Elimination of sensory inputs induces growth and synaptic changes in crayfish motor axons

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Damage to motor neurons induces regeneration processes including axonal growth and change of synaptic properties. Sensory axons that run along the motor axons are also damaged, but their possible role in the motor neuron's regeneration is generally ignored. Here, the effect of eliminating some sensory inputs from intact motor axons on the motor axon's properties was studied. Micro-dissecting one of the segmental, bilateral, sensory stretch receptor pairs of the crayfish abdomen induced the deep extensor abdominal motor axons to grow and changed their synaptic properties. The results demonstrate directly, probably for the first time, that change in sensory neuron activity can induce motor axons to grow, form new synapses, and change their synaptic properties.

Keywords: adult; axonal growth; crayfish; neuromuscular junction; stretch receptor; synapse formation

1. INTRODUCTION

The structure and function of the adult nervous system and its ability to rearrange and adapt to new circumstances is important to animal survival. Damage to motor neurons, for instance, leads to a cascade of regeneration events that include axonal growth and change in synaptic properties (Clarke & Richardson 1994). Regeneration of motor axons has been studied extensively, and many of the parameters controlling this process are known (Kuffler 1994; Lundborg *et al.* 1994). Studies of motor axon regeneration are typically conducted after damage or treatment to a peripheral motor nerve. The sensory axons that run along the motor axons in the same nerve usually receive the same treatment. Central projections of sensory neurons were shown to sprout and change their synaptic connections and efficacy after damage (Manabe *et al.* 1990; Woolf *et al.* 1992). However, the possible role of the altered sensory inputs in motor axon regeneration is usually ignored. Several studies conducted in the past decade suggested that central signals are indeed involved in motor axon regeneration (Atwood *et al.* 1988; Rotshenker 1988). Motor axons were shown to sprout, form novel synapses, and enhance transmitter release after damage to contralateral homologue nerves (Herrera & Grinnel 1981; Steinbach 1981; Rotshenker 1988). Moreover, immobilization of the crayfish claw changed the neuromuscular synapses of the fast closer excitor to produce higher initial release of transmitter and more rapid depression, whereas the contralateral axon showed the opposite changes (Pahapill *et al.* 1985). That only the homologue contralateral motor axons responded to the damage in these studies suggests that specific central mechanisms may

contribute to the change, rather than growth factor secretion (Easter *et al.* 1985).

The unique array of the segmental, bilateral, muscle receptor organ (MRO; Alexandrowitz 1951) and the dorsal extensor abdominal (DEA) muscle (Parnas & Atwood 1966) in the crayfish, *Procambarus clarkii*, is a good model system for studying regeneration-related processes (figure 1). In the most dorsal position of each abdominal segment, two bilateral pairs of MROs can be found. Each MRO is comprised of a receptor muscle (RM) and a sensory stretch receptor (SR) neuron. The SR neuron's activity regulates the abdominal muscle's tone and stretch (Alexandrowitz 1951; Kuffler 1958). The cell body of the SR is located in the periphery near the RM and its dendrites are embedded within the muscle. Each SR axon is sent to the central nervous system (CNS) where it has a complex morphology (Alexandrowitz 1951; Bastiani & Mulloney 1988). The same abdominal segment contains bilateral DEA muscles. Each DEA muscle is innervated by six motor neurons (five excitatory and one inhibitory), originating from the corresponding abdominal ganglion. In each lateral hemi-segment, the sensory axons of the two SR neurons and the motor neurons of the DEA run in the same nerve, but carry signals in opposite directions. Several millimetres from the SRs cell bodies (figure 1), the SR axons and the DEA axons bifurcate and completely diverge. The DEA axons run deep to innervate the DEA muscle and the SR axon runs in the dorsal branch with the superficial extensor motor axons toward their cell bodies (Fields 1966). None of the DEA axons runs in the dorsal branch. However, the medial RM (RM2) is found in close proximity to the medial muscle of the DEA (DEAM) and one or two branches of the DEAM motor axon may cross to innervate RM2 as well (Fields & Kennedy 1965). Among many other synaptic connections, the SR axons form excitatory synapses with three of the DEA excitatory motor neurons in their original segmental abdominal ganglion (Kuffler 1958; Wine 1977b).

