Invertebrate Neuroscience, 3, 27-40 (1997)

Regeneration of central and peripheral synaptic connections in the locomotor system of the pteropod mollusc *Clione limacina*

YURI V. PANCHIN[1, 2], PAVEL V. ZELENIN[2] and LYUDMILA B. POPOVA[2]

[1]Institute of Problems of Information Transmission, Russian Acad. Sci., Bolshoi Karetny pereulok 19, Moscow, Russia; [2]A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia

ABSTRACT The neural network underlying rhythmic wing movements in the mollusc *Clione limacina* is well-studied. Two different groups of motoneurons innervate two distinct groups of wing muscles. The locomotor rhythm generated in the left and right pedal ganglia is synchronized by interneurons. When the axons of the locomotor motoneurons are crushed, numerous fine neurites sprout towards the denervated muscles and reach them in 8-15 days. At this stage motoneurons project to and synapse on not only correct but equally incorrect muscle targets. After 2 weeks of regeneration the number of incorrect neurites and synaptic connections begins to decrease and following 1.5-2 months all incorrect connections are eliminated, incorrect axons are withdrawn and the behavioral deficit is compensated. In this study the regeneration of interneurons and the growth profiles of inter- and motoneurons were also studied *in vitro*. Two individually isolated pedal ganglia were co-cultured in three different configurations: a) the wing nerve stump from one ganglion was fixed against the commissural stump from another ganglion; b) the wing nerve stumps were fixed against each other; c) the commissural stumps were fixed against each other. Under the above experimental conditions we found that the interneurons were able to cross only the contact between two commissural stumps, and in this case found their original targets, restored correct connections and synchronized the rhythm in two pedal ganglia. In contrast, motoneurons were able to cross all types of contacts.

KEY WORDS: *Clione limacina*; regeneration; sprouting; growth restrictions; neuromuscular junction; synapse elimination

Introduction

Gastropod molluscs have a remarkable capacity to regenerate their axotomized axons which not only re-innervate their appropriate target tissues but also compensate for the behavioral deficit in the whole animal (Moffett, 1995). The central ganglia from a variety of molluscan species contain a relatively small number of neurons, many of which are individually identifiable. Detailed investigations of regeneration of the central and peripheral connections have been carried out on identified neurons of *Helisoma trivolvis* (Bulloch and Kater, 1982; Cohan *et al.*, 1987) and some other molluscan species (Allison and Benjamin, 1985; Chiasson *et al.*, 1994; Fredman and Nutz, 1988; Ross *et al.*, 1994; Scott *et al.*, 1995). These studies have provided insight into mechanisms underlying regeneration and specific synapse formation. The well-studied swimming CPG of pteropod mollusc *Clione* (Fig. 1A), with most neurons and their connections identified (Arshavsky *et al.*, 1985a-d; Arshavsky *et al.*, 1989; Satterlie, 1985; Panchin *et al.*, 1996), is an attractive model for neuronal regeneration studies. In the present study we investigated central (interneurons) and peripheral (motoneurons) patterns of regeneration and re-innervation in either the organ culture or whole animal.

Clione is a planktonic animal; it swims by using rhythmical movements (~1 Hz) of two wings. Each locomotor cycle consists of two phases, a dorsal wing flexion (D-phase) and a ventral wing flexion (V-phase), which are produced by alternating contractions of antagonistic muscles. The swimming rhythm is generated centrally by the CPG neurons primarily located in the paired pedal ganglia. Figure 1A illustrates the network organization of *Clione*'s swimming CPG. Symmetrical sets of neurons are located in the left (L) and right (R) pedal ganglia. Functionally, the network can be divided into two 'half-centers', consisting of neurons that are active during either D- or V-phases of the swimming cycle (D-phase and V-phase half-centers). Neurons involved in the V-phase of the swimming cycle are given even numbers whereas D-phase neurons have odd numbers. Interneurons 7 and 8, active during D- and V- phases of the cycle respec-

Corresponding author: Dr. Yuri V Panchin. e-mail: panchin@neuro.genebee.msu.su

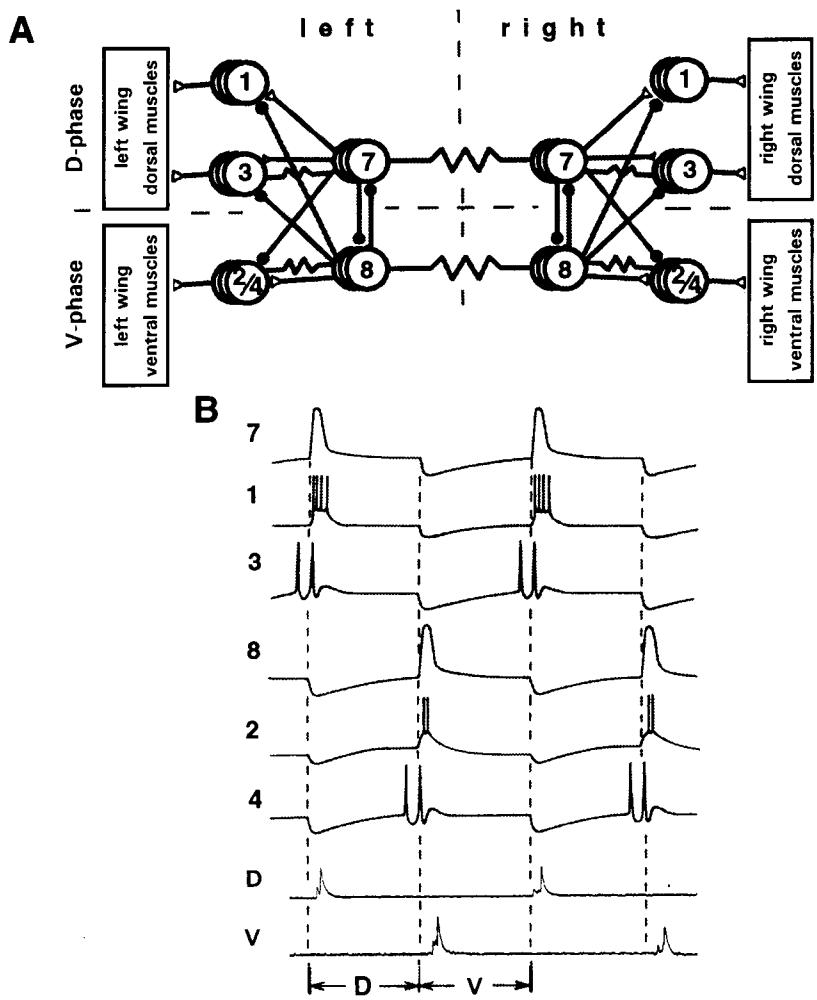

Fig. 1. (A) Neural network controlling rhythmical wing movements in *Clione*. *Subsets of neurons in left and right ganglia and muscles in left and right wing are shown. Interneurons of types 7 and 8 span the commissure and produce connections in both ganglia. Electrical connections are shown by resistor symbols, excitatory chemical connections by white triangles, and inhibitory connections by black dots. All neurons of the same group are electrically coupled.* **(B)** *Diagram showing phases of the activity of different groups of neurons and muscles in the swimming cycle. See text for details.*

tively, play a crucial role in the rhythm generation. There are about 10 cells of each group per ganglion. Axons of the interneurons project through the pedal commissure into the contralateral ganglion (Fig. 3A, 9A). These interneurons generate a single prolonged (about 100 ms) action potential per swimming cycle. The two groups of interneurons inhibit each other. All interneurons of a given group from both ganglia are electrically interconnected. As a result, interneurons are excited simultaneously, so that rhythmic output on the left and right side is synchronous. If the pedal commissure is cut, the two wings beat independently (Arshavsky *et al.*, 1985e; Panchin, 1984). Interneurons 7 and 8 control wing muscle motoneurons. They excite motoneurons of the same half-center and inhibit antagonistic motoneurons. The motoneurons in turn excite corresponding wing muscles. Figure 1B shows the temporal activity pattern of different groups of neurons and muscles during the swimming cycle.

In the present study we investigated the regeneration of two different types of connections in *Clione*'s locomotor system, specifically: chemical synaptic connections between the motoneurons and the wing muscles and the electrical connections between locomotor interneurons from the left and right pedal ganglia. We addressed the following questions: do adult *Clione* neurons restore correct innervation and function after axotomy; do they form inappropriate and transient connections? What are the patterns of growth and of connection formation if the normal pathway is not available?

Some preliminary results have been reported previously (Arshavsky *et al.*, 1985e; Panchin, 1984; Panchin *et al.*, 1995b, 1996).

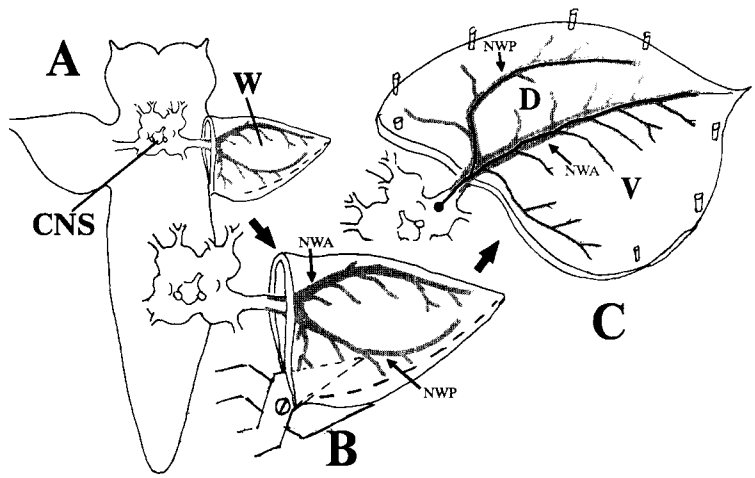

Fig. 2. Making the split wing preparation in *Clione*. **(A)** *The wing (W) and the central nervous system (CNS) were dissected from the whole animal (dorsal view).* **(B)** *The caudal rim of the wing was cut by small scissors. The wing nerve with two main branches: anterior (NWA) and posterior (NWP) and secondary branches are shown in grey inside the wing.* **(C)** *The wing is split open, the intermediate retractor muscles are removed and two muscle layers: dorsal (D) and ventral (V) are separated. The main branch NWA is connected with both muscle layers via secondary branches and in the split wing preparation lies right between dorsal and ventral muscle layers. NWP is separated from the ventral layer and retains connections only with dorsal muscle. The axon morphology of the ventral 2A motoneuron is shown. 2A's axon reaches only the ventral muscles. Note that the NWP-branch of 2A has no exits to the muscles in this preparation.*

Methods

Experiments were carried out at the White Sea Marine Biological Station, Kartesh. Different types of preparations were used, ranging from whole animals to *in vitro* preparations. In the first case the animal was put in sterile sea water containing antibiotics (final concentrations: 50 U/ml of penicillin, 0.05 mg/ml of streptomycin), and a small incision in the skin was made at the ventral surface. The CNS was exposed and the wing nerve was crushed with fine forceps or cut with scissors close to the exit from the ganglion. A thin nerve from the contralateral side was cut in all experiments. The animals were then put in tanks containing sea water. After 2-60 days postsurgery the animals were dissected and prepared for intracellular recordings and staining. If the nerve was cut, only the CNS was used. If the nerve was crushed the wing-CNS preparation was made. The wing was split open so that the D and V muscles were separated (see Fig. 2, 'split wing preparation'). The intermediate retractor muscles were removed. The wing nerve has two main trunks which carry axons projecting towards both D and V muscle layers (Fig. 2, NWA and NWP- anterior and posterior wing nerve trunks). These two main trunks give rise to a number of secondary branches which are attached only to D or V muscles and carry motoneuron axons of only appropriate phase. When the wing is opened as in Fig. 2, it is impossible to preserve all the secondary branches and in all preparations used in this paper ventral secondary branches of NWP were cut.

The preparations were treated for 1 minute in 0.5% solution of Pronase E in sea water to soften the ganglion sheath and rinsed in a large volume of sterile sea water. The ganglia with or without the wing muscle were pinned on a layer of agarose gel and fixed with a drop of warm liquid agarose. This procedure restrained muscle twitching and improved intracellular recordings of the neurons and muscle cells. The largest D-phase motoneuron 1A and the largest V-phase motoneuron 2A (Arshavsky *et al.*, 1985a) are easily identified on the dorsal side of the pedal ganglia on the basis of their size, position and activity. These motoneurons were used in most experiments to monitor the rhythmic locomotor activity and to study the morphology of regenerating motoneurons.

For *in vitro* organ culture, the ganglia were either cultured as a paired pedal ganglia preparation (PPG) (Fig. 3B) or used for other types of preparations (see below). The ganglia were put into 35-mm polystyrene dishes (Falcon 3001) lined with 1% agarose gel in sea water (volume of the gel was 2 ml) and fixed with a thin layer of thickening agarose gel. The gel was covered with 2 ml of salt-adjusted 100% L15 medium containing antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) (Panchin *et al.*, 1995a). For other types of preparation, the pedal and thin subpedal commissures were cut, and two isolated ganglia were put close together on an agarose layer and fixed with a drop of agarose gel and small glass pins, and cultured as described above. In several experiments, single pedal ganglia were cultured (PG preparation). In CC prepa-

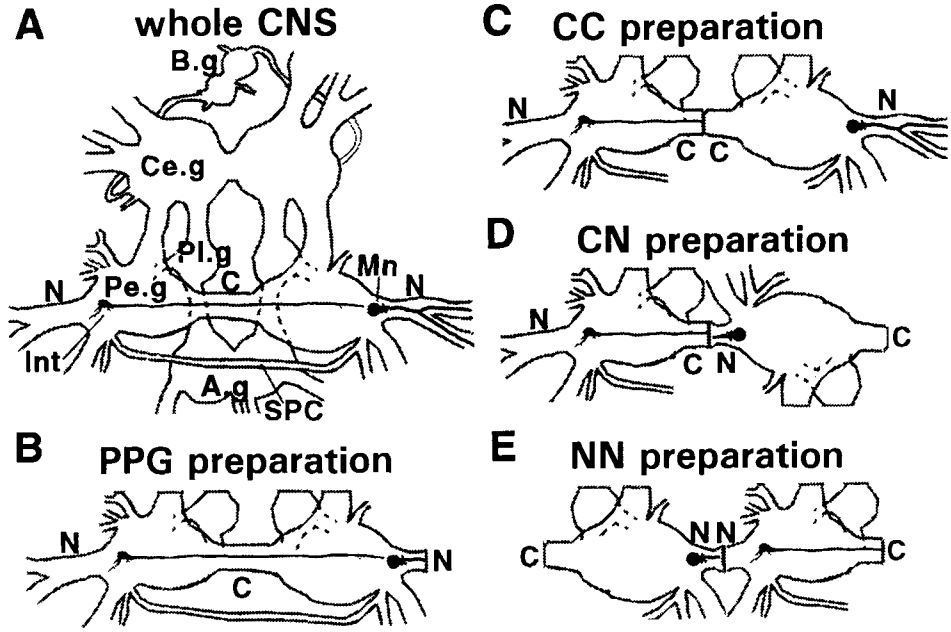

Fig. 3. Schematic representation of *Clione*'s CNS (A) and of the four main types of preparations (B-E). *Int-interneuron, Mn-motoneuron.* **(B)** *Paired pedal ganglia preparation (PPG).* **(C)** *CC preparation with commissure (C) stumps put in contact.* **(D)** *CN preparation with commissure stump put in contact with the wing nerve stump (N).* **(E)** *NN preparation with two nerve stumps contacting each other. B.g.-buccal ganglia, Ce.g.-cerebral ganglia, Pl.g.-pleural ganglia, Pe.g.-pedal ganglia, A.g.-abdominal ganglia, Spc.-subpedal commissure.*

ration, the cut ends of pedal commissure were juxtaposed against each other (Fig. 3C). In CN preparation, the wing nerve of one ganglion was cut short and put against the stump of commissure of another ganglion (Fig. 3D). In NN preparation, the stump of the wing nerve of one pedal ganglion was put against the stump of the wing nerve from another ganglion. (Fig. 3E)

Intracellular recordings of two to four cells (neurons and muscle fibers) were carried out with 3M KCl-filled glass microelectrodes having tip resistance of 20-40 MΩ. Current was injected into neurons through the recording electrode. A bridge circuit was used to compensate partly for the artifact caused by the polarizing current.

For morphological studies, neurons were injected with Lucifer yellow CH and photographed under fluorescence microscope (LOMO, Dorozhny). Because of technical limitations, i.e. the large number of long thin neurites of the stained cells that laid in different optical planes, *camera lucida* drawings were made.

All drugs were from Sigma.

