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Primary culture and characteristic morphologies of neurons from the cerebral ganglion of the mud crab, *Scylla paramamosain*

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Abstract Crustacean neurons, obtained from the cerebral ganglion of the mud crab *Scylla paramamosain*, were successfully cultured in vitro. They maintained typical morphological characteristics and showed better outgrowth in modified Medium 199 (M199) medium than that in Liebowitz's L-15 medium. Fetal bovine serum (FBS), muscle extracts, and hemolymph of the mud crab *S. paramamosain* were added as supplements. Only 20% FBS could promote neuron outgrowth, while muscle extracts and hemolymph of *S. paramamosain* did not improve neuron outgrowth. For cell dissociation, both collagenase type I and trypsin worked well as determined by initial cell viability and following cell outgrowth potential. More than six kinds of cells with different morphological characteristics were identified in the neuron outgrowth. They were "small cells", "veilers", "branchers", "multipolar cells", "super-large cell", and "bipolar cells". Among all of the cells, bipolar cells were identified for the first time in crustacean neurons culture and they could live longer than other cells. The neurons could grow for more than a week before retraction and eventual degradation.

Keywords Cerebral ganglion · Neuron · Cell culture · *Scylla paramamosain* · Crustacean

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Introduction

Over the past half-century, little progress in marine invertebrate cell and tissue culture had been reported. No established cell line from any marine invertebrate are available yet, although there is a substantial demand for cell cultures from marine invertebrates for both basic research and applied research. In crustacean, there were some reports of primary cell culture from various tissues, including lymphoid, neuron, ovary, hemocytes, pharyngeal, and hepatopancreas (David et al. 1990; Elpidio et al. 1999; Walton and Smith 1999; Sashikumar and Desai 2008; Zeng et al. 2010). Most of them were working with short-term culture. As neuron culture of crustacean is concerned, there were reports about successful culture of the peptidergic neurons of the X-organ and the stomatogastric ganglion from the land crab *Cardisoma carnifex* (Cooke et al. 1989; Graf and Cooke 1990; Grau and Cooke 1992).The cardiac ganglion neurons in shore crab *Carcinus maenas* not only survived in vitro but also maintained their intrinsic membrane and transmitter response (Saver et al. 1999). The culture method of medulla terminalia X-organ from the eyestalks of Chinese mitten crab *Eriocheir sinensis* had also been developed (Sun et al. 2000). In addition, culturing medulla terminalis neurons from the Chinese shrimp, *Fenneropenaeus chinensis* was successfully established (Gao et al. 2003). There are also reports about primary culture of the lobster *Homarus americanus* olfactory sensory neurons in a defined medium (Stephanyan et al. 2004).

Although many attempts have been made for neuron cell culture in crustacean, there were very few reports about cerebral ganglion. The cerebral ganglion of crustacean, located above the esophagus, also known as supraesophageal ganglion, consisted of protocerebrum, deurocerebrum,

and tritocerebrum. The cerebral ganglion of crustacean, not only plays a role on neuromodulation, but also works on hormonal coordination such as controlling the release of hormones. Besides that, cerebral ganglion can also produce some important hormones, such as gonad-stimulating hormone which stimulates gonad maturation (Ostu 1963; Gomez 1965; Eastman-Reks and Fingerman 1984; Kulkarni et al. 1984; Kulkarni et al. 1991). Some studies showed that the protocerebrum of some invertebrate might also synthesis molt inhibiting hormone synthesis, a substance essential to maintain the integrity of the male genital system and promote serotonin (5-HT) production (Mocquare et al. 1971; Touir 1977; Kulkami and Fingerman 1986). It was also found that the cerebral ganglion might play an important role on regulating the osmolarity of the body fluid (Kamemoto and Tullis 1972; Tullis and Kamemoto 1974; Van den Bosch de Aguilar 1976; Davis and Hagadorn 1982).

Available data have shown that immunostaining signal of serotonin (5-HT), neuropeptide Y, follicle stimulating hormone (FSH), and luteinizing hormone (LH) could be located in the cerebral ganglion of *Scylla paramamosain*. It has been shown that FSH and LH may play a role in regulation of gonad development (Huang et al. 2005; Ye et al. 2006). Besides that, estrogen receptor (ER) and androgen receptor (AR) has also been identified in the cerebral ganglion of *S. paramamosain* (Ye et al. 2008). This suggests that the cerebral ganglion of *S. paramamosain* may also be involved in the feedback regulation of crustacean neuroendocrine by ER and AR.

Culturing crustacean neurons in vitro will provide the possibility of undertaking experimental manipulations under controlled conditions. In this study, we reported for the first time methods for in vitro culture of neurons separated from the cerebral ganglion of *S. paramamosain*. Successful culture of crustacean neurons can facilitate further study of their function by electrophysiology analysis as well as biochemical approach.

Materials and Methods

Animals and dissection. Adult, healthy *S. paramamosain*, were purchased from local vendors. The crabs were submerged in potassium permanganate solution (potassium permanganate/sterilized seawater=1:50) for 15 min before scarification. The crabs were then rinsed twice with sterilized seawater to remove the residual potassium permanganate and all the following dissections were performed in a horizontal-flow super clean bench. Cerebral ganglion were removed and rinsed with three changes of sterile crab saline (440 mM NaCl, 11.3 mM KCl, 13.3 mM $CaCl_2$, 26 mM $MgCl_2$, 23 mM Na_2SO_4, and 10 mM Hepes; pH 7.4; supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO 15140, New York, NY)). The tissues were then cut aseptically into smaller fragments with sterile ophthalmic scissors and used for enzymatic cell dissociation.

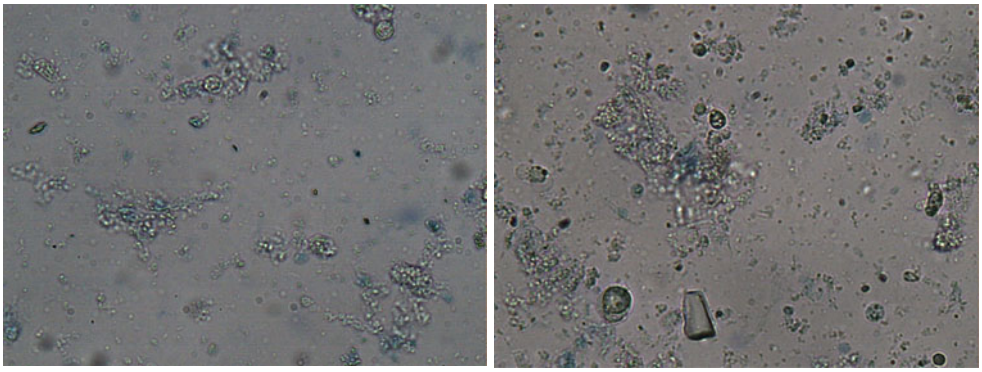
Development of primary cell culture. Cell isolation and culture conditions—in this study, collagenase type I (Sigma, C0130, New York, NY) and trypsin was used for cell dissociation. The tissues were treated with 0.1% Trypsin (Solarbio, T8150, Beijing, China) in Ca^{2+} and Mg^{2+} free crab saline for 90 min at room temperature or 0.2% collagenase type I (Sigma, C0130) for 30 min at room temperature. After enzyme digestion, tissue samples were rinsed thoroughly with crab saline, followed by poking with a sterile glass pipet for roughly 100 times until the cell suspension became turbid. The cell suspension was then filtrated through sterile 200 mesh cell strainers to remove the tissues fragments and large cell aggregates and transferred to sterile culture dishes (Corning 3055, Corning, NY). After culturing for 30 min at room temperature to allow cells to adhere to the culture dish, 10 ml culture medium with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine were added and cells were cultured at 25°C in a biochemical incubator. The culture medium was changed when there are many cell debris floating on the culture medium that might inhibit the cell outgrowth.

A portion of dissociated cells were subjected to trypan blue dye exclusion assay to test cell viability after enzyme digestion.

Different culture media support the cell outgrowth. Liebowitz L-15 medium and modified M199 medium were used in our study. L-15 powder (GIBCO, 511955) was reconstituted and mixed with an equal volume of crab saline of 1.75× normal concentration, buffered with 20 mM Hepes (Cooke et al. 1989). The modified M199 culture medium consisted of Media 199 (GIBCO, 417006) was reconstituted with the saline ($MgSO_4 \cdot 7H_2O$ 12.17 mM, L-glutamine 1.03 mM, NaCl 188.23 mM, $Na_2HPO_4 \cdot 12H_2O$ 0.28 mM, $CaCl_2$ 8.11 mM, KCl 5.37 mM, $NaHCO_3$ 26.19 mM, Hepes 1 mM, $MgCl_2 \cdot 6H_2O$ 14.9 mM, and sodium pyruvate 1 mM).

Effects of supplements. Fetal bovine serum (FBS), *S. paramamosain* muscle extracts and hemolymph were added to the culture medium in order to enhance viability and growth of the neurons. FBS was added to a final concentration of 20%.

For crab muscle extracts, 25 g *S. paramamosain* muscle was homogenized with an equivalent volume of crab saline and were heated to 60°C for 30 min followed by centrifugation at 7,000×*g* for 50 min at 4°C. Resulting



C T

Figure 1. Different enzyme treatment on cell isolation. *C*, The tissues were treated with Collagenase (type I) to treat after trypan blue dye exclusion test. *T*, The tissues were treated with Trypsin after trypan blue dye exclusion test.

supernatant fluid was filtrated through 0.22 μm filter membranes and store at −80°C until later usage.

The hemolymph of *S. paramamosain* was collected from the animal's pleopod as described earlier (Tong and Miao 1996).

Estimation of outgrowth. The outgrowth of cultured cells was monitored daily and microphotographed with a phase-contrast microscope under 200× magnification for up to 2 wks.

Results

Effects of different enzyme treatments on cell isolation. In this study, collagenase type I and trypsin were used for cell isolation. Both of them worked well for cell dissociation from the dissected cerebral ganglion mass. Trypan blue dye exclusion test was also performed to determine cell viability after enzymatic digestion. Both collagenase type I and trypsin showed different initial survival rate (Fig. 1). Thus, collagenase type I was used for all other following experiments.

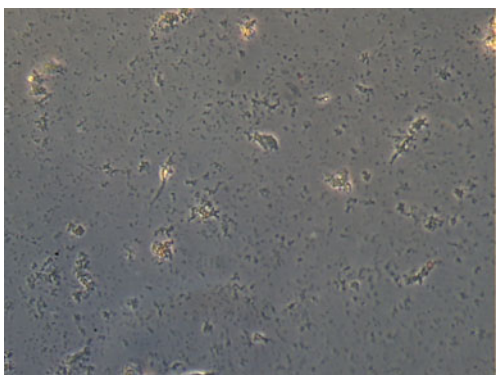


Figure 2. Photomicrographs of neurons isolated from the cerebral ganglions of *Scylla paramamosain* in L-15 medium with 20% FBS, many cells withdraw to cell debris. This photo was recorded at the second d.

Effect of different culture media effects on the outgrowth of neurons. Liebowitz L-15 medium and modified M199 medium was used to support the growth of dissociated cells from the crab cerebral ganglion. Cell growth and viability was monitored daily after dissection. With modified M199 medium, most of the cells out-grew well from the tissue, attached to the surface of the culture dish and maintained distinct morphological characteristic of neurons. In contrast, with Liebowitz L-15 medium, most outgrowth cells retracted and formed debris on the second d (Fig. 2).We concluded that Liebowitz L-15 medium is not suitable for supporting neurons outgrowth after enzyme digestion. Thus, M199 medium was used for the following experiment.

Effect of different supplements on the outgrowth of neurons. Fetal bovine serum, the *S. paramamosain* muscle extracts, and hemolymph were added to M199 medium as supplements to test if they can further enhance the outgrowth of neurons. Only FBS showed obvious stimulating effect on cells outgrowth. Muscle extracts and hemolymph of *S. paramamosain* showed no promoting effect as compared with the control of M199 medium alone. The neuron cells in culture medium with muscle extracts or hemolymph of *S. paramamosain* formed many debris.

Outgrowth of cerebral ganglion neurons of S. paramamosain. Outgrown cells from the mud crab cerebral ganglion were monitored under phase-contrast microscope and microphotographed daily for consecutively 2 wks or until the cells lost typical morphology of neurons and diminished eventually. The cells with axonal stumps showed distinct changes in size and form after cultured for several days. The size of the soma and the number of dendrite got bigger. In contrast, the cells without axonal stumps did not show similar changes and the cells withdrew within several d. Most of cells in the outgrowth can be

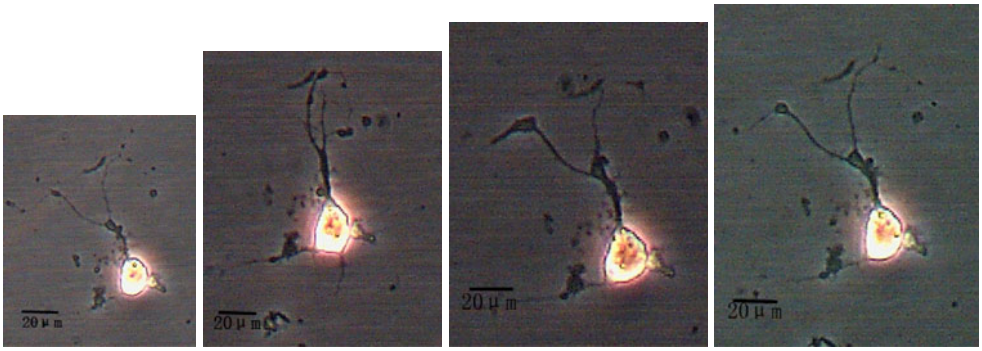


Figure 3. Morphological types of neurons. "Small cell": a spherical soma (20 μm) with an axon (30 μm). *3* Day 3, *4* day 4, *5* day 5, *6* day 6. Photomicrographs of these neurons isolated from the cerebral ganglions of *Scylla paramamosain* in modified M199 medium with 20% FBS.

classified into seven categories by their size and morphological difference during the first few d in culture. The first six types cells were similar to the cells outgrown from the land crab *C. carnifex* X-organ as described earlier (Grau and Cooke 1992).

Seven morphological types of neurons:

1. "Small cells" (Fig. 3): The soma of these neurons was roughly 20 μm in diameter. The cells always produced an axon at one polar. The axons would grow up to three to five times longer than the soma diameter during the first 5 d after the cells dissociation. The small cells showed slow outgrowth. On the sixth day, most of the axons retracted rapidly, which was usually coupled with the cell withdrawal. This suggests that the neurons without axon are difficult to maintain in vitro culture or the retraction of the axon is the first step in the cell degradation in vitro.

2. "Veilers" (Fig. 4): This type of cells has a spherical soma about 10 μm. Each of the cells has a single neurite, which is thick and protruded from the whole soma. The neurite, also known as filopodia, usually grows rapidly into a bigger one during the culture. This type of cell can grow for about 6 d in culture medium and then withdraw rapidly after that.

3. "Branchers" Two kinds of cells with different morphologies are classified into this type in our observations. These two had some common characteristics: ovoid soma of about 20 μm in diameter—unipolar neurite—some branches extending from the soma in the middle or the end of the neurite. Many filopodia extruding from the neurite at the middle or the end of the neurite would continue to grow and form a new branch. Besides that, each of this sub-group also has its own characteristics.

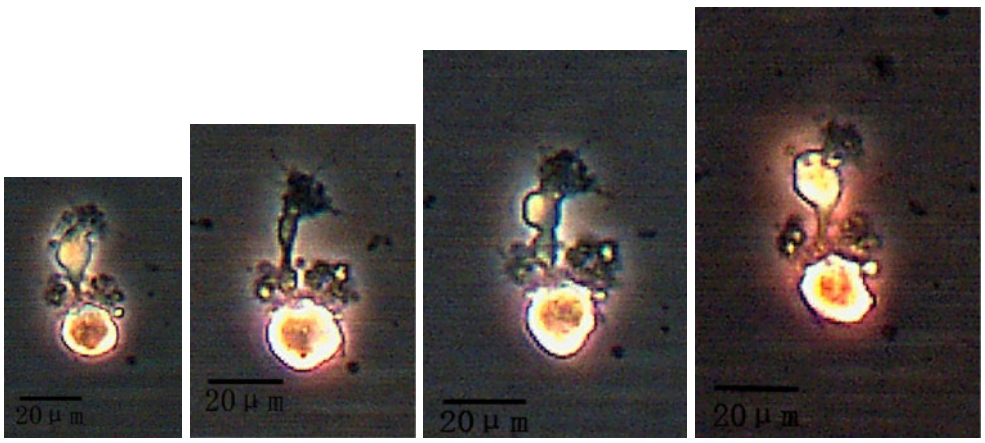
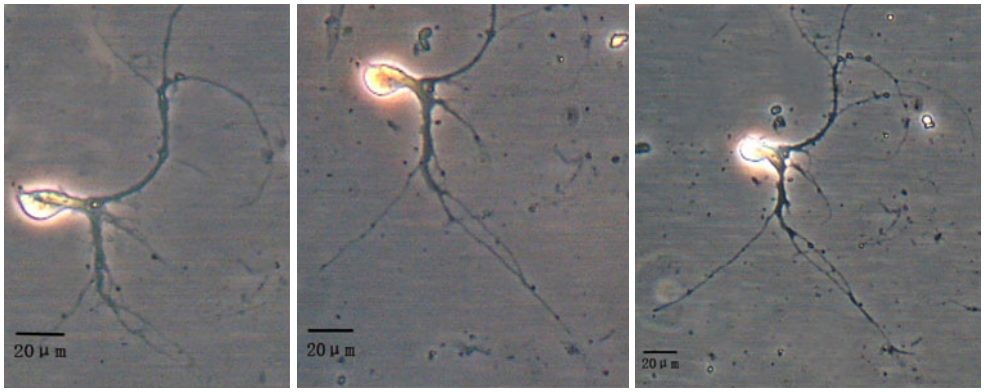
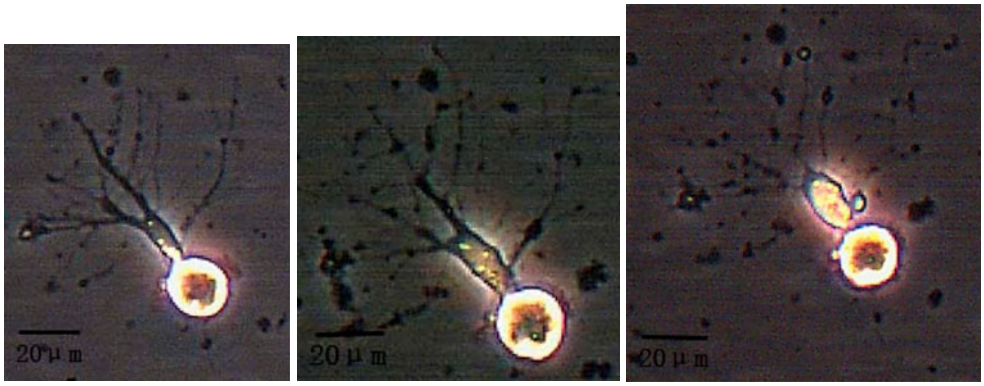


Figure 4. Morphological types of neurons. "Veilers": a spherical soma about (10 μm), the cell has a single neurite, which is thick and is produced from the whole soma. *2* Day 2, *3* day 3, *4* day 4, *6* day 6. Photomicrographs of neurons isolated from the cerebral ganglions of *Scylla serrata* in modified M199 medium with 20% FBS.



3 4 6

Figure 5. Morphological types of neurons. Photomicrographs of neurons isolated from the cerebral ganglions of *S. paramamosain* in modified M199 medium with 20% FBS. "Branchers": ovoid soma (20 μm), unipolar neurite, some branch extending from the soma, at the middle or the end of the neurite, many filopodia occurring from the neurite and continue to grow and developed a new branch. *3* Day 3, *4* day 4, *6* day 6.



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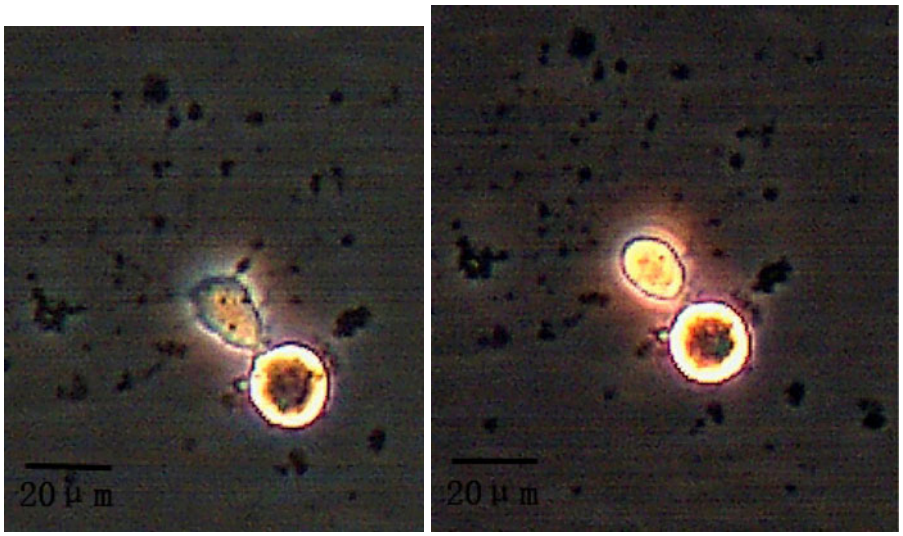

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Figure 6. Morphological types of neurons. "Branchers": ovoid soma (20 μm), unipolar neurite, some branches extending from the soma or at the middle or the end of the neurite, many filopodia occurring from the neurite and continue to grow and developed a new branch. *4–2* A single thick major neurite occurring from the soma. The branches occurred at the end of the thick neurite. *2* Day 2, *3* day 3, *4* day 4, *5* day 5, *6* day 6. After the sixth day, the cell withdraws obviously. Photomicrographs of neurons isolated from the cerebral ganglions of *S. paramamosain* in modified M199 medium with 20% FBS.

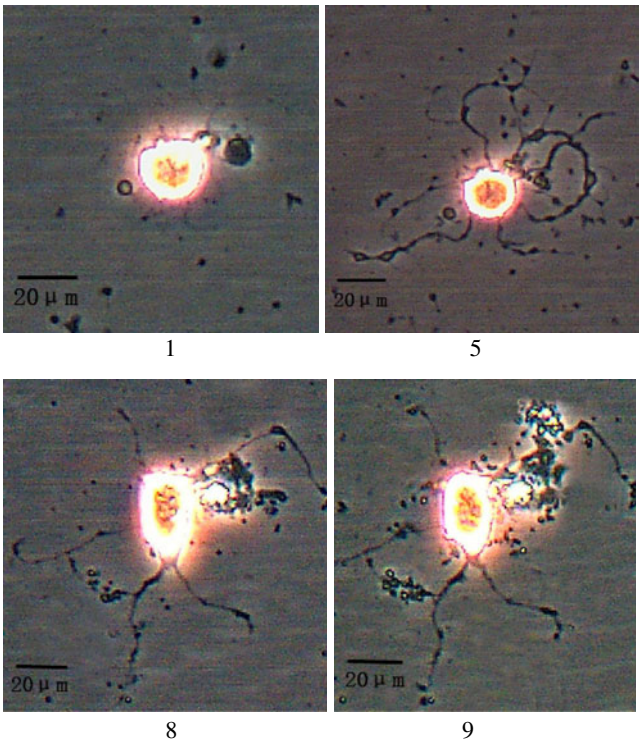


Figure 7. Morphological types of neurons. “Multipolar cells”: a spherical soma (20¦Ìm), many processes extended from the soma, no major neurite. *1* Day 1, *5* day 5, *8* day 8, *9* day 9. Photomicrographs of neurons isolated from the cerebral ganglions of *S. paramamosain* in modified M199 medium with 20% FBS.

(a) "Branchers a" (Fig. 5): In this cell type, many protruding branches grow cone ramified at their ends. The filopodia showed excellent outgrowth in the culture. Typically, this cell type was observed on day 3 of in vitro culture and withdrawn rapidly after day 6.

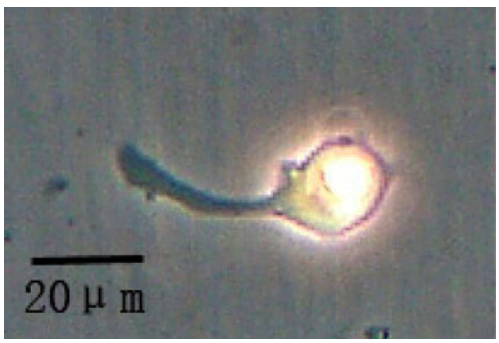


Figure 8. Morphological types of neurons. This type of cells often have a spherical soma (20 μm) with a single neurite. But there is no filopodia or branch. The cells do not show outgrowth, day 2. Photomicrographs of neurons isolated from the cerebral ganglions of *S. paramamosain* in modified M199 medium with 20% FBS.

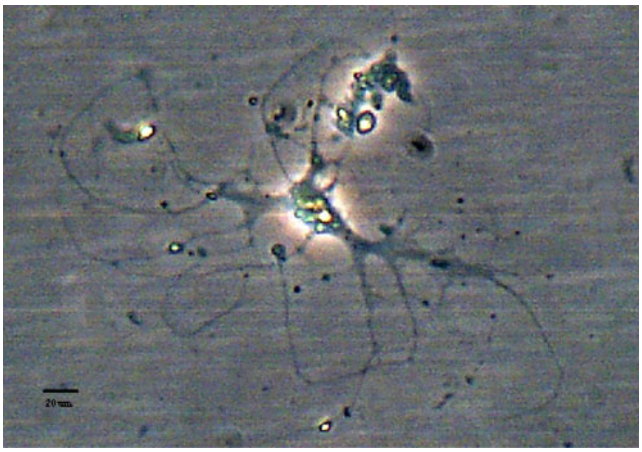


Figure 9. Morphological types of neurons. “Super-large cell” the super-large cell had a large soma (60 μm), observed on the second day. Photomicrographs of neurons isolated from the cerebral ganglions of *S. paramamosain* in modified M199 medium with 20% FBS.

(b) "Branchers b" (Fig. 6): For this cell type, there was only one thick major neurite from the soma. These cells were observed on day 2 of in vitro culture. After day 4, the filopodia usually retracted dramatically. However, the thick neurite would continue to extend and get thicker and become spherical. On day 6, the neurite would separate itself from the soma and the cell deteriorated eventually.

4. "Multipolar cells" (Fig. 7): For this cell type, each cell had a spherical soma about 20 μm in diameter. There are also many processes extended from the soma and most of them are thin branches with no major neurite observed for this cell type.

5. "Unipole cells" (Fig. 8): This type of cells typically had a spherical soma of more than 30 μm with a single neurite. But there was no filopodia or branch. These cells usually did not show outgrowth.

6. "Super-large cell" (Fig. 9): The super-large cell had a soma about 60 μm in diameter, which is about two to three times larger than the other cell types. It was phase-bright when observed under phase-contrast microscope.

7. "Bipolar cells" (Fig. 10): Many cells from the outgrowth can be classified into this type in our study. These cells had two neurites, growing in opposite directions. The cells had a spherical or ovoid soma about 15–20 μm. Usually they

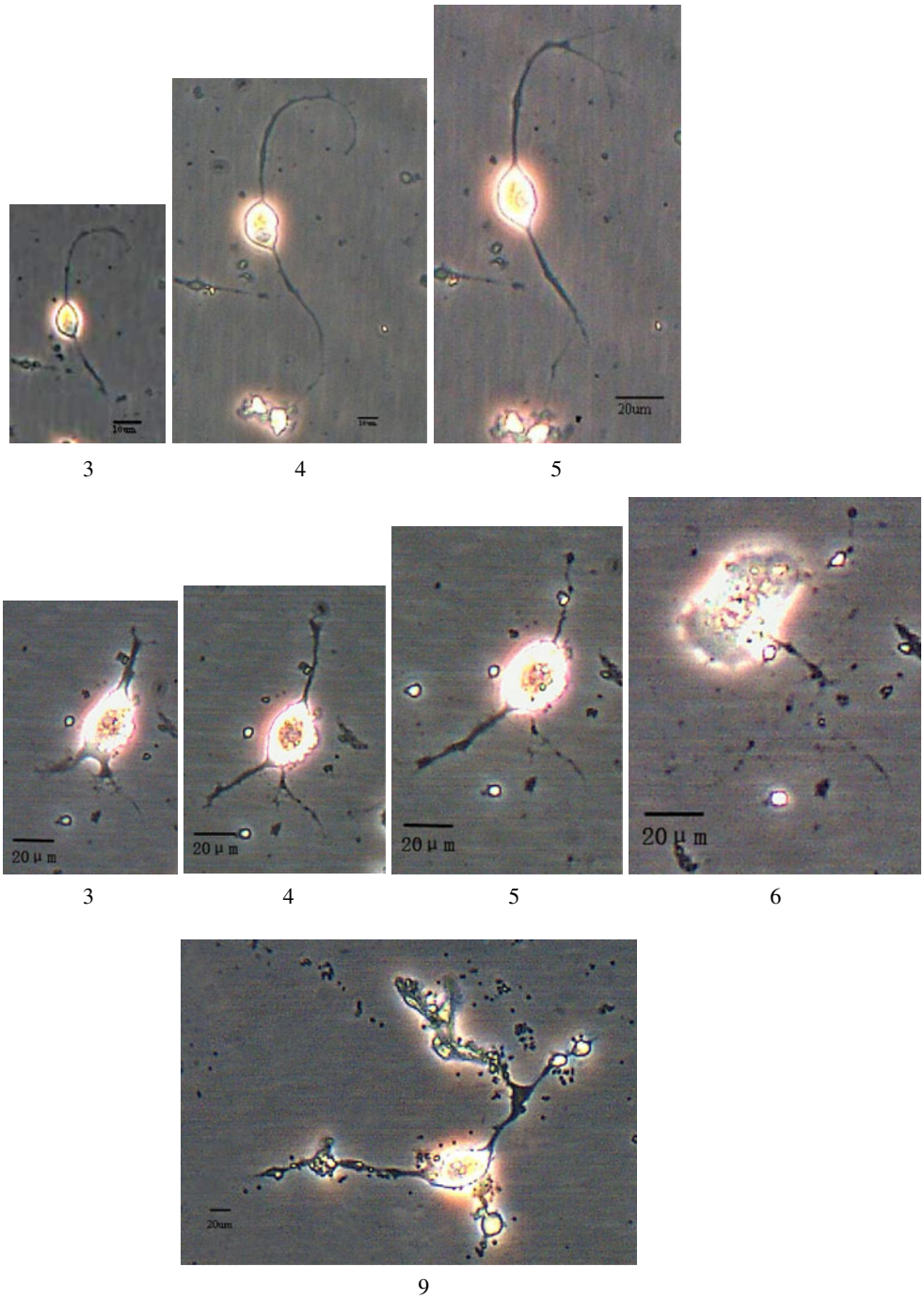


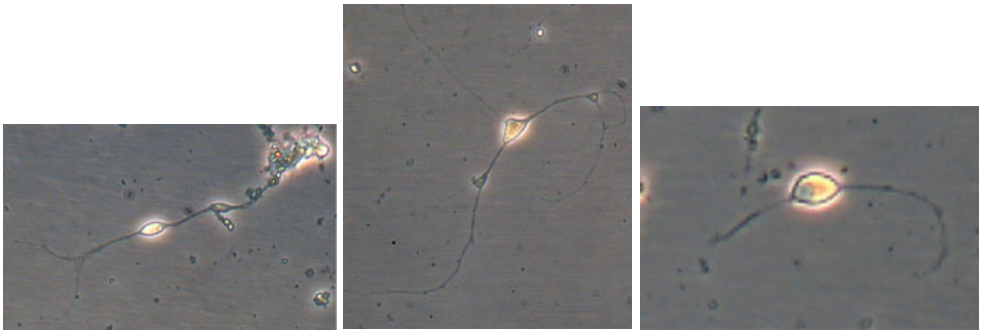
Figure 10. Morphological types of neurons. "Bipolar cells": ovoid soma (15–20 μm), neurite grow in two opposite directions. *3* Day 3, *4* day 4, *5* day 5. *9* This figure is recorded on the ninth d. Photomicrographs of neurons isolated from the cerebral ganglions of *S. paramamosain* in modified M199 medium with 20% FBS.

appeared in the first day of in vitro culture. Some of them maintained outgrowth until day 9 or even longer.

Discussion

In this report a method for culturing cerebral ganglion neurons from *S. paramamosain* was first established. Neurons were cultured for up to 9 d in modified M199 medium supplemented with 20% FBS. The neurons kept good morphologies and showed outgrowth in vitro. More than six kinds of neurons with different morphologies characterized were observed in our study (Fig. 11).

We first tested which enzyme is suitable for dissociation of cells from *S. paramamosain* cerebral ganglion by using collagenase type I and trypsin, which are commonly used for primary cell preparation in both vertebrate cells and invertebrate cells. Both of collagenase type I and trypsin worked well for cell dissociation from the dissected



3 3 3

Figure 11. Morphological types of neurons. "Biopolar cells" the biopolar cells had two axons occurring from the soma at the opposite sides. Photomicrographs of neurons isolated from the cerebral ganglions of *S. paramamosain* in modified M199 medium with 20% FBS.

cerebral ganglion mass. Trypan blue dye exclusion assay was also performed to determine cell viability after enzymatic digestion. Both of collagenase type I and trypsin used in our studies showed similar initial survival rate after treatment of tissues (Fig. 1). The observations were similar to those reported for in vitro culture of peptidergic neurons of *C. carnifex* (Cooke et al. 1989). In general, collagenase type I can hydrolyze the amphipathic helix of collagen at physiological pH and temperature, however, it does not hydrolyze other proteins and tissues. Compared to the trypsin, collagenase type I is mild in enzymatic digestion. Our results have shown the neurons cells from *S. paramamosain* cerebral ganglion can tolerate both trypsin and collagenase type I digestion. To lessen the experiment time, we used collagenase type I for all other experiments that followed.

Many studies had been done to establish methods for in vitro culture of crustacean cells. Historically, different media were used for different crustacean species to support their outgrowth. In the early studies, L-15 media were widely used, which was reconstituted by mixing L-15 with an equal volume of crab saline of 1.75× normal concentration buffered with 20 mM Hepes (pH 7.6~7.8; Cooke et al. 1989), modified L-15 medium has also been developed and showed better support for in vitro culture of the crustacean cells (Cooke et al. 1989; Sun et al. 2000; Sashikumar and Desai 2008). A medium for penaeid shrimp MPS, a newly designed medium for penaeid shrimp, has been reported to support the cell culture of penaeid shrimp (Tong and Miao 1996). In this study, we found that the modified M199 medium works better than L-15 medium in supporting cell culture of cerebral ganglion neurons of *S. paramamosain*. This result suggests that neurons of *S. paramamosain* may have unique nutrient requirement for in vitro culture, which cannot be provided by L-15 medium. When the neurons of *S. paramamosain* were cultured in L-15 medium, many cells degenerated in day 2. This did not happen when the cells were cultured with modified M199 medium. It was also possible that the osmolarity and pH of L-15 medium is not compatible with the neurons of *S. paramamosain*, while the osmolarity and pH of M199 medium resembles the in vivo physiological conditions and can provide better support for in vitro culture of neurons of *S. paramamosain*.

Previous works have shown different supplements to the basic culture medium can stimulate crustacean cell growth in vitro, such as FBS, crustacean muscle extracts, and hemolymph (Tong and Miao 1996). In our study, we determined if FBS, muscle extracts, and hemolymph of *S. paramamosain* neurons from cerebral ganglion. Our results have shown that 20% FBS can improve the cell growth in M199 medium, while no significant stimulating effect was observed when the muscle extracts and hemolymph of *S. paramamosain* were added as the supplements.

The effect of FBS on invertebrate neuron cell culture is still of controversy. The peptidergic neurons of *C. carnifex* cultured in L-15 medium without FBS kept good morphologies and showed outgrowth (Cooke et al. 1989). The thoracic neurons of the migratoria locusts, *Locusta migratoria*, remained viable for up to 3 wks in a serum-free defined culture medium (Kirchhof and Bicker 1992). However, there are also evidences that FBS could increase neurons outgrowth in tissues cultures of the prawn, *Penaeus chinensis* (Tong and Miao 1996), and penaeid shrimp (Chen and Wang 1999). Our results have shown that FBS was essential for outgrowth of neuron cells from the cerebral ganglion of *S. paramamosain*. Without FBS, the neurons could grow for 2 d in M199 medium and then degenerated quickly. In contrast, the neurons cultured in M199 supplemented with 20% FBS kept viable and showed outgrowth up to 9 d. It is possible that some key nutrient required for culture of *S. paramamosain* neurons in M199 medium will be depleted after short-term culture. While supplementing of FBS can replenish the supply of these key nutrients and further support the growth of *S. paramamosain* neuron up to 9 d.

There are also reports that crustacean muscle extacts and hemolymph can affect crustacean cell growth in vitro, either positively or negatively. Lobster muscle extracts and hemolymph has been shown to prolong the survival time of lymphoid tissue cells (Chen and Wang 1999). While the

extracts of shrimp muscles were less effective and the hemolymph of the mantis shrimp, *Squilla oratoria*, was inhibitory in tissue culture of the prawn, *Penaeus chinensis* (Tong and Miao 1996). We found that *S. paramamosain* muscle extracts and hemolymph cannot significantly promote outgrowth of its neurons. Instead, neuron cell growth was restrained in the presence of *S. paramamosain* muscle extracts and hemolymph. This result suggests that different crustacean species may have different nutrient requirement for in vitro cell growth, which also underline the importance of tailoring the culture meida formulation for different crustacean species.

Whereas the histology and the ultra-structure of neurosecretory cells in cerebral ganglion of *S. paramamosain* had been reported before (Huang et al. 2001; Huang et al. 2003), neurons in our study displayed a great variety in morphology. Most of the cell types identified in our study were similar to that reported for *C. carnifex* and *Panulirus marginatus* previously (Cooke et al. 1989; Grau and Cooke 1992). They are "small cells", "veilers", "branchers", "multipolar cells", and "super-large cell". Differences in outgrowth form may reflect difference in abundance and distribution of granules and microtubules in concert with signals derived from surface membrane contact with the substrate (Cooke et al. 1989). We have also identified "bipolar cell" in the neuron cell outgrowth of *S. paramamosain* for the first time. "bipolar cell" has only been found in insect neurons culture before (Weigel 2005; Smith and Howes 1996) and has not been reported in crustacean neurons culture to the best of our knowledge. Bipolar cells could be identified in culture on day 3 and kept alive for up to 9 d. The biological function of this cell type needs further study.

Our successful culture of cerebral ganglion neurons can help us to understand the outgrowth and withdrawal mechanism of *S. paramamosain* neurons in vitro. It provides an important tool for further research on the neurosecretory cells in cerebral ganglion. With this in vitro culture system, it is possible to determine the accurate location and function of the specific cell types in the cerebral ganglion of *S. paramamosain* and understand the endocrine metabolism and regulation on the single cell level. Further study of the characteristics of the neurons by biochemical and electrophysiological means are under way in our lab. The long-term goal is to establish a cell line with particular function which can excrete specific a kind of hormone in order to study the neurophysiology in vitro.

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Ethical standards The experiments comply with the current laws of the country in which they were performed.

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