Animal Cells and Systems 13: 267-274, 2009

Animal Cells
and
Systems

http://www.AnimalCells.or.kr

Effects of Protease Treatment and Animal Behavior on the Dissociative Culture of *Aplysia* Neurons

Nuribalhae Lee, Young-Soo Rim, and Bong-Kiun Kaang*

National Creative Research Initiative Center for Memory, Department of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 151-747, Korea

Abstract: The dissociative culture technique of *Aplysia* neuron is one of the key methods that have been used for studies of cellular and molecular mechanisms of neuronal functioning. However, despite the advantages this method offers as an experimental model, its technical efficiency has had room for improvement. In this study, we examined certain putative factors that might affect the culture quality. The effects of neuronal damage induced by physical injuries, heat shock, and surface protein degradation were evaluated along with the correlation between the culture quality and animal behavior. As a result, we found that physical injury can be a critical factor that affects culture quality, whereas the heat shock and surface protein degradation had negligible effect on it. In addition, we discovered that siphon retraction time was not a good measurement for healthy neurons. Based on these findings, we suggest here an improved method in which the degree of physical injury is reduced by means of multiple protease treatment.

Keywords: *Aplysia*, dissociative culture, heat shock, protease treatment, siphon withdrawal reflex

INTRODUCTION

The central nervous system of invertebrates, including leeches and snails, has been a useful model for neurophysiologists. *Aplysia, Helisoma, Helix, Limax, Lymnaea,* and many other invertebrates of the phylum Mollusca were used in various neurophyiological studies which contributed greatly to our understanding of neuronal physiology (Kandel, 1976, Hoyle, 1977, Goodman and Pearson, 1982). This was mainly because of two advantages: simplicity of the nervous system and large size of the neurons. These two features enabled better characterization and manipulation of invertebrate nervous systems, making them extraordinary experimental models for neurophysiology. The nervous system of sea mollusca *Aplysia* is a particular example of these invertebrate models. With its detailed map of well identified neurons and their connectivity (Kandel et al., 1967), *Aplysia* has been used as a powerful tool for studying the cellular and molecular mechanisms of neuronal functioning (Kandel, 1976; 1979).

Dissociative cell culture technique, which also relies greatly on these advantages, extended the value of *Aplysia* nervous system as an experimental model (Schacher and Proshansky, 1983). Because of the convenience of identification and manipulation, investigators could make cultures of neurons in specific partnerships as they were *in vivo*. Combined with microinjection techniques (Kaang et al., 1992; 1993, Kaang, 1996), dissociative culture became a very powerful tool for studying neuronal physiology under extremely controlled experimental conditions. For this reason, the dissociative culture of *Aplysia* neuron has been used in a number of experiments showing the monosynaptic modifications (Rayport and Schacher, 1986), changes in cellular level and composition of mRNAs and proteins (Montarolo et al., 1986), or role of a specific molecule or a signaling pathway during the process of memory formation (Jang et al., 2005, Kim et al., 2006, Lee et al., 2006, Lee et al., 2007; Lee et al., 2008).

Despite all these advantages, dissociative culture technique still has some room for improvement. Broad variation of the culture quality is one of the major areas that require methodological improvement. Even though this variation has been disturbing the efficient production of qualified cultures, until now the only ways to overcome this were to improve the skill of the experimenters or sacrifice more animals. This was mainly because very little was known about the factors that affect the culture quality.

*To whom correspondence should be addressed.
Tel: +82-2-880-7525; Fax: +82-2-884-9577
E-mail: kaang@snu.ac.kr

In this study, we tested some putative factors that may affect the quality of the dissociative culture of *Aplysia* neurons: mechanical injuries, heat shock, surface protein degradation, and healthiness of the animal. Experiments were performed based on the previous protocol which has been used for the *Aplysia kurodai* (Lim et al., 1997). We found that mechanical injuries are the predominant factor that affects the culture quality. Also, based on the results of the experiments, we suggest here some modified methods that can be useful in specific experimental conditions.

MATERIALS AND METHOD

Animals

Aplysia kurodai was purchased from professional sea-divers in Pusan and Yung-duck, Korea. Animals were maintained in a recirculating sea water bath at 14 and exposed to a 12 h light/night cycle before use.

Protease solutions

Protease solutions were freshly prepared by dissolving protease in isotonic L-15 medium to the final concentration of 10 unit/mL. Four types of proteases were used for the experiments: protease from *Bacillus polymixa* type IX (Sigma), proteinase from *Aspergillus mellius* type XXIII (Sigma), protease from *Aspergillus sojae* type XIX (Sigma), and protease from *Bacillus licheniformis* (Sigma). Prepared protease solutions were filtered with 0.2 μm syringe filter (Sartorius) before usage.