Results

Morphology and synaptic connections of the swim motoneurons

Both the wing structure and the morphology and physiology of wing motoneurons are well studied (Arshavsky *et al.*, 1985b; Panchin *et al.*, 1996; Satterlie, 1991; Satterlie, 1993). The motoneurons have several short (not exceeding 50 micrometers) central branches and a long axon that exits the ganglion through ipsilateral nerve branching in the wing. The split wing preparation used in this study revealed some new details of the nerve arborization and allowed us to discriminate the dorsal and ventral branches of the wing nerve. Fig. 4A and 4B illustrate the branching patterns of two largest neurons of the D- and V-phases (1A and 2A correspondingly) in a split wing preparation (see Methods and Fig. 2). Axon branches of the 1A motoneuron reach muscles via dorsal secondary branches of the nerve, while motoneuron 2A uses ventral ones. Note that although NWP (which is located on the dorsal muscle layer) contains the axon of a motoneuron 2A, no axon branches terminate on the D muscles. Secondary branches of this axon which were connected with V muscles via secondary nerve branches were cut during preparation. Fig. 4C demonstrates simultaneous recording of the V-phase motoneuron 2A and the ventral and dorsal muscle cells during the locomotor rhythmic activity. All ventral phase motoneurons, including 2A, receive excitatory postsynaptic potentials (EPSP) in the V-phase and inhibitory postsynaptic potentials (IPSP) in the D-phase. The dorsal phase motoneurons, including 1A (not shown), receive IPSP in the V-phase and EPSP in the D-phase. Correspondingly the ventral muscle cell (VWM) is excited in the V-phase, and the dorsal one

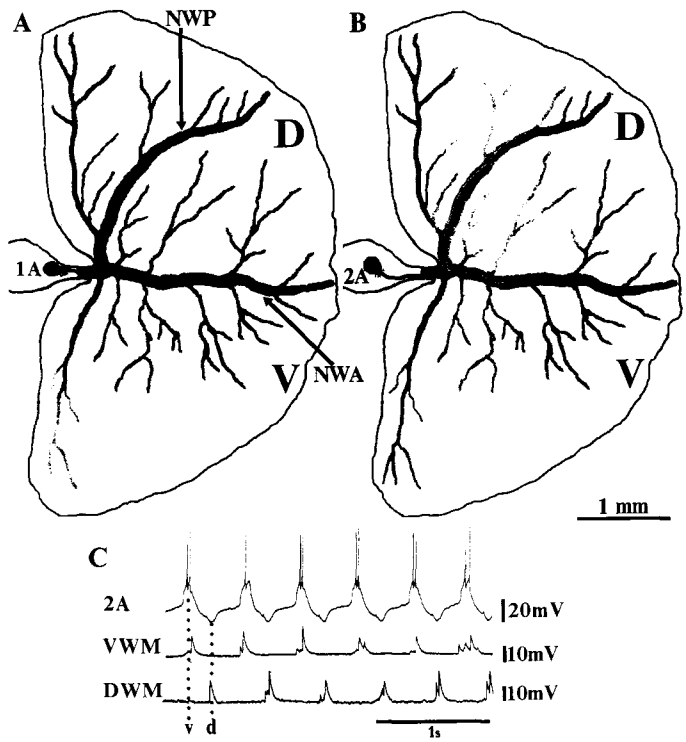

Fig. 4. Normal morphology and physiology of two biggest locomotor motoneurons of the D- and V-phases and wing muscles. **(A)** *The D-phase motoneuron 1A.* **(B)** *The V-phase motoneuron 2A. Axon branches of motoneuron 1A reach muscles via dorsal secondary branches of the wing nerve while motoneuron 2A uses ventral ones. Note that although NWP which is laying on the dorsal muscle contains an axon of motoneuron 2A none of its axon branches terminates on D muscles. They were connected with V muscles via secondary nerve branches that were cut during preparation. Neurons in A and B were successively stained in split wing preparation with Lucifer Yellow CH (see Methods). The dorsal muscle layer is above the NWA and the ventral muscle layer is below the NWA.* **(C)** *Simultaneous recording of the V-phase motoneuron 2A, ventral (VWM) and dorsal (DWM) muscle cells during the locomotor rhythmic activity. Motoneuron 2A receives EPSP in the V-phase and IPSP in the D-phase. Correspondingly VWM is excited in the V-phase (shown with dotted line and letter v), and DWM is excited in the D-phase (shown with dotted line and letter d). D- dorsal muscle layer of the wing; V- ventral muscle layer of the wing.*

(DWM) is excited in the D-phase. We have never observed excitation in dorsal muscles during the V-phase or excitation of ventral muscles during the D-phase of locomotor cycle.

As all the motoneurons of the same group are excited synchronously it was difficult to decide which motoneuron(s) was (were) responsible for excitation of any given recorded muscle unit. Thus the motoneuronal activity shown in Fig. 4-7 merely represents the phase of the muscle excitation and does not show that the synapses are monosynaptic.

Restoration of neuromuscular connections and the swim motoneuron morphology after the wing nerve crushing

When motoneuron axons were crushed in the nerve and the thin nerve from the contralateral side was cut the wing movements stopped. In 6 preparations made 1-6 days after nerve crushing no locomotor excitatory junction potentials (EJPs) were detected in the wing muscles. The dye, Lucifer Yellow, was injected into the swim motoneurons and the preparations were subsequently prepared for fluorescence microscopy and *camera lucida* drawing. Numerous fine neurites start to sprout mainly from the stump of the cut axon of the swim motoneurons. In 2-3 days several fine branches pass the crushed site of the nerve. At that time, it was clear that the majority of the growth cones of the regenerating motoneurons were projecting along the wing nerve in the direction of the denervated muscles and reached them in 7-10 days. Fig. 5 demonstrates the morphology of the dorsal phase motoneuron, 9 days after the axotomy, and a ventral phase motoneuron 10 days postaxotomy. At this stage, motoneurons display no preference for either the dorsal or the ventral muscles. For instance, motoneuron 2A (Fig. 5B) had neurites projecting to the ventral muscles as well as to the dorsal muscles. All motoneurons of the D- (N = 7) and V-phase (N = 6) stained at this stage had not only branches projecting to their correct muscle targets but also approximately the same number of branches projecting to the antagonistic muscles.

Intracellular recordings from the muscle fibers at

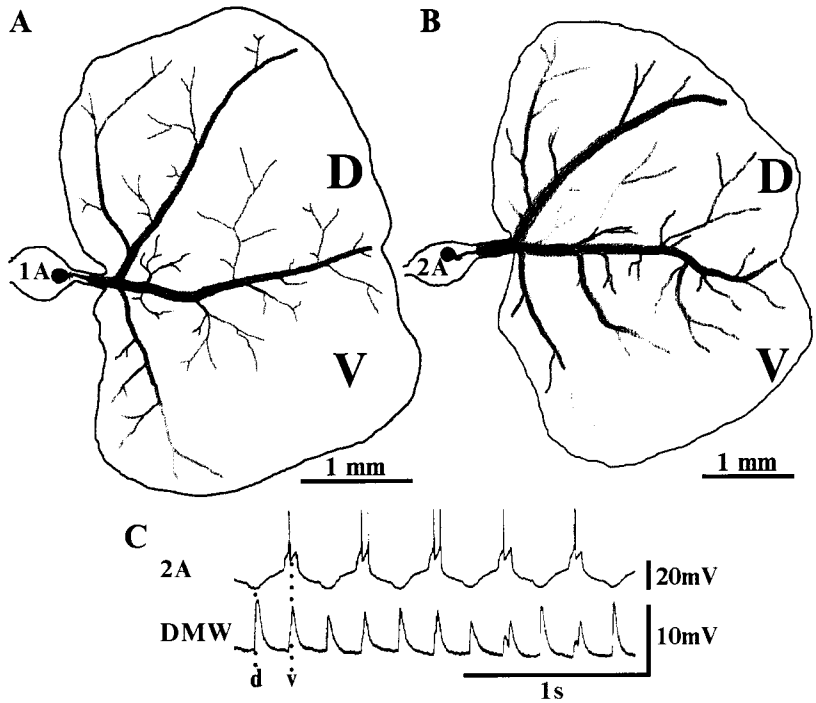

Fig. 5. Early stage of regeneration. *Morphology of the D-phase motoneuron 1A 9 days after crushing of wing nerve* **(A)** *and the V-phase motoneuron 2A 10 days after crushing of wing nerve.* **(B)** *Motoneurons at this stage have neurites that project indiscriminately into the nerve branches going to the dorsal and ventral muscles.* **(C)** *Simultaneous recordings of activity of the V-phase motoneuron 2A and a muscle cell from the dorsal muscle layer of the wing 10 days after the crushing of the wing nerve. Dorsal muscle fiber shown here receives excitatory junction potentials (EJPs) of approximately equal amplitude in both locomotor phases. Abbreviations are the same as in Fig 2 and Fig. 4.*

this stage demonstrated that new neurites had established functional neuromuscular connections. The ventral muscle fiber (Fig. 5C) received excitatory junction potentials (EJPs) in both locomotor phases. In each of 6 different animals, the muscles recorded 7-10 days after operation displayed correct, incorrect and mixed (both from dorsal and ventral motoneurons) innervation in different fibers. About 50% of the recordings showed mixed innervation, while another half of the recordings was almost equally divided between correct and incorrect innervations.

Motoneuronal activity and their synaptic connections in the CNS did not change in the regenerated preparations. No aberrant electrical or chemical connections were detected, and all the locomotor motoneurons were found to be active during their normal phase, never displaying activation in antagonist phase (data not shown.) This indicated that the muscles in these preparations indeed receive mixed innervation from the motoneurons of both D- and V-phases.

After 10 days' regeneration, both the number of aberrant neurites and their synaptic connections began to decrease. Fig. 6 demonstrates the morphology of the dorsal phase motoneuron 19 days after the axotomy, and a ventral phase motoneuron 22 days after the axotomy. At the 11-25-day stage, in all 8 animals examined, the number and length of correct neurites had increased while incorrect fibers gradually had withdrawn compared to the previous stage.

Intracellular recordings from the muscle fibers at this stage demonstrated that the majority of the connections were correct (73%). In all cases of mixed innervation the amplitude of the incorrect EJPs was substantially smaller than that of the correct EJPs. Only in a single animal at this stage one muscle fiber with a wrong innervation was detected. Fig. 6C depicts an example of the mixed innervation of the ventral muscle fiber 12 days after the nerve crushing. In this case, the D-phase EJPs were much smaller than those of the V-phase. Behaviorally, at this stage animals looked as if they had recovered. As a rule the operated wing moved almost normally; however in some animals the most distal part of the wing bent more weakly than the intact wing.

Fig. 7 shows the morphology of 1A and 2A motoneurons 56 days after axotomy. Only correct neurites were present. The overall number of neurites was reduced and they became thicker when compared with the 11-25 days stage. The morphology of the 1A and 2A motoneurons in 2 month old preparations looked normal. The main difference was that in the control preparations only single axons were present in each nerve branch, while at the late stage of the regeneration several thinner fibers could be observed. The overwhelming majority of the muscle cells recorded in

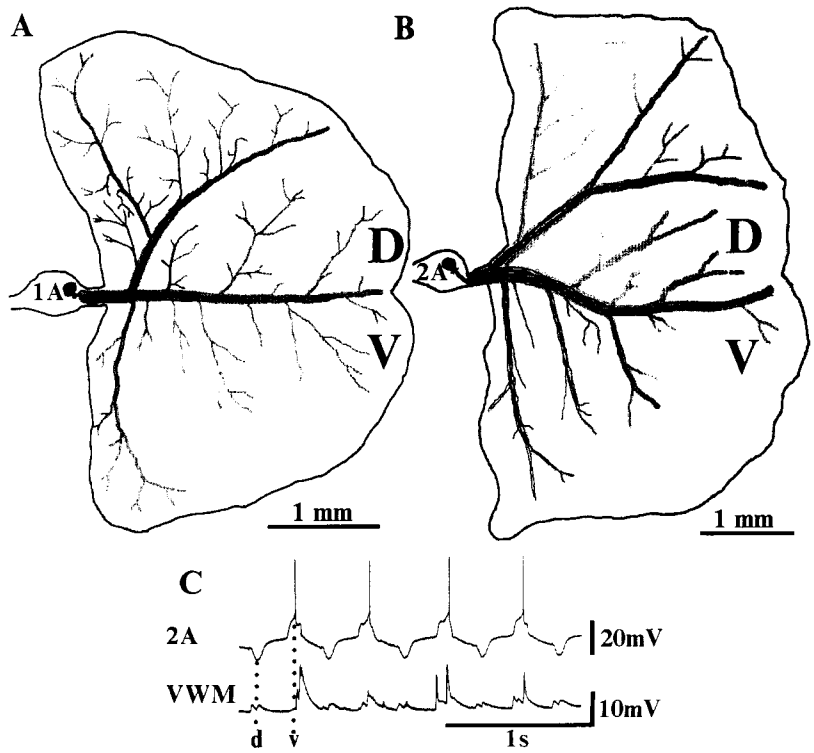

Fig. 6. Intermediate stage of regeneration. *Morphology of the dorsal phase motoneuron 1A 19 days after the axotomy* **(A)** *and morphology of the ventral phase motoneuron 2A 22 days after the axotomy* **(B)**. *The number of neurites in the incorrect nerve branches is smaller than in the correct branches and they are shorter. During this stage the number and the length of correct neurites is increased while incorrect fibers are gradually withdrawn.* **(C)** *An example of the mixed innervation. Simultaneous recordings of activity of the V-phase motoneuron 2A and a muscle cell from the ventral muscle layer of the wing 12 days after the crushing of the wing nerve. In this case the D-phase EJPs are much smaller than those of the V-phase. Abbreviations are the same as in Fig 2 and Fig. 4.*

this animal received EJPs only in appropriate phase (Fig. 7C). We detected, however, a single muscle unit in the dorsal muscle layer that was excited in contraphase with neighboring muscle units. Apparently, it was not innervated by motoneuron 2A but probably by some other ventral motoneurons.

Morphology of the swimming motoneurons in the absence of the muscle targets

To study the growth of the locomotor motoneurons in the absence of their normal targets (wing muscles) either the wing nerve was cut in the whole animals or the pedal ganglia were cut out from the animal and cultured (for 1-20 days) in a Petri dish.

When the wing nerve and the thin nerve from the contralateral side were cut in the whole animals, the wing movements stopped and were not restored even after 2 months. The morphology of motoneurons as revealed by Lucifer Yellow 1-20 days after the nerve transsection showed sprouting, gradually increasing with time, that was similar to that in cultured pedal ganglia. In the cultured ganglia the growth was more pronounced than in the preparations from the whole animals with the same postaxotomy time. Fig. 8B shows the sprouting of a 1A motoneuron in an isolated pair of pedal ganglia, after 6 days in culture (compare with the normal morphology, Fig. 8A). Numerous new neurites, originating from the cut stump, and probably from the cell body, spread all over ipsi- and contralateral ganglia. These neurites were found in all the peripheral nerves and central commissures. In some experiments, one to twelve neurites were present in every nerve.

In cultured pedal ganglia, the degree of central sprouting was dependent on the site of the nerve transsection. If the wing nerve was left longer, sprouting was much less pronounced, and usually restricted to the wing nerve itself.

In the experiment shown in Fig. 8C two isolated pedal ganglia were cultured for 3 days in a position where the stumps of their wing nerves (N) were put against each other (NN-preparation). Motoneuron 1A stained in this preparation had neurites crossing the contact site. In 3 out of 4 of such preparations, the motoneurons sprouted into another ganglion.

Similarly in 4 of 5 preparations, motoneurons outgrew into another pedal ganglion through the contact between the nerve and commissure stumps (CN preparations; Fig. 8D).

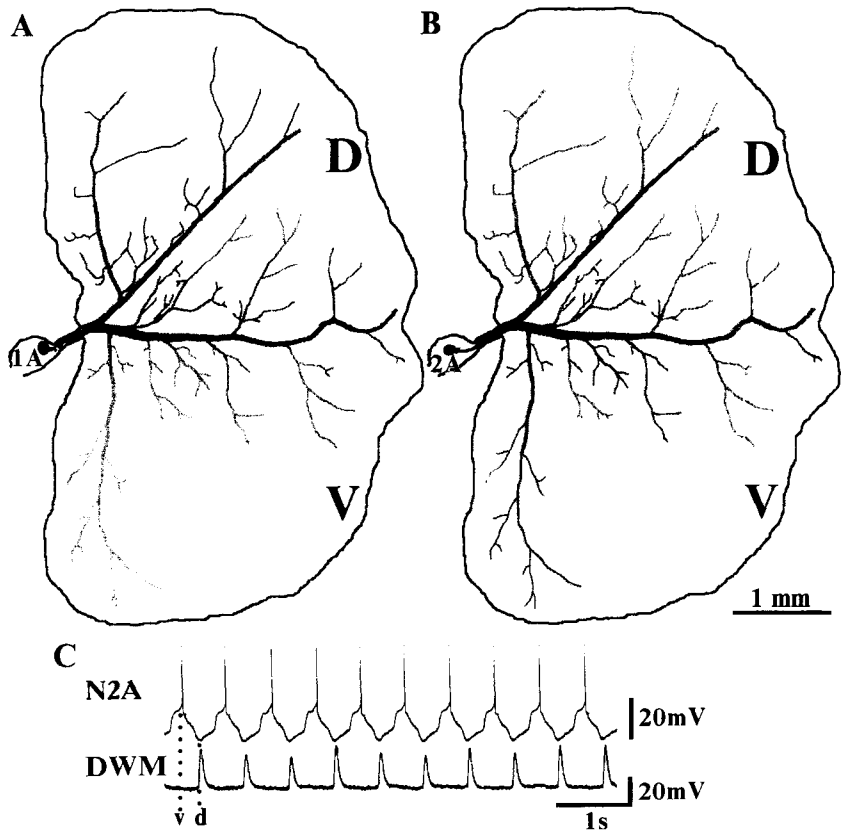

Fig. 7. Late stage of regeneration. *Morphology of 1A* **(A)** *and 2A* **(B)** *motoneurons in 56 days after axotomy. Only correct neurites are present. The overall number of neurites is reduced and they become thicker in comparison to the 11-25 days stage. Neurons in A and B are from the same preparation.* **(C)** *Simultaneous recordings of activity of the V-phase motoneuron 2A and a muscle cell from the dorsal muscle layer of the wing 56 days after the crushing of the wing nerve. Abbreviations are the same as in Fig 2 and Fig. 4.*

