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Authors: Kawai, Ryo, Yasuoka, Takashi, and Sakakibara, Manabu

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Dynamical Aspects of *in vitro* Conditioning in *Hermissenda* Type B Photoreceptor

Ryo Kawai[1], Takashi Yasuoka[1] and Manabu Sakakibara[2]*

[1]*Department of Chemistry, Graduate School of Science, Tokai University, Kita-Kaname, Hiratsuka 259-1292, Kanagawa, Japan*
[2]*Department of Biological Science and Technology, School of High-Technology for Human Welfare, Tokai University, Numazu 410-0321, Shizuoka, Japan*

ABSTRACT—Dynamics of changes in physiology and morphology were studied in *Hermissenda* photoreceptors after *in vitro* conditioning with paired light and vibration. An increase in input resistance of the type B photoreceptor was observed following 5 paired presentations of light and vibration. It peaked at 10 min after *in vitro* conditioning, then decreased to a level twice the pre-conditioning level for more than 60 min. Contraction of the terminal branches along centro-lateral direction was initiated 5 min after conditioning and reached its final state at 10 min after conditioning. The pairing specific contraction of the axon terminal was not observed in ASW containing anisomycin. The dynamics in physiology and morphology were completely parallel 30 min after conditioning. These findings suggested that *in vitro* conditioning induced contraction was dependent on protein synthesis dependent process initiated within 5 min after training trials and that the change of cell morphology is a form of short-term synaptic plasticity that involves changes in macromolecular synthesis. Present findings that functional remodeling at the terminal branch of the type B photoreceptor occurred within 10 min after conditioning was the fastest modification process reported so far.

Key words: *in vitro* conditioning, temporal sequence, terminal branch arborization, type B photoreceptor, morphological and physiological modification

INTRODUCTION

Morphological changes of the terminal region or dendritic spine are part of functional remodeling of neural circuits during recovery from injury and acquisition of learning. Nothing has known about the dynamical aspects of functional remodeling, what is the trigger or how to maintain modifications after remodeling. In rat hippocampal neurons, morphological changes in the numbers and motility of dendritic spines depends on synaptic activity, or involvement of specific receptors (Korkotian and Segal, 1999; Fischer *et al*., 2000). In *Aplysia* abdominal ganglia, the axonal branches of some neurons elongates and increase the number of varicosities in response to 5HT application (Sun and Schacher, 1998; Hatada *et al*., 2000). Though the mechanism for organizing neural circuits in culture (Merz and Fromherz, 2002) provides clues to the mechanisms for rearrangement of neural circuits during acquisition of learning, it is not sufficient for understanding the neural mechanism of circuit remodeling after acquisition of learning and retention of memory because cultural system is over simplified due to lacks of delicate humoral environment, for example.

Hermissenda crassicornis, which exhibit positive phototactic behavior prior to training, contract their foot in response to a light stimulus after paired presentation of light and rotation (Alkon, 1974; Crow, 1985; Lederhendler and Alkon, 1989; Matzel *et al*., 1990). After acquisition of learning, physiological modifications are observed at the type B photoreceptor such as an increase of input resistance, prolongation of neuronal excitability and inactivation of K^+ currents (West *et al*., 1982; Alkon *et al*., 1985). In addition to these physiological modifications, morphological changes are observed as a contraction at the terminal branch of the type B photoreceptor. The enclosing volume of the axon terminal in the type B photoreceptor decreases by 50% three days after Pavlovian conditioning. In this previous study, only the axonal branching arborization appears to shrink (Alkon *et al*., 1990). They interpreted this as the elimination of useless synaptic contacts in long-lasting memory formation; thus, they termed this modification “focusing”. Though

* Corresponding author: Tel. +81-55-968-1111;
Fax +81-55-968-1156.
E-mail: manabu@tokai.ac.jp

the neuronal modification caused by *in vitro* conditioning is basically assumed to be the identical process as to *in vivo*, we would like to examine whether morphological changes proceeds to be parallel as physiological changes. The dynamical analysis of physiology and morphology will provide a clue what is the trigger of the morphological modification occurring at the terminal B photoreceptor.

These physiological and morphological findings are observed also in the *in vitro* conditioning preparation after 5 paired presentations of light and rotation trