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Axotomized neurons of the pteropod mollusc *Clione limacina* develop novel sites of transmitter release in the absence of their normal muscle target

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Abstract

Neural network for rhythmic wing movements in the swimming mollusc *Clione limacina* is a well-studied system. After nerve transection the efferent wing neurons cannot reach muscles and consequently display intensive central sprouting. In the present work it was shown that two types of efferent neurons with different neurotransmitters: acethylcholinergic locomotor motoneurons and serotonergic modulatory efferent neurons – when deprived of their normal targets, release their neurotransmitter intended for peripheral muscles, in the unusual compartment—neuropile. Such 'unauthorized' release of neurotransmitter may cause nervous system dysfunctions in the damaged brain of other animals. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introduction

The central ganglia of gastropod molluscs 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* [10,12] and some other molluscan species [1,11,13,17,22].

Swimming central pattern generator (CPG) of pteropod mollusc *Clione* is a well-studied system (Fig. 1**A**) with most neurons and their connections identified [2–5,7,25,20]. Therefore, it is an attractive model for studying neuronal regeneration, target selection, formation and elimination of connections, and plasticity of synapses. During rhythmic locomotor activity interneurons of the swimming CPG drive two populations of the wing motoneurons reciprocally. When the ventral (V) phase motoneurons are excited, the dorsal (D) phase motoneurons are inhibited, and vice versa. Correspondingly, the V- and D-phase motoneurons activate corresponding ventral and dorsal wing muscles (Fig. 1**B**). The neuromuscular connections in *Clione*'s wing have been shown to be acethylcholinergic [19]. The wing muscles are also innervated by serotonergic modulatory efferent neurons that increase the amplitude of muscle contraction [28,26]. *Clione*'s locomotor motoneurons display different growth patterns after axotomy in different experimental conditions [21]. When the nerve is crushed in the whole animal, new neurites grow predominantly through the damage site to the periphery and initial innervation is restored. It has been shown that formation of correct neuromuscular connections depends on the presence of appropriate muscle. If the normal targets are not available, motoneurons may connect to incorrect muscle (Zelenin

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and Panchin, in preparation). If the nerve is cut, the motoneurons can not reach the muscle and display intensive central sprouting [21]. In the present paper, the formation of novel connections in these conditions is considered. We show that axotomized efferent neurons deprived of their normal peripheral targets sprout inside the central nervous system (CNS), develop the capacity to influence other pedal neurons and the activity of the locomotor generator. Two types of efferent neurons with different neurotransmitters: acethylcholinergic motoneurons and serotonergic modulatory efferent neurons, will be considered.