The normal interneuron morphology

The interneurons' morphology has previously been described, (Arshavsky *et al.*, 1985a-e; Panchin, 1984; Satterlie and Spencer, 1985; Satterlie *et al.*, 1985). Additionally, in this study, we intracellularly stained 21 interneurons in freshly isolated ganglia to reveal a more detailed picture of their normal neurite branching and related position. Most interneurons had a simple morphology with a single long neurite crossing the commissure, and numerous short branches in each ganglion (Fig. 9A). Nine of twelve type 7, and seven of nine type 8 interneurons had this kind of morphology. Three of type 7 and two of type 8 interneurons had a distinct neurite pattern with an additional, relatively long neurite branch in each pedal ganglion (Fig. None of the interneurons were seen to leave the pedal ganglia through other connectives or peripheral nerves. Some branches were directed to the wing nerves, but never spread further than 100 micrometers down the nerve.

All type 8 neurons are located on the ventral surface of the ganglion, while type 7 neurons are present on the ventral and dorsal sides. In this study all the cultured preparations were positioned with their dorsal side up, so we concentrated on type 7 interneurons located on the dorsal side. All the dorsal interneurons stained in control preparations had morphological type as in Fig. 9A (n=5). Only these interneurons' sprouting was studied in cultured ganglia (see below).

Interneuron outgrowth *in vitro*

In all types of preparation, where the axons were lesioned by cutting the commissure, the intracellular staining of type 7 interneurons revealed intense sprouting with a number of new fine branches originating apparently from the damaged site (Fig. 9C-F). In contrast, in PPG (n=5) preparations' interneuron morphology remained unchanged after culturing for up to 15 days (n = 5). We studied interneurons sprouting in 13 CC preparations (see Methods and Fig. 3). One interneuron of type 7 was stained in each experiment after two to nine days in culture. All interneurons revealed new growing fibers that turned back from the damaged site and grew in the same ganglion along the original axon, forming a 'return loop'. In 10 out of 13 CC preparations studied, 1 to 6 new neurites crossed the border between the stumps of two commissures, entering the opposite ganglion at different distances. Fig. 9C illustrates the 'return loop' and a bunch of neurites, crossing the contact in CC preparation in which

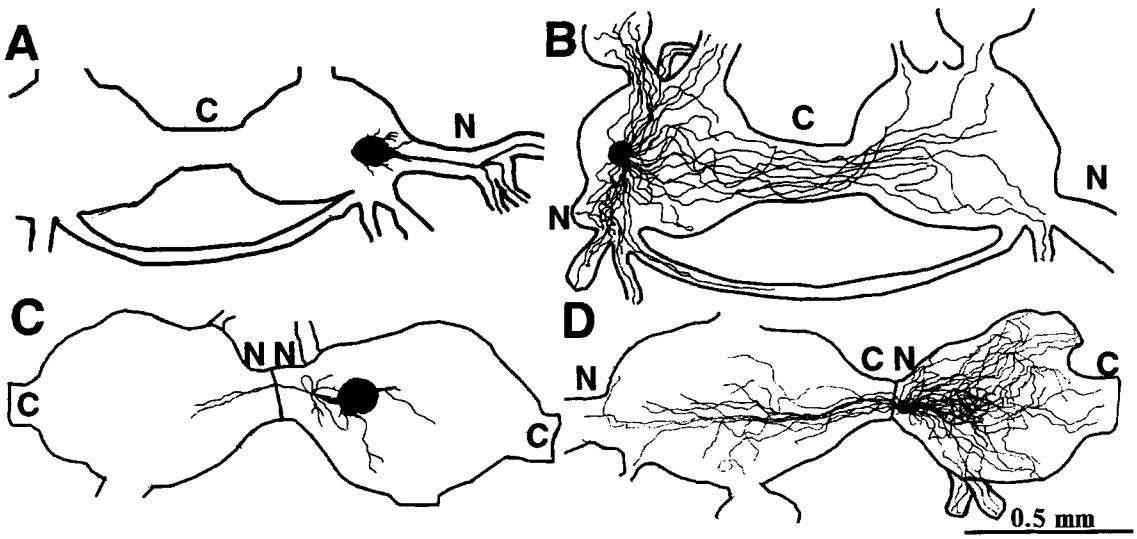

Fig. 8. Morphology of 1A motoneuron in the absence of the muscle target. **(A)** *Normal central morphology of motoneuron 1A in freshly isolaed pair of pedal ganglia (PPG). Sprouting of motoneuron 1A in culture* **(B-D).** **(B)** *PPG preparation 6 days in culture.* **(C)** *NN preparation 3 days in culture.* **(D)** *CN preparation 6 days in culture (cell body was destroyed during fixation and only initial axon is present). C-pedal missure, N- the wing nerve.*

two ganglia were attached to each other by spearing them with a thin glass pin. (A regular microelectrode was used to string two ganglia in a close contact. Two preparations of this type were made). In preparations, where no spearing was used, the result was essentially the same (Fig. 9D). The number of fibers crossing the border between two ganglia varied from 1 to 5-6 (Fig. 9C-E). In three CC preparations the pedal ganglia were taken from different animals. In two of them interneurons outgrew the contact. In CN (n = 5), NN (n = 3) and single pedal ganglion preparations (n = 4), the 'return loop' was similar to that in CC preparations, but no growth into the opposite ganglion was observed (Fig. 9F). In most cases, where interneuron sprouting was present, it was restricted to a limited area of the ganglia, forming rather compact bundle of neurites. Peripheral projections from these interneurons were not observed.

Functional regeneration of interneurons

In cultured pedal ganglia preparations, it was possible to record from identified moto- and interneurons several times to check whether the synchronization which is typical to intact CNS took place. No synchronization was observed in NC and NN preparations. In 7 of 10 CC preparations, after three to nine days in culture, left and right pedal ganglia re-established some extent of synchronization of their locomotor rhythms. Fig. 10 depicts the recordings of neurons from the same CC preparation taken after 3 and 5 days in culture. Locomotor rhythmical generation was present in both ganglia, and the locomotor neurons from the same ganglion were oscillating in the same rhythm and phase relations as seen in the control preparations (Fig. 3B). 1AL and 2AL motoneurons were firing in antiphase. In contrast, locomotor neurons from left and right ganglia were oscillating independently. In the first cycle on Fig. 10A, 2AR neuron received excitatory potentials in phase with 2AL, while in the seventh cycle its excitation coincided with IPSP in 2AL. Two days later, these two ganglia became well synchronized (Fig. 10B). In this preparation, the synchronized pattern of activity continued for hours, without failure, and it resembled very much that in the ganglia with intact commissure. However the connections between these two ganglia were not as strong as seen in the intact pairs of ganglia. When interneurons were hyperpolarized, the probability of their activation was reduced and occasionally single cycles were skipped (Arshavsky *et al*, 1985c). Fig. 10C illustrates an example where one swimming cycle was missing on the left side, while the generation continued on the right side. In motoneuron 2AL, during this missing cycle, there was no trace of postsynaptic potentials corresponding to PSPs in the motoneuron from the right ganglion. This indicates that interneurons from the right ganglion did not form direct synapses on motoneurons from the opposite ganglion at this stage of regeneration. In contrast, in 7L interneuron, a small depolarization was apparent during the missing cycle which occurred in synchrony with the IPSP in 2AR (this reflects the firing of 7R interneurons). This indicates that excitatory connections were formed between the interneurons of the same swimming phase from different ganglia.