Cell culture

Cell Culture was done basically following the protocol as described by Lim et al. (1997). The hemolymph was collected from the body cavity of *A. kurodai* (150-300 g) and filtered through a 0.45 μm syringe filter (Sartorius). The culture medium was made by mixing the hemolymph with equal volumes of 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 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 6.24 mg/mL dextrose.

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 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). Protease treatment was performed by incubating prepared ganglia in protease solutions described above. Incubation time for each experimental condition was chosen by preliminary experiments.

Ganglia were washed several times with ASW after the protease treatment and desheathed carefully by using microscissors and microforceps. After pinning the ganglia on a sylgard (Dow Corning) plate, neurons were dissociated with a long glass capillary as described in Schacher and Proshansky (1983).

For sensory neuron cultures, 16 intact neurons were dissociated from the sensory cluster of left pleural ganglion. Dissociated neurons were transferred and plated carefully onto poly-L-lysine (Sigma)-coated culture dishes (P50G-0-14-F, MatTek Corp.) containing isotonic L-15 with hemolymph. For sensory to motor cocultures, LFS motor neurons were dissociated from the abdominal ganglion and plated on the first day. Sensory neurons were attached to the motor neuron to make synapses on the following day, as in the sensory neuron cultures. All cultures were maintained in 18°C incubator for 3 or 4 days before experiments.

Microinjection

Microinjection into *Aplysia* neurons was carried out as previously described (Kaang, 1996). Sensory neurons in sensory to motor cocultures were microinjected with an injection solution containing 0.5 μg/ μL of pNEXδ-EGFP.

Electrophysiology

The membrane potential of cultured sensory neurons was measured 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 were recorded 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 neurons, 5-hydroxytryptamine creatine sulfate (5-HT) (Sigma) was applied by introducing a small volume of a concentrated stock solution to yield the final concentration of 10 μM in the bath.

Excitatory post-synaptic potential (EPSP) recordings for sensory to motor cocultures were performed as described previously (Lee et al., 2007). LFS motor neurons were impaled with glass microelectrodes to measure the EPSP. For the induction of long-term facilitation (LTF), five pulses of 5-HT (10 mM) were applied with an inter-stimulus interval of 15 min. 24 h after the 5-HT treatment, final EPSP was measured. All physiological recordings were performed at 18°C.

Siphon retraction time measurement

The measurement of the siphon retraction time (SR) was performed before body weight measurement and anesthetization. A fully stretched siphon was stimulated

