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Selective regeneration of the neuromuscular connections in the pteropod mollusc *Clione limacina*

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Abstract

In the pteropod mollusc *Clione limacina*, two different groups of motoneurons innervate two physiologically identical wing muscles (dorsal and ventral). When motoneuron axons are crushed in the nerve of whole animals regeneration starts. In its course motoneurons initially project to both the correct and incorrect muscles. Then incorrect connections and neurites are eliminated and the original innervation is restored. Here we investigated neuromuscular regeneration when one of the muscles was removed or the muscle size was reduced. Motoneurons formed both correct and incorrect connections not only *in vitro* when the pedal ganglion was attached to only one of two wing muscles, but also in whole-animal 'no choice' experiments when only one muscle was available for reinnervation. In these experiments incorrect connections were stable and were not eliminated at the later stages, as happened in experiments in which both muscles were accessible. In whole-animal experiments with reduced size of the muscles, a normal pattern of regeneration was conserved although not all incorrect connections were eliminated. Thus, in the course of regeneration: (i) locomotor motoneurons make connections with both correct and incorrect muscles; (ii) if for some group of motoneurons the correct targets are unavailable, the incorrect connections survive and become stable; (iii) if both groups of motoneurons have a choice between the correct and incorrect targets, initial mixed innervation is replaced by purely correct innervation; (iv) elimination of incorrect synapses could be a result of the competition between correct and incorrect synapses of the same neuron.

Introduction

Since the classical works of Redfern (1970), neuromuscular connections are a popular system for studying target selection, formation of connections, and maturation and plasticity of synapses. He has shown that, initially, mammalian embryonic skeletal muscles are innervated by several motoneurons. In the normal course of development, excess connections are withdrawn, so that each muscle fibre is innervated by exactly one motoneuron (for review, see Jansen & Fladby, 1990; Hall & Sanes, 1993). This sequence of events is also conserved during neuromuscular regeneration (Gorio *et al*., 1983; Ribchester & Taxt, 1983). It was concluded that synapse elimination is a result of competitive interactions between terminals of different motoneurons formed on the same muscle fibre (Thompson *et al*., 1979; Brown *et al*., 1981; Ridge & Betz, 1984; Callaway *et al*., 1987). A similar situation is described for reinnervation of phasic and tonic muscles in frogs and birds (Feng & Lu, 1965; Hnik *et al*., 1967). On the other hand, it has been demonstrated that motoneurons are able to support and maintain only a limited number of synapses and that the size of the available field of innervation may influence synapse removal (Thompson & Jansen, 1977; Fladby & Jansen, 1987; Jansen & Fladby, 1990). This suggests another model for neuromuscular synapse withdrawal based on competition between different synapses of the same motoneuron on different muscle cells (Van Essen *et al*., 1989). In this paper we demonstrate that in pteropod mollusc *Clione limacina* the competition between synapses of the same neuron is implicated in the process of neuromuscular regeneration.

Clione (Fig. 1A) swims by rhythmical movements (1–2 Hz) of two wings. The wing motoneurons located in the pedal ganglia reach the muscles via the wing nerve. Two main populations of motoneurons (1 and 2) control dorsal (D) and ventral (V) wing muscles, respectively (Arshavsky *et al*., 1985a; Satterlie, 1993; Panchin *et al*., 1996). Each population comprises a dozen smaller cells and one large cell called 1A or 2A, respectively. During rhythmic locomotor activity, the two populations of wing motoneurons are active reciprocally. When the V-phase motoneurons are excited the D-phase ones are inhibited and *vice versa*. All motoneurons innervating the ventral muscles are strongly electrically coupled. The motoneurons supplying the dorsal muscles are coupled similarly. Due to this electrical coupling, and to the common input from pacemaker interneurons, the motoneurons of a given muscle fire synchronously. Thus, motoneurons provide alternating excitation of the wing muscles (Satterlie, 1993) (Fig. 1C). The neurotransmitter of these motoneurons has been shown to be acetylcholine (Panchin *et al*., 1996).

When axons of motoneurons are crushed in the nerve, regeneration starts and results in a complete restoration of function in 1–2 months (Panchin *et al*., 1998). After axotomy, numerous fine neurites start growing in the direction of the denervated muscles, and reach them in 8–15 days. At this stage, motoneurons display no preference for

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the dorsal or ventral muscles. Accordingly, muscle fibres display correct, incorrect and mixed innervation (Fig. 1D). After 2 weeks of regeneration, selection starts and numbers of incorrect neurites and neuromuscular junctions begin to decrease (Fig. 1E) so that in 1.5–2 months all incorrect axons are withdrawn and the muscles are innervated only by appropriate motoneurons (Fig. 1F). This sequence of events (Fig. 1D–F) will be referred to as 'standard regeneration'. The use of an *in vitro* preparation together with surgical modifications of whole-animal regeneration experiments reported below allow investigation of how the presence of the muscle target and its size affect selective formation and elimination of neuromuscular connections in *Clione*.

Materials and methods

This work was done at the White Sea Marine Biological Station 'Kartesh'. The experiments were done either on *in vitro* preparations or on whole animals.

In vitro *preparations*

For *in vitro* culturing, the dorsal or ventral muscle layer and CNS were dissected from the animals, briefly treated with 0.5% solution of Pronase E (1 min), rinsed in sea water and placed in 35-mm polystyrene dishes (Falcon 3001,) lined with 1% agarose gel in sea water (volume of the gel was 2 mL). The wing nerve of one pedal ganglion was cut short, put in a close contact with the muscle layer and fixed with a thin layer of thickening agarose gel. The gel was covered with 2 mL of modified Leibovitz-15 (L-15,) medium. Preparations were incubated at +5 °C. Most of the preparations were tested several times during culturing. Modified L-15 medium had the salinity adjusted to that of White Sea water, and 1 L of the final medium contained 14.8 g of L-15 powder medium, 50000 U Penicillin, 50 mg streptomycin, 200 mL distilled water and 800 mL sea water. All drugs were from Sigma.

Whole-animal preparations

For surgery, an animal was fixed in a Sylgard-coated dish in sterile sea water with antibiotics. A small incision in the skin was made above the wing nerve and different nerve branches were cut with scissors according to the type of experiment (the procedure enables cutting nerve branches without injuring muscles). Then the wing nerve was crushed with fine forceps close to the exit from the ganglion. In some experiments the distal two-thirds of the wing were removed. Then the animals were kept in a sterile tank with fresh sea water. In 2–60 days, the animals were dissected and preparations for intracellular recording and staining with Lucifer Yellow were made. To make the wing–CNS preparation, the wing was split open so that the D and V muscles were separated (see Fig. 1A–C). The intermediate retractor muscles were removed. The wing nerve has two main trunks, which carry axons to both D and V muscle layers [Fig. 1, anterior and posterior wing nerve trunks (NWA and NWP)]. These two main trunks give rise to secondary branches. All the secondary branches are attached only to D or V muscles and carry motoneuron axons of only appropriate phase. When the wing is opened as shown in Fig. 1, it is impossible to preserve all the secondary branches and in all preparations used in this work the 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. Then, ganglia with the wing were pinned on a layer of agarose gel and fixed with a drop of warm liquid agarose. These procedures restrained muscle twitching and improved the intracellular recordings from neurons and muscle cells.

Electrophysiology and morphology

Simultaneous intracellular recordings of two to four cells (neurons and muscle fibres) were carried out with 3 M KCl-filled glass microelectrodes having tip resistances of 30–70 MΩ.

The motoneurons innervating the same muscle are tightly electrically coupled and driven synchronously by interneurons. As a result, they fire closely although not quite simultaneously. Thus, each neuron of a given phase accurately mirrors the activity of the whole population. The largest D-phase motoneuron, 1A, and the largest V-phase motoneuron, 2A, are easily identifiable on the dorsal side of the pedal ganglia by 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. Other motoneurons have very similar physiological and morphological properties, yet are less suitable experimentally (See also Panchin *et al*., 1998). Normally, and during regeneration, the swimming muscles in *Clione* are innervated by more than one motoneuron of the same population (Satterlie, 1993; Panchin *et al*., 1998). The excitatory junction potential (EJPs) induced by activity in the motoneurons innervating the given muscle cell overlap. Thus, in muscle fibre recordings we observed compound EJPs in phase with activation of the motoneurons (Figs 1C–F, 2C–E).

During *in vivo* regeneration and *in vitro* culturing, there were no new connections between the neurons generating swimming pattern and no changes in the output of these neurons (Panchin *et al*., 1998). In particular, all wing motoneurons were active at the same phase of the swimming cycle as in controls. Thus all EJPs in the given phase of the cycle could be induced only by the motoneurons of the same phase, and the origin of EJPs in a given phase of the swimming cycle could be unambiguously attributed to one of the two populations of motoneurons (dorsal or ventral).

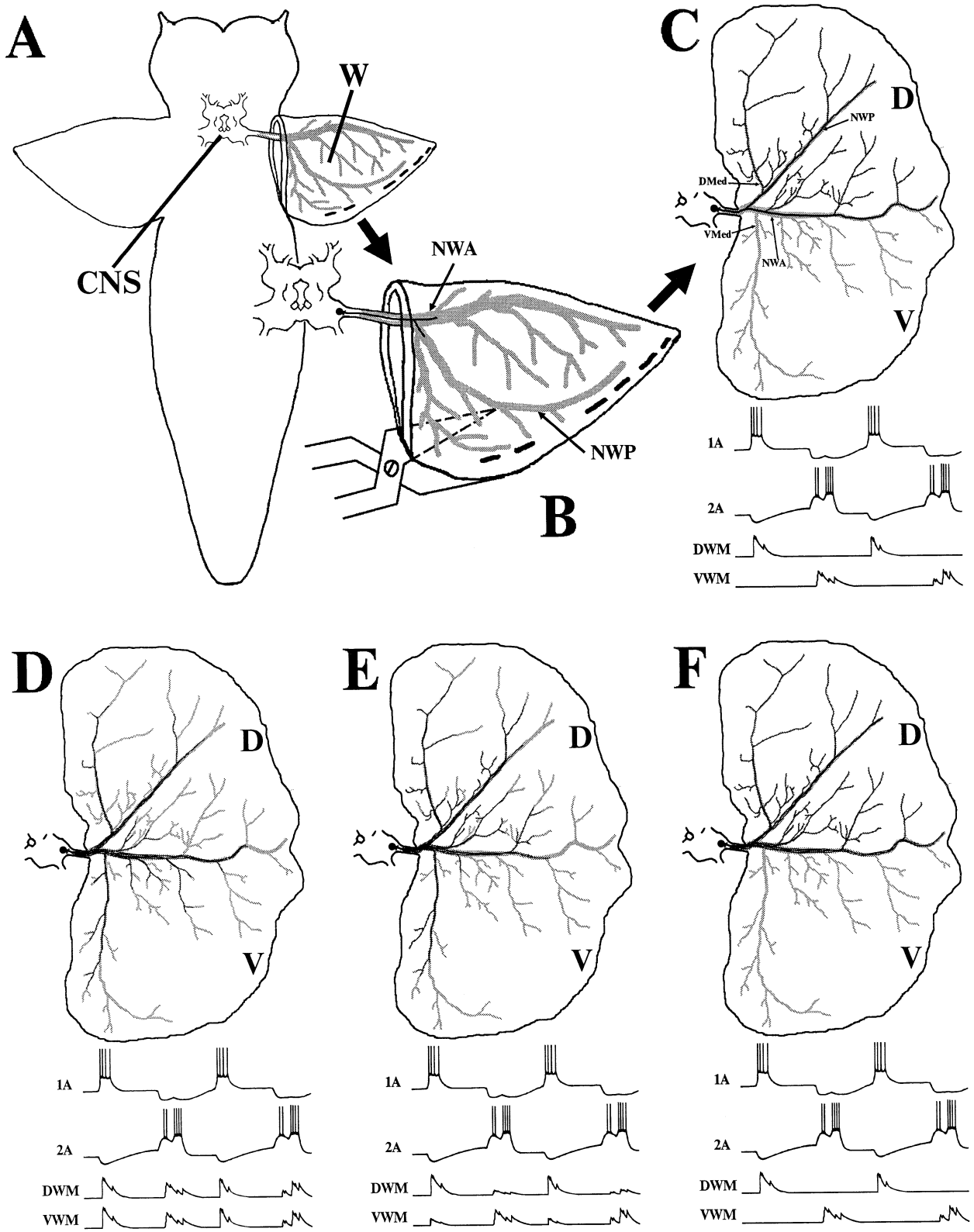
For morphological studies, neurons were injected with the fluorescent dye Lucifer Yellow CH, and photographed under a fluorescent microscope. Because of technical limitations, i.e. a large number of long thin neurites in stained cells that passed through different optical planes, so that some of them were not clear in photographs, drawings were made from the photographs and by use of a *camera lucida*.

