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Projection Pattern and Target Selection of *Clione limacina* Motoneurons Sprouting Within an Intact Environment

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ABSTRACT

In the pteropod mollusc *Clione limacina*, two groups of locomotor motoneurons, located in the pedal ganglion, innervate the dorsal and ventral muscle layers of the ipsilateral wing through the wing nerve. Separate branches of this nerve go either only to the dorsal muscle layer or only to the ventral one. In the present study, growth of novel neurites of the wing motoneurons was induced by cutting the wing nerve. In addition, all other peripheral nerves and connectives of the pedal ganglion were cut, except for the pedal commissure to the contralateral pedal ganglion. Thus, the neurites were allowed to grow only towards the contralateral pedal ganglion. We have found that the novel neurites, entering the contralateral pedal ganglion, were capable of growing everywhere inside the central nervous system (CNS) and into any peripheral nerve. However, they preferred the wing nerve. This finding suggests that the preference is caused by the guiding cues in the wing nerve or the attractive influence of the wing muscles. Because the contralateral pedal ganglion and nerves were left intact, the growth direction of the new neurites could be determined only by factors permanently existing in the CNS, rather than induced by nerve injury or muscle denervation. Within the wing nerve, the neurites could not discriminate between the nerve branches going to the dorsal and ventral muscle layers. They formed synapses on muscles of both layers, despite the fact that the muscles were innervated by their own motoneurons. J. Comp. Neurol. 423:220–226, 2000.

Indexing terms: neuromuscular regeneration; pathfinding; simple nervous system

One of the central problems in neuroscience is the formation and maintenance of the nervous system structure. How do neuronal processes find the correct way? How do they make the choice between correct and incorrect targets? Do the neurons make erroneous connections and, if they do, can the connections be eliminated? A capacity for neuronal regeneration after damage in some animals with a simple nervous system makes them a convenient model for such investigations (Moffett, 1995).

As a model system for studies of neurite growth and synapse formation, we used regeneration of neuromuscular connections in the pteropod mollusc *Clione limacina. Clione* (Fig. 1A) is a planktonic animal. It swims due to synchronous rhythmical movements (1–2 Hz) of two wings (Arshavsky et al., 1985a). The structure of the wings, and the morphology and function of wing motoneurons are well characterized (Satterlie, 1991, 1993; Arshavsky et al., 1995b; Panchin et al., 1996). Two main muscle layers are responsible for wing beating (Fig. 1A–C): dorsal (D) and ventral (V). Muscles of each layer are innervated by approximately 30 motoneurons driven by the rhythm-generating interneurons (Arshavsky et al., 1995a–c; Panchin et al., 1996). Due to this input, the motoneurons can be divided into two groups: 1) the neurons active during the dorsal flexion of the wing (D-phase of the cycle); and 2) the neurons active during the ventral flexion of the wing (V-phase), respectively (Fig. 1D). The groups include two large cells, 1A and 2A, that are active in the D- and

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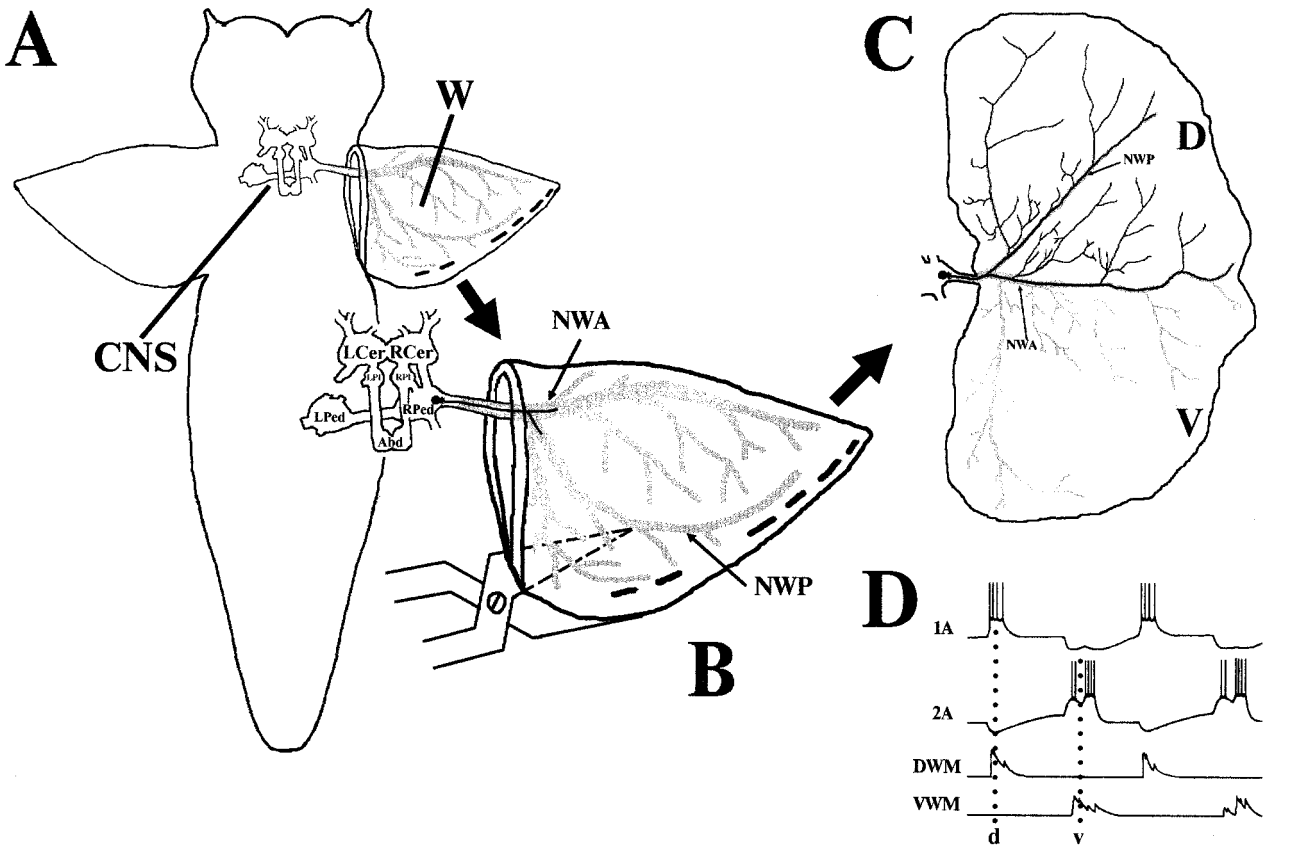

Fig. 1. Schematic representation of the wing motoneuron morphology and neuromuscular connections in *Clione* in an intact animal. **A,B:** A wing (W) and the central nervous system (CNS) were dissected from the whole animal (dorsal view). The caudal rim of the wing was cut by small scissors. The wing nerve with anterior (NWA) and posterior (NWP) main branches and a number of thinner secondary branches in Figure 1 are shown in gray. All nerves and connectives leaving the left pedal ganglion are cut so that the ganglion is connected to the rest of the CNS only by the pedal commissure. RCer, right cerebral ganglion; LCer, left cerebral ganglion; RPed, right pedal ganglion; LPed, left pedal ganglion; RPl, right pleural ganglion; LPl, left pleural ganglion; Abd, abdominal ganglia. **C:** The wing is split open, the intermediate retractor muscles are removed and two muscle layers, dorsal (D) and ventral (V), are separated. The anterior main branch (NWA) of the wing nerve is connected with both muscle layers via secondary branches and in the split wing preparation lies right between dorsal and ventral muscle layers. The posterior main branch (NWP) is separated from the ventral layer and retains connections only with dorsal muscle. The axon morphology of the dorsal phase motoneuron 1A on the unoperated side, i.e., normal morphology of intact motoneuron, is shown. 1A motoneuron's axon contacts only the dorsal muscles. **D:** A scheme of the normal activity of the D-phase motoneuron 1A, the V-phase motoneuron 2A, ventral (VWM) and dorsal (DWM) muscle cells during the locomotor rhythmic activity. Motoneurons of type 1 receive excitatory postsynaptic potentials (EPSP) and fire spikes in the D-phase (denoted by a dotted vertical line and d) and inhibitory postsynaptic potentials (IPSP) in the V-phase (denoted by a dotted vertical line and v). Motoneurons of type 2 receive EPSP and fire spikes in the V-phase and IPSP in the D-phase. Correspondingly, DWM is excited in the D-phase and VWM is excited in the V-phase.

V-phases, respectively. The pattern of their rhythmic activity is schematically shown in Figure 1D, together with the activity of dorsal and ventral muscle cells. The 1A and all other dorsal phase motoneurons receive excitatory postsynaptic potentials (EPSP) in the D-phase and inhibitory postsynaptic potentials (IPSP) in the V-phase. The 2A and all other ventral phase motoneurons receive IPSP in the D-phase and EPSP in the V-phase. Correspondingly, the dorsal muscle cells (DWM) are excited in the D-phase and the ventral ones (VWM), in the V-phase. The 1A and 2A motoneurons innervate the whole area of the wing, whereas the smaller motoneurons innervate limited parts of the muscle layers. All motoneurons within a group (D or V) fire in a close synchrony because they simultaneously receive inputs from the rhythm-generating interneurons and also because most of them are electrically coupled. The wing nerve (NW) bifurcates into the anterior (AntNW) and the posterior (PostNW) main branches immediately after entering the wing (Fig. 1B). Axons of both D- and V-phase motoneurons transit inside these main branches. The main branches of the wing nerve give rise to thinner secondary branches. Each secondary branch projects to only one of the two muscle layers and carries axons of only one of the two classes of motoneurons (either only D or only V; Fig. 1C; Panchin et al., 1998).