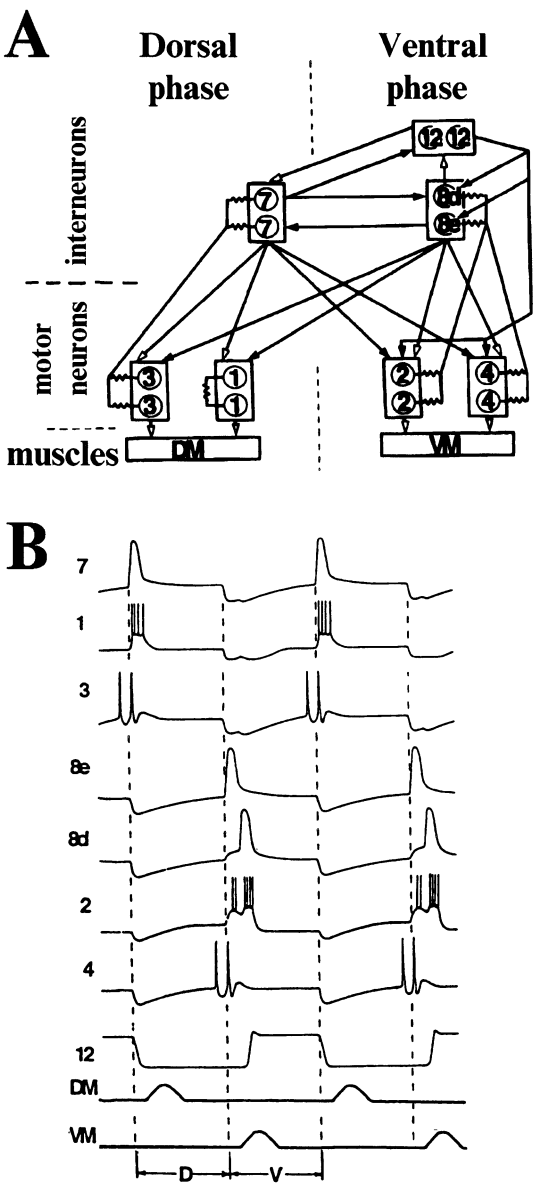

Fig. 1. **A**: Neuronal network controlling rhythmical wing movements in *Clione*. Main types of motoneurons (types 1, 3, 2, 4) and interneurons (7, 8e, 8d, 12) are indicated. Electrical connections are shown by resistor symbols, excitatory chemical connections by white arrows, and inhibitory connections by black arrows. Dorsal (D) and ventral (V) wing muscles. **B**: Diagram showing phases of the activity of different groups of neurons in the swimming cycle. Mechanograms of dorsal and ventral wing muscles are also shown. D—a dorsal phase of locomotor cycle, V—a ventral phase of locomotor cycle.

2. Materials and methods

Experiments were carried out at the White Sea Marine Biological Station, Kartesh. The experiments were made either on whole animals or on in vitro preparations. In the first case the animal was put in sterile sea water with antibiotics, a small incision in the skin was made above the CNS and the wing nerve was cut with scissors close to the exit from the ganglion. After that, the animal was hold in a sterile tank with fresh sea water. In 2–20 days the animals were dissected and preparations for intracellular recordings and staining with fluorescent dye Lucifer Yellow were made. The preparations were treated for 1 min in 0.5% solution of Pronase E in sea water to soften the ganglion sheath and then rinsed in a large volume of sterile sea water. After that the ganglia were pinned on a layer of agarose gel and fixed with a drop of warm liquid agarose.

For in vitro culturing the CNS was removed from the animal and the pedal ganglia were dissected. The ganglia were treated for 2 min in 0.5% solution of Pronase E in sea water to soften the sheath and rinsed in a large volume of sterile sea water. Then ganglia were either cultured as a paired pedal ganglia preparation (PPG) or the pedal and thin subpedal commissures were cut and a single pedal ganglion was cultured (PG preparation). The ganglia were placed 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 100% L15 medium balanced with sea water.

The largest D-phase motoneuron 1A and the largest V-phase motoneuron 2A are easily identified 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 sprouting motoneurons. Serotonergic efferent neurons are located in the medial part of the pedal ganglia and could be identified by their size and position.

Intracellular recordings of two to four cells were carried out with 3 M KCl-filled glass microelectrodes having tip resistance of 20–40 MOhm. 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.

All drugs were from Sigma.

3. Results

3.1. The growing swimming motoneurons develop novel connections in the absence of the muscle target

Normally locomotor motoneurons of *Clione* can affect other neurons only through electrical connections (Fig. 1**A**). V-phase motoneurons of type 2 are coupled to the interneurons of the same phase of the locomotor cycle. Therefore, the effects of their stimulation would mimic the effects of the V-phase interneuron polarization, i.e. depolarization of these motoneurons would increase the swimming rhythm, and hyperpolarization would block or slow down the rhythm. Type 1 motoneurons are electrically connected only to each other and thus produce no effects on the locomotor rhythm or other locomotor neurons [2,3]. In this work, this result was reproduced in all freshly dissected pedal ganglia ($n = 6$). We also tested effects of 1A polarization on 135 unidentified nonlocomotor pedal neurons and detected no effects (1A motoneurons are the biggest motoneurons of type 1, 2A motoneurons are the biggest motoneurons of type 2). This result suggests that 1A motoneurons indeed have no central connections except for mutual electrical coupling. In the previous paper [21] it was shown that in the regenerated preparations where the nerve was crushed but motoneurons were able to reach the muscle, branching of the motoneurons and their connections inside the CNS were not changed. No aberrant electrical or chemical connections were detected [21]. In contrast to the crushing of the nerve, 48 h after wing nerve transection, or later, the motoneurons that displayed intensive central sprouting developed the ability to inhibit locomotor rhythm generation and to affect some nonlocomotor neurons. Fig. 2 shows simultaneous recording of three locomotor motoneurons from the PPG preparation cultured for 4 days with the right wing nerve cut close to the ganglion and the left nerve left long. It was shown earlier that proximal axotomy induces intense central sprouting, whereas in long nerves the growth is restricted to the ends of the nerves. Correspondingly, depolarization of the right side motoneurons (1AR and 2AR) with intense central sprouting resulted in a temporary arrest of the locomotor rhythm (Fig. 2**A**,**C**), while depolarization of the left side motoneuron (1AL) had no effect (Fig. 2**B**). Seven of ten 1A and four of eight 2A motoneurons tested at the side of PPG preparation where the nerve was cut short displayed this kind of effect. None of the 1A ($n = 9$) or 2A ($n = 7$) motoneurons with long initial axons had inhibitory effects on locomotor generation. Their effects were the same as in controls.