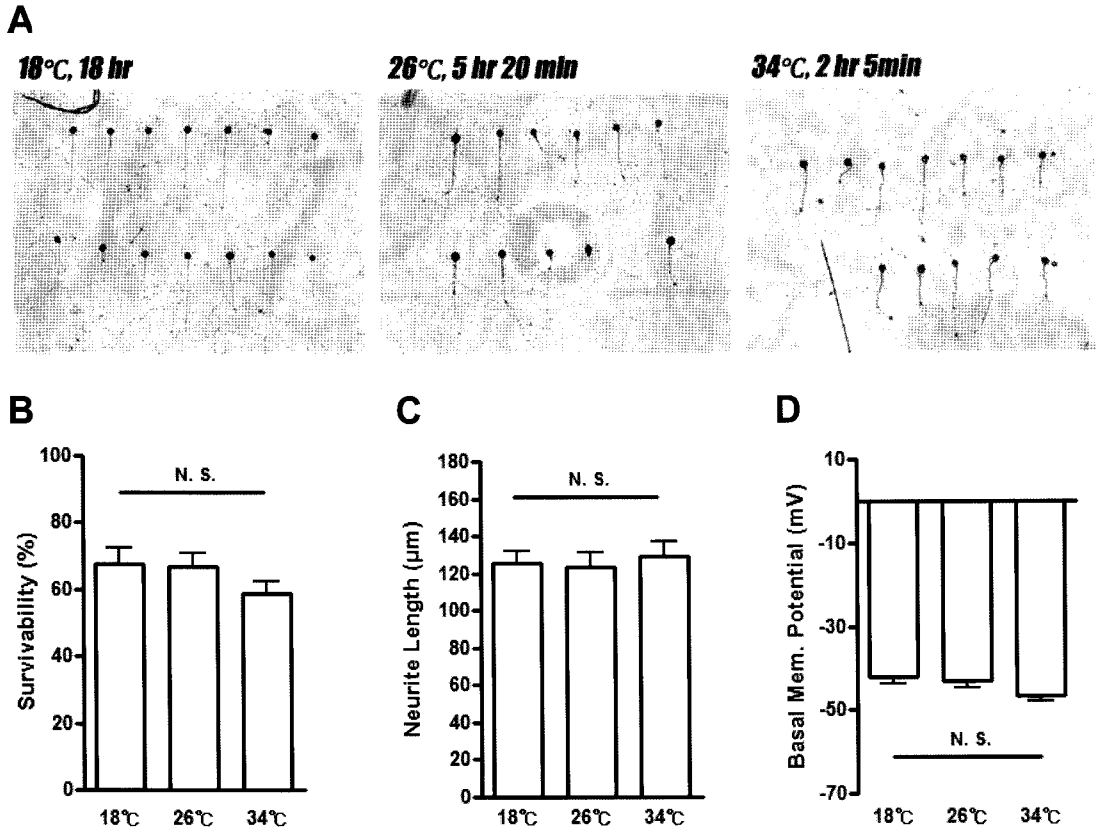

Fig. 1. Comparison between the cultures made under different protease treatment conditions. Three parameters showing properties of three different groups, including under 18 for 18 h (18), 26 for 5 h and 20 min (26), and 34 for 2 h and 5 min (34), were measured and analyzed. (A) Appearance of the plates cultured under each condition. The neurons are aligned in two rows for comparison. The scale bar shows 100 μm. Plates showed no difference in (B) survivability (18, n=10, 26, n=10, 34, n=10, $P>0.05$, one-way ANOVA and Newman-Keuls multiple comparison test), (C) neurite length (18, n=108, 26, n=107, 34, n=94, $P>0.05$, one-way ANOVA and Newman-Keuls multiple comparison test), and (D) basal membrane potential (18, n=35, 26, n=35, 34, n=35, $P>0.05$, one-way ANOVA and Newman-Keuls multiple comparison test). All data are expressed as a mean±the standard error of the mean (SEM). Error bars indicate SEM.

mildly to induce the retraction, and the time between complete retraction and full restoration was measured.

RESULTS

The amount of heat shock does not affect the culture quality

Heat shock is the effect of subjecting a cell to a temperature higher than that of the ideal body temperature of the organism from which the cell was derived. In such high temperature, cells experience destabilization of proteins and nucleic acids that causes homeostatic unbalance. This destabilization is known to be controlled by the heat shock response, which is initiated by the activity of heat shock transcription factors and heat shock element that also respond to various other stresses, unless the amount of destabilization exceeds the threshold (I. Shamovsky and E. Nudler, 2008).

In previous methods for dissociative culture, the dissected ganglia were incubated under 34°C, which is 16°C higher than the ideal temperature, during 2 h of the protease treatment step. In the first experiment, we tried to figure out whether the heat shock induced here is critical for the culture quality.

We tested three different treatment temperatures using protease type IX, which has been used in previous methods (Schacher and Proshansky, 1983, Lim et al., 1997). Cell-favorable temperature (18°C), room temperature (26°C), and optimal protease activity temperature (34°C) were selected for the representative test. Since protease activity varied according to the temperature, incubation time was adjusted for each condition. Time-serial incubation under each temperature was performed to find the time point that guarantees proper single cell dissociation, which implies similar level of surface protein degradation and similar degree of physical injuries. 18 h under 18°C, 5 h and 20 min under 26°C, and 2 h and 5 min under 34°C were selected according to the test (Data not shown).

After 4 days of stabilization, survival ratio, basal membrane potential, and the neurite length of surviving cells were measured (Fig. 1A). The different protease treatment conditions showed similar rates of survival, mean lengths