If the wing nerve is crushed near the pedal ganglion in whole animal, the axotomized motoneurons start sprouting. Most of the new neurites pass the crushed site, reach the wing muscles and form new synapses. During the early stages of the regeneration, neurites of each motoneuron grow indiscriminately into the secondary branches of both types (D and V) and form synapses on the muscle of

both layers. Later, the incorrect synapses and neurites are withdrawn. This leads to restoration of the correct morphology of motoneurons and correct muscle innervation (Panchin et al., 1998). Additional data on the pathfinding and target selection of the sprouting motoneurons during wing reinnervation were obtained in the experiments on regenerating animals and during in vitro culturing of muscles and ganglia. In the later set of experiments, either the target for reinnervation was restricted to one muscle, or the size of the target muscle was reduced (Zelenin and Panchin, 1999). These studies led to the conclusion that correct innervation is produced as a result of competition between different axonal branches of the same motoneuron synapsing on correct and incorrect muscle targets. It was also shown that in the conditions when muscle targets are not available, motoneurons sprout inside the central nervous system (CNS) and form novel central connections that are absent in the intact ganglia (Panchin et al., 1999). In the present study, we used an experimental approach that allowed us to study the growth and synapse formation of the sprouting motoneurons in the intact environment of the contralateral pedal ganglion and normally innervated contralateral wing. This provided an opportunity to answer questions regarding regeneration, which were difficult to address in the previous regeneration and organ culture experiments. That is, do the newly formed neurites grow to the contralateral wing nerve? If so, is the growth to the wing nerve more intensive than to other peripheral nerves? Is the growth to the secondary wing nerve branches selective? Can the neurites make new neuromuscular synapses on the normally innervated muscles? If so, is the synapse formation selective, that is, do the neurons form synapses on the correct muscles only?

MATERIALS AND METHODS

Experiments were carried out at the White Sea Marine Biological Station *Kartesh* during the summer-autumn period. Animals were collected locally and kept in seawater at +5°C.

Initial surgery

For surgery, an animal was fixed on a Silgard-coated bottom of a Petri dish in sterile seawater. A small incision was made in the body wall on the ventral side above the site of the wing nerve entrance into the wing. All nerves and connectives leaving one of the pedal ganglia were cut with scissors through this incision, except the pedal commissure. As a result, the ganglion was denervated, and was connected to the rest of the CNS only by the pedal commissure (Fig. 1, left pedal ganglion). Cutting a nerve in *Clione* prevents outgrowth to the periphery from the stump, although such outgrowth is possible in other gastropods. A growth of novel neurites was thus channeled to the contralateral pedal ganglion and contralateral nerves. After the operation, the animals were held at +5°C in sterile containers with seawater.

In vitro preparation

In 1.5–2 months after the operation, the animals were used for experiments. A CNS-"split wing" preparation was made (Fig. 1C; Panchin et al., 1998). The posterior edge of the wing contralateral to the denervated ganglion was cut with scissors and the two layers of swimming muscles were separated (Fig. 1B,C). To facilitate the intracellular recording of the muscle cells and for better flattening of the wing muscles, the retractor muscles between the dorsal and ventral swimming muscle layers were removed. Not all connections between the wing nerve branches and muscles could be preserved in this preparation: the posterior main branch (PostNW) of the wing nerve could be left attached either only to the dorsal or only to the ventral muscle. In all experiments, the posterior branch was left on the dorsal muscle layer and the secondary branches running from it to the ventral layer were cut (Fig. 1C). The ganglion sheath was softened with solution of pronase E (3 minutes, 0.5% solution in seawater) to facilitate the intracellular recording of the motoneurons. Then the CNS with the muscles were pinned onto agarose gel layer (2% agarose in seawater) in the Petri dish and covered with a drop of thickening agarose gel. This procedure restricted muscle twitches and facilitated intracellular recording of the muscles.

Motoneuron identification

Motoneurons 1A and 2A, located on the dorsal side of the pedal ganglia, can be easily identified by their size, position, and activity. These motoneurons were used to monitor the rhythmic activity of the locomotor network and to study the morphology of regenerating motoneurons. The other locomotor motoneurons have very similar physiological and morphological properties, yet are less suitable experimentally.

Electrophysiology and morphology

As the role of any single motoneuron in wing muscle innervation is comparatively weak, we used the locomotor activity that recruits many motoneurons to monitor the muscle cell innervation. A synchronous burst of motoneurons of the same locomotor phase produces a strong excitatory junction potential (EJP) in the innervated muscle. Intracellular recordings of two to four cells (neurons and muscle fibers) were carried out with 3 M KCl-filled glass microelectrodes having tip resistance of 30–70 MOhm. For morphological studies, the neurons were injected with Lucifer yellow CH and photographed under a fluorescent microscope. Because of technical limitations, i.e., a large number of long, thin neurites of the stained cells that lie in different optical planes, some of them were not clear in photographs, so drawings were made from the photographs and by use of camera lucida.