In the PG preparations the effects were stronger than in the PPG preparations. 1A cells inhibited rhythm in five cases and 2A cells did this in four cases out of six

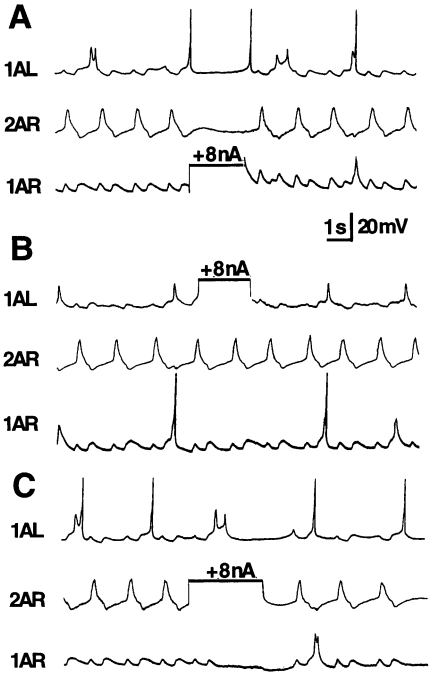

Fig. 2. Effects of motoneuron depolarization on the locomotor rhythm generation in cultured paired pedal ganglia (PPG) preparation in 4 days after isolation. Simultaneous recording of two motoneurons from the right side (1AR, 2AR) and a motoneuron from the left side (1AL). The wing nerve on the right side is cut short and motoneurons 1AR and 2AR had strong central sprouting and showed a clear inhibitory action on rhythm generation (**A** and **C**). The nerve of the left pedal ganglion was left long and 1AL depolarization (**B**) did not affect the rhythm. The periods of current injection are marked by solid lines the strength and polarity of the current being indicated.

PG preparations tested (Fig. 3**A**,**C**). Hyperpolarization of the 1A motoneuron had no effect on the rhythm or other locomotor neurons (Fig. 3**B**), implying that 1A motoneurons established no new electrical connections with other locomotor neurons.

Effects of 1A motoneuron stimulation were also tested on non-locomotor neurons in PPG and PG preparations. On Fig. 4, three unidentified neurons demonstrate different responses to 1A depolarization (Fig. 4**A**), while 1A hyperpolarization has no effect (Fig. 4**B**). About 50% of unidentified neurons ($n = 40$) demonstrated some response to depolarization of 1A motoneuron in PG and PPG preparations.

The central effects of the motoneurons on the frequency of the locomotor generator and on nonlocomotor cells were also present in preparations of the CNS dissected from the whole animals 3–15 days after nerve transection. In this case the effects were weaker and expressed rarely (in 3 preparations of 10). It correlates with the fact that the central sprouting is more expressed in cultured ganglia than in whole animals [21].

3.2. Inhibitory effects of growing motoneurons are likely to be mediated by acethylcholine

It was found that locomotor motoneurons in *Clione* are acethylcholinergic [19]. For that reason, we have tested the effects of acethylcholine antagonists on inhibition produced by depolarization of the sprouting locomotor motoneurons in five PG preparations. As type 1 motoneurons have no electrical connections to other cells and their effects are thus more reliable, only 1A neuron effects were studied. In all preparations, atropine and Flaxedil at concentration as low as 10^{-6} M reversibly suppressed the inhibitory action of 1A motoneuron depolarization in 5–10 min after application (Fig. 5). D-tubocurarine and α-bungarotoxin had no effects on inhibition produced by 1A motoneurons.

The experiments described above imply that in cultured ganglia acethylcholine normally destined for peripheral muscles is released by sprouting motoneurons within the CNS, and causes the inhibition of locomotion and de- or hyperpolarization of some nonlocomotor neurons. It is not clear whether consistent inhibition of the locomotor rhythm is due to abnormal yet precise connections from motoneurons to particular command or CPG neurons, or whether random connections and 'volume release' of transmitter are sufficient for this effect. If the inhibitory effect of regenerating motoneurons is a result of random connections and 'volume release' then we can expect that the bath application of acethylcholine will result in supression of locomotion. To check this possibility in three preparations a short puff of acethylcholine was applied to a pair of freshly isolated pedal ganglia. Indeed, as shown in Fig. 6, the general effect of acethylcholine was the inhibition of locomotion.