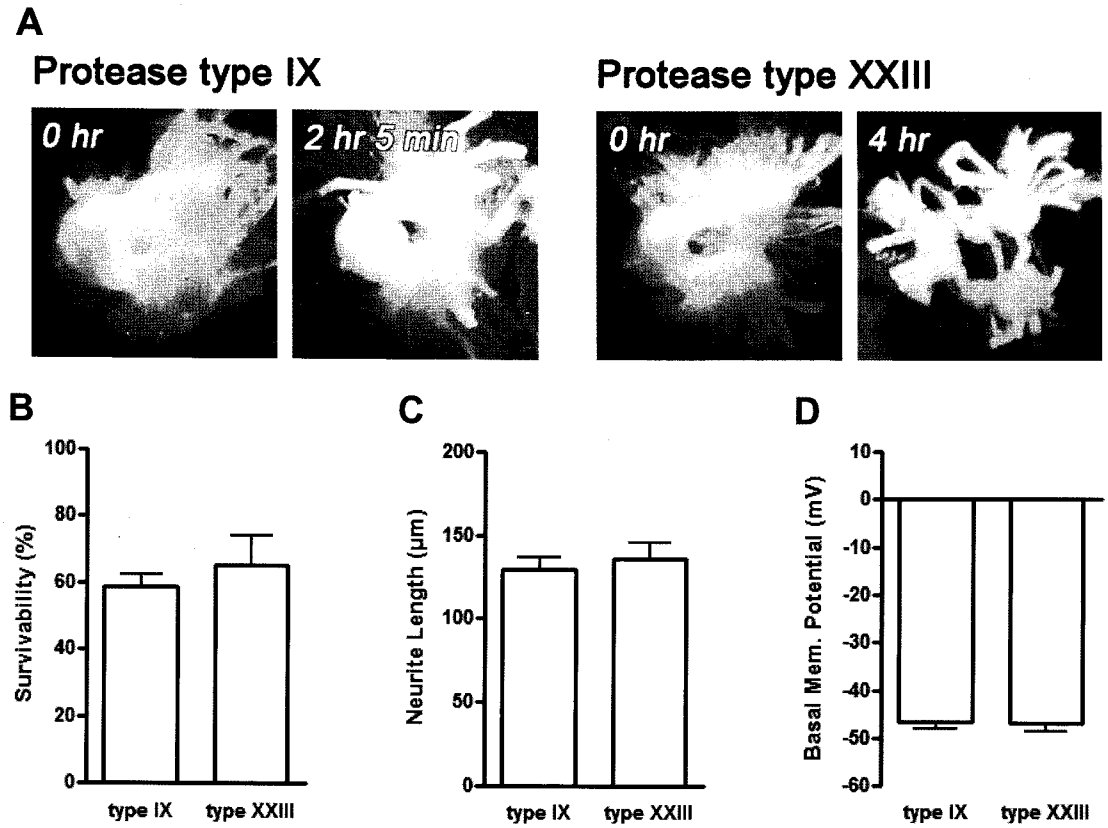

Fig. 2. (A) Treatment of protease type XXIII under 34 for 4 h showed a very different pattern of degradation compared to that of protease type IX under 34 for 2 h and 5 min. But the comparison between groups of protease type XXIII treatment and protease type IX does not show any significant difference. (B) Comparison between survivabilities (Type IX, n=10, Type XXIII, n=5, p>0.05; unpaired t test), (C) Comparison between neurite lengths (Type IX, n=94, Type XXIII, n=42, P>0.05; unpaired t test), (D) Comparison between basal membrane potentials (Type IX, n=35, Type XXIII, n=15, P>0.05; unpaired t test). All data are expressed as a mean±SEM. Error bars indicate SEM.

of neurites, and basal membrane potential (P>0.05; one-way analysis of variance (ANOVA) and Newman-Keuls multiple comparison test) (Fig. 1B-D).

Differential damages from protease-induced surface protein degradation do not affect the culture quality

Although the purpose of the protease treatment is to weaken the structures that mediate cell to cell or cell to sheath linkages, destruction of the proteins exposed on the surface of the cell is also inevitable. Destruction of these proteins can be lethal for the cell, since these proteins can be channels, transporters, or scaffolding proteins for the maintenance of cytoskeletal structures or signaling cascades. The cell death, which is observed when the protease treatment time is increased, shows that this damage actually affects the cell condition during dissociative cell culture.

In the second experiment, we examined if these damages can affect the culture quality even after 4 days of stabilization, and if so, whether the type-dependent target specificity of a protease can be a factor that alters the effect. Two different types of protease with different origins and different target specificities were selected for the representative test: protease from *Bacillus polymixa* type IX, which has been used for previous methods and proteinase from *Aspergillus mellius* type XXIII, which is known to have strong collagenase activity (Molla et al., 1989).

Treatment of protease type XXIII showed differents effect on both the sheath and the extracellular matrix compared to protease type IX. Its activity on the sheath was much stronger than that of protease type IX, degrading most of the sheaths in an hour, while its effect on the extracellular matrix was much weaker (Fig. 2A). Since the activity of protease type XXIII was totally different from protease type IX, time-serial incubation for the selection of a proper incubation time was performed as in the first experiment. Under the temperature of 34°C, 4 h of incubation was selected as the time period which guarantees proper single cell dissociation (Data not shown).

After the whole process of dissociative culture and 4 days of stabilization that followed, the survival ratio, basal membrane potential, and neurite length were measured as in the previous experiment. All parameters indicated no significant difference between the two groups (P>0.05; unpaired t test) (Fig. 2B-D).

In addition to two proteases tested above, two additional proteases, protease from *Bacillus licheniformis* and

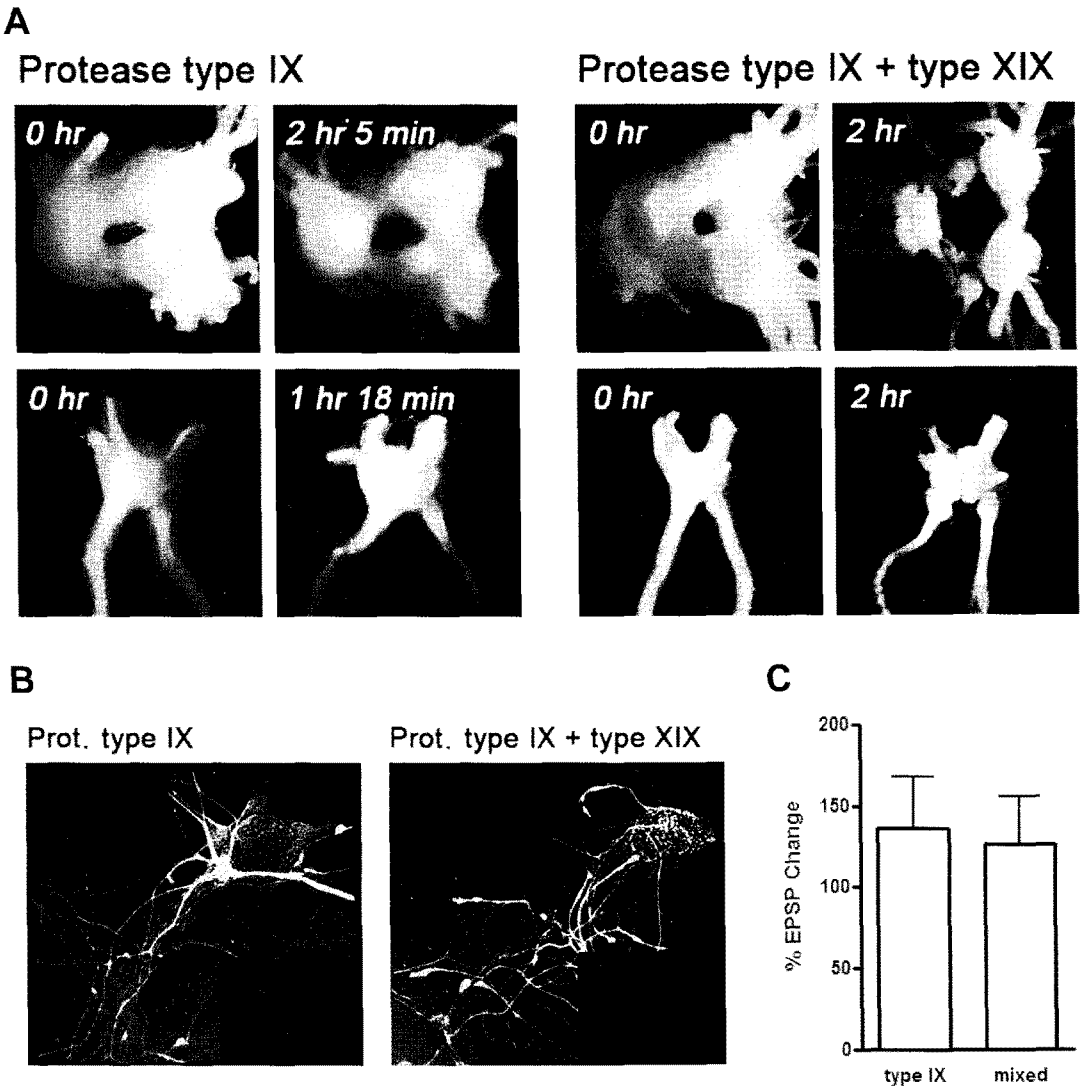

Fig. 3. Treatment of mixed solution of protease type IX and XIX was sufficient for dissociative culture of *Aplysia* neurons. (A) After 2 h of incubation, both central and abdominal ganglia lost most of their sheath. Single neurons were dissociated easily. (B) Sensory to motor coculture made under protease type IX and mixed protease conditions. Cultured sensory neurons have no defects that compromised expression of microinjected enhanced green fluorescent protein (eGFP). The soma of motor neurons are located in the center of the images and the neurites from the adjacent sensory neurons, labeled with eGFP, are entangled around the neurite of Motor neurons. (C) In response to 5x 5-HT treatment, LTF was induced in both cocultures made under different conditions.

protease from *Aspergillus sojae* type XIX were tried for the dissociative culture. The effect of each protease was similar to that of the previously tested one with a similar origin (Data not shown).

Treatment of multiple proteases is effective for the qualified culture production

Since the results of preceding experiments showed that the damages from heat shock or surface protein degradation are not critical for good culture quality, we hypothesized that control of the mechanical injury alone can be sufficient for producing cultures with good quality. To confirm this idea, we tested if a new protease treatment method that just controls the amount of mechanical injuries, and is designed to increase the convenience of the experimenter, would be a plausible option.

For the new method, we chose two types of proteases for the mixed protease solution: Protease from *Aspergillus sojae* type XIX, which shows strong collagenase activity, and protease from *Bacillus polymixa* type IX, which has stronger effect on the degradation of the extracellular matrix. Both types of protease were dissolved in isotonic media to the concentration of 10 unit/mL each. As in the previous experiments, time-serial incubation for the selection of a proper incubation time was performed for both abdominal ganglion and central ganglion. Under the temperature of 34°C, 2 h of incubation time was selected for both abdominal and central ganglia (Data not shown).