RESULTS

Growth of the axotomized motoneurons into the contralateral wing nerve

In a previous study (Panchin et al., 1998) where only a wing nerve was cut in the whole animal, the neurite growth toward the contralateral side was weak, and most neurites grew out of the ganglion to the ipsilateral peripheral nerves. To facilitate the growth of new sprouts to the contralateral ganglion and wing, all exits from one of the pedal ganglia were severed (see Materials and Methods). As a result, the only place for the novel neurites to grow was within their own pedal ganglion and across the pedal commissure. In 1.5–2 months after the operation, in all animals (n = 7), neurites of the axotomized motoneurons passed through the pedal commissure to the contralateral

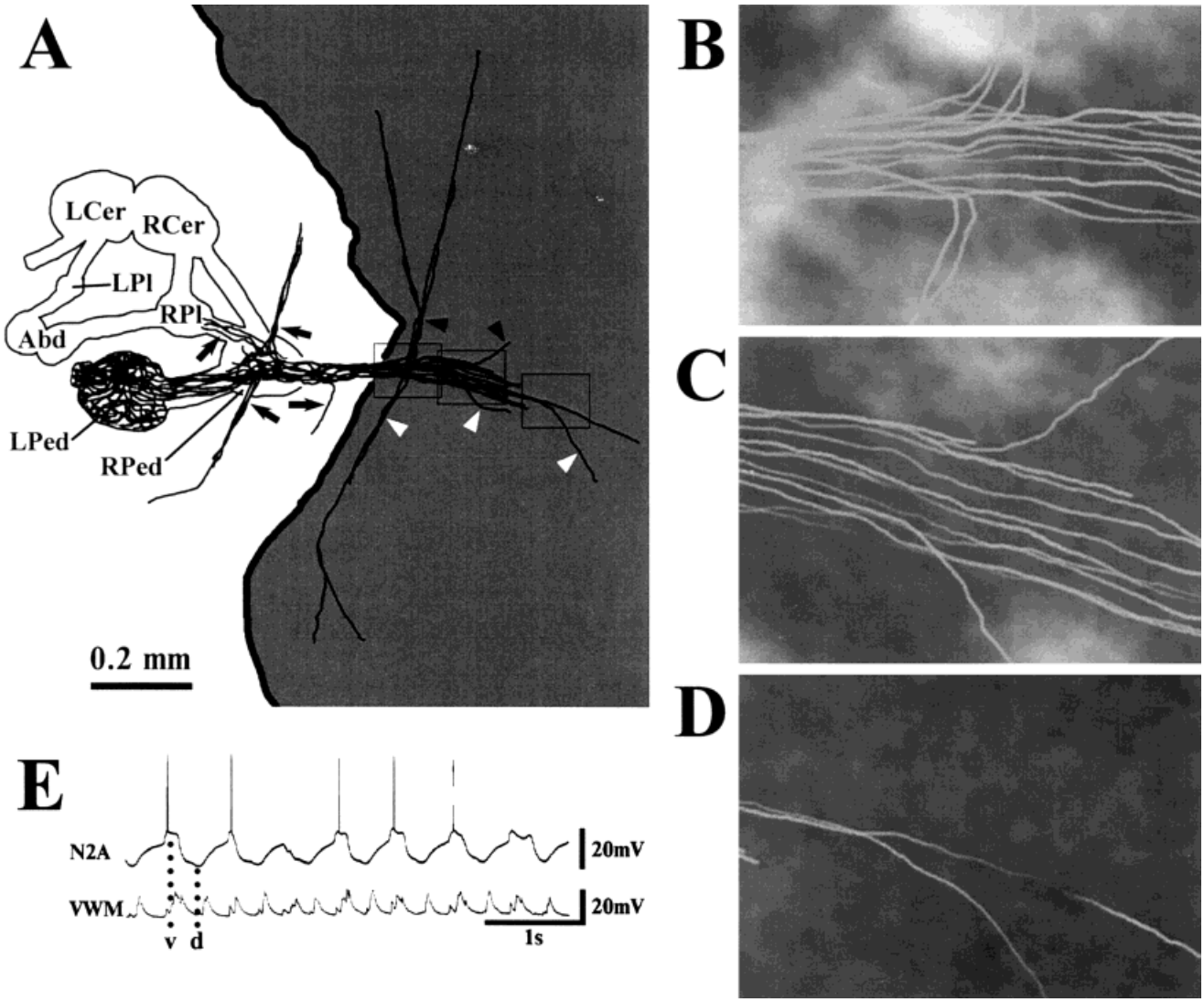

Fig. 2. **A:** Morphology of the 2A ventral motoneuron 2 months after all nerves and connectives of the ipsilateral pedal ganglion were cut. Numerous new thin neurites grow out of the motoneuron. (For clarity the neurites are show considerably thicker than they were in reality.) Most of them pass through the pedal commissure into the contralateral pedal ganglion and enter the peripheral nerves (shown by arrows) including the wing nerve. (The wing muscle layers are indicated by gray color. Split wing preparation is shown; for details see Materials and Methods and Fig. 1B,C.) Inside the wing a part of the neurites grow into correct secondary wing nerve branches (shown by white arrowheads) to the ventral muscles and another part of the neurites grow into incorrect secondary wing nerve branches (shown by black arrowheads) to the dorsal muscle layer (three sites where the neurites grow into secondary wing nerve branches are shown in **B–D** and their positions in A are indicated by black boxes). At this stage of regeneration, the mean lengths and numbers of the correct and incorrect neurites are equal (see also Table 2). **E:** An example of double correct-incorrect innervation of muscles at this stage of regeneration. V-phase motoneuron 2A was recorded simultaneously with a ventral wing muscle fiber (VWM). Note that there are EJPs in the muscle fiber coinciding both with spike bursts in the motoneuron during V-phase of the locomotor cycle (denoted by a dotted vertical line and v) and with IPSPs in the motoneuron during D-phase of the locomotor cycle (denoted by a dotted vertical line and d). Compare this figure with Figure 1D where all EJPs in a ventral muscle cell coincide only with spike bursts in a ventral motoneuron and there are no EJPs in the muscle when V-phase motoneurons are inhibited. For abbreviations in A, see Figure 1.

pedal ganglion. There they were able to enter any peripheral nerves and connectives and reach any ganglion in the CNS. Figure 2A–D shows the morphology of the V-phase motoneuron 2A 2 months after all nerves and connectives of the ipsilateral pedal ganglion were cut. Numerous novel neurites originated from the cell body and stump of the axon. Many of them grew through the pedal commissure into the contralateral pedal ganglion and entered different peripheral nerves including the wing nerve. When in the wing nerve, the neurites grew to both ventral (correct) and dorsal (incorrect) wing branches. This growth pattern was typical for both type 1A dorsal (n = 4) and type 2A ventral (n = 3) motoneurons. We calculated the lengths and numbers of neurites in different nerves for each motoneuron (Table 1) and found that the wing nerve was a preferred direction for growth of the new neurites compared to all

TABLE 1. Neurites of the Contralateral Motoneurons Grow Preferably Into the Wing Nerve[1]

	Neurites exiting the PedG through the NW[2]	Neurites exiting the PedG through all other nerves and connectives	All neurites exiting the PedG
Percentage of such neurites	60	40	100
Mean length of such neurites, μm	4,320 ± 210	1,770 ± 160	3,330 ± 240

[1]The mean length of the neurites exiting the pedal ganglion through the wing nerve differ significantly from the mean length of the neurites exiting the pedal ganglion through all other nerves and connectives ($P < 0.0001$, two-tailed t-test). This implies that the neurites of locomotor motoneurons recognize the wing nerve and prefer it for growth.
[2]PedG, pedal ganglion; NW, wing nerve.

TABLE 2. Preferences of Neurites of the Contralateral Motoneurons for Growth Into the Secondary Branches of the Wing Nerve[1]

	Neurites going into the correct secondary branches of the NW[2]	Neurites going into the incorrect secondary branches of the NW	All neurites going into the secondary branches of the NW
Percentage of such neurites	54	46	100
Mean length of such neurites, μm	4,020 ± 340	4,300 ± 410	4,150 ± 270

[1]The mean length of the neurites growing into correct secondary branches does not differ significantly from the mean length of the neurites growing into incorrect secondary branches ($P < 0.50$; two-tailed t-test). This demonstrates that the neurites do not discriminate between the correct and incorrect branches of the wing nerve.
[2]NW, wing nerve.

TABLE 3. Innervation of the Intact Muscle by Contralateral Motoneurons

	Correct EJPs[1]	Incorrect EJPs	All registered muscle cells
Mean amplitude of EJPs in proximal muscles, mV	5.6 ± 0.3	1.67 ± 0.25	—
Number of muscle cells with such type of EJPs in proximal muscles	35	31	35
Mean amplitude of EJPs in distal muscles, mV	5.7 ± 0.6	0 ± 0	—
Number of muscle cells with such type of EJPs in distal muscles	26	0	26

[1]EJP, excitatory junction potential.

other nerves and connectives that originate from the pedal ganglion. At the same time, no tendency for preferential growth to the dorsal or ventral muscle layers was revealed at this stage of regeneration. The mean lengths and number of the correct and incorrect neurites were found to be equal (Table 2).