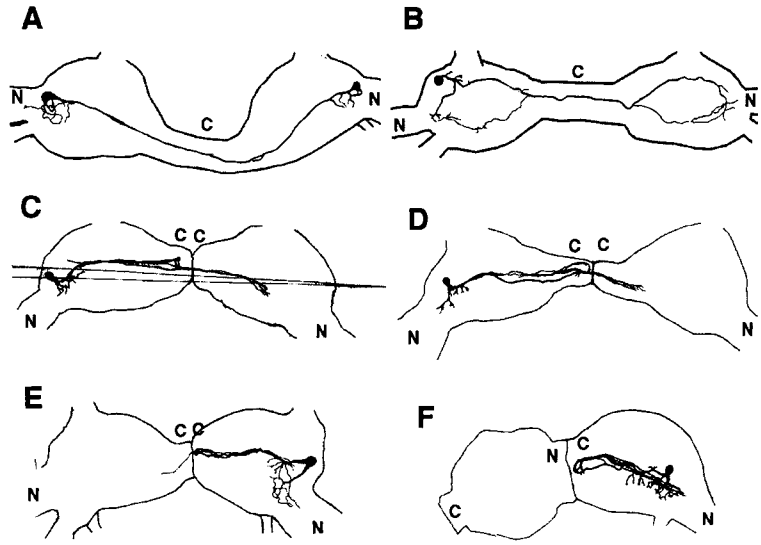

Fig. 9. Normal morphology (A,B) and sprouting (C-F) of interneurons. **(A)** *Two type 7 neurons from the dorsal surface were stained in the freshly dissected preparations. Neurons from cultured ganglia in* **C** *to* **F** *were of the same morphological type as shown in* **A.** **(B)** *Type 7 interneuron with a different morphology from the ventral side of the pedal ganglion from freshly dissected preparation.* **(C-E)** **CC** *preparations. A microelectrode was used to fix two ganglia together in* **C.** **(C, D)** *5 days in culture,* **(E)** *3 days.* **(F)** *CN preparation cultured for 7 days.*

In most CC preparations, it was not necessary to hyperpolarize interneurons to demonstrate that the synchronization was not absolutely reliable. Fig. 10D shows an example of such CC preparation, after 5 days in culture. A type 7 interneuron was recorded with 3L motoneuron from the opposite ganglion. Synchronous firing of both ganglia was interrupted when one cycle was missing spontaneously on the right side. The arrowhead shows EPSP in D-phase interneuron 7R in response to D-phase activation at the left ganglion as indicated by the firing of 3L motoneuron. Another example of partial synchronization is shown at Fig. 10E. In this case, V-phases of two ganglia were apparently synchronized by type 8 interneurons as IPSP of type 7R neurons coincided with 2AL excitations, although a short period of dissynchronization was present in this recording. In the right ganglion, an unusual pattern of generation with D-phase interneurons firing twice in one cycle was present and there was no detectable correlation between 7R excitations and 2AL IPSPs. In some experiments, both phases appeared to be equally synchronized, while in others, one half center (V or D) from left and right ganglia was synchronized better than another.

Discussion

Moto- and interneurons of an adult *Clione* can regenerate their functional connections

In this paper the regeneration of the central and peripheral connections and the neurite outgrowth of the identified locomotor neurons after axotomy were studied.

When motoneuron axons were crushed in the nerve regeneration occurred which resulted in a complete restoration of function. (Although in this study we did not show that the newly formed synapses were indeed monosynaptic but, based on previously published studies of normal neuromuscular synapses in *Clione* (Satterlie, 1993), they are most likely to be direct.) Earlier studies in *Clione* have demonstrated that in whole animals following the wing nerve transection the motoneurons grow new neurites and project at some distance towards the periphery (up the body wall). These studies suggested that these processes may reach the wing and restore the innervation. (Arshavsky et al., 1985e). This study however shows that if the nerve is cut, reinnervation does not occur even 2 months after operation.

We also showed that when two isolated pedal ganglia were co-cultured *in vitro* in a position with pedal commissure reconnected, swimming interneurons were able to form correct connections and they synchronized the locomotor rhythm in two pedal ganglia which resembled that seen *in vivo*. Since regeneration occurred in the cultured ganglia in the absence of muscles, actual movements are not necessary for correct connectivity of interneurons. It would be interesting to know whether the activity is required at all for correct regeneration. This kind of *in vitro* preparation may

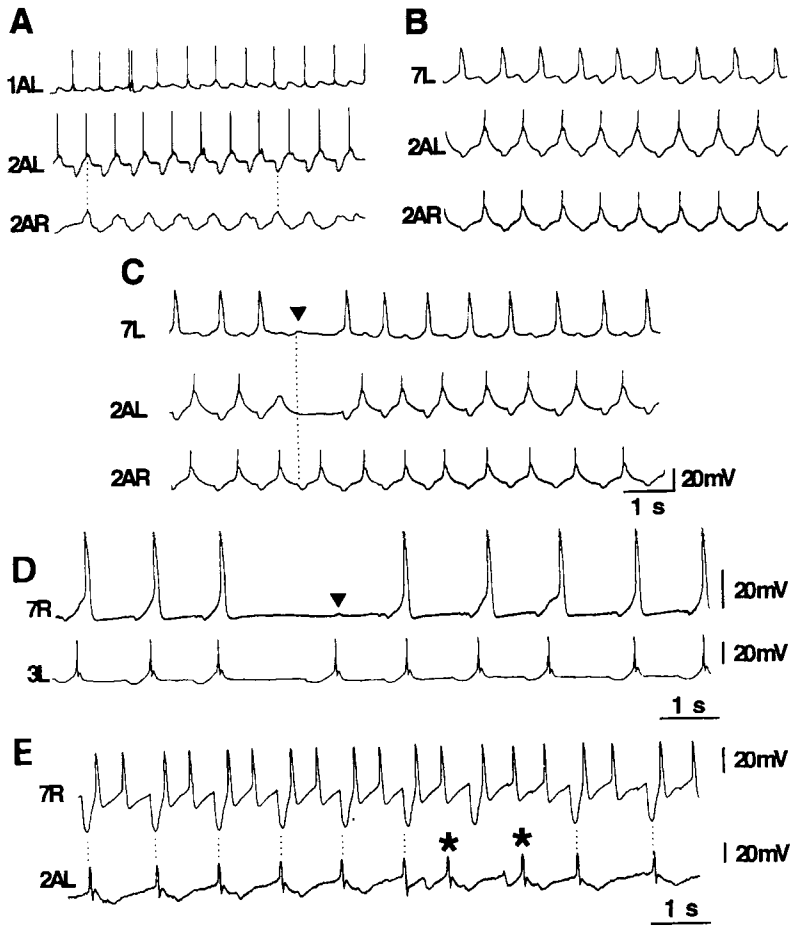

Fig. 10. The synchronization of locomotor rhythm in CC preparation. (A) *The lack of synchronization in CC preparation after 2 days in culture.* **(B)** *Strong functional regeneration after 5 days.* **(C)** *Constant injection of hyperpolarizing current (-2 nA) applied to 7L interneuron disrupts the synchronization. The arrowhead indicates a small EPSP in 7L interneuron in phase with IPSP in 2AR motoneuron (dotted line). Scalebars in* **A** *and* **B** *are the same as in* **C**. **(D-E)** *Examples of instability in synchronization in CC preparations. Two CC preparations cultured for 5 days display a different degree of synchronization between V and D half-centers of the left and right ganglia. The arrowhead in* **D** *indicates a small depolarization in type 7 interneuron from the right ganglion in phase with excitation of D-phase motoneuron (3 L) from the left ganglion. Asterisks in* **E** *indicate cycles in which V-phase in the left ganglion (monitored by 2AL motoneuron excitation) is asynchronous with V-phase in the right ganglion (as monitored by IPSPs in 7R interneuron). Dotted lines indicate V-phase of locomotor cycle synchronized in both ganglia.*

rect regeneration. This kind of *in vitro* preparation may allow us to approach this question. The synchronization is re-established due to the regeneration of positive (probably electrical) connections of synergistic interneurons 7 to 7 and 8 to 8 from the two ganglia. Thus, although morphological data presented in this paper (due to technical limitations) were restricted to type 7 interneurons, electrophysiological analysis suggested that type 8 interneurons also regenerate their connections. Another type of neuron that might participate in synchronization of the left and right sides is type 12 (Arshavsky *et al.*, 1985d; Arshavsky *et al.*, 1989). Preliminary results obtained from whole animals with crushed commissure showed that type 12 neurons failed to cross the site of damage even after 3 months (Panchin, unpublished data). We suggested that they may not be required for synchronization of rhythmical activity in the cultured pedal ganglia.

