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Short communication

Formation of connections between cultured identified neurones from the pleural ganglion of the pteropod mollusc *Clione limacina*

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Abstract

A cluster of electrically interconnected neurosecretory cells (the 'white cells') involved in the control of reproductive behavior was identified in the right pleural ganglion of the marine mollusc, *Clione limacina*. Pleural ganglia also contain large neurons (PL1 and PL2) having no connections with each other and with the white cells. Most isolated white cells put into the simple unconditioned medium (50% L-15) adhered to the bottom of uncoated dishes and demonstrated neurite outgrowth for 7–10 days. If growing processes overlapped, the white cells formed electrical connections with each other, but they formed no connections with the PL1 and PL2 neurons. It is concluded that in the case which was under study cellular intrinsic properties were sufficient for the formation of 'correct' connections between neurones.

Keywords: Mollusc; Clione limacina; Pleural ganglia; Neurone culture; Cell recognition

Understanding of mechanisms of specific connection formation in the nervous system is among the most important problems of the modern neurobiology. A part of this problem is a question of sufficiency of neurone intrinsic properties for the establishment of precise connections. One of the approaches to the problem is the study of neurone interconnections in cellular culture. This approach was widely used for studying the formation of connections between molluscan neurones. It has been found that, in culture, molluscan neurones are capable of forming both electrical and chemical interconnections. However, the data concerning specificity of neuronal connectivities in culture are rather contradictory. The establishment of specific connections between cultured neurones was observed in some cases, while in other cases neurones which were not connected in situ formed connections in culture [5–12,16,21].

Neural networks controlling different functions were extensively studied in the pteropod mollusc *Clione limacina*. Many neurones and connections between them were identified [1,2,4,14,18,19]. This makes *Clione* a good object for studying mechanisms of forming interconnections between neurons. In previous work it was found that unidentified cultured neurones from *Clione*'s nervous system demonstrated neurite outgrowth and formation of electrical and chemical connections [3]. To check how specific these connections are, we started to culture the identified neurones from different networks. In this work we dealt with formation of connections between neurones from the pleural ganglia containing several groups of large, easily identifiable cells.

The work was carried out at the White Sea Biological Station *Kartesh*. The adult mollusks (3–5 cm long) were used. For cell isolation, the central nervous system was treated with proteolytic enzymes (0.5% pronase E, 20–30 min. and then 0.5% subtilisin, 20–30 min.); in the course of the enzymatic treatment, the pleural ganglia were desheathed. The identified pleural neurones were isolated using the method described earlier [15]. Three micromanipulators supplied with glass tools were used. The thin ends of two tools (T1 and T2 in Fig. 1B) were put around a cell axon be-

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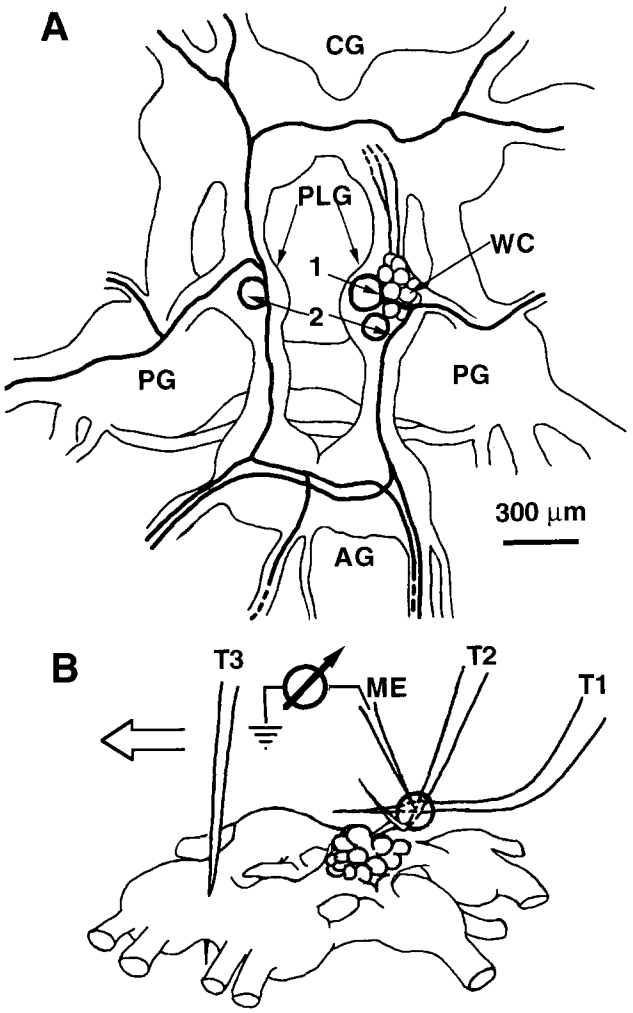