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Figure 1. Schematic drawing of longitudinal section of one segment of the crayfish abdomen. The thick black lines represent the ventral and the dorsal abdominal skeleton. Each segment of the crayfish abdomen (S2, S3 and S4: segments 2, 3 and 4 accordingly) is supplied by two bilateral pairs of muscle receptor organs, two bilateral DEA muscles and two bilateral superficial muscles between them. Each muscle receptor organ is comprised of a muscle receptor (RM) fibre attached on one side to the dorsal skeleton of its own segment and on the other to the dorsal skeleton of the segment posterior to it. A sensory stretch receptor (SR) neuron supplies each RM. The SR cell body is found near the RM and its dendrites are embedded in it. The axon of each SR is sent through the second root to the segmental ganglion (N2) in which the ipsilateral motor DEA axons are running. The location of the SR cell body allowed us to cut its axon without any damage to the DEA motor neurons (Cut, the location at which the SR axon was cut). Inset: schematic drawing of the DEA muscle and the excitatory motor axons innervating the DEAL1 muscle (L1, DEAL1 muscle; M, medial part of the muscle). Note that the DEAL1 muscle is divided again into anterior (A) and posterior (P) parts that receive different neuronal inputs (Ins, the partition between segments 3 and 4 dividing the segments mechanically and electrically; C. Ex., common excitor, innervating all muscle parts; L1 Ex., L1 excitor innervating the posterior part of its own segment and the anterior part of the succeeding segment).

The lobster's and the crayfish's DEA neuromuscular junction has been studied extensively (Parnas & Atwood 1966; Parnas *et al.* 1984; Dudel & Parnas 1987; Nguyen & Atwood 1992). It was found that upon elimination of one of the DEA motor neurons by pronase injection, the synergistic motor neuron innervating the same territory became stronger and released more transmitter. It is possible that the CNS participated in inducing the remaining neuromuscular synapses to strengthen by changing the pattern of sensory inputs to the DEA motor neurons. To address this possibility, the sensory inputs of one of the bilateral pairs of SRs of abdominal segment 3 in adult crayfish was eliminated by micro-dissecting its axons near its peripheral cell body (2–3 mm from the divergence bifurcation; figure 1), and the ensuing changes which occurred in both the ipsilateral and contralateral lateral 1 DEA muscles

(DEAL1) of this segment were studied. The results demonstrate that although the DEA axons were not treated nor harmed in any way, they showed changes typically described for regeneration processes. The axons grew new branches, the distance between neighbouring synapses decreased, the quantal content of the individual release sites increased, and transmitter was released for longer periods. This is the first direct evidence that sensory inputs can activate regeneration-like processes of axonal growth and formation of new synapses in an intact adult neuromuscular system.

2. MATERIALS AND METHODS

Experiments were conducted using adult crayfish, *Procambarus clarkii*, of both sexes. In total, 60 crayfish were used. Half an hour before treatment, the crayfish were anaesthetized by cooling on crushed ice, and then fastened to a small Plexiglass stretcher by rubber bands, dorsal side up. Using a scalpel blade, a small window (3 mm × 3 mm) was cut in the dorsal skeleton, slightly lateral to the abdominal midline. The SR axons were then identified and cut at the hillock region, and the piece of skeleton was immediately glued back in place with dental glue (Duralon, EPSE). The whole procedure did not last more than 5 min, and survival rate was about 90%. The crayfish were killed 2 ($n=10$ animals), 7 ($n=9$ animals), 14 ($n=10$ animals) and 45 ($n=16$ animals) days post-treatment and the effect of axotomy on the DEAL1 motor axons was studied (for schematic drawing of the DEA muscle and the excitatory DEAL1 innervation pattern, see inset of figure 1). Ten animals were used as untreated controls and five as surgical controls.

At the time of sacrifice, the crayfish's abdominal segments 2–4 were isolated and the DEA muscles exposed leaving a long piece of motor axon attached to it. The preparation was then pinned to the Silgard-coated (Dow Corning) bottom of a small plexiglass chamber (6 cm × 3 cm × 1 cm). During the experiment, the preparation was exposed to a constant flow of modified Ringer solution in which CaCl_2 and MgCl_2 concentration was adjusted to prevent muscle contraction (NaCl , 220 mM; KCl , 5.4 mM; Trizma maleate, 10 mM; CaCl_2 concentration was halved to 6.25 mM and MgCl_2 concentration was tripled to 7.5 mM, $\text{pH} = 7.4$, 10°C).