Formation and elimination of abundant connections during motoneuron regeneration

The process of restoration of the correct neuromuscular connections in Clione included an intermediate stage when muscle fibers display correct, incorrect and mixed innervation. Later the selection started and the number of wrong neurites and neuromuscular junctions began to decrease so that finally all the wrong axons were withdrawn and the muscles were innervated only by appropriate motoneurons. The situation in which initial abundant connectivity is shaped into the final pattern by elimination of incorrect or redundant synapses has been described in several cases of regeneration and development of the nervous system of invertebrates and vertebrates. In cockroach, at an early stage of regeneration, many axotomized motoneurons of the leg nerve send axonal processes into the muscle 178. In intact animals this muscle receives innervation from only one motoneuron. At later stages of regeneration these inappropriate axonal branches become eliminated until this muscle is again innervated by its single motoneuron (Denburg, 1982). Formation and subsequent elimination of redundant connections were also shown in crayfish neuromuscular regeneration (Goransson *et al.*, 1988; Hunt and Velez, 1989). Formation of the transient novel connections between regenerating neurons was observed in molluscs (Bulloch and Kater, 1982; Cohan *et al.*, 1987). In other cases in molluscs, however, the restoration of initial innervation occurred without aberrant connections (Benjamin and Allison, 1985; Murphy and Kater, 1978; Ross *et al.*, 1994).

The best known examples of synapse elimination come from the development and regeneration of the motor and visual systems in vertebrates. In neonatal skeletal muscles each muscle fiber is innervated by several motoneurons. The normal adult pattern of innervation in which each muscle fiber receives input from only one motoneuron subsequently emerges using activity-dependent mechanisms; blockage of activity prevents or delays this process of synapse elimination (Thompson *et al.*, 1979; Brown *et al.*, 1981a,b; for a review see Van Essen *et al.*, 1990). Mammalian motoneuron axons reach and synapse with their correct muscle targets without producing aberrant branches and connections to other muscles. The mechanism of synapse elimination is used only for the refinement of projections within correctly selected muscle (Jansen and Fladby, 1990). In contrast, during the intermediate stage of *Clione*'s neuromuscular regeneration each motoneuron can innervate both dorsal and ventral muscles and elimination of incorrect processes and synapses provides precise final specificity of innervation of two antagonistic muscles and the correct functioning of the wing. As the elimination of synapses in motor and visual systems of vertebrates involves activity-dependent mechanisms (Ribchester and Taxt, 1983; Ribchester, 1988) it is possible that a similar mechanism could play some role in synapse elimination in *Clione*'s neuromuscular regeneration. The motoneurons of the dorsal and ventral pools fire reciprocally while the activity within the same pool is strongly synchronized. Thus the electrical activity could serve as a good base for the competition between two different pools and elimination of aberrant synapses and branches. Since we observed correct reinnervation of initially totally denervated muscles, the hypothesized activity-dependent mechanism cannot be the single mechanism working in this system.

Growth restrictions for moto- and interneurons

We have previously shown in experiments on the whole animals, that in the course of regeneration interneuron neurites never leave the CNS and grow into peripheral nerves (Arshavsky *et al.*, 1985e; Panchin, 1984). Present data on cultured ganglia confirm this observation. In whole animals peripheral nerves were attached to their targets. This could be why the interneurons ignored them. This, however, was not the case as in *in vitro* preparations, the pattern of growth of the interneurons was the same as in whole animals. Moreover, when the nerve stump contacted the stump of the commissure in CN preparations and thus was located immediately next to the origin of interneurons' sprouting, no fibers crossed the contact. At the same time, CC contacts produced no obstacle for growing fibers. A possible explanation of these results is the existence of repulsive signals in the peripheral nerves. The present data demonstrate that the pattern of outgrowth of interneurons is very similar in different preparations and is restricted to limited areas of neuropile, suggesting that growing fibers follow the same predetermined routes in the ganglia. These routes might be delineated by attractive or repulsive cues, or new fibers may fasciculate to particular pre-existing pathways. In contrast to interneurons, motoneurons showed no visible restrictions to their growth. They outgrew into all possible nerve tracts and crossed the border between two adjacent ganglia in all types of preparations used in this paper.

The growth of neurons during regeneration was also studied in other invertebrates. In some cases new processes of neurons follow the routes of their original axons (motoneuron B15 in *Aplysia* (Ross *et al.*, 1994), giant serotoninergic cerebral cell in *Achatina* (Chiasson *et al.*, 1994) and in *Helisoma* (Murphy *et al.*, 1985)). In other cases the neurons can produce aberrant branches (RPeD1 cell in *Lymnaea* (Allison and Benjamin, 1985), buccal neurons in *Helisoma* (Murphy and Kater, 1980a,b; Bulloch and Kater, 1982; Hadley *et al.*, 1982), motoneurons in cockroach (Denburg, 1982)), although some predominant growth directions were observed. The neurons mentioned above originally grow into peripheral nerves and their behavior is similar to that of motoneurons in *Clione*. Such a case was reported for interneurons when regenerating cells outgrew to peripheral nerves (medial giant interneuron in cricket (Roederer and Cohen, 1983)). In another system (S-interneuron in leech (Muller and Carbonetto, 1979)) the growth of the neuron was restricted to CNS and new neurites could only grow along their surviving separated axon. Our experiments in *Clione* with pedal ganglia co-cultured in close contact were similar to those made previously in *Helisoma*. In *Helisoma* the cut peripheral nerve trunks from the buccal ganglia were attached by their distal ends (Hadley *et al.*, 1982). The B4 motoneurons formed electrical connections in the region of attached stumps. Thus the contact of two nerves was not an obstacle for motoneuron outgrowth.

Novel electrical connections

No unusual electrical connections were found in this study, although they have previously been reported in molluscan ganglia (Cohan *et al.*, 1987; Hadley *et al.*, 1982). These 'incorrect' electrical connections are rather common in culture of the isolated cells (Bodmer et al., 1984; Carrow and Levitan, 1989; Hadley *et al.*, 1983; 1985). Preliminary study of *Clione*'s identified motoneurons 1A and 2A in cell culture shows that

electrical connections could be formed in 1A-2A pairs as well as in 1A-1A and 2A-2A pairs (Popova *et al.*, 1994), but they make no electrical connections to some other identified neurons (Panchin *et al.*, 1995).

Acknowledgements

This work was supported by RFBR grants 96-04-49115 and 96-04-63106 and by Priority trends in genetics grant 2-044. We thank C. Page and N. Panchina for useful assistance and Dr. P. Katz for comments on the manuscript.

References

Allison, P. and Benjamin, P. R. (1985) Anatomical studies of central regeneration of an identified molluscan interneuron. *Proc. R. Soc. Lond.*, **B226**, 135-157.

Arshavsky, Yu. I., Beloozerova, G. N., Orlovsky, G. N., Panchin, Yu. V. and Pavlova, G. A. (1985a) Control of locomotion in marine mollusc *Clione limacina*. I. Efferent activity during actual and fictious swimming. *Exp. Brain Res.*, **58**, 255-262.

Arshavsky, Yu. I., Beloozerova, G. N., Orlovsky, G. N., Panchin, Yu. V. and Pavlova, G. A. (1985b) Control of locomotion in marine mollusc *Clione limacina*. II. Rhythmic neurons of pedal ganglia. *Exp. Brain Res.*, **58**, 263-272.

Arshavsky, Yu. I., Beloozerova, G. N., Orlovsky, G. N., Panchin, Yu. V. and Pavlova, G. A. (1985c) Control of locomotion in marine mollusc *Clione limacina*. III. On the origin of locomotor rhythm. *Exp. Brain Res.*, **58**, 273-284.

Arshavsky, Yu. I., Beloozerova, G. N., Orlovsky, G. N., Panchin, Yu. V. and Pavlova, G. A. (1985d) Control of locomotion in marine mollusc *Clione limacina*. IV. Role of type 12 interneurons. *Exp. Brain Res.*, **58**, 285-293.

Arshavsky, Yu. I., Gelfand, I. M., Orlovsky, G. N., Pavlova, G. A., Panchin, Yu. V. and Popova, L. B. (1985e) Regeneration of pedal ganglion neurons in the sea butterfly *Clione limacina*. *Nejrofiziologia*, **17**, 449-455. (in Russian).

Arshavsky, Yu. I., Orlovsky, G. N., Panchin, Yu. V. and Pavlova, G. A. (1989) Control of locomotion in marine mollusc *Clione limacina*. VII. Reexamination of type 12 interneurons. *Exp. Brain Res.*, **78**, 398-406.