Morphology of wing motoneurons ipsilateral to the intact wing is not affected by the other nerve injury

It has been reported that muscle denervation may affect morphology of motoneurons whose axons were not damaged and whose targets retain intact innervation (Rotshenker, 1979; Rotshenker and Tal, 1985). Taking into account these data, we checked the morphology of the intact (i.e., contralateral to the operated side) motoneurons in the same type of experiments as described above. Three motoneurons of type 1A, and 4 motoneurons of type 2A on the side of the intact wing nerve were stained with Lucifer yellow 1.5 months after operation. No difference from their normal morphology was detected. All branches of type 2A motoneurons projected to the ventral muscles, whereas all branches of 1A motoneurons terminated on the dorsal muscles. It was also found that physiological properties of the wing motoneurons in both intact and operated pedal ganglia were not changed upon regeneration. The patterns of their rhythmic activity and the phase relations in the locomotor cycle were the same as in the control animals.

New neuromuscular connections on the muscles with intact innervation

As far as the novel neurites of the motoneurons had grown into the secondary branches of the wing nerve coming directly to the swimming muscles, one could expect the formation of new neuromuscular connections. Innervation of the wing muscles by regenerating motoneurons was studied by paired recordings from the muscle cells and the wing motoneurons. In all experiments, the muscle cells recorded in the proximal part of the wing muscle layers displayed the excitatory junction potentials (EJPs) not only during the correct locomotor phase but also during the incorrect locomotor phase, as illustrated in Figure 2E, where the muscle cell from the ventral layer exhibits two bursts of EJPs in each locomotor cycle. One of them coincides with excitation of the ventral phase motoneuron 2A, and another (of smaller amplitude) coincides with the dorsal phase, monitored by inhibition of the 2A motoneuron. Most of the recorded muscle cells had similar double innervation (Table 3). The amplitude of incorrect EJPs was, on average, significantly lower than the amplitude of correct EJPs.

No incorrect EJPs were found in the muscle cells located in the distal areas of the wing muscle layers (Table 3). This corresponded to the observation that there were no novel neurites of the regenerating motoneurons growing that far to the wing at the term of regeneration studied.

DISCUSSION

Novel neurites of the locomotor motoneurons grow selectively to the contralateral nerve of the wing

When *Clione's* wing nerve is crushed in whole animals, the majority of newly formed neurites grow across the crush site directly into the wing nerve to innervate their targets. Such selective growth under similar conditions

was reported also in other species (Van Essen and Jansen, 1977; Murphy and Kater, 1980b; Hunt and Velez, 1982; Allison and Benjamin, 1985; Croll and Baker, 1990; Ross et al., 1994; Moffett, 1995). Several basic mechanisms could direct this apparently specific growth: 1) new neurites of the motoneurons may start to grow directly forward from the stump of the axon and automatically follow the closest (i.e., original) nerve; 2) the crushed nerve could be more suitable for the growth of novel neurites. For example, it is possible that the separated axons degenerate and leave free space for the growth of new neurites, as proposed by Brown and Hopkins (1981), or the surviving separated axons guide the growth of new neurites as shown in leech by Van Essen and Jansen (1977); 3) the third explanation suggests that even an undamaged wing nerve is a preferable place for the growth of the motoneuron neurites. Some preferences of this kind were observed in the study by Allison and Benjamin (1985); 4) the denervated wing muscles may attract the growing neurites as proposed by several authors (Brown and Ironton, 1978; Wigston, 1980; Haimann et al., 1981); 5) the wing muscles may attract the neurites of motoneurons regardless of their state of innervation. In order to choose among these possibilities, we used the *Clione* neuromuscular regeneration model to study the growth of novel neurites of wing motoneurons into the intact contralateral wing nerve.

If only the wing nerve was cut in the whole animal, the majority of newly formed neurites grew within the ipsilateral part of the CNS, rarely invading the contralateral pedal ganglion (Panchin et al., 1998). To enhance the growth towards the contralateral part of the CNS and into the contralateral wing nerve, we deprived the growing neurites of all possible tracts except for the tract to the contralateral pedal ganglion. We have found that in 1.5–2 months after the operation, novel neurites of the motoneurons not only grew into the contralateral pedal ganglion but also entered peripheral nerves, in particular, the wing nerve and its secondary branches. At this stage of regeneration, we found a considerable predominance in number and total length of the neurites growing into the wing nerve over the neurites growing into all other peripheral nerves and connectives. This finding suggests that even an intact wing nerve is a more preferable area for growth of motoneuron neurites than any other nerve or connective. Thus, mechanisms 1), 2), and 4) listed above are not likely to guide the preferential growth into the wing nerve. Presumably, the growth is determined either by the wing nerve itself (intact or damaged) as a more suitable substrate for growth of the neurites of the locomotor motoneurons, or by the attractive influence of the wing muscles independent of whether or not they are innervated.

