity of axon branches of CI_1 in nerve N3 and in the terminals on M92 are given.

N, number of animals.

A, number of cases in which a CI_1 branch was present; B, number of cases in which CI_1 was no longer stained at the branching point of N3, i.e. changes in immunoreactivity were evident; C, number of cases in which the CI_1 branch was absent.

The electron micrographs were obtained from cross sections of nerve N3C1 innervating muscle M92.

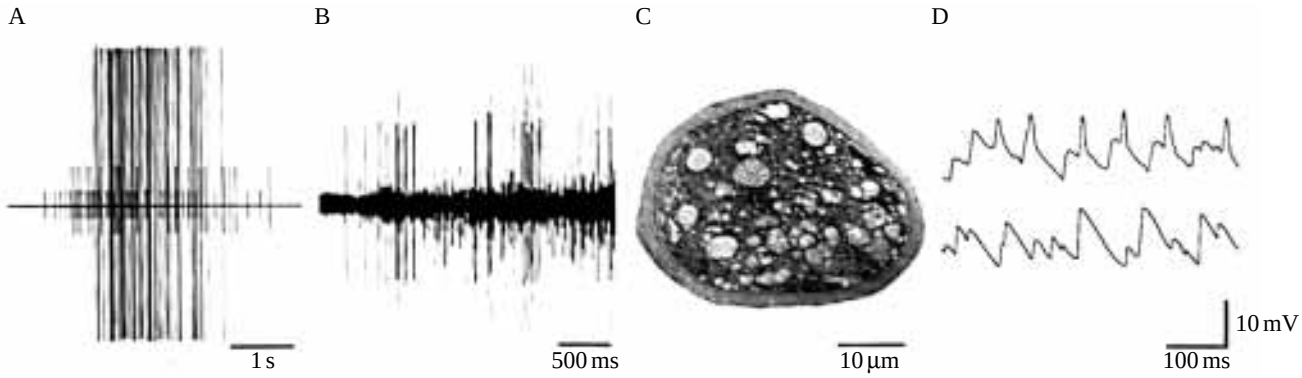

Fig. 4. (A) Extracellular recording from N3C1 (amplification 100×) of a control animal after mechanical stimulation of the tarsi. Large-amplitude signals are from the fast motor axon, medium-amplitude signals are from the slow motor axon and small-amplitude signals are from CI_1. (B) Extracellular recording from a regrown nerve in an adult after axotomy of N3 in a fourth instar upon mechanical stimulation of tarsi (amplification 1000×). (C) Electron micrograph of a cross section of a regrown nerve N3C1. Compare with the control in Fig. 3G. (D) Intracellular recordings from two fibres of M92 in an adult after axotomy of N3 in the fifth instar. Upper trace: EPSPs from a dorsal muscle fibre, innervated in controls only by the fast motoneurone. Lower trace: EPSPs from a muscle fibre of a deeper layer, in control muscles with double or triple innervation. The membrane potential of both fibres was −24 mV.

permanently eliminated by photoinactivation even in instars, thus selectively depriving the muscle of its inhibitory input.

Denervation of M92 by axotomy

In a comparative analysis, we also denervated M92 of the middle leg by cutting its supplying nerve N3C1 or nerve N3 at its point of exit from the ganglion. The length of the remaining distal stump was about 2 mm. Again, animals at different developmental stages (fourth and fifth instars or adults) were used. In control animals, the activity of the three axons innervating M92 can be recorded extracellularly from N3C1. The neurones can be identified from the amplitude of their efferent signals. Fig. 4A shows a typical recording from N3C1 of an unoperated control animal upon mechanical stimulation of the tarsi.

In all denervation experiments (N=28), gradual reinnervation of M92 was observed starting several days after cutting the motor nerve. In adult animals (N=18), the nerve stump distal to the lesion was still present 6 days after severing the nerve. However, it became apparent from electron micrographs that the stump consisted of the nerve sheath devoid of distinct axon profiles and filled with compact material similar to structures described for other degenerating insect axons (Rees and Usherwood, 1972*b*). Spontaneous and evoked synaptic activity in the form of EPSPs and IPSPs, normally present in intracellular recordings from muscle fibres upon stimulation of mechanoreceptors on the animal's tarsi, was also absent. However, activity reappeared in some fibres as early as 8 days after severing the nerve. This was taken as the first sign of functional reinnervation. Within 20 days after axotomy, the number of muscle fibres exhibiting evoked synaptic responses had increased, i.e. reinnervation had apparently progressed. However, no distinct nerve supplying M92 could be detected morphologically in these animals. The outgrowing motor axons to M92 had apparently taken different routes (e.g. along tracheae) and were not detectable in the preparations.