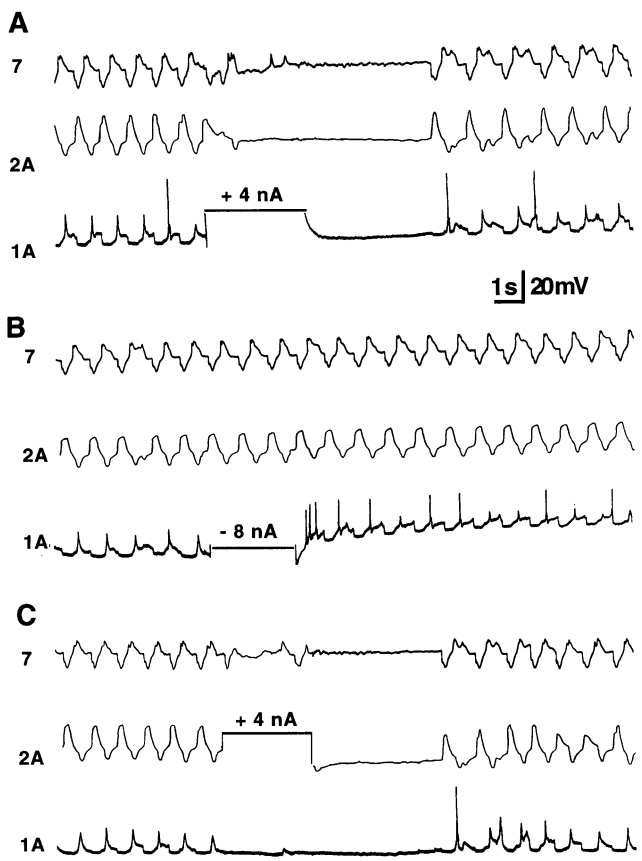

Fig. 3. Effects of motoneuron polarization on the locomotor rhythm generation in cultured single pedal ganglion (PG) preparation. Simultaneous recording of 1A and 2A motoneurons and type 7 the D-phase interneuron. 1A and 2A motoneurons depolarization inhibits the locomotor rhythm in PG preparation (**A**,**C**). The hyperpolarization of motoneuron (1A) has no effect (**B**). 3 days in culture, wing nerve cut short.

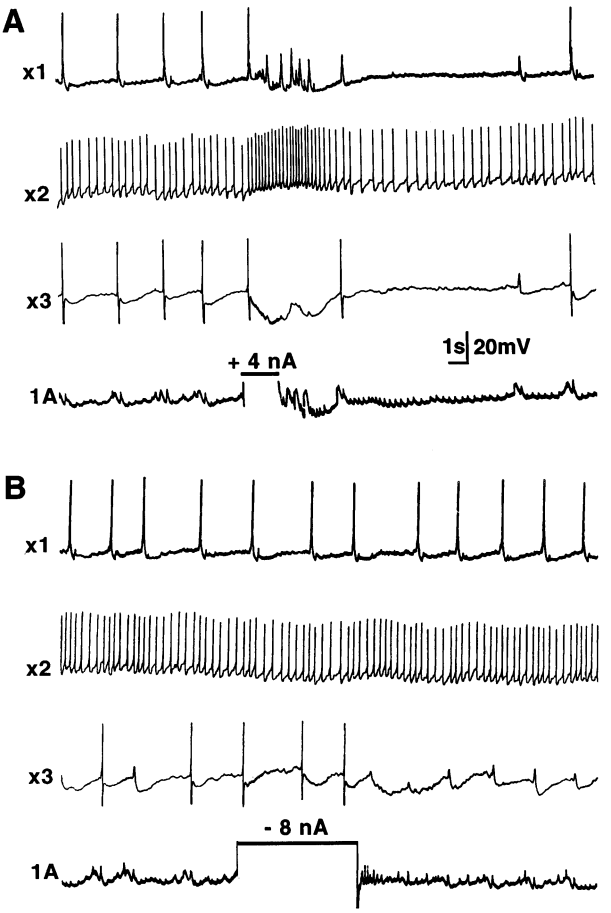

Fig. 4. Effects of motoneuron polarization on unidentified neurons in cultured PG preparation. Three unidentified neurons (x1, x2, x3) were recorded in PG preparation after 4 days in culture together with 1A motoneuron. De- (**A**) but not hyperpolarization (**B**) of 1A results in diverse responses in these neurons.