Individual axons were stimulated by applying weak suction to a glass electrode (40 μm opening) to attach it to the axon surface, and then passing current through it (isolated stimulator 2533, Devices). Extracellular excitatory postsynaptic currents (EPSCs) were recorded from the DEAL1 neuromuscular junctions using the macro patch-clamp technique (Dudel 1981) with electrodes (15 μm in diameter) filled with the bath solution (for example, of raw EPSCs recorded from control and treated DEAL1 muscles; see figure 3). By using a hydraulic manipulator (Narishige, MO 203) the technique allowed us to slide the glass pipette gently above the DEAL1 muscle fibres without damaging them, and locate all available release sites. The distance between neighbouring release sites along a given muscle fibre was measured by visually inspecting the position of the recording pipette with a calibrated eye piece grid. Synaptic currents were recorded in areas not further than 3 mm from both sides of the main nerve trunk. Sometimes, the synapses released more than one transmitter quantum per stimulation pulse, which would summate on the recording trace. Because each quantum is usually released at a different time delay after the stimulation, a distinct difference in the kinetics of the synaptic current rise time can be observed at

the summation point, thus allowing a reliable count of the number of quanta released for the number of stimulation pulses given. Subsequently, the quantal content of this synapse could be calculated directly as the number of quanta released divided by the number of stimulation pulses given. The EPSCs were digitized at 20 μ s, stored online, and later analysed automatically by a computer (OnLine, Hovav). The OnLine software automatically detected changes in the EPSC rise-time kinetics and measured the delay in time from the beginning of stimulation to the beginning of the quantal current. From these measurements, two parameters of release could be calculated: (i) the quantal content (using the method described above); and (ii) the kinetics of transmitter release, after constructing a histogram of all the quantal delays of a specific synapse. Intracellular excitatory post-synaptic potentials (EPSPs) were recorded using a 15 M Ω electrode filled with 3 M KCl solution connected via a silver wire to a Sugimuri bridge amplifier. The morphological observations were conducted following the electrophysiological experiments after the preparation was stained with methylene blue, using a dissecting microscope (Wild M5A) equipped with attached photographic apparatus. The data gathered from the synapses of the ipsilateral treated muscles were averaged and compared with control muscles (using Student's *t*-test), whereas a paired *t*-test was used for comparisons between contralateral sides of the same animal.

3. RESULTS

(a) Morphology

The innervation pattern and the arborization of the crayfish DEA motor neurons are very conservative (figure 2*a*; Parnas & Atwood 1966). Cutting the SR axons induced the ipsilateral DEA motor neurons of the treated segment to grow and form new axonal branches. Forty-five days after the treatment, axonal growth was apparent by microscopic inspection, especially in the primary bifurcation area (figure 2*b*). These branching patterns were observed neither in untreated control animals, nor in surgical control animals in which a carapace window was opened in the same manner as the experimental animals and then glued back, without cutting their SR axons. Stimulation of the new axonal branches produced EPSPs in the normal innervation field of the original axons. These results indicate that the new branches probably stemmed from the original DEA axons and were not new axons that grew to innervate the muscle.

(b) Synaptic properties

In addition to axonal growth, elimination of the sensory inputs from the DEAL1 motor neurons induced many significant changes at the synaptic level (see figure 3 for typical EPSCs recorded from control (*a*) and treated (*b*) animals). The mean distance between neighbouring release sites decreased gradually on both sides of the treated segment (distance between normal release sites = $155.46 \pm 6.73 \mu\text{m}$, $n=238$ release sites). On the treated side, the mean distance between two adjacent release sites was about 80% of controls after one week (figure 4*a*). After two weeks, the distance decreased even further and reached a new steady-state level of about 50% of the control values (2 d, $n=204$; 7 d, $n=198$; 14 d, $n=214$; 45 d, $n=238$). On the contralateral side the distance reduction began a little later, but after 14 days it