Denervation experiments were also performed on 10 juvenile animals (fourth instar N=6; fifth instar N=4). The operated animals were tested only as adults, at which time all fibres of M92 examined were reinnervated (Fig. 4D). In most preparations, no distinct regrown nerve could be detected. However, in three of the six animals denervated in their fourth instar, a distinct nerve supplying M92 was present. In cross sections, this regrown nerve contained a large number of small axon profiles (Fig. 4C). Accordingly, extracellular recordings from the regenerated nerve showed activity of small amplitude from a large number of units (Fig. 4B). Larger signals, probably produced by a fast excitatory motorneurone, were often present and can be seen in the recording shown.

Effects of denervation by axotomy or photoinactivation of CI_1 on fibre composition of M92

Muscle M92 is composed of about 80–120 fibres belonging to three different types (I, IIa and IIb) according to Müller *et al.* (1992). The different types are distributed in a consistent and distinctive pattern within the muscle (see controls in Figs 5, 6) which is most evident in cross sections. The slow-contracting fibres form a core surrounded by layers of fast-contracting fibres. The shortening velocity of the fibres is positively correlated with their mATPase activity. Since the mATPase activity of different isoforms can be distinguished histochemically, the intensity of the staining at two pH values (at pH 8.4 and after preincubation of the sections at pH 10.2) serves as a convenient indirect means of distinguishing slow- and fast-contracting fibres (for muscle M92, see Müller *et al.* 1992). The slow type I fibres stain light at pH 8.4 and dark after treatment at pH 10.2. The reverse pattern is obtained for the fast type IIb fibres. The type IIa fibres stain medium to dark at both pH values and they cannot always be histochemically

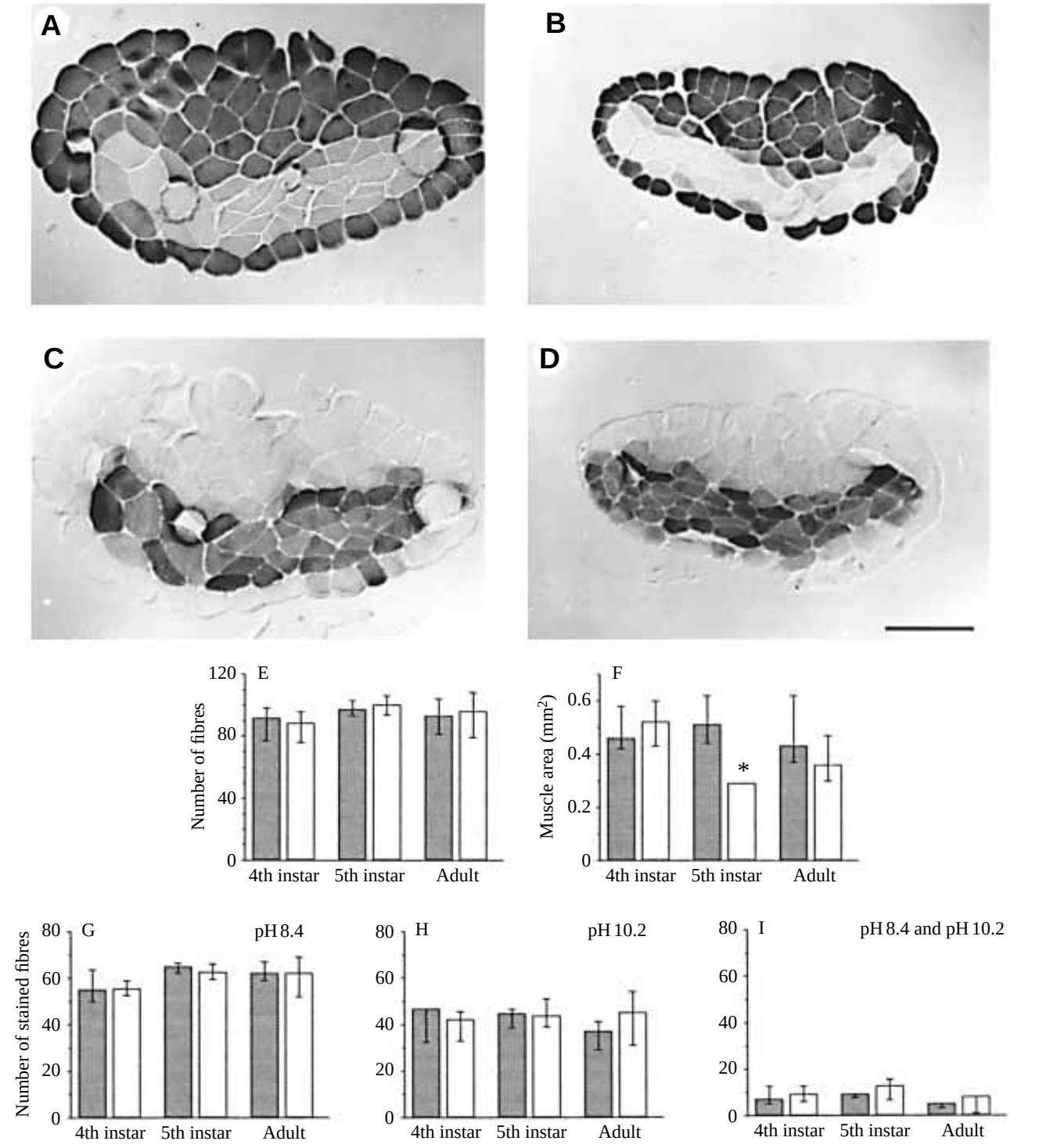

Fig. 5. Histochemical staining for mATPase activity in cross sections of M92 after axotomy of N3 in the fifth instar, investigated 3 weeks after the animal had moulted to become an adult. Scale bar, 250 μm. (A,B) mATPase activity at pH 8.4. The fast-contracting fibres stain medium to dark, the slow-contracting fibres stain light. (C,D) mATPase activity after preincubation of the sections at pH 10.2. Most fast-contracting fibres stain light, the slow-contracting fibres stain dark. (A,C) Control muscle with intact innervation. (B,D) Contralateral muscle after denervation through axotomy and subsequent reinnervation. (E,F) Number of fibres and cross-sectional area of M92 in 15 adult animals after axotomy in fourth instars (N=4), in fifth instars (N=4) and in adults (N=7). (G) Number of fibres with medium to high mATPase activity at pH 8.4 (fast-contracting fibres type IIb and some IIa fibres). (H) Number of fibres with medium to high mATPase activity after preincubation of sections at pH 10.2 (slow-contracting fibres type I and some IIa fibres). (I) Number of fibres with medium to high ATPase activity at pH 8.4 and 10.2 (rest of type IIa fibres). The median (represented by the column) and the 25 %/75 % quartile (lines) are given. Shaded column, control side; open columns, denervated side. The asterisk marks a statistically significant difference ($P \leqslant 0.05$, Mann–Whitney test) from the unoperated control side.

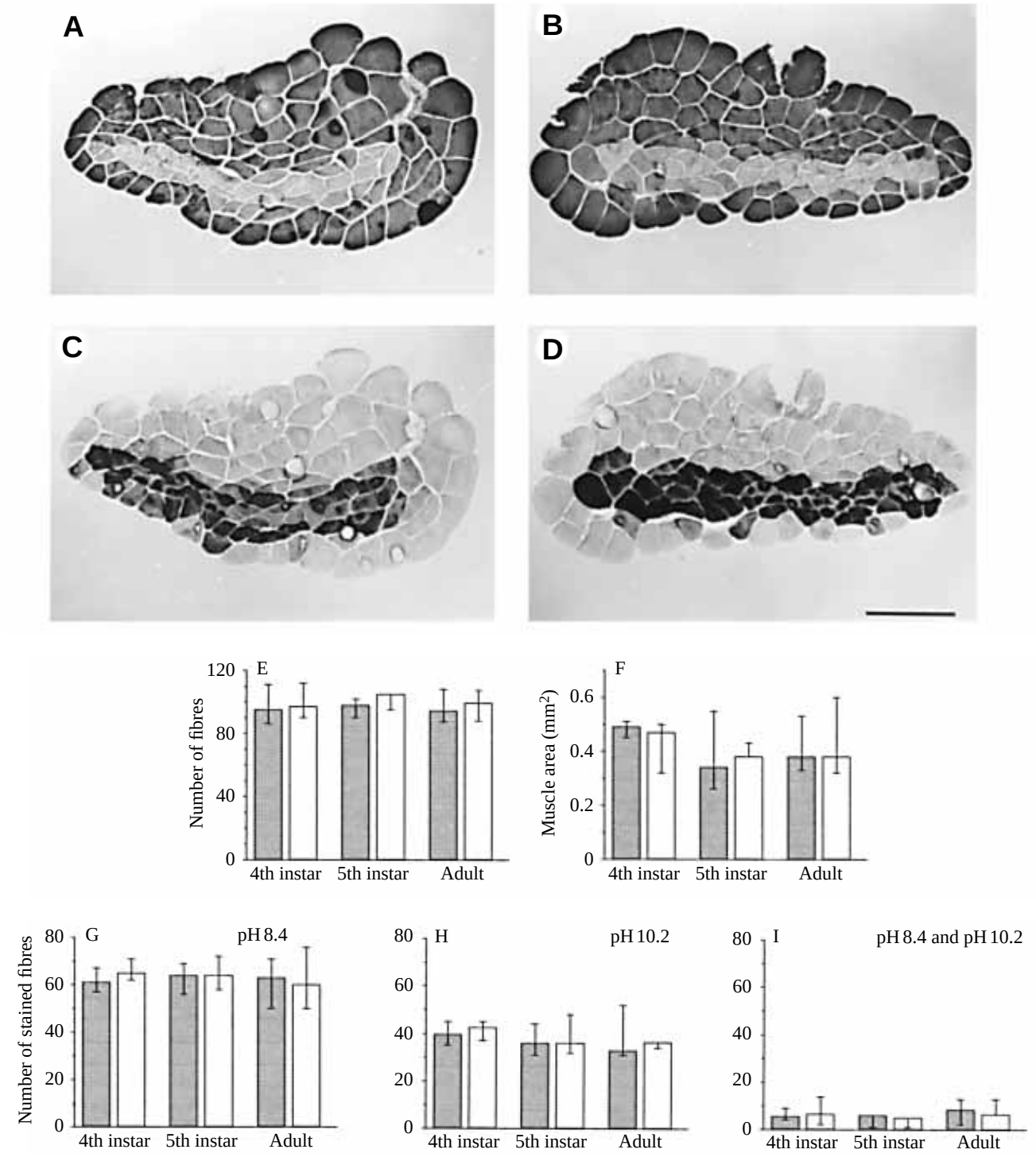

Fig. 6. Histochemical staining of mATPase activity in cross sections of M92 31 days after photoinactivation of CI_1 in an adult locust. Scale bar, 250 µm. (A,B) mATPase activity at pH 8.4. The fast-contracting fibres stain medium to dark, the slow-contracting fibres stain light. (C,D) mATPase activity after preincubation of the sections at pH 10.2. Most fast-contracting fibres stain light, the slow-contracting fibres stain dark. (A,C) Control muscle with intact innervation. (B,D) Contralateral muscle without CI_1 innervation. (E,F) Number of fibres and cross-sectional area of M92 in 18 adult animals after photoinactivation in fourth instars (N=7), fifth instars (N=3) and adults (N=8). (G) Number of fibres with medium to high mATPase activity at pH 8.4 (fast-contracting fibres type IIb and some IIa fibres). (H) Number of fibres with medium to high mATPase activity after preincubation of the sections at pH 10.2 (slow-contracting fibres type I and some IIa fibres). (I) Number of fibres with medium to high ATPase activity at pH 8.4 and 10.2 (rest of type IIa fibres). The median (represented by the column) and the 25 %/75 % quartile (lines) are given. Shaded columns, control side; open columns, photoinactivated side.

identified as a specific type. However, all three fibre types can be clearly distinguished by their innervation patterns (Müller *et al.* 1992): CI_1 innervates the type I and type IIa fibres together with the slow excitatory neurone. The fast motoneurone innervates type IIa (the only fibre type with triple innervation) and IIb fibres.

In a statistical analysis (Mann–Whitney test, $P \leqslant 0.05$), we compared the number and distribution of the fibres (based on their mATPase activity at pH 8.4 and after treatment at pH 10.2) after denervation of muscle M92 by axotomy and after photoinactivation of CI_1. For the statistical analysis, we distinguished between stained and unstained fibres at each pH value. Therefore, the slow-contracting type I fibres and fast-contracting fibres were identified, but it was not possible to discriminate between the fast-contracting IIa and IIb fibres. In all experiments (N=33), the muscle was examined 3–4 weeks after the final moult of the animals into adulthood.

The denervation experiments employing axotomy did not affect the total number of fibres of M92 (Fig. 5E). Also, the number of fibres stained in the different histochemical regimes did not differ significantly in operated and control animals (Fig. 5G–I). The pattern of distribution of different fibre types and the composition of the muscle (Fig. 5B,D) were similar to those of controls (Fig. 5A,C), regardless of whether axotomy was performed in instars or adults. In most operated fourth instars and adults, the total cross-sectional area of M92 on the operated side was comparable to that of the contralateral muscle on the intact side (Fig. 5F). However, in some fourth instars and most fifth instars, the total cross-sectional area of the muscle on the operated side was smaller. This could be due to some atrophy or to the fact that muscle fibre growth had been delayed until reinnervation began.

When denervation of M92 was achieved through photoinactivation of CI_1, the number of muscle fibres and also the cross-sectional area of the muscle remained unchanged (statistically significant according to Mann–Whitney test) 3–4 weeks after the operation (Fig. 6E,F). The pattern of distribution of the different fibre types was very similar in the muscles lacking CI_1 (Fig. 6B,D) and in the contralateral control muscle of the non-operated side (Fig. 6A,C). In addition, the numbers of slow- and fast-contracting fibres exhibiting opposite staining properties at pH 8.4 and 10.2, respectively (Fig. 6G,H), as well as the number of fibres which showed medium to dark staining at both pH values (Fig. 6I) were very similar in operated and control muscles. This was also true when photoinactivation of CI_1 was performed as early as in the fourth or fifth instar (N=10) and the animals were allowed to grow to the adult stage (Fig. 6G–I). These data show that within the approximately 40–60 days of postoperative life available for observation, elimination of the inhibitory synaptic input and the resulting change in mechanical performance of the muscle had no influence on the muscle fibre properties investigated in this study.

Discussion

We have shown that, in the locust, photoinactivation is a suitable technique for permanently depriving a muscle of the efferent synaptic input from a single, identified motoneurone. To achieve this, it is sufficient to irradiate the dye-filled central portions of the neurone, i.e. the soma and the primary neurite within the ganglion. In the case of the common inhibitory neurone CI_1, the resulting degeneration spread into the peripheral axon and, within 20 days, had reached the neuromuscular junctions of a coxal target muscle. This is documented by the loss of GABA immunoreactivity first in the axon and later at the neuromuscular terminals. After 16 days, the profiles of CI_1 had disappeared from cross sections of most peripheral motor nerves that normally contain collaterals of this neurone. The time required for complete degeneration varied slightly, probably reflecting different degrees of damage to the dendritic branches during illumination. The ability of CI_1 to elicit IPSPs at M92 upon antidromic electrical stimulation of its collaterals was lost about 7 days after photoinactivation of CI_1, well before the morphological changes became apparent.

In contrast to other studies involving axotomy of motor axons in locusts (Usherwood, 1963; Rees and Usherwood, 1972*a*; Lakes and Kalmring, 1991), we always found rapid reinnervation of muscle fibres of M92 after cutting its motor nerve. In adults, EPSPs elicited upon stimulation of tarsal mechanoreceptors reappeared in some fibres of M92 as early as 8 days after the operation. In a study by Lakes and Kalmring (1991) on a tergite nerve, sprouting of the axotomized axons occurred proximal to the lesion. Reinnervation of muscles was not observed but the examination was based on morphological criteria alone. We also found there was no morphological evidence for reinnervation in adults (in contrast to some instar preparations), although synaptic activity could be evoked in the muscle fibres. This suggests that reinnervation begins with only a few outgrowing axons, which may follow routes different from those of the original axons (e.g. along tracheae). Usherwood (1963) has studied the extensor tibiae muscle in the hindleg of locusts. In this specialized leg, the motor nerve must traverse a constriction adapted for autotomy of the leg. This constriction might have posed a particular obstacle for the outgrowth of regenerating nerve fibres. In the case of the retractor unguis muscle, no apparent reinnervation of the muscle occurred, even 68 days after the motor nerves had been severed (Rees and Usherwood, 1972*b*). The rapid functional reinnervation we observed could simply be due to the short distance (approximately 2 mm) between the lesion and the target muscle. However, the rapid reinnervation of a leg muscle in the locust after axotomy is in agreement with similar findings in cockroaches and in crickets (Jacklet and Cohen, 1967; Denburg, 1988; Washio, 1989; Chiba and Murphy, 1990).

The fate of the distal portions of cut axons varies greatly in arthropods. In the cockroach, cricket and locust, the distal stumps of axotomized leg motoneurones lose their capability

for evoked and, later, spontaneous transmitter release. They degenerate progressively for up to 3 weeks and eventually become phagocytozed (Usherwood, 1963; Usherwood *et al.* 1968; Jacklet and Cohen, 1967; Rees and Usherwood, 1972*b*; Clark, 1976*a*,*b*; Clark *et al.* 1979). However, very slow degeneration of the distal portions of cut motor axons has also been reported in the locust: 45 days after sectioning a tergite nerve, the distal stumps of the cut motor axons still persisted, although functionality was not determined (Lakes and Kalmring, 1991). In contrast, in the present study, rapid degeneration of the distal stump of nerve N3C1 within 6–8 days was observed. This short time could be correlated with the rapid onset of reinnervation of the target muscle. The fate of distal stumps of cut motor axons is strikingly different in crustaceans: the axons survive functionally, retaining the ability to release transmitter upon stimulation for 200 days and occasionally even longer (Hoy *et al.* 1967; Bittner, 1988; Atwood *et al.* 1989; Parnas *et al.* 1991).

The fibres of muscle M92 can be grouped into three different types on the basis of the combination of several parameters, i.e. innervation pattern, activity and pH-stability of their myofibrillar ATPase isoforms (Müller *et al.* 1992). In the present study, we have evaluated only the histochemical parameter, i.e. the activity of the mATPase at pH 8.4 and after preincubation at pH 10.2. We counted the number of stained fibres in cross sections of the muscle. In controls, the different staining patterns of the mATPase isoforms serve as a marker for the slow-contracting fibres (which are lightly stained at pH 8.4 and darkly stained at pH 10.2) and for the fast-contracting fibres (which are darkly stained at pH 8.4 and lightly stained at pH 10.2). A small number of fibres stain medium to dark at both pH values. They represent a population of type IIa fibres. Considering only the histochemical staining results, we cannot type a small group of IIa fibres which histochemically resemble IIb fibres (dark at pH 8.4, light after treatment at pH 10.2). Since they are fast-contracting fibres, we have refrained from a further separation within this group into type IIa and IIb fibres.

Photoinactivation of CI_1 and also denervation of the muscle through axotomy changed neither the number of lightly and darkly stained fibres at both pH values nor their pattern of distribution within the muscle. Therefore, we are confident that there was little or no effect on the phenotype expression of the fibres after partial denervation. When denervation through axotomy was performed in instars, the growth of the muscle was sometimes reduced, but the distribution of the different fibre types was not affected. The possibility remains, however, that a transformation from slow- to fast-contracting fibres could have been counterbalanced by a transformation from fast- to slow-contracting fibres. Since the pattern of differently stained fibres remained very similar within the muscle in operated and control animals, this possibility is rather unlikely.

The finding that the muscle parameters investigated in this study were not influenced by elimination of the inhibitory synaptic input to the muscle for up to a month is perhaps not surprising because the operation caused only partial denervation of the type I and type IIa fibres. The excitatory motoneurones (the slow in the type I fibres, and the slow and the fast in type IIa fibres) remained intact. In contrast, CI activity is essential for the mechanical performance of the whole muscle in promoting rapid contractions during locomotion (Ballantyne and Rathmayer, 1981; Wolf, 1990). CI prevents the slow-contracting fibres from participating in rapid contraction and relaxation cycles of the muscle. In locusts walking freely on a treadwheel, Wolf (1990) has shown that reducing the discharge of CI_1 by hyperpolarizing the neurone through current injection results in a slowing of the swing phase of the leg. The lack of inhibitory input to M92 due to photoinactivation of CI_1 should therefore influence the mechanical performance of this muscle in a similar way by making it slower. The slow-contracting fibres can now participate fully in every contraction cycle and the fast-contracting fibres will be slowed by the parallel load imposed by the co-activated slow fibres. This anticipated change in the mechanical performance of the muscle, however, had no effect on the fibre properties studied in this investigation.

In contrast to most insect muscle fibres, which are innervated polyneuronally, the fibres of mammalian skeletal muscles receive input from a single motoneurone. A large body of evidence indicates that, in mammalian muscle, the type of activity induced by the motoneurones controls the phenotype expression of different muscle fibre types (for a review, see Pette and Vrbová, 1992). Stimulation of the motoneurones supplying a fast-twitch muscle in the rat (e.g. the extensor digitorum longus) with tonic impulse patterns characteristic for the activation of slow muscles (e.g. the soleus) results in a change of the contractile properties of the fibres innervated by these motoneurones, making the twitch contractions slower. Concomitantly, a sequential transformation of the myosin heavy chain (MHC) isoforms from MHC IIb (characteristic of fast-contracting fibres) into MHC IId, MHC IIa and ultimately into MHC I (characteristic of the slowest fibres) was observed (Termin and Pette, 1992). In the rabbit, this transition begins within a few days and progresses steadily. After 60 days, two-thirds of the MHCs are of the slow MHC I isoform (Leeuw and Pette, 1993).

The fact that the slowing of fast contractions in a locust muscle by eliminating the inhibition of the slow-contracting fibres had no influence on the parameters investigated in this study could mean that these changes in mechanical performance are not essential for the maintenance of the histochemically determined phenotype properties of the fibres. It could also mean that the time required for transformation of muscle fibre properties exceeds the life span of the operated animals. Since the first stage accessible for the operation was the fourth instar, observation was limited to a period of only 40–60 days. Within this period, however, biosynthesis of myofibrillar proteins is still going on as the muscle grows from larval to adult.

A further possibility is that, in the locust, a hemimetabolous insect whose larval stages show locomotory behaviour very

similar to that of the adults, the different phenotypes of muscle fibres are permanently determined at a very early stage. An analysis of the effects of photoinactivation of identified excitatory motoneurones would provide valuable new information. Such a study is not feasible with M92, but could be carried out on other muscles in the locust.

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References

ATWOOD, H. L., DUDEL, J., FEINSTEIN, N. AND PARNAS, I. (1989). Long-term survival of decentralized axons and incorporation of satellite cells in motor neurons of rock lobsters. *Neurosci. Lett.* **101**, 121–126.

BALL, E. E., HO, R. K. AND GOODMAN, C. S. (1985). Development of neuromuscular specificity in the grasshopper embryo: Guidance of motoneuron growth cones by muscle pioneers. *J. Neurosci.* **5**, 1808–1819.

BALLANTYNE, D. AND RATHMAYER, W. (1981). On the function of the common inhibitory neuron in the walking legs of the crab, *Eriphia spinifrons*. *J. comp. Physiol.* A **143**, 111–122.

BÄSSLER, D., KREISSL, S. AND RATHMAYER, W. (1993). Changes in fibre composition of a locust leg muscle during development and after denervation. In *Gene – Brain – Behaviour, Proceedings of the 21st Göttingen Neurobiology Conference* (ed. N. Elsner and M. Heisenberg), p. 187. Stuttgart, New York: Thieme Verlag.

BÄSSLER, D. AND RATHMAYER, W. (1992). Time course of degeneration of an identified locust motoneurone after photoinactivation of the soma. In *Rhythmogenesis in Neurons and Networks, Proceedings of the 20th Göttingen Neurobiology Conference* (ed. N. Elsner and D. W. Richter), p. 481. Stuttgart, New York: Thieme Verlag.

BENTLEY, D. AND CAUDY, M. (1983). Pioneer axons lose directed growth after selective killing of guidepost cell. *Nature* **304**, 62–65.

BITTNER, G. D. (1988). Long-term survival of severed distal axonal stumps in vertebrates and invertebrates. *Am. Zool.* **28**, 1165–1179.

BROOKE, M. H. AND KAISER, K. K. (1970). Three 'myosin adenosine triphosphatase' systems: The nature of their pH lability and sulfhydryl dependence. *J. Histochem. Cytochem.* **18**, 670–672.

BÜSCHGES, A., KOCH, U. T. AND BREUTEL, G. (1989). A simple and low-cost illumination system for *in vivo* fluorescent visualization of neurones. *J. exp. Biol.* **141**, 461–464.

CHIBA, A. AND MURPHY, R. K. (1990). Connectivity of identified central synapses in the cricket is normal following regeneration and blockade of presynaptic activity. *J. Neurobiol.* **22**, 130–142.

CLARK, R. D. (1976*a*). Structural and functional changes in an identified cricket neurone after separation from the soma. I. Structural changes. *J. comp. Neurol.* **170**, 253–266.

CLARK, R. D. (1976*b*). Structural and functional changes in an identified cricket neurone after separation from the soma. II. Functional changes. *J. comp. Neurol.* **170**, 267–278.

CLARK, R. D., GRATION, K. A. F. AND USHERWOOD, P. N. R. (1979). Relative 'trophic' influences of excitatory and inhibitory innervation of locust skeletal muscle fibres. *Nature* **280**, 679–682.

COHAN, C. S., HADLEY, R. D. AND KATER, S. B. (1983). 'Zap axotomy': Localized fluorescent excitation of single dye-filled neurones induces growth by selective axotomy. *Brain Res.* **270**, 93–101.

DENBURG, J. L. (1988). Cell–cell recognition in the regenerating neuromuscular system of the cockroach. *Am. Zool.* **28**, 1135–1144.

EDWARDS, J. S. (1969). Postembryonic development and regeneration in the insect nervous system. *Adv. Insect Physiol.* **6**, 97–137.

ELY, P. AND VELEZ, S. J. (1982). Regeneration of specific neuromuscular connections in the crayfish. I. Pattern of connections and synaptic strength. *J. Neurophysiol.* **47**, 656–665.

GUTH, L. AND SAMAHA, F. J. (1970). Procedure for the histochemical demonstration of actomyosin ATPase. *Exp. Neurol.* **28**, 365–367.

GUTHRIE, D. M. (1967). The regeneration of motor axons in an insect. *J. Insect Physiol.* **13**, 1593–1611.

HALE, J. P. AND BURROWS, M. (1985). Innervation patterns of inhibitory motor neurones in the thorax of the locust. *J. exp. Biol.* **117**, 401–413.

HOMBERG, U., KINGAN, T. G. AND HILDEBRAND, J. G. (1987). Immunocytochemistry of GABA in the brain and suboesophageal ganglion of *Manduca sexta*. *Cell Tissue Res.* **248**, 1–24.

HOY, R. R., BITTNER, G. D. AND KENNEDY, D. (1967). Regeneration in crustacean motorneurons: Evidence for axonal fusion. *Science* **156**, 251–252.

HUNT, W. P. AND VELEZ, S. J. (1989). Regeneration of an identifiable motoneuron in the crayfish. I. Patterns of reconnection and synaptic strength established in normal and altered target areas. *J. Neurobiol.* **20**, 703–717.

JACKLET, J. W. AND COHEN, M. J. (1967). Nerve regeneration: Correlation of electrical, histological and behavioral events. *Science* **156**, 1640–1643.

JACOBS, G. A. AND MILLER, J. P. (1985). Functional properties of individual neuronal branches isolated *in situ* by laser photoinactivation. *Science* **228**, 344–346.

JELLIES, J. (1990). Muscle assembly in simple systems. *Trends Neurosci.* **13**, 126–131.

LAKES, R. AND KALMRING, K. (1991). Regeneration of the projection and synaptic connections of tympanic receptor fibers of *Locusta migratoria* (Orthoptera) after axotomy. *J. Neurobiol.* **22**, 169–181.

LEEUW, T. AND PETTE, D. (1993). Coordinate changes in the expression of troponin subunit and myosin heavy chain isoforms during fast-to-slow transition of low-frequency stimulated rabbit muscle. *Eur. J. Biochem.* **213**, 1039–1046.

LOER, C. M. AND KRISTAN, W. B., JR (1989). Peripheral target choice by homologous neurons during embryogenesis of the medicinal leech. I. Segment-specific preferences of Retzius cells. *J. Neurosci.* **9**, 513–527.

MADISON, R. D., MACKLIS, J. D. AND FROSCH, M. P. (1988). Non-invasive laser microsurgery selectively damages populations of labeled mouse neurons: dependence on incident laser dose and absorption. *Brain Res.* **445**, 101–110.

MARANTO, A. R. (1982). Neuronal mapping: A photooxidation reaction makes Lucifer Yellow useful for electron microscopy. *Science* **217**, 953–955.

MILLER, J. P. AND SELVERSTON, A. I. (1979). Rapid killing of single neurons by irradiation of intracellularly injected dye. *Science* **206**, 702–704.

MÜLLER, A. R., WOLF, H., GALLER, S. AND RATHMAYER, W. (1992). Correlation of electrophysiological, histochemical and mechanical

properties in fibres of the coxa rotator muscle of the locust, *Locusta migratoria*. *J. comp. Physiol.* B **162**, 5–15.

NÜESCH, H. (1985). Control of muscle development. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology, Postembryonic Development*, vol. 2 (ed. G. A. Kerkut and L. I. Gilbert), pp. 425–452. Oxford, New York, Toronto, Sydney, Paris, Frankfurt: Pergamon Press.

PADYKULA, H. A. AND HERMAN, E. (1955). Factors affecting the activity of adenosine triphosphatase and other phosphatases as measured by histochemical techniques. *J. Histochem. Cytochem.* **3**, 161–167.

PARNAS, I., DUDEL, J. AND ATWOOD, H. L. (1991). Synaptic transmission in decentralized axons of rock lobster. *J. Neurosci.* **11**, 1309–1315.

PARNAS, I., DUDEL, J. AND GROSSMAN, Y. (1982). Chronic removal of inhibitory axon alters excitatory transmission in a crustacean muscle fiber. *J. Neurophysiol.* **47**, 1–10.

PEARSON, K. G. AND BERGMAN, S. J. (1969). Common inhibitory motoneurones in insects. *J. exp. Biol.* **50**, 141–169.

PEARSON, K. G. AND BRADLEY, A. B. (1972). Specific regeneration of excitatory motoneurons to leg muscles in the cockroach. *Brain Res.* **47**, 492–496.

PETTE, D. AND VRBOVÁ, G. (1992). Adaption of mammalian skeletal muscle fibers to chronic electrical stimulation. *Rev. Physiol. Biochem. Pharmac.* **120**, 115–202.

REES, D. AND USHERWOOD, P. N. R. (1972*a*). Effects of denervation on the ultrastructure of insect muscle. *J. Cell Sci.* **10**, 667–682.

REES, D. AND USHERWOOD, P. N. R. (1972*b*). Fine structure of normal and degenerating motor axons and nerve–muscle synapses in the locust, *Schistocerca gregaria*. *Comp. Biochem. Physiol.* **43**A, 83–101.

SCHMIDT, J. AND RATHMAYER, W. (1993). Central organization of common inhibitory motoneurons in the locust: role of afferent signals from leg mechanoreceptors. *J. comp. Physiol.* A **172**, 447–456.

SCHNEIDERMANN, H. A. (1967). Insect surgery. In *Methods in Developmental Biology* (ed. F. H. Wilt and N. K. Wessels), pp. 753–766. New York: Crowell.

SELVERSTON, A. I. AND MOULINS, M. (1987). *The Crustacean Stomatogastric System*. Berlin, Heidelberg: Springer Verlag.

SNODGRASS, R. E. (1929). The thoracic mechanism of a grasshopper and its antecedents. *Smithson misc. Collns* **82**, 1–111.

STERNBERGER, L. A. (1979). *Immunocytochemistry*. New York: J. Wiley and Sons.

TERMIN, A. AND PETTE, D. (1992). Changes in myosin heavy-chain isoform synthesis of chronically stimulated rat fast-twitch muscle. *Eur. J. Biochem.* **204**, 569–573.

USHERWOOD, P. N. R. (1963). Response of insect muscle to denervation. II. Changes in neuromuscular transmission. *J. Insect Physiol.* **9**, 811–825.

USHERWOOD, P. N. R., COCHRANE, D. G. AND REES, D. (1968). Changes in structural, physiological and pharmacological properties of insect excitatory nerve–muscle synapses after motor nerve section. *Nature* **218**, 589–591.

WASHIO, H. (1989). Time course of failure and resumption of excitatory and inhibitory transmission in the denervated muscle fibres of the cockroach. *J. exp. Biol.* **142**, 257–266.

WATSON, A. H. D., BURROWS, M. AND HALE, J. P. (1985). The morphology and ultrastructure of common inhibitory motor neurones in the thorax of the locust. *J. comp. Neurol.* **239**, 341–359.

WOLF, H. (1990). Activity patterns of inhibitory motoneurones and their impact on leg movement in tethered walking locusts. *J. exp. Biol.* **152**, 281–304.