Benjamin, P. R. and Allison P. (1985) Regeneration of excitatory, inhibitory and biphasic synaptic connections made by a snail giant interneuron. *Proc. R. Soc. Lond.*, **B226**, 159-176.

Bodmer, R., Dagan, D. and Levitan, I. B. (1984) Chemical and electrotonic connections between *Aplysia* neurons in primary culture. *J. Neurosci.*, **4**, 228-233.

Brown, M. C., Holland, R. L. and Hopkins, W. G. (1981a) Restoration of focal multiple innervation in rat muscles by transmission block during a critical stage of development. *J. Physiol.*, **318**, 355-364.

Brown, M. C., Holland, R. L. and Hopkins, W. G. (1981b) Motor nerve sprouting. *Ann. Rev. Neurosci.*, **4**, 17-42.

Bulloch, A. G. and Kater, S. B. (1982) Neurite outgrowth and selection of new electrical connections by adult *Helisoma* neurons. *J. Neurophysiol.*, **48**, 569-583.

Carrow, G. M. and Levitan, I. B. (1989) Selective formation and modulation of electrical synapses between cultured *Aplysia* neurons. *J. Neurosci.*, **9**, 3657-3664.

Chiasson, B. J., Baker, M. W. and Croll, R. P. (1994) Morphological changes and functional recovery following axotomy of a serotonergic cerebrobuccal neuron in the land snail *Achatina fulica*. *J. Exp. Biol.*, **192**, 147-167.

Cohan, C. S., Haydon, P. G., Mercier, A. J. and Kater, S. B. (1987) Formation, maintenance, and functional uncoupling of connections between identified *Helisoma* neurons *in situ* (published erratum appears in *J. Neurobiol.*, (1987) **18**, 583). *J. Neurobiol.*, **18**, 329-341.

Denburg, J. L. (1982) Elimination of inappropriate axonal branches of regenerating cockroach motoneurons as detected by the retrograde transport of horseradish peroxidase conjugated wheat germ agglutinin. *Brain Res.*, **248**, 1-8.

Fredman, S. M. and Nutz, P. G. (1988) Regeneration of identified neurons and their synaptic connections in the central nervous system of *Aplysia*. *Am. Zool.*, **28**, 1099-1108.

Goransson, L. G., Hunt, W. P. and Velez, S. J. (1988) Regeneration studies on a crayfish neuromuscular system. II. Effect of changing the nerve entry point into the muscle field on the gradient of innervation. *J. Neurobiol.*, **19**, 141-152.

Hadley, A. D., Bodnar, D. A. and Kater, S. B. (1985) Formation of electrical synapses between isolated, cultured *Helisoma* neurons requires mutual neurite elongation. *J. Neurosci.*, **5**, 3145-3153.

Hadley, A. D., Kater, S. B. and Cohan, C. S., (1983) Electrical synapse formation depends upon interaction of mutually growing neurites. *Science*, **221**, 466-468.

Hadley, R. D., Wong, R. G., Kater, S. B., Barker, D. L. and Bulloch, A. G. (1982) Formation of novel central and peripheral connections between molluscan central neurons in organ cultured ganglia. *J. Neurobiol.*, **13**, 217-230.

Hunt, W. P. and Velez, S. J. (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.

Jansen, J. K. and Fladby, T. (1990) The perinatal reorganization of the innervation of skeletal muscle in mammals. *Prog. Neurobiol.*, **34**, 39-90.

Moffet, S.B. (1995) Neural regeneration in gastropod molluscs. *Prog. Neurobiol.* **46**, 286-330.

Muller, K. J. and Carbonetto, S. (1979) The morphological and physiological properties of a regenerating synapse in the CNS of the leech. *J. Comp. Neurol.*, **185**, 485-516.

Murphy, A. D. and Kater, S. B. (1978) Specific reinnervation of a target organ by a pair of identified molluscan neurons. *Brain Res.*, **156**, 322-328.

Murphy, A. D. and Kater, S. B. (1980a) Sprouting and functional regeneration of an identified neuron in *Helisoma*. *Brain Res.*, **186**, 251-272.

Murphy, A D. and Kater, S. B. (1980b) Differential discrimination of appropriate pathways by regenerating identified neurons in *Helisoma*. *J. Comp. Neurol.*, **190**, 395-403.

Murphy, A. D., Barker, D. L., Loring, J. F. and Kater, S. B. (1985) Sprouting and functional regeneration of an identified serotonergic neuron following axotomy. *J. Neurobiol.*, **16**, 137-151.

Panchin, Yu. V. (1984) Synchronization of activity in pedal ganglia of pteropodal mollusc during locomotion. *Nejrofiziologia*, **16**, 540-543. (in Russian).

Panchin, Y. V., Popova, L. B., Pavlova, G. A., Zelenin, P. V. and Arshavsky, Y. I. (1995a) Formation of connections between cultured identified neurons from the pleural ganglion of the pteropod mollusc *Clione limacina*. *Brain Res.*, **669**, 315-319.

Panchin Y., Popova, L., Zelenin, P. and Sadreev, R. (1995b) Formation of synaptic connections in the pteropodal mollusc *Clione limacina*: regeneration and cell culture studies. *Society for Neuroscience. Abstracts*, **21**, part 3. 707.17.

Panchin, Y. V., Sadreev, R. I. and Arshavsky, Y. I. (1996) Control of locomotion in marine mollusc *Clione limacina*., X. Effects of acetylcholine antagonists. *Exp. Brain Res.*, **109**, 361-365.

Panchin, Y. V., Zelenin, P. V. and Popova, L. B. (1996) Regeneration of the neuromuscular connections in the locomotor system of the pteropod mollusc *Clione limacina*. *Society for Neuroscience. Abstracts*, **22**, part 1. 259.3.

Popova L. B., Panchin Y. V., Pavlova G. A., Zelenin P. V., Sadreev R.I. and Arshavsky Y.I. (1994) Formation of synaptic connections in the pteropod mollusc *Clione limacina*: regeneration and cell culture studies. *Abstracts of International Symposium on Intercellular Communications. Puschino*, RAS, Moscow 27.

Ribchester, R. R. and Taxt, T. (1983) Motor unit size and synaptic

competition in rat lumbrical muscles reinnervated by active and inactive motor axons. J. Physiol., **344**, 89-111.

Ribchester, R. R. (1988) Activity-dependent and -independent synaptic interactions during reinnervation of partially denervated rat muscle. *J. Physiol.*, **401**, 53-75.

Roederer, E. and Cohen, M. J. (1983) Regeneration of an identified central neuron in the cricket. I. Control of sprouting from soma, dendrites, and axon. *J. Neurosci.*, **3**, 1835-1847.

Ross, T. L., Govind, C. K. and Kirk, M. D. (1994) Neuromuscular regeneration by buccal motoneuron B15 after peripheral nerve crush in *Aplysia californica*. *J. Neurophysiol.*, **72**, 1897-1910.

Satterlie, R. A (1985) Reciprocal inhibition and postinhibitory rebound produce reverberation in a locomotor pattern generation. *Science*, **229**, 402-404.

Satterlie, R. A., LaBarbera, M. and Spencer, A. N. (1985) Swimming in the pteropod mollusc *Clione limacina*. 1. Behavior and morphology. *J. Exp. Biol.*, **116**, 189-204.

Satterlie, R. A. and Spencer, A. N. (1985) Swimming in the pteropod mollusc *Clione limacina*. 2. Physiology. *J. Exp. Biol.*, **116**, 205-222.

Satterlie, R. A (1991) Electrophysiology of swim musculature in the pteropod mollusc *Clione limacina*. *J. Exp. Biol*, **159**, 285-301.

Satterlie, R. A (1993) Neuromuscular organization in the swimming system of the pteropod mollusc *Clione limacina*. *J. Exp. Biol.*, **181**, 119-140.

Scott, M. L., Li. Y. and Kirk, M. D. (1995) Functional neural regeneration in the feeding system of *Aplysia*: behavioral recovery correlated with changes in buccal motor output. *J. Neurophysiol.*, **73**, 39-55.

Thompson, W., Kuffler, D. P. and Jansen, J. K. (1979) The effect of prolonged, reversible block of nerve impulses on the elimination of polyneuronal innervation of new-born rat skeletal muscle fibers. *Neuroscience*, **4**, 271-281.

Van Essen, D. C., Gordon, H., Soha, J. M. and Fraser, S. E. (1989) Synaptic dynamics at the neuromuscular junction: mechanisms and models. *J. Neurobiol.*, **21**, 223-249.

Accepted 6 January 1998