Fig. 1. A: schematic diagram of *Clione's* central nervous system. Localization of different types of pleural neurones (see text) is shown. Morphology of neurone axons was revealed by intracellular staining with Lucifer yellow. CG, cerebral ganglia; PLG, pleural ganglia; PG, pedal ganglia; AG, abdominal ganglia; WC, white cells; 1, PL1 neurone; 2, PL2 neurones. B: method of neurone isolation. T1 and T2, glass tools holding the neurone; T3, glass tool used to 'spear' the CNS; ME, microelectrode; the arrow shows direction of T3 movement.

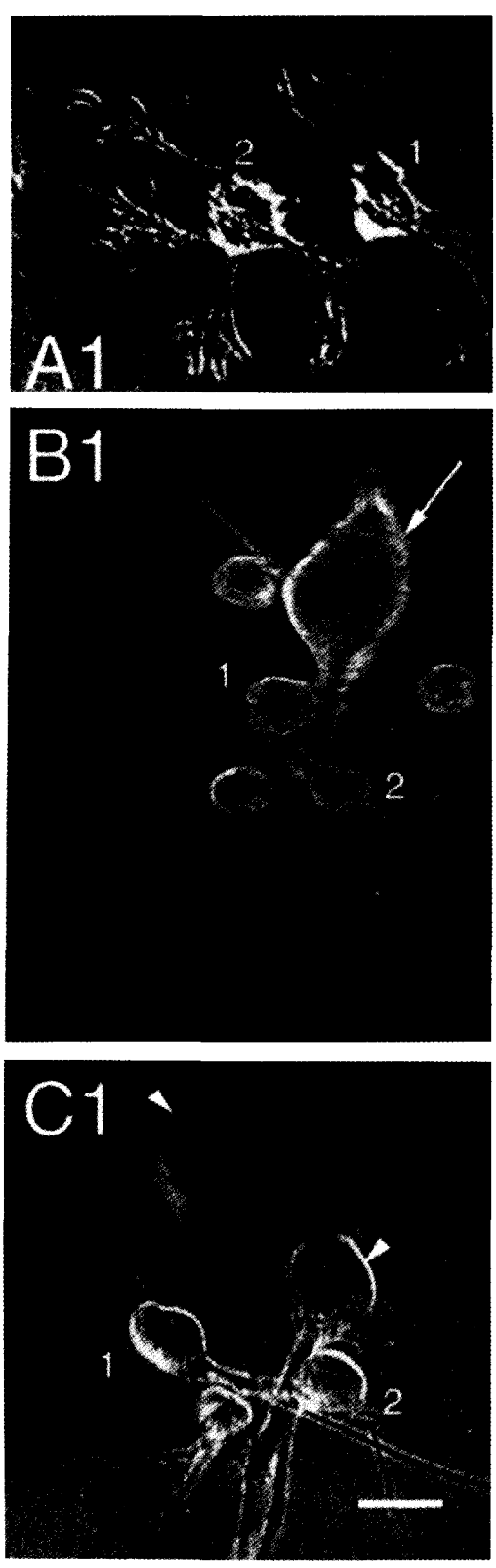
tween a cell body and the ganglion. They formed a cleft, the width of which was narrower than the diameter of a cell body. The cell was isolated by moving the CNS with the third tool as shown in Fig. 1B. Two other micromanipulators supplied with 3 M KCl microelectrodes (tip resistance of 40–60 MΩ) were used for intracellular recording the cell before isolation and for checking its connections with other pleural neurones. For intracellular staining, microelectrodes filled with a solution of Lucifer yellow were used [20]. Isolated cells were put in sterile 35-mm uncoated polystyrene dishes (Falcon 3801) filled with about 4 ml of the salt-adjusted 50% Leibovitz-15 medium containing antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 mg/ml gentamicin; all chemicals used were from Sigma). The medium was replaced every 2–3 days. To study whether cultured neurones form interconnections, they were placed in groups of several cells in close proximity to each other. When testing for connections between neurones, current was injected into cells either through the recording electrode using the bridge circuit or through a second electrode inserted in the same cell. Cells were photographed with phase contrast using an inverted microscope. The results presented here are based on recordings from more than 100 pairs of neurones.