Mathematical procedures

To measure the prevalence of the correct or incorrect innervation we used a parameter of 'asymmetry' *A*. For individual muscle cell it was calculated as:

$$A = (\Sigma U^{+}_{i} - \Sigma U^{-}_{i})/(\Sigma U^{+}_{i} + \Sigma U^{-}_{i}) \quad (1)$$

where U^{+}_{i} is the amplitude of correct EJPs and U^{-}_{i} is the amplitude of incorrect EJPs. Sums of EJP amplitudes from 10 sequential cycles (ΣU^{+}_{i}, ΣU^{-}_{i}, i taking values from 1 to 10) are used in Eqn 1 because EJPs in each phase are produced by several motoneurons and their amplitudes may vary from cycle to cycle. The parameter of asymmetry may range from $A = -1$ (only incorrect inputs on the given muscle cell, i.e. 'asymmetric purely incorrect innervation') to $A = +1$ (only correct inputs on the given muscle cell, i.e. 'asymmetric purely correct innervation'). $A < 0$ when incorrect innervation is dominating and the mean amplitudes of EJPs in correct phase are smaller than in incorrect. Accordingly when $A > 0$, correct innervation is dominating. $A = 0$ corresponds to equal values of the mean amplitudes


A
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CNS
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in both phases of locomotor cycle, i.e. 'totally symmetrical innervation'.

Asymmetry is linked to the mean amplitudes of correct and incorrect EJPs by a simple equation:

$$U^{+}_{m}/U^{-}_{m} = (1 + A)/(1 - A) \quad (2)$$

where U^{+}_{m} and U^{-}_{m} are, respectively, the mean amplitudes of correct and incorrect EJPs. We prefer to use A rather than U_m because the ratios U^{+}_{m}/U^{-}_{m} and U^{-}_{m}/U^{+}_{m} take infinite values when innervation is purely correct or incorrect.

Parameter A is also applicable to a group of muscle cells (muscle layer):

$$A = (\Sigma U^{+}_{ik} - \Sigma U^{-}_{ik})/(\Sigma U^{+}_{ik} + \Sigma U^{-}_{ik}) \quad (3)$$

where U^{+}_{ik} and U^{-}_{ik} are the amplitudes of correct and incorrect EJPs in muscle cell number k (k takes values from 1 to N; where N is the number of cells in the group).

The degree of competition between the two inputs to a given muscle cell was measured by the parameter 'purity of innervation' P.

$$P = |A| \quad (4)$$

For a group of cells,

$$P = \Sigma P_k/N \quad (5)$$

where N is the number of cells in the group and P_k is a purity of innervation of the muscle cell number k. P may take values from $P = 0$ (for each cell of the group correct and incorrect inputs are equal) to $P = 1$ (all cells in the group display either pure correct or pure incorrect innervation).

All the data are presented as means (± SD).

Results

In vitro *experiments*

Twenty eight *in vitro* preparations were examined after 2–34 days in culture. The first EJPs which were synchronous with locomotor rhythm in the ganglia could be recorded in muscle cells after 3 days in culture. At that time Lucifer Yellow staining of the motoneurons reveals that growing neurites span the contact area between the ganglion and the muscle layer and grow out into the muscle (Fig. 2A and B). Initially EJPs were recorded only in the muscle cells in the nearest vicinity from the contact. The innervated area gradually expanded with time. Nevertheless, even in the oldest preparations (34 days in culture), the innervated area covered only a portion of the muscle layer. In both modifications of *in vitro* preparations (with dorsal or with ventral muscle layers grafted to the pedal ganglion) muscle cells displayed EJPs in dorsal phase, the ventral phase or both mixed. No preference was noticed for correct or incorrect connections. Figure 2C–E represents some examples of the muscle recordings from the preparation in which the ventral muscle was cultured in contact with a wing nerve of the pedal ganglion. Some ventral muscles received EJPs synchronously with activation of the V-phase motoneuron 2A ('correct' innervation, Fig. 2C), some received EJPs during the dorsal phase of the locomotor cycle ('incorrect' innervation, Fig. 2D), others displayed different degrees of mixed innervation (Fig. 2E). Dorsal muscles were innervated in the same way as ventral. The averaged parameter of asymmetry A (see methods) for all muscles from all the preparations at any given period of culture was close to zero ($A = 0$). Yet in individual preparations one type of connection, correct or incorrect, could be more pronounced ($A > 0$ or $A < 0$, respectively; see Fig. 3A). With longer times in culture, the values of correct and incorrect connections, even for any individual preparation become closer and asymmetry approached zero (Fig. 3A). Asymmetry of innervation also declined in individual muscle fibres (Fig. 3B). Thus, the 'purity of innervation' parameter, P, was ≈ 0.75 at early stages of regeneration (that means that, on average, for a given muscle fibre one type of EJP was ≈ 7 times larger than another; Fig. 3C). At late stages it became ≈ 0.33 (on average the amplitude of one type of EJPs was just two times larger than that of another type). Present data shows that there was no domination of correct over incorrect connections at any stage of the *in vitro* experiments with only one muscle layer present. In standard regeneration in whole animals such symmetrical innervation took place only at the initial stage of regeneration and was later replaced by correct innervation. In *in vitro* preparations there were no signs of elimination of incorrect connections at any time. Moreover, if the connections from motoneurons of different phases competed for the same muscle cell we would expect that eventually one source of innervation would take over and the muscle cells would be innervated predominantly by the motoneurons of one phase (parameter of the innervation purity P would increase). Experimental data shown on Fig. 3C indicates that P is actually decreasing with time. That indicates that synapses of motoneurons of different phases peacefully coexist on the same muscle cells with no apparent interactions.