A separate question is what time sequence leads to apparently selective distribution of the neurites in the CNS and the peripheral nerves. One possibility is that the initial growth to all nerves including the wing nerve is indiscriminate and the neurites in the wing nerve are preferentially stabilized relative to those that grow into incorrect peripheral nerves. Another possible explanation is that the intensity of growth into the wing nerve is higher than into other nerves. The unambiguous choice between these two interpretations demands examination of earlier stages of the neurite growth than reported in this work. However, indirect evidence favors the second hypothesis. During regeneration after the wing nerve crush there is a small number of neurites growing not to the crushed nerve, but into the CNS and the other nerves. Such neurites stopped their growth at the early stages of regeneration, and later, no more changes in their appearance were observed (unpublished observations).

The dorsal and ventral branches of the intact wing nerve are indistinguishable to the growing neurites of axotomized motoneurons

When the wing nerve was crushed in a whole animal, at the early stages of regeneration novel neurites grew indiscriminately into correct and incorrect secondary branches of the wing nerve and established connections both with correct and incorrect muscles (Panchin et al., 1998). On the other hand, there are many well-documented cases in which neurites grow straight to their final targets (Van Essen and Jansen, 1977; Murphy and Kater, 1980a; Allison and Benjamin, 1985; Mackler et al., 1986). In our previous regeneration studies, it was possible to attribute the lack of selectivity to the wing nerve damage. Present data, however, indicate that motoneurons do not discriminate between secondary branches even in the intact wing. This implies that dorsal and ventral branches are indistinguishable for a growing neurite and that the contact of the growing neurite with muscles is a necessary condition to identify whether it has reached a correct or incorrect target. At later stages, this information leads to selective withdrawal of incorrect synapses and fibers (Panchin et al., 1998).

Growing neurites form neuromuscular connections on muscles with intact innervation

The possibility of hyperinnervation of intact muscles needs to be explored. Some authors suggest that a normally innervated muscle can not be subjected to further innervation (Frank and Jansen, 1976; Robbins et al., 1977; Slack, 1978) but some show that the hyperinnervation is possible (Bixby and Van Essen, 1979; Hunt and Velez, 1982, 1989). As demonstrated in the present study, new innervation occurred in the wing muscles and was superimposed on their initial innervation, when the contralateral motoneurons were induced to sprout. That the contralateral motoneurons were responsible for this additional innervation was supported by the fact that the morphology of the ipsilateral motoneurons and their rhythmic locomotor activity was not altered. We have not shown directly that the motoneurons not only grow indiscriminately to the correct and incorrect muscles but also make synapses on them without any preference. The presence of correct connections from the contralateral motoneurons was difficult to detect on the background of the strong ipsilateral EJPs. However, almost all muscle cells in the proximal wing area had both correct and incorrect innervation, and the mean amplitudes of incorrect innervation was similar to the mean amplitude of incorrect and correct innervation at the early stages of regeneration after the wing nerve crush (Panchin et al., 1998; Zelenin and Panchin, 1999). This suggests that the process of innervation goes on similarly in both these cases, and probably in both cases correct and incorrect muscles are equally innervated.

We can not tell at present whether the elimination of incorrect connections and neurites takes place in the con-

tralateral wing, as it occurs in ipsilateral wing regeneration of sea angel (Panchin et al., 1998), as well as demonstrated in some other models (Denburg, 1982; Wigston and Kennedy, 1987; Hennig and Dietrichs, 1994). To address this question, a study of regeneration over a longer term is required and will be the subject of future experiments. Even though some details of reinnervation of the initially innervated muscle in *Clione* wing are not clear, it is shown that motoneurons deprived of their normal target can innervate muscle cells that are already innervated by their normal neurons. Thus, denervation is not an absolute requirement for synapse formation. The natural tendency of motoneurons to make new connections when deprived of their normal target seems to be sufficient to drive hyperinnervation of any available muscle target.

LITERATURE CITED

Allison P, Benjamin PR. 1985. Anatomical studies of central regeneration of an identified molluscan interneuron. Proc R Soc Lond B 226:135–157.

Arshavsky YI, Beloozerova GN, Orlovsky GN, Panchin YuV, Pavlova GA. 1985a. Control of locomotion in marine mollusc *Clione limacina*. I. Efferent activity during actual and fictious swimming. Exp Brain Res 58:255–262.

Arshavsky YI, Beloozerova GN, Orlovsky GN, Panchin YuV, Pavlova GA. 1985b. Control of locomotion in marine mollusc *Clione limacina*. II. Rhythmic neurons of pedal ganglia. Exp Brain Res 58:263–272.

Arshavsky YI, Beloozerova GN, Orlovsky GN, Panchin YuV, Pavlova GA. 1985c. Control of locomotion in marine mollusc *Clione limacina*. III. On the origin of locomotor rhythm. Exp Brain Res 58:273–284.