Figure 2. The axonal tree of the DEA motor neurons in segment 3 of the crayfish abdomen in control (*a*), and 45 days (*b*) after the SR axons were cut (the preparation was stained with methylene blue). In controls, the axonal tree is very conservative. After entering the muscle, the axons bifurcate into two main branches in a primary bifurcation zone (x). One branch is sent toward the DEAM muscle (m) and the other posteriorly (p). Note the additional branches in the treated animal (marked 'br' in (*b*)). Such additional bifurcations were seen neither in control animals, nor in surgical controls. L1, DEAL1 muscle. Calibration bar, 1 mm.

reached the treated side levels (7 d, $n=166$; 14 d, $n=213$; 45 d, $n=245$; for significance levels, see figure 4*a*).

The mean quantal content (m) of these synapses increased on both sides as well, although on the contralateral side it was augmented to a lesser extent (figure 4*b*; normal $m=0.53 \pm 0.03$, $n=329$ release sites). The increase in quantal content showed a time-course similar to the decrease in distance between the synapses, and reached a new steady-state level after 14 days. On the ipsilateral side, m was augmented after a week by about 25%, and after two weeks by 85% (2 d, $n=264$; 7 d, $n=260$; 14 d, $n=293$; 45 d, $n=319$). On the contralateral side, m was augmented after a week by about 8%, and after two weeks by 30% (7 d, $n=258$; 14 d, $n=277$; 45 d, $n=327$; for significance levels, see figure 4*b*).

Figure 3. Six typical EPSCs recorded from normal DEAL1 muscle (*a*) and 45 days after treatment (*b*). The synapses occasionally released multiple quanta, as indicated by the change in the kinetics of the current rise time (marked by the arrows in the second and fourth traces from the top in (*b*)). In addition, note the differential time delay (marked by D in the first trace of (*b*)) between the start of the stimulation artefact and the beginning of the quantal current.

(c) *Kinetics of transmitter release*

A histogram reflecting the kinetics of transmitter release can be constructed after a large enough number of quanta is recorded from a specific synapse (Katz & Miledi 1965; see figure 3 for an example of different EPSC time delays; see also figure 5*a* for typical control and treated delay histograms). From such histograms, three parameters of the kinetics of transmitter release can be derived: (i) minimal delay after which transmitter release starts (MD); (ii) the time interval from start of release to the peak (D2P); and (iii) the time-interval from peak of release to 90% of the total release (0.9D). Delay histograms were constructed from the synapses collected for the above quantal content analysis (see figure 3*b*). Only the 0.9D of the ipsilateral synapses was changed after the treatment and was prolonged by about 15% (for significance levels, see figure 5*b*). Again the same time-course of changes as for the above synaptic properties was observed, and after 14 days the 0.9D reached a new steady-state level (figure 5*b*).

4. DISCUSSION

Taken together, the results of the present study indicate that elimination of sensory inputs from the motor neurons of the DEAL1 muscle leads to processes previously described for regeneration of damaged motor axons (Dudel & Parnas 1987; Rotshenker 1988; Landmesser 1994; Lundborg *et al.* 1994). Although no direct treatment was applied to the DEAL1 motor axons, the ipsilateral axonal tree grew new branches, more release sites per axon were found, and the neurotransmitter release almost doubled. Smaller changes occurred in the synapses of the segmental contralateral side, even though no apparent new axonal branches were seen on this side. In addition, the ipsilateral kinetics of release were prolonged by 15% compared with normal DEA synapses. On the whole, DEAL1 synapses of both ipsi- and contralateral sides of the treated segment released more transmitter than control synapses. It is interesting to note that cutting the SR axons involves cutting the edges of the two accessory inhibitor axons, which have their cell bodies located in

Figure 4. Time-course of distance reduction between neighbouring release sites (a), and increase in quantal content (b; stimulation = 1 Hz). The treated side is represented by the solid line and open squares (in (a) $\pm$ s.e.m. and in (b) $\pm$ s.e.m.), and the contralateral side by the dashed line and filled squares (in (a) $\pm$ s.e.m. and in (b) $\pm$ s.e.m.). The horizontal lines represent control values of untreated animals ($\pm$ s.e.m.). Both the distance and the quantal content of the synapses reached a new steady-state level 14 days after the treatment. Significance levels: Student's *t*-test, * $p > 0.001$, ▼ $p > 0.01$; paired *t*-test, ▲ $p > 0.001$.

the abdominal ganglion. This might have caused their soma to be hyperexcitable, as was described for other crayfish neurons (Kuwada & Wine 1981). This hyperexcitability most probably was not the origin of the changes reported here, as it lasts only one week and because the innervation fields of the accessory neurons and the DEA motor neurons are completely segregated and do not influence each other directly (Wine 1977a,b).