Under this condition, both types of ganglia lost most of their sheaths, allowing the omission of the desheathing step (Fig. 3A).

One day after the dissociative culture of LFS motor neurons from abdominal ganglia, sensory neurons were dissociated from the left pleural ganglia and cultured to make synapses. After 3 days of stabilization, sensory neurons were microinjected with pNEXδ-EGFP construct. Cultured neurons showed no defects that compromise the overexpression of microinjected EGFP (Fig. 3B), and also on the synapse formation and 5x 5-HT-induced LTF induction (P>0.05, unpaired t test) (Fig. 3C).

Correlation between animal behavior and cultured cell condition

Health of the animal is one of the key factors that are practically known to affect the efficiency of the dissociative culture. In the fourth experiment, we tested the possibility of animal behavior reflecting health of the animal, thereby providing an effective criterion for better animal selection.

We chose siphon withdrawal reflex, which is one of the most widely used behavior model in the study of learning and memory of *Aplysia* (Pinsker et al., 1970, Castellucci et al., 1970, Carew et al., 1983, Hawkns et al., 1983), as the behavior to be tested, since the sensitivity of this behavior can be measured by a very simple test, without causing any significant change on the properties of the neurons involved. Supporting our hypothesis, siphon retraction time showed a tendency to increase with the period time the animal was kept in the aquarium (3-month breeding group, 36.54±3.160 sec; n=8 vs. 2-week breeding group, 23.10± 1.757 sec; n=8, $^{**}P$<0.005, unpaired t test), which means that the overall condition of an animal is actually reflected by the sensitivity of its behavior, at least on the siphon withdrawal reflex.

Immediately after the measurement of siphon retraction time, animals were anesthetized and the ganglia were dissected for dissociative culture. The dissociative culture was performed following the previous method (Lim et al., 1997) and three parameters that represent the responsiveness of an *Aplysia* sensory neuron were measured: basal membrane potential, minimal current to generate an action potential (AP), and the excitability increase in response to 5-HT bath application. The measurements were statistically analyzed for any correlation. We could not find any significant correlation either between the siphon retraction time and basal membrane potential (R=0.277, P=0.382) and minimal current to generate an AP (R=0.025, P=0.939), or between the siphon retraction time and excitability increase (R=0.037, P=0.919) (Fig. 4A-C).