Bixby JL, Van Essen DC. 1979. Competition between foreign and original nerves in adult mammalian skeletal muscle. Nature 282:726–728.

Brown MC, Hopkins WG. 1981. Role of degenerating axon pathways in regeneration of mouse soleus motor axons. J Physiol 318:365–373.

Brown MC, Ironton R. 1978. Sprouting and regression of neuromuscular synapses in partially denervated mammalian muscles. J Physiol 278: 325–348.

Croll RP, Baker MW. 1990. Axonal regeneration and sprouting following injury to the cerebral-buccal connective in the snail *Achatina fulica*. J Comp Neurol 300:273–286.

Denburg JL. 1982. Elimination of inappropriate axonal branches of regenerating cockroach motor neurons as detected by the retrograde transport of horseradish peroxidase conjugated wheat germ agglutinin. Brain Res 248:1–8.

Frank E, Jansen JK. 1976. Interaction between foreign and original nerves innervating gill muscles in fish. J Neurophysiol 39:84–90.

Haimann C, Mallart A, Tomas i Ferre J, Zilber-Gachelin NF. 1981. Interaction between axons from two different nerves reinnervating the pectoral muscle of *Xenopus laevis*. J Physiol 310:257–272.

Hennig R, Dietrichs E. 1994. Transient reinnervation of antagonistic muscles by the same motoneuron. Exp Neurol 130:331–336.

Hunt WP, Velez SJ. 1982. Regeneration of specific neuromuscular connections in the crayfish. II. Effects of changes in target area. J Neurophysiol 47:666–676.

Hunt WP, Velez SJ. 1989. Regeneration of an identifiable motoneuron in the crayfish. II. Patterns of reconnection and synaptic strength established in the presence of an extra nerve. J Neurobiol 20:718–730.

Mackler SA, Yin HS, Selzer ME. 1986. Determinants of directional specificity in the regeneration of lamprey spinal axons. J Neurosci 6:1814–1821.

Moffett SB. 1995. Neural regeneration in gastropod molluscs. Prog Neurobiol 46:289–330.

Murphy AD, Kater SB. 1980a. Differential discrimination of appropriate pathways by regenerating identified neurons in *Helisoma.* J Comp Neurol 190:395–403.

Murphy AD, Kater SB. 1980b. Sprouting and functional regeneration of an identified neuron in *Helisoma.* Brain Res 186:251–272.

Panchin YV, Sadreev RI, Arshavsky YI. 1996. Control of locomotion in marine mollusc *Clione limacina,* X. Effects of acethylcholine antagonists. Exp Brain Res 109:361–365.

Panchin YV, Zelenin PV, Popova LB. 1998. Regeneration of central and peripheral synaptic connections in the locomotor system of the pteropod mollusc *Clione limacina.* Inv Neurosci 3:27–40.

Panchin YV, Zelenin PV, Popova LB. 1999. Axotomized neurons of the pteropod mollusc *Clione limacina* develop novel sites of transmitter release in the absence of their normal muscle target. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 123:185–191.

Robbins N, Antosiak J, Gerding R, Uchitel OD. 1977. Nonacceptance of innervation by innervated neonatal rat muscle. Dev Biol 61:166–176.

Ross AL, Govind CK, Kirk MD. 1994. Neuromuscular regeneration by buccal motoneuron B15 after peripheral nerve crush in *Aplysia californica.* J Neurophysiol 72:1897–1910.

Rotshenker S. 1979. Synapse formation in intact innervated cutaneous-pectoris muscles of the frog following denervation of the opposite muscle. J Physiol (Lond) 292:535–547.

Rotshenker S, Tal M. 1985. The transneuronal induction of sprouting and synapse formation in intact mouse muscles. J Physiol (Lond) 360:387–396.

Satterlie RA. 1991. Electrophysiology of swim musculature in the pteropod mollusc *Clione limacina.* J Exp Biol 159:285–301.

Satterlie RA. 1993. Neuromuscular organization in the swimming system of the pteropod mollusc *Clione limacina.* J Exp Biol 181:119–140.

Slack JR. 1978. Interaction between foreign and regenerating axons in axolotl muscle. Brain Res 146:172–176.

Van Essen DC, Jansen JKS. 1977. The specificity of re-innervation by identified sensory and motor neurons in the leech. J Comp Neurol 171:433–454.

Wigston DJ. 1980. Suppression of sprouted synapses in axolotl muscle by transplanted foreign nerves. J Physiol 307:355–366.

Wigston DJ, Kennedy PR. 1987. Selective reinnervation of transplanted muscles by their original motoneurons in the axolotl. J Neurosci 7:1857–65.

Zelenin PV, Panchin YV. 1999. Selective regeneration of the neuromuscular connections in the pteropod mollusc *Clione limacina.* Eur J Neurosci 11:1800–1808.