3.3. Axotomized serotonergic efferent neurons develop novel excitatory connections in the absence of their muscle target

In contrast to acethylcholine, serotonin is a powerful stimulator of the locomotor rhythm in *Clione* [3,24] and

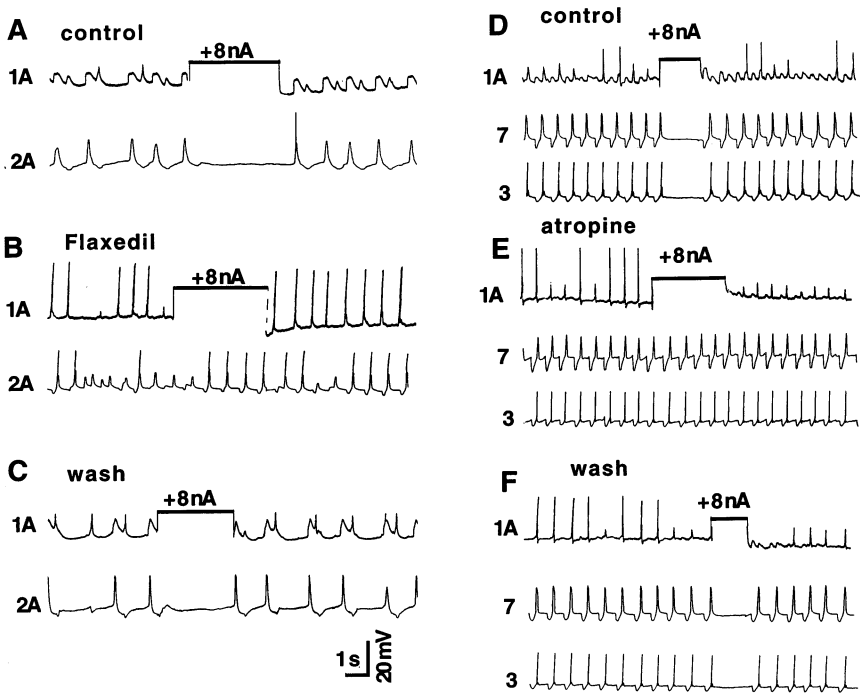

Fig. 5. Flaxedil and atropine blockade of inhibitory effects of 1A motoneuron on the locomotor rhythm in PG preparation after 4 days in culture. **A** and **D**: Control. **B** and **E**: 5 min after acethylcholine antagonists (10^{-6} M) application. **C** and **F**: Restoration of polarization effect upon washout. Simultaneous recording of 1A, 2A motoneurons (**A**–**C**) and 1A, type 3 the D-phase motoneurons and type 7 the D-phase interneuron (**D**–**F**).

is an excitatory neurotransmitter for many pedal ganglion neurons [19]. Normally serotonin is delivered to the pedal ganglia by identified cerebro-pedal neurons [18,27], while the pedal serotonergic neurons (Pd-SW) release serotonin only peripherally and their stimulation has no effect on the activity of the locomotor CPG [28]. However, 5 days after axotomy or later the pedal serotonergic neurons in all tested preparations ($n = 5$) developed the ability to activate locomotor rhythm generation. Fig. 7 shows simultaneous recording of Pd-SW cell and type 7 locomotor interneuron from the PG preparation cultured for 8 days with the wing nerve cut close to the ganglion. Depolarization of pedal serotonergic neuron resulted in acceleration of the locomotor rhythm (Fig. 7). The extent of this acceleration was slightly different for different Pd-SW cells from different preparations. On average the frequency of the locomotor rhythm was $19 \pm 2\%$ higher after Pd-SW stimulation than before it ($n = 12$). Hyperpolarization of Pd-SW cells produced no effects on locomotor rhythm generation. This implies that the effects of Pd-SW stimulation were mediated by chemical transmission rather than by novel electrical synapses.