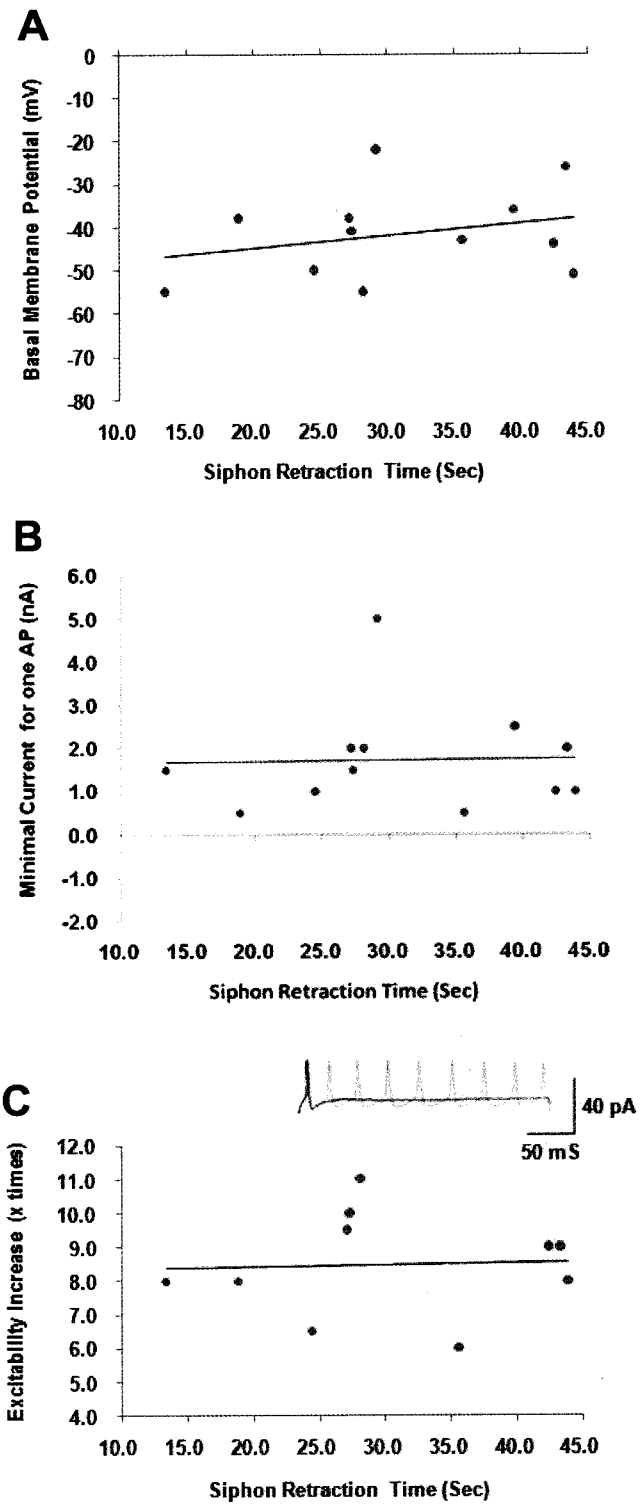

Fig. 4. Correlations between behavioral responsiveness and cultured neuronal properties. No correlations were observed between the siphon retraction time and (A) basal membrane potential (n=12, R=0.277, P=0.382), (B) minimal current to generate an AP (n=12, R=0.025, P=0.939), and (C) excitability increase after 5-HT treatment (n=12, R=0.037, P=0.919).

DISCUSSION

Single cell dissociability is one of the most prominent factors that determines the quality of cultured neurons

In the present study, we examined the effect of two major damaging factors involved in the protease treatment procedure, heat shock and surface protein destruction, on the culture condition. We compared the properties of neuron cultures made under three different temperatures and with two different types of proteases, to find out that these damages cannot be major factors that degrade the quality of cultured neurons.

This does not mean that these factors never affect the neuronal conditions. However, at least in the range of applications we performed to make dissociative cultures of *Aplysia* neurons, the damages induced by them do not exceed the physical damage introduced, mostly by the mechanical force provided during the dissociation of single neurons. Our results which demonstrate that guaranteeing the dissociability is sufficient for making healthy cultures strongly support this idea. It appears that the damages induced by the heat shock, surface protein degradation, and some other factors can be recovered during several days of stabilization, unless the damage is critical or deadly.

In light of this, we suggest dissociability of single neurons as a good criterion for those who are trying to find a novel protease treatment condition under specific temperature, pH or even a specific protease type, according to the requirement of their experiments.

Usage of multiple proteases can vary the culture method for more efficient experiments

In this study, we found that the usage of several type of proteases can be sufficient for dissociative culture, just as the usage of protease type IX, which has been the only protease used until now. The target specificity of each protease does not affect the final culture quality, and this was also the case of multiple protease treatment. This result opens the possibility of various culture methods specifically designed for each set of experimental conditions.

The usage of collagenases we suggest here is a particular example of these methodological variations. Applying protease type XIX and type XXIII, which degrade the sheath specifically, we could omit the desheathing step without any negative effect. The desheathing step, in which the half-degraded sheath is removed manually, is highly damage inducible, so that it requires a well trained experimenter who can minimize the mechanical injuries. We expect that the omission of this step will reduce these difficulties and turn dissociative culture technique into one that is easier to perform stably.

Not being a metaloenzyme, which requires a specific metal ion for the proper activity, protease type XXIII and the protocol we suggest here can also be used in Ca^{2+} deprived conditions, in which protease from *Bacillus polymyxa* type IX is inactive. Also, it seems possible for this type of protease to be used the sheath-specific elimination when access to a neuron in an intact ganglion is required, such as in ganglion *in situ* hybridization and so on.

It is unlikely that the proteases we tested here are the only ones that enable various applications. By using the multiple protease treatment, possibly the mixture with protease type IX, which helps efficient degradation of extracellular matrix and cell dissociation, one can find various ways to make *Aplysia* neuron cultures more efficiently.

Siphon retraction time is not likely to be a reliable criterion for healthy sensory neurons

In the fourth experiment, we attempted to find a way to discriminate an animal in a better condition for the dissociative culture by observing its behavior. We found that alterations made on the animal health can be reflected by its behavior, but this altered behavior did not show any correlation with the condition of cultured neurons.

Since little is known about the health-related factors that may cause behavioral alterations, it is hard to address the exact mechanism underlying this irrelevance. However, this result seems to be a partial evidence supporting that the potential of neurons to maintain and recover their responsiveness can remain intact, through changes on the health condition, and during the process of dissociative culture.

We do not exclude the possibility that more crucial, or chronic changes on the health condition may damage the potential of a neuron, causing unrecoverable defects in some of its properties, when it is dissociated and cultured. Reproduction of *Aplysia*, accompanied by dramatic humoral and behavioral changes, leads to the death of the animal (Kandel, 1976), can possibly be an example of these cases. However, if the animals were kept in a near-optimal environment for less than several months of time, behavioral alterations and underlying health conditions do not seem to work as critical factors on the cultured neuronal condition.

ACKNOWLEDGMENTS

This work was supported by National Creative Research Initiative Program of the Korean Ministry of Science and Technology and BK fellowship.

REFERENCES

Carew TJ, Walters ET, and Kandel ER (1981) Classical conditioning in a simple withdrawal reflex in *Aplysia* californica. *J Neurosci* 1: 1426-1437.