FIG. 1. Schematic representation of the wing motoneuron morphology and neuromuscular connections in *Clione* in a normal animal (A–C) and during 'standard regeneration' (D–F). (A and 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 using small scissors. The wing nerve with anterior (NWA) and posterior (NWP) main branches and a number of thinner secondary branches are shown in grey. (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 it 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 dorsal phase motoneuron 1A is shown. The axon of the 1A motoneuron contacts only the dorsal muscles. Below the view of the preparation is a scheme of the normal activity of the D-phase motoneuron 1A, the V-phase motoneuron 2A, and ventral (VWM) and dorsal (DWM) muscle cells during the locomotor rhythmic activity. Motoneurons of type 1 receive EPSPs in the D-phase and IPSPs in the V-phase. Motoneurons of type 2 receive EPSPs in the V-phase and IPSPs in the D-phase. Thus, DWM is excited in the D-phase and VWM is excited in the V-phase. Abbreviations used in this figure: D, dorsal muscle layer of the wing; V, ventral muscle layer of the wing; Dmed, dorsal medial secondary branch of the wing nerve; Vmed, ventral medial secondary branch of the wing nerve. (D–F) Schemes of motoneuron 1A's morphology and activity of neurons and muscles at different stages of regeneration are shown as in C. (D) Early stage of 'standard regeneration'. Motoneurons at this stage have neurites that project indiscriminately into the nerve branches that go to the dorsal and ventral muscles. At this stage muscle fibres of both muscle layers display excitatory junction potentials (EJPs) of approximately equal amplitude in both locomotor phases. (E) Intermediate stage of 'standard regeneration'. The number of neurites in the incorrect nerve branches is smaller than in correct branches and they are shorter. During this stage the number and the length of the correct neurites are increased, while incorrect fibres are gradually withdrawn. The incorrect EJPs are much smaller than correct ones. (F) Late stage of 'standard regeneration'. Only correct neurites are present. The overall number of neurites is reduced and they become thicker compared with the 11–25 days stage. Innervation looks quite normal; no trace of incorrect EJPs is present.

