Mol. Cells, Vol. 7, No. 3, pp. 399-407

Partial Anatomical and Physiological Characterization and Dissociated Cell Culture of the Nervous System of the Marine Mollusc *Aplysia kurodai*

Chae-Seok Lim, Do Young Chung and Bong-Kiun Kaang*
Molecular Neurobiology Laboratory, Institute for Molecular Biology and Genetics, and Program in Cognitive Sciences, Department of Biology, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

(Received on March 5, 1997)

Snail nervous systems are powerful tools for neurobiological studies as the biophysical properties of the giant neurons and their neural circuits can be examined in relation to specific behaviors of animals. The marine mollusc *Aplysia californica* is particularly useful for analyzing the components of learning and memory at the molecular and cellular levels. Here we partially examined the nervous systems of two species (*A. kurodai* and *A. juliana*) commonly found along the Korean coast in comparison with that of *A. californica*, one of the American marine snails. *A. kurodai* appeared to be identical to *A. californica* in both anatomical and physiological properties of the nervous system. *A. juliana* could be distinguished from *A. californica* in certain morphological aspects of the nervous system. The hemolymph either from *A. kurodai* or from *A. juliana* was required for effectively elongating neurite outgrowth of *A. kurodai* neurons in dissociated cell culture. The cultured cells retained neuronal properties such as neurite outgrowth, synapse formation, and generation of action potentials. The sensory cells of *A. kurodai* in dissociated cultures showed a response to serotonin (5-HT) of spike broadening and enhanced membrane excitability as in intact ganglia. Therefore, the nervous system and dissociated neuronal culture of *A. kurodai* may be useful for studying learning and memory in the context of well-defined neural circuits of *A. californica*.

The phylum Mollusca, second only to the arthropods in number of species (Abbott, 1958) includes many genera that attract the attention of neurobiologists: *Achatina, Anisodoris, Aplysia, Helisoma, Helix, Hermissenda, Limax, Loligo, Lymnaea, Octopus, Pleurobranchaea, Sepia, and Tritonia*. Particularly, the opisthobranch mollusc *Aplysia* has been an interesting snail for neurobiology. The large identifiable neurons and numerical simplicity of the nervous system have enabled *Aplysia californica* to be used as a model to elucidate molecular and cell biological mechanisms of neuronal functioning. The ability to identify specific neurons and manipulate their physiology has turned out to be a powerful approach for studying learning and memory (Kandel and Schwartz, 1982). In addition, the ability to culture these giant neurons in the laboratory makes *A. californica* particularly useful in examining regeneration and specificity of synapse formation among neurons (Schacher and Proshansky, 1983).

In this study, we partially characterized both the anatomy and physiology of the central nervous systems of *A. kurodai* and *A. juliana* (synonym, *A. sibogae*), both of which are commonly found in Eastern Asian Pacific coastal lines. Their nervous systems were compared with that of *A. californica*, which has been well studied at the levels of both behavior and cell biology (Kandel, 1976; 1979). We have applied the neuronal culture technique originally developed for *A. californica* to the neurons of *A. kurodai*. Primary cell culture would provide a enormous tool, making individual living cells accessible for direct visualization and manipulation of entire neuron structures and for pharmacological and molecular studies of single neurons. We show that *A. kurodai* and *A. californica* have nearly identical nervous systems and that *in vitro* cultured neurons from *A. kurodai* retain the properties of living nerve cells in the presence of the hemolymph of *A. kurodai* or *A. juliana*.

* To whom correspondence should be addressed.

Materials and Methods

Animals

Aplysia kurodai and *A. juliana* were purchased from professional sea-divers in Pusan, Pohang, and Sokcho, Korea. Identification of animals was based on criteria described by Choe and Lee (1994) and

Okada (1967). *A. kurodai* was easily distinguished from *A. juliana* by the presence of irregular whitish spots on the body and the secretion of purple ink when disturbed. Although *A. juliana* looked similar to *A. californica* in body color pattern, it did not have inking behavior. Animals were maintained in a recirculating sea water bath at 14 ℃ and exposed to a 12-h light/dark cycle before use.

Cell culture

Cell culture was done basically following the protocol as described in Schacher and Proshansky (1983). The hemolymph was collected from the body cavity of *A. kurodai* or *A. juliana* (150-300 g) and filtered through a 0.45 μm syringe filter (Gelman). The culture medium was made by mixing the hemolymph with equal volumes of an isotonic L-15. Isotonic L-15 was made as described in Schacher and Proshansky (1983) by adding appropriate salts to L-15 (Sigma Co.) so that the final salt concentrations were the following: 400 mM NaCl, 27 mM $MgSO_4$, 27 mM $MgCl_2$, 11 mM $CaCl_2$, 10 mM KCl, and 2 mM $NaHCO_3$. Isotonic L-15 also contained 0.1 mg/ml glutamine and 6 mg/ml glucose. Before dissection, animals were anesthetized by injection of isotonic $MgCl_2$ equal to approximately half of the body volume. Ganglia were removed and rinsed three times in a cold artificial sea water (ASW: 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, 10 mM HEPES, pH 7.6). Ganglia were incubated in isotonic L-15 containing 1% protease (type IX, Sigma) at 34 ℃ for 2-3 h and washed several times with ASW. The ganglia were pinned down on a Sylgard (Dow Corning) plate and desheathed carefully by using microscissors and tweezers. Individual cells with axon segments more than 500 μm in length were removed with a long glass capillary as described in Schacher and Proshansky (1983). Sensory neurons were dissociated from the sensory cluster in the pleural ganglion and random populations of dissociated neurons were obtained from the pedal ganglion. Dissociated cells were plated onto poly-L-lysine (Sigma)-coated culture dishes (P50G-0-14-F, MatTek Corp.) containing isotonic L-15 with or without hemolymph. Cultures were maintained at room temperature for the first day and subsequently transferred into an 18 ℃ incubator. The media were replaced every other day. Neurite growth was observed and measured using an inverted phase contrast microscope (model Optiphot-2, Nikon).

Electrophysiology

The membrane potential of cultured cells or of neurons in the intact abdominal ganglia was measured by using an Axoclamp 2B amplifier (Axon Instruments) with microelectrodes filled with 3 M KCl. The impedence of microelectrodes ranged from 5 to 15 MΩ. Data was registered by using a conventional video cassette recorder through a digital data recorder (Model VR-10B, Instrutech Corp.). The current injection with a square pulse of 500 ms to elicit spikes from neurons and acquisition of the resulting profile of membrane potential were carried out by a computer using a pCLAMP program (Axon Instruments). To examine the pharmacological response of sensory cells, we applied 5-HT (5-hydroxytryptamine creatine sulfate, Sigma) by introducing a small volume of a concentrated stock solution to yield the final concentration of 10 μM in the bath. All physiological recordings were conducted at room temperature.

Results

The nervous system of A. kurodai *and* A. juliana

The gross plan of the nervous system of two *Aplysia* species (Fig. 1) conforms to that of *A. californica* (Kandel, 1979). The nervous system is detorted and the connectives uncrossed. It can be divided into two parts, the head ganglia and the abdominal ganglion. The circumesophageal ring forms around the esophagus and consists of eight head ganglia: two fused cerebrals, two pedals, two pleurals, and two buccals. They are arrayed in symmetrical pairs with nerve connectives. They are known to be mainly involved in the somatic functions of controlling movements of the musculature. The visceral connectives between the pleural and abdominal ganglia are long and the abdominal ganglion lies some

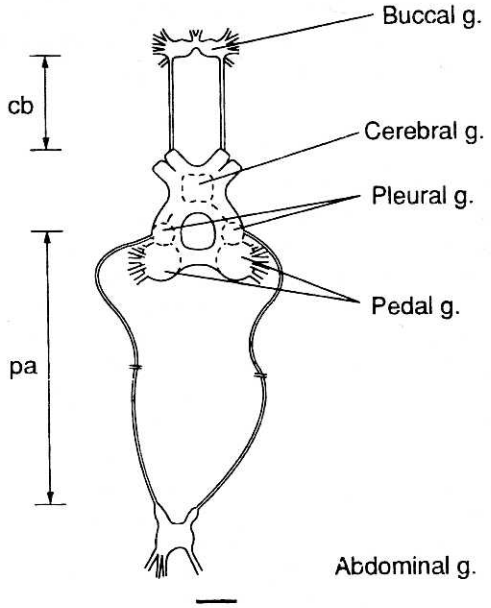

Figure 1. The nervous system of *Aplysia kurodai*, showing the asymmetrical abdominal ganglion and the paired buccal, cerebral, pleural, and pedal ganglia. It is detorted and the connectives uncrossed. The nervous system of *A. juliana* is essentially identical to that of *A. kurodai* except for the structure of abdominal ganglion (see text and Figs. 2A and B). The pleuroabdominal connectives (abbreviated to pa) is drawn as shortened and they are actually about 5 times longer than the cerebrobuccal connectives (cb). Scale bar, 2 mm.