Castellucci V, Pinsker H, Kupfermann I, and Kandel ER (1970) Neuronal mechanisms of habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*. *Science* 167: 1745-1748.

Goodman CS and Pearson KF (1982) Neuronal development: Cellular approaches in invertebrates. *Neurosci Res Program Bull* 20: 773-942.

Hawkins RD, Abrams TW, Carew TJ, and Kandel ER (1983) A cellular mechanism of classical conditioning in *Aplysia*: activity-dependent amplification of presynaptic facilitation. *Science* 219: 400-405.

Hoyle G (1977) Identified Neurons and Behavior of Arthropods. Plenum Press, New York.

Jang DH, Han JH, Lee SH, Lee YS, Park H, Lee SH, Kim H, and Kaang BK (2005) Cofilin expression induces cofilin-actin rod formation and disrupts synaptic structure and function in Aplysia synapses. *Proc Natl Acad Sci USA* 102(44): 16072-16077

Kaang BK, Pfaffinger PJ, Grant SG, Kandel ER, and Furukawa Y (1992) Overexpression of an Aplysia shaker K^+ channel gene modifies the electrical properties and synaptic efficacy of identified Aplysia neurons. *Proc Natl Acad Sci USA* 89(3): 1133-1137.

Kaang BK, Kandel ER, and Grant SG (1993) Activation of cAMP-responsive genes by stimuli that produce long-term facilitation in Aplysia sensory neurons. *Neuron* 10(3): 427-435.

Kaang BK (1996) Parameters influencing ectopic gene expression in Aplysia neurons. *Neurosci Lett* 221(1): 29-32.

Kandel ER (1976) Cellular Basis of Behavior, W. H. Freeman & Co., San Francisco.

Kandel ER (1979) Behavioral Biology of *Aplysia*. W. H. Freeman & Co., San Francisco.

Kandel ER, Frazier WT, Waziri R, and Coggeshall RE (1967) Direct and common connections among identified neurons in *Aplysia*. *J Neurophysiol* 30(6): 1352-1376.

Kandel ER and Gardner D (1972) The synaptic actions mediated by the different branches of a single neuron. In Neurotransmitters: Proceedings, Association for Research in Nervous and Mental Diseases, *E J Kopin* 50: 91-144.

Kandel ER and Schwartz JH (1982) Molecular biology of learning: modulation of transmitter release. *Science* 218(4571): 433-443.

Kim H, Lee SH, Han JH, Lee JA, Cheang YH, Chang DJ, Lee YS, and Kaang BK (2006) A nucleolar protein ApLLP induces ApC/EBP expression required for long-term synaptic facilitation in aplysia neurons. *Neuron* 49(5): 707-718.

Lee JA, Lee SH, Lee C, Chang DJ, Lee Y, Kim H, Cheang YH, Ko HG, Lee YS, Jun H, Bartsch D, Kandel ER, and Kaang BK. (2006) PKA-activated ApAF-ApC/EBP heterodimer is a key downstream effector of ApCREB and is necessary and sufficient for the consolidation of long-term facilitation. *J Cell Biol* 174(6): 827-838.

Lee SH, Lim CS, Park H, Lee JA, Han JH, Kim H, Cheang YH, Lee SH, Lee YS, Ko HG, Jang DH, Kim H, Miniaci MC, Bartsch D, Kim E, Bailey CH, Kandel ER, and Kaang BK (2007) Nuclear translocation of CAM-associated protein activates transcription for long-term facilitation in *Aplysia*. *Cell* 129: 801-812

Lee YS, Bailey CH, Kandel ER, and Kaang BK (2008) Transcriptional regulation of long-term memory in the marine snail Aplysia. *Mol Brain* 1(1): 3

Lim CS, Chun DY, and Kaang BK (1997) Partial Anatomical and Physiological Characterization and Dissociated Cell Culture of the Nervous System of the Marine Mollusc *Aplysia Kurodai*. *Korean J Zool* 37: 352-376.

Montarolo PG, Goelet P, Castellucci VF, Morgan J, Kandel ER, and Schacher S (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. *Science* 234(4781): 1249-1254.

Pinsker H, Kupfermann I, Castellucci V, and Kandel ER (1970) Habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*. *Science* 167: 1740-1742.

Rayport SG and Schacher S (1986) Synaptic plasticity in vitro: cell culture of identified *Aplysia* neurons mediating short-term habituation and sensitization. *J Neurosci* 6(3): 759-763.

Schacher S and Proshansky E (1983) Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. *J Neurosci* 3(12): 2403-2413.

Shamovsky I and Nudler E (2008) New insights into the mechanism of heat shock response activation. *Cell Mol Life Sci* 65(6): 855-861.

[Received May 29, 2009; accepted July 1, 2009]