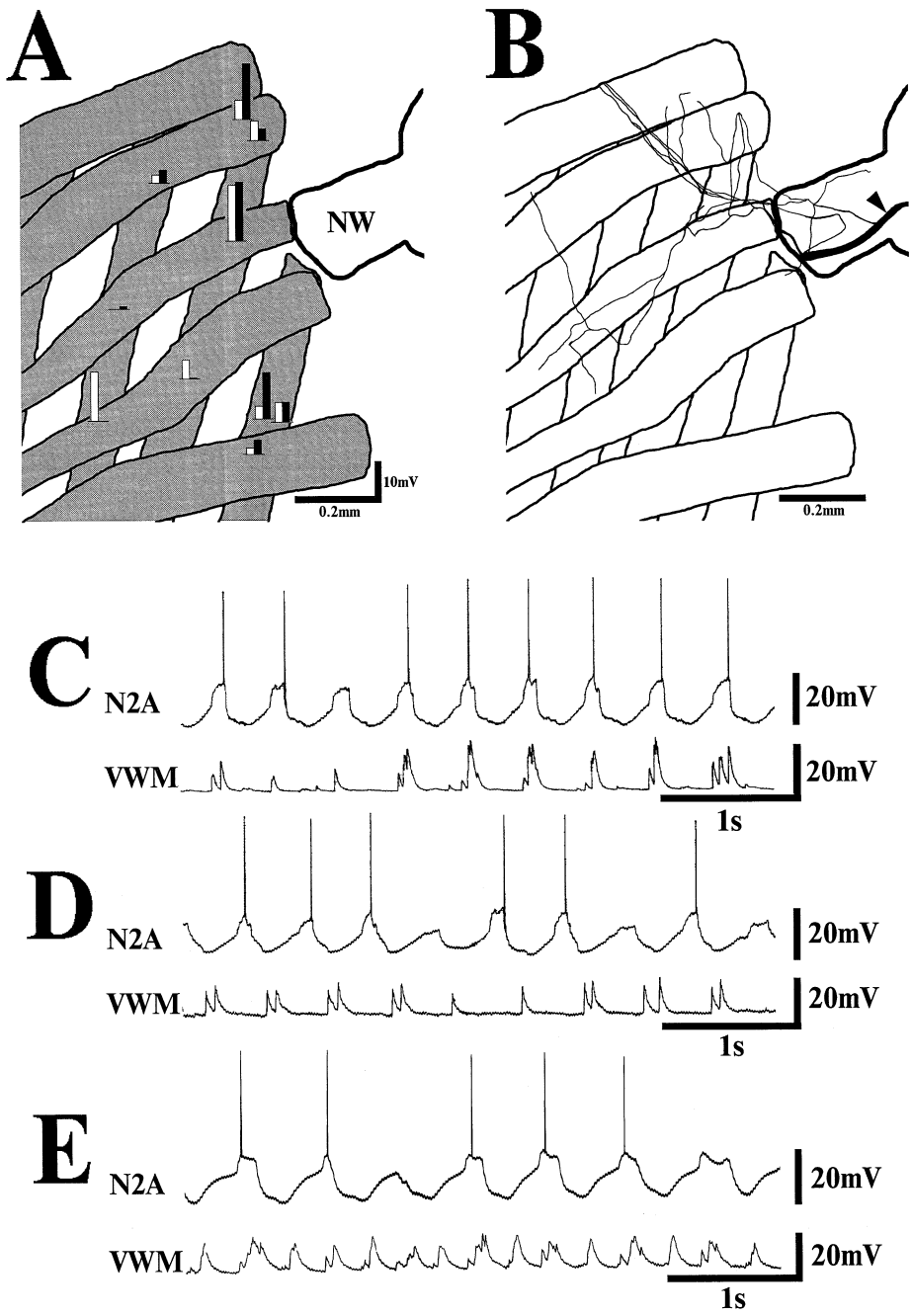

FIG. 2. *In vitro* reinnervation of muscles. (A) The stump of the wing nerve (NW) placed close to the dorsal wing muscle layer (bundles of muscle fibres are shown in grey). In 7 days, new connections are recorded. Mean amplitudes of correct and incorrect EJPs are shown by white and black columns, respectively. Each pair of columns is placed at the point where a given muscle cell was recorded. (B) The 2A motoneuron sprouting in the same preparation. A number of new thin neurites have crossed the site of contact between the wing nerve and the wing muscle layer and spread over the muscles. The distal part of the 2A motoneuron axon is marked by an arrowhead. (C–E) Examples of purely correct ($A = +1$), purely incorrect ($A = -1$) and mixed ($A \approx 0$) innervation from an *in vitro* preparation with the ventral muscle layer grafted to the pedal ganglion after 10-days in culture. Different ventral muscle cells (VWM) recorded together with the same 2A motoneuron.

There are two possible explanations of this data. One is trivial, and assumes that in *in vitro* conditions the mechanism of synapses withdrawal is, for some reason, impaired. Another explanation implies that withdrawal of the incorrect connections can take place only when the correct connections are also formed, so that synapses from the same neuron compete and correct connections induce elimination of incorrect synapses. The direct way to distinguish between these alternatives would be to culture ganglia with both muscle layers grafted to the same wing nerve. Unfortunately, these experiments are too difficult for technical reasons. Another way to check whether a correct target is required for elimination of incorrect connections is to leave only one muscle layer in the whole-animal regeneration experiments.

Whole-animal regeneration experiments with only one muscle layer available

Two secondary medial nerve branches (ventral and dorsal) leave the main nerve trunks proximally to the wing nerve entrance. When the main nerve trunks and one of the medial secondary branches are cut the nerve would be attached to only one muscle layer by the remaining medial branch (Fig. 4A). In the whole-animal preparations the growing fibres of regenerating motoneurons can reach the muscles only via the nerves. Therefore in the animals with all wing nerve branches but one cut off, only one muscle layer is accessible for reinnervation after the axons in the wing nerve are crushed. Thus, as with *in vitro* experiments, a situation could be created in the whole animals where only one muscle target is available. These whole-animal experiments will be called ‘no