The neuromuscular synapses of the fast closer axon of the crayfish claw change their properties to produce larger EPSPs that fatigue faster after immobilization of

Figure 5. Typical delay histogram (a) of control and treated synapses, and time-course of change in the kinetics from peak to 90% of release of the ipsilateral synapses (b). In (a) two histograms are superimposed (normalized to 100% of transmitter release; control filled bars and treated empty bars, 0.9D is marked by the arrows). Each synapse was stimulated by 1200 pulses. (b) After the treatment the 0.9D has changed in the treated side only (solid line, open squares, $\pm$ s.e.m.). The 0.9D was prolonged and reached a new steady-state level after 14 days. The horizontal line represents control values of untreated animals ($\pm$ s.e.m.). Significance level: * $p > 0.001$.

the claw. Upon external stimulation of the axon, these changes could not be reversed to produce normal activity patterns. It was postulated that changes in the level of synaptic inputs to central parts of the fast closer excitor might be responsible for the altered physiological properties of the peripheral neuromuscular synapses (Pahapill *et al.* 1985). It is possible that the signal for the changes reported here originated from the CNS as well.

In order for peripheral trophic factors to produce the described specific effects, they would have to diffuse through the relatively large spatial distance separating the treated RMs and both contralateral DEALI muscles without being washed or diluted by the circulating haemolymph.

The SR axon is mainly ipsilateral (Wine 1977*b*) and in each ganglion it forms chemical synapses that become weaker as they travel further from the original ganglion (Kennedy *et al.* 1966; Bastiani & Mulloney 1988). In its ganglion, the axon forms excitatory monosynaptic connections with three of the ipsilateral DEA motor neurons and with some thin dendrites of the contralateral DEA motor neurons (Kuffler 1958; Wine 1977*a*; Bastiani & Mulloney 1988). The synapses with the ipsilateral DEA motor neurons produce EPSPs of about 7 mV in the cell bodies (Wine 1977*b*); eliminating these inputs had probably reduced the electrical activity of the cell bodies of the DEA motor neurons. Neely & Nicholls (1995) proposed that electrical activity of axons modulates their growth by regulating calcium entry into the growth cones through voltage-sensitive calcium channels. Elevated amounts of calcium flowing into the neurites' growth cones cause their cytoskeleton structure to collapse. It is possible that by reducing the electrical activity of the DEA motor neurons in the present study, the calcium levels in the neuromuscular terminals were reduced, 'allowing' the axons to grow.

Increase in quantal content as a result of reduced impulse activity was demonstrated in both central and peripheral synapses of invertebrates and vertebrates. In the lobster, eliminating one of the two synergistic excitatory axons innervating the DEALI muscle culminated in fewer net synaptic inputs to the muscle, but the quantal content in the remaining axon was augmented (Parnas *et al.* 1984). Enhancement of synaptic transmission in spinal neurons was demonstrated in both motor (Gallego *et al.* 1979) and sensory (Manabe *et al.* 1990) synapses after blockage of nerve impulse activity by chronic TTX application. Similar treatment of vertebrate motor nerves leads to enhancement of transmitter release in the corresponding neuromuscular synapse (Snider & Harris 1979; Robbins & Fischbach 1971). Here, transmitter release was augmented due to three factors: more synapses, higher quantal content, and slight prolongation in the kinetic of release. Enucleated DEA motor axons of the lobster show a 10–20-fold prolongation of the 0.9D (Parnas *et al.* 1991). The kinetics of transmitter release are neither calcium-dependent (Hochner *et al.* 1989) nor activity-dependent (Parnas *et al.* 1991). These findings indicate that the prolongation of transmitter release must have resulted from changes in the kinetics of the molecular cascade underlying the process of transmitter release.

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