4. Discussion

In the previous publication [21] it was shown, that axotomized motoneurons are able to regenerate their normal neuromuscular connections if the axons are crushed in the wing. In this case no central aberrant connections are formed. At the same time it was shown that when usual muscle target is absent, incorrect neuromuscular junctions are formed and become stable (Zelenin and Panchin, in preparation). It was concluded that the presence of choice between different targets is an important condition that promotes the correct pattern of motoneuron connectivity. Here we investigate the case when all muscle targets both correct and incorrect are absent. Our data imply that efferent neurons which are deprived of targets and display a strong central sprouting, develop unusual chemical connections inside the pedal ganglia. For motoneurons the case is supported by sensitivity of these connections to acethylcholine antagonists. The same acethylcholine antagonists Flaxedil and atropine that block the effects of

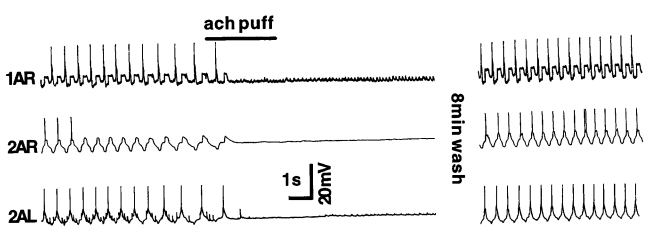

Fig. 6. A short puff of acethylcholine (500 nl; 5×10^{-5} M in sea water) onto a surface of freshly isolated pedal ganglia inhibits ongoing locomotor rhythm.

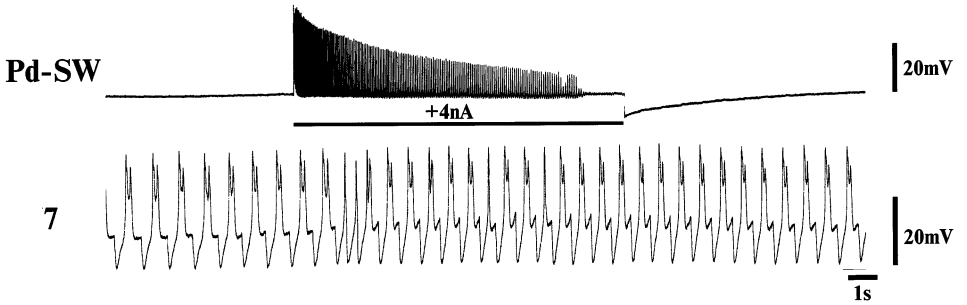

Fig. 7. Effects of serotoninergyc efferent neurons depolarization on the locomotor rhythm generation in cultured pedal ganglion (PG) preparation in 8 days after isolation. Simultaneous recording of serotonergic modulatory cell Pd-SW and type 7 locomotor interneuron. Activation of the Pd-SW neuron (depolarizing current is shown with a black bar) accelerates the locomotor rhythm.

sprouting motoneuron stimulation also effectively suppress putative central acethylcholinergic receptors in normal animals [19]. We suggest that in the absence of the normal peripheral targets, acethylcholine synthesized by motoneurons is now released in the CNS. In other molluscan species it was shown that during the process of motoneuron regeneration, neurons restore their original connections or form new connections [9,12,15,17]. Novel chemical connections were described in the buccal ganglia of Helisoma during regeneration. Acethylcholinergic efferent neuron B5 after axotomy formed chemical synapses on neuron B4, that were not observed in intact animals. These synapses were blocked by acethylcholine blocker [14].

In *Clione* bath application of acethylcholine on pedal ganglia results in inhibition of locomotion, and thus mimics the effects of regenerating motoneuron stimulation. Hence the motoneuron action is not necessarily mediated by precise connections to particular neurons but might be produced by random connections or 'nonaddressed' release of the neurotransmitter into the neuropile [16,23,8,29]. The possible role of nonsynaptic transmission in *Clione* have been discussed previously [6].

Although regeneration and sprouting of the serotonergic efferent neurons was not studied in detail, our data suggest that after axotomy these neurons release within the CNS their neurotransmitter, normally designated to peripheral target. The sign of the effects produced on locomotor rhythm generation by sprouting Pd-SW and by the bath application of serotonin was the same [3,24]. Thus, we conclude that two types of efferent neurons with different neurotransmitters: acethylcholinergic motoneurons and serotonergic modulatory efferent neurons release their neurotransmitter in the central neuropile when deprived of their normal targets.

Although central sprouting in adult vertebrates is strongly suppressed, it would be interesting to know if any similar events take place in higher animals and humans after nerve and brain damage. Such 'unauthorized' release of neurotransmitters may cause a wide spectrum of nervous system dysfunctions. These effects may probably also take place in normal development and in the damaged immature brain.

Acknowledgements

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