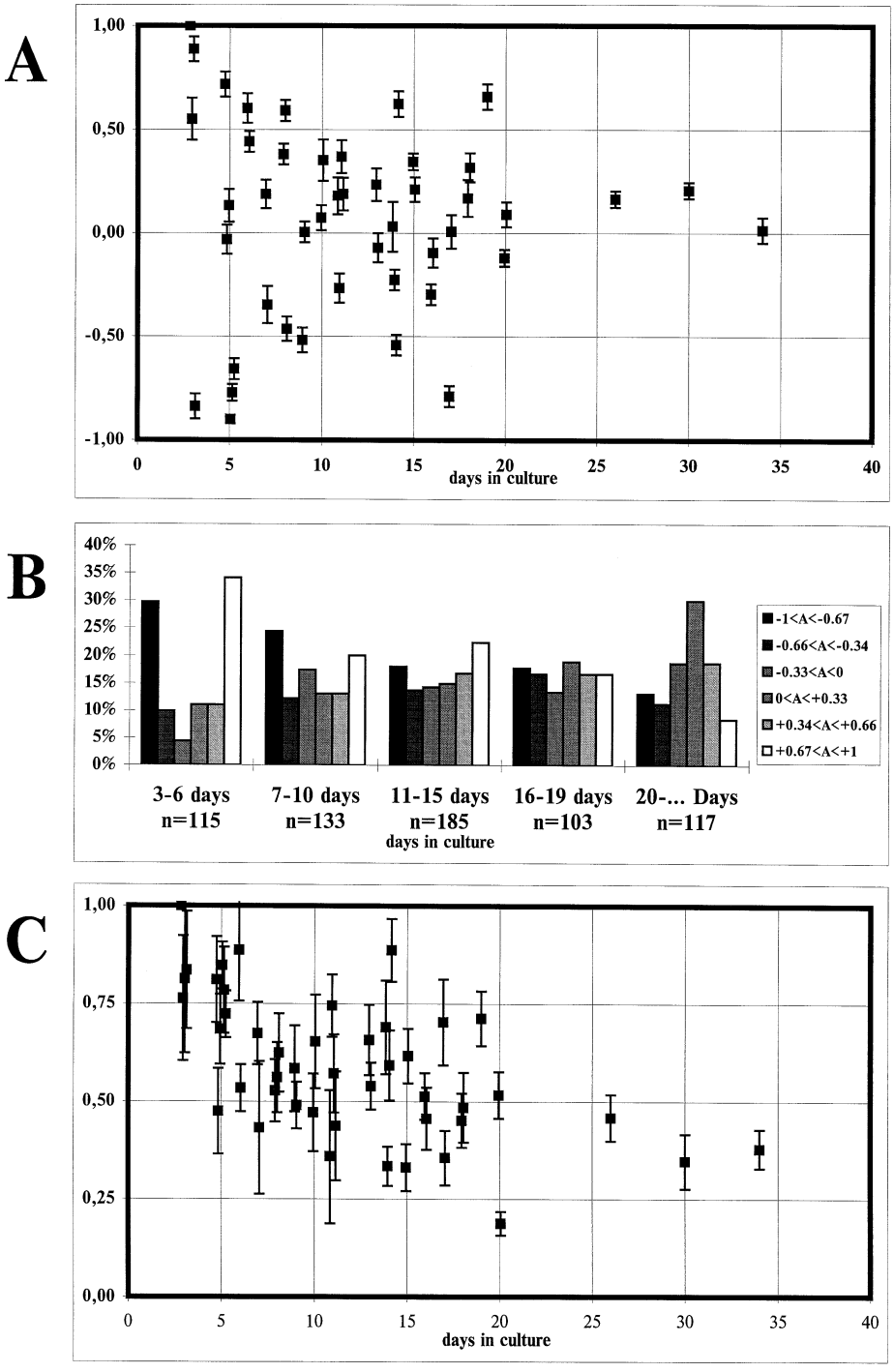

FIG. 3. Time dependence of innervation in *in vitro* experiments. (A) Time dependence of asymmetry of muscle cell innervation in the *in vitro* experiments. Each dot represents a mean value (± SD) of asymmetry of innervation, *A*, for all registered muscle cells from a given preparation. With time, the asymmetry approaches zero. This means that the efficacies of correct and incorrect innervation for any given preparation become closer to one another. (B) Distribution of asymmetry of innervation in the *in vitro* experiments. Six ranges of the parameter *A* are shown on the right. Each column represents a percentage of the muscle cells within a given range of asymmetry, *A*, at the stages indicated under histograms (*n* is the number of recorded muscle cells). More and more muscle cells become symmetrically innervated at the later stages, i.e. the mean amplitudes of incorrect and correct EJPs become closer for every muscle cell. (C) Time dependence of purity of muscle cell innervation in the *in vitro* experiments. Each dot represents a mean value (± SD) of purity of innervation, *P*, for all registered muscle cells from a given preparation. With time, purity approaches zero.

choice' experiments. At early stages of regeneration in 'no choice' experiments, as in standard regeneration, both correct and incorrect connections are equally produced. However, at later stages these two types of preparations look very different. At 20–25 days after surgery, during standard regeneration, the elimination of incorrect connections and neurites is prominent ($A = 0.85 \pm 0.06$) and only 27% of the muscle cells retained some incorrect innervation (Fig. 1E, Table 1). In contrast, 'no choice' preparations displayed no difference between correct and incorrect connections at the same regeneration time ($A = 0.11 \pm 0.07$, Table 1) and both inputs were present on all the recorded muscle units. No differences in morphological patterns were detected between motoneurons growing towards correct or incorrect muscle targets. When the regeneration time was very long (35–65 days) a degree of domination of the correct connections over incorrect was detected ($A = 0.50 \pm 0.03$). Even at such a late stage of regeneration, asymmetry of the 'no choice' preparations was considerably lower than that in standard regeneration at even earlier (see Table 1) stages. In 'no choice' preparations at 35–65 days of regeneration, 73% of the muscles cells recorded had retained incorrect EJPs, and the morphology of the motoneurons innervating correct or incorrect muscle targets was similar

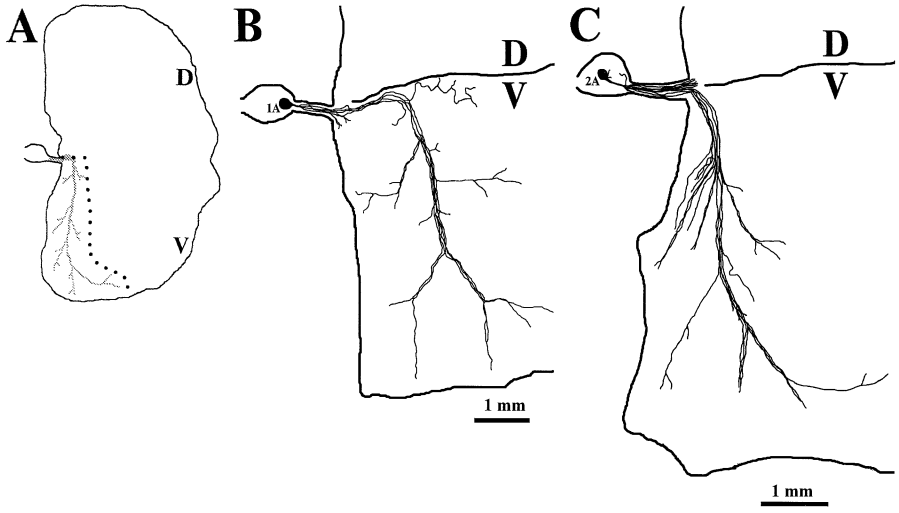

FIG. 4. Whole-animal regeneration experiments with only one muscle layer available. When all branches of the wing nerve except for the ventral medial secondary branch (shown in grey) are cut, only a part of the ventral muscle layer (outlined with black dots) is available for reinnervation (A). Dorsal motoneuron 1A after 65 days of regeneration following the wing nerve crush (B) and ventral motoneuron 2A at 55 days of regeneration (C) have similar morphology. D, V indicate dorsal and ventral muscle layers, respectively.

TABLE 1. Asymmetry of innervation and proportion of correctly innervated muscle fibres in different types of *in vivo* experiments at intermediate and late stages of regeneration

	Regeneration for 20–25 days			Regeneration for 35–65 days		
Type of experiment	*A* (mean ± SE)	$N_{Correct}$ (%)	(*n*)	*A* (mean ± SE)	$N_{Correct}$ (%)	(*n*)
Both intact muscles	0.85 ± 0.06	73	30	0.97 ± 0.02	90	10
Medial part of only one muscle	0.11 ± 0.07	0	18	0.50 ± 0.03	23	60
Medial parts of both muscles	0.50 ± 0.06	32	31	0.69 ± 0.08	43	7

A is the value of asymmetry of innervation; $\boldsymbol{N_{Correct}}$ is the percentage of muscle fibres with purely correct innervation; *n* is the number of muscle cells examined in each situation. All mean values of *A* are statistically different at different times and comparing the different types of experiments ($P < 0.01$, two-tailed *t*-test).

(Fig. 4B and C), suggesting that no neurite retraction took place in these experiments. This result is similar to that obtained in *in vitro* experiments.

Whole-animal neuromuscular regeneration with reduced size of the muscle target

In the 'no choice' experiments described above the size of the muscle layers available for regeneration was smaller than in the standard regeneration preparation. If the motoneurons are programmed to produce an intrinsically determined number of connections, the reduction of the target size may distort the normal pattern of synapse elimination. To study the influence of this factor in whole animals, the distal two-thirds of the wing were amputated, or the main trunks of the wing nerve were cut like those in the 'no choice' preparations, leaving only the medial branches intact. Then, the main wing nerve was crushed as usual. In both cases, the area of correct wing muscles available for reinnervation was reduced to about one-third of the normal size, but for both groups of motoneurons normal targets were preserved (Fig. 5A). The outcome of these experiments was similar to that with standard regeneration. In particular, at 25 days of regeneration the share of the correct synapses was about three times higher than of incorrect ones ($A = 0.50 \pm 0.06$, Table 1). At this time the morphology of motoneurons was also strongly asymmetrical. However, some differences from the standard regeneration were revealed at very late stages (>50 days of regeneration). A small number of branches was still present on the inappropriate muscle layer (Fig. 5B and C) and some incorrect connections were still present, although their relative proportion was strongly reduced ($A = 0.69 \pm 0.08$, Table 1).

Discussion

Neuromuscular connections in culture and standard regeneration in whole animals

In our previous publication (Panchin *et al*., 1998) it was shown that when motoneuron axons are crushed in the nerve of the whole animal, regeneration starts and eventually results in a complete restoration of function. The process of restoration of the correct connections includes an intermediate stage when muscle fibres display symmetrical correct, incorrect and mixed innervation. Later the selection starts, and the number of inappropriate neurites and neuromuscular junctions begins to decrease. That results in asymmetrical, entirely correct, axon morphology and innervation; all the wrong axons are withdrawn and the muscles are innervated only by appropriate motoneurons. In this study of an *in vitro* system it was shown that in culture, as in early stages of the whole-animal regeneration, both correct and incorrect connections of motoneurons are formed, leading to symmetrical innervation. In contrast to the regeneration in the whole animals, this symmetrical innervation in culture does not become asymmetrical at the later stages. This difference could be attributed to the absence of choice (only one muscle layer is present) or to the deficiency of the *in vitro* conditions. Certainly, as mentioned above, the most satisfactory way to choose one of these two possibilities would be to culture pedal ganglia with both muscle layers grafted to the same wing nerve. Unfortunately, these experiments are too difficult for technical reasons. However, there is evidence that regeneration in culture is similar to that *in vivo*. First, the physiological properties of the pedal neurons *in vitro* were indistinguishable from normal activity in the locomotor generator, and lasted for the whole culture time (up to 34 days).

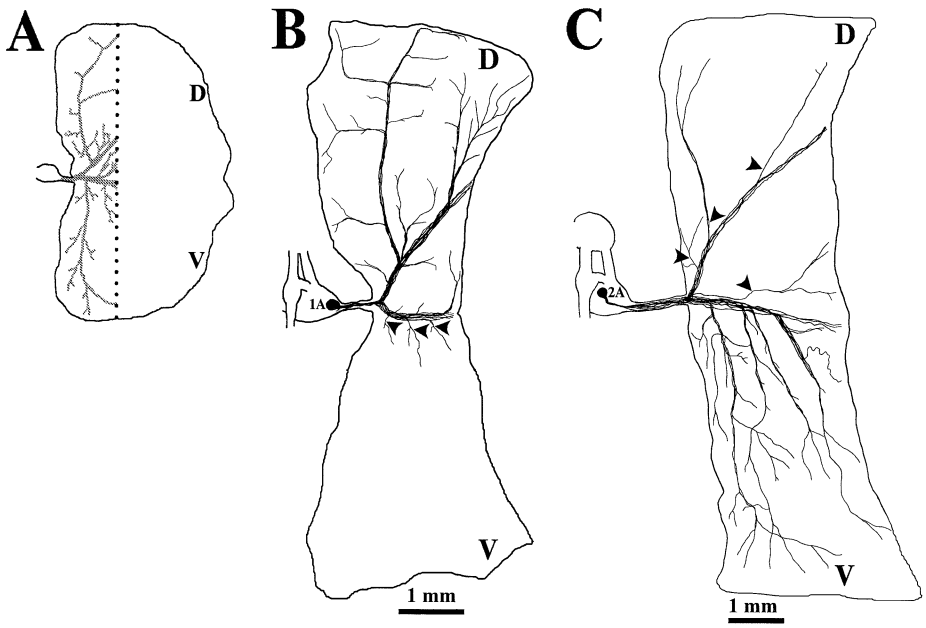

FIG. 5. Whole-animal neuromuscular regeneration with reduced size of the muscle target. (A) Schematic drawing of the wing muscles available for reinnervation if the distal two-thirds of the wing are cut off prior to the wing nerve crush. In this case only medial parts of both dorsal and ventral muscle layers are available for reinnervation. (B and C) Morphology of motoneuron 1A after 64 days of regeneration and motoneuron 2A after 57 days of regeneration. For both neurons, most of the neurites are on the correct muscle. However, there are still several short incorrect neurites (marked with arrowheads). Abbreviations are the same as in Fig. 4.

Secondly, EJPs in cultured muscles were similar to those during whole-animal regeneration. Thirdly, 'no choice' experiments in whole animals gave virtually the same outcome as the *in vitro* experiments. The possible role of injury is small in the *in vitro* preparation and virtually eliminated in 'no choice' whole-animal experiments because only nerve branches were cut, leaving muscles intact. In both cases (*in vivo* and *in vitro*) when only one of two target muscles was available, the withdrawal of neurites and synapses was strongly suppressed. Thus, it is likely that an *in vitro* system is an adequate model for neuromuscular regeneration studies.

Elimination of neuromuscular connections and the choice of target

Clione experiments with only one of the muscle targets presented to the growing motoneurons indicate that there is no competition between synapses of different motoneurons on the same muscle cell. Connections from correct and incorrect motoneurons may peacefully share the muscle cell in these conditions, probably indefinitely long. Actually, in *in vitro* experiments the innervation became even more symmetrical with time. In this respect neuromuscular regeneration in *Clione* is different from that in vertebrates. In the normal course of development, and during regeneration, vertebrate neuromuscular synapses from different motoneurons compete for the target, so that finally each muscle cell is innervated only by one motoneuron (Brown *et al*., 1976; Taxt, 1983). In *Clione*, the lack of competition between inputs from two different groups of motoneurons may not be so surprising, considering that several motoneurons of the same group normally innervate the same muscle fibre (see Introduction section and also Satterlie, 1993). At the same time we know that ventral and dorsal muscle layers are different and in the course of normal neuromuscular regeneration (standard regeneration) only one type of input remains on each muscle target, while all inputs of the other type are withdrawn. If muscle cells do not discriminate between correct and incorrect inputs how is appropriate innervation established during 'standard regeneration'? It is possible that competition occurs among the synapses of the same neuron on different muscle targets. If, for instance, all synapses of one neuron utilize the same limited resources and if synapses on appropriate targets have some advantage in getting or using these resources, the correct synapses may grow and develop at the expense of synapses on the wrong muscle and induce their elimination. Apart from the actual mechanisms of synapse selection (which are not yet known) we can conclude that the presence of the choice between correct and incorrect targets is a necessary condition for neuromuscular synapse elimination and neurite withdrawal.

The role of physiological properties of neurons and muscles in target selection

In the case of elimination of multiple neuromuscular innervation in mammals (mentioned above) the selection occurred between physiologically identical targets. A different situation arises during the elimination of the aberrant neuromuscular connections of the frog and bird motoneurons after reinnervation of phasic and tonic muscles by appropriate nerve fibres (Feng *et al*., 1965; Hnik *et al*., 1967; Miledi & Stefani, 1970). It was shown that the different physiological properties of these two muscle types (Gordon *et al*., 1977; Gordon *et al*., 1981) play a key role in this selection.

The differences in physiological properties of neurons and muscles are hardly responsible for the selection in *Clione*'s regeneration of neuromuscular connections. Dorsal and ventral muscle layers seem to be identical in their anatomical, morphological, histological and physiological properties (Huang & Satterlie, 1989; Satterlie, 1991). Although both tonic and phasic muscle fibres are found in *Clione*'s wing, they are equally presented in both dorsal and ventral muscle layers (Huang & Satterlie, 1989). On the other hand, the properties of D-phase and V-phase motoneurons are similar. Their firing patterns in the swimming cycle are actually the same (Arshavsky *et al*., 1985b). Both groups of motoneurons release the same neurotransmitter acethylcholine (Panchin *et al*., 1996). Their branching patterns on the corresponding muscle layer are identical (Panchin *et al*., 1998).

The role of the target size in neuromuscular regeneration

Is the availability of the correct target for the motoneuron not only necessary but also a sufficient condition for incorrect synapse and neurite elimination? We have already considered the situations where

only one muscle layer was available to regenerating motoneurons (*in vitro* and 'no choice' *in vivo* preparations) or both normal full-size muscle targets were present ('standard regeneration'). What happens if both normal targets for motoneurons are present but strongly reduced in size? The present study shows that regardless of the smaller size of muscle the correct motoneurons innervate it more strongly than the incorrect ones. On the other hand, the motoneurons with reduced target size retain some incorrect connections and wrong projections at the period that exceeds the time of complete retraction of aberrant axon branches in the standard regeneration. That means that the neurite withdrawal depends not only on the presence and type of the target muscles but also on the size of the area available for innervation. These results are in agreement with the hypothesis that synapses from the same cell on different muscle fibres compete for common factors and give it a quantitative basis. If the reduction of the muscle size restricts the number of correct synapses of the given motoneuron, these correct synapses will utilize less common factor, leave more of it for synapses on incorrect muscle and allow them to persist longer. No actual mechanism for suggested competition is known but we hope that the combination of different approaches, including *in vitro* culturing, will allow us to study this question.

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Abbreviations

1A, the largest D-phase motoneuron; 2A, the largest V-phase motoneuron; CNS, the central nervous system; D, dorsal; DWM, dorsal wing muscle; EJP, excitatory junction potential; EPSP, excitatory postsynaptic potentials; IPSP, inhibitory postsynaptic potentials; NW, the wing nerve; NWA, the anterior main branch of the wing nerve; NWP, the posterior main branch of the wing nerve; V, ventral; VWM, ventral wing muscle.

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