The sizes of ganglia were measured in about 150 molluscs ranging from 1 to 60 mm long. Ganglia or the CNS were homogenized as described by Ram et al. [17]. The homogenate was injected into the body cavity in the vicinity of the heart.

The right and left pleural ganglia in *Clione* are different in size. This difference arises in the course of *Clione*'s growth. In young mollusks (under 1 cm long) both pleural ganglia are of the same size (from about 70 μm in diameter in mollusks 2–3 mm long to 150 μm in mollusks 10 mm long). In adult animals the right ganglion is larger (about 350 μm in diameter) than the left one (250 μm). The right ganglion, unlike the left one, grew even more (up to 450 μm) in animals which were ready for egg-laying. The asymmetry between pleural ganglia is mainly determined by the fact that they contain cells (the 'white cells') which increase in size and possibly in number in the course of *Clione* growth and sex maturation. The white cells are located mainly in the right ganglion where we could recognize up to several dozens of them. In contrast, the left ganglion contains few white cells. Diameter of most of the white cells in reproductively mature *Cliones* ranges from 60 to 90 μm, reaching 150 μm in some cells. The correlation between *Clione* sex maturation and sizes of the white cells suggests that the latter are neurosecre-

Fig. 2. Formation of electrical connections between pleural neurones in culture. A1, B1 and C1: photomicrographs of cultured neurones; calibration, 100 μm. A2, B2 and C2: recordings from neurones shown in (A1), (B1), and (C1), respectively. A: two white cells (WC) after 24 h in culture. For current injection two microelectrodes were inserted into the WC2. B: the PL1 neurone (marked by an arrow in B1) cultured with five WCs. Simultaneous recording of the PL1 and two WCs (marked in B1) is shown in (B2). Injection of current in one of the WCs resulted in a deflection of the membrane potential in the 2nd WC, but not in the PL1. C: Two PL2 neurones (marked by arrowheads in C1) cultured with three WCs. Simultaneous recording of two PL2s and two WCs (marked in B1) is shown in (C2). Injection of hyperpolarizing current in one of the WCs resulted in a deflection of the membrane potential in the 2nd WC, but not in the PL2s. The periods of current injection are marked by solid lines the strength and polarity of the current being indicated.

tory neurones involved in the control of reproductive behavior. This suggestion was supported by the fact that injection of a homogenate of the pleural ganglia taken from one or two adult mollusks into the reproductively mature *Clione* ($n = 8$) provoked egg-laying within 65 to 110 min. In contrast, injection of a ho-



mogenate of any other ganglia or the central nervous system without pleural ganglia ($n = 12$) did not produce such an effect.

A cluster of the white cells located in the rostral part of the right ganglion was identified. Intracellular staining of the neurones has revealed that their axons project into the cerebral ganglia (Fig. 1A). Like neurosecretory neurones producing the egg-laying hormone (the bag cells in *Aplysia* [13] and CDC cells in *Lymnaea* [22]), the white cells within the cluster are electrically coupled. Usually the white cells had no background activity. In response to injection of depolarizing current they generated single spikes or short spike discharges; in some cases we were not be able to excite the white cells even by injection of a strong depolarizing current.

In addition to the white cells, the pleural ganglia contain a number of large neurones. Among them there are the visually identifiable unpaired giant neurone PL1 of 150–200 μm in diameter located in the right ganglion and the symmetrical neurones PL2 of 100–150 μm in diameter (Fig 1A; see also Fig. 2A in [4]). An axon of the PL1 neurone divides into two branches within the pleural ganglion; one branch projecting into the pedal ganglion and further into one of the pedal nerves, the second branch projecting into the abdominal ganglia and then into right and left abdominal nerves (Fig. 1A). We could not find any effects produced by stimulation of the PL1 neurone. The PL2 neurones have numerous processes which project into cerebral, pedal and abdominal ganglia and then go into peripheral nerves (Fig. 1A). The PL2 neurones produce retraction of the tail and an inhibition of locomotion [2,14]. We did not find any electrical or chemical connections of the PL1 and PL2 neurones either with each other or with other neurones from the pleural ganglia.

Most isolated white cells put into the simple unconditioned medium adhered to the bottom of the dish and demonstrated neurite outgrowth at least during 7–10 days. Neurite outgrowth started after cell adhesion and appeared to be especially intensive during the first 2–3 days (Fig. 2 A1, B1, C1). If the white cells were located close enough and their growing processes overlapped each other, the cells formed electrical connections (Fig. 2 A2, B2, C2). In most cases a coupling coefficient between cells ranged from 0.3 to 0.7 (Fig. 2A2). As a result, spikes arising in one of the cells produced 1: 1 transient potentials in the coupled cell (Fig. 2A2–C2). In some experiments an electrical coupling between cells could be detected the next day, that is within 24 hours after the beginning of culturing (Fig. 2 A2). Similar results were obtained in studies of *Aplysia* bag cells; in culture they formed strong electrical connections with a coupling coefficient ranged from 0.02 to 0.79 [5,11].

The PL1 and PL2 neurones also demonstrated a neurite outgrowth in culture (Fig. 2 B1, C1). However, unlike the white cells, the PL1 and PL2 neurones never formed electrical or chemical connections with each other or with the white cells. Figures 2B and 2C show the PL1 and PL2 neurones cultured together with the white cells. One can see that the white cells are electrically coupled with each other but not with the PL1 and PL2 neurones. Since we did not find the PL1 and PL2 neurones to be electrically coupled with any pleural neurones in vivo, we could suggest that the PL1 and PL2 neurones are not able to establish electrical connections at all because, for example, connexins participating in forming gap junctions are not present in their membrane. To check this suggestion, control experiments were performed in which the white cells were cultured together with the pedal motoneurones 1A and 2A which were found to have electrical connections with their synergists [1]. In culture the white cells never formed any connections with the 1A and 2A motoneurones as they did with the PL1 and PL2 neurones (unillustrated). Therefore, the cells which contain connexins in their membrane also did not form electrical connections with the white cells.

Results obtained in this work have shown that in the case which was under study cellular intrinsic properties were sufficient for the formation of 'correct' connections between neurones. On the other hand, it was found by many investigators that, in culture, molluscan neurones can lose their capability of specific cell–cell recognition. As a result, neurones, which were not connected in vivo, formed connections in vitro [7,9,10]. The goals of next studies are: (i) to verify why some cells form specific connections, while others form unspecific ones, and (ii) to find factors influencing the selectivity of neuronal connections in culture. Answering these questions is important for understanding factors determining a specific cell–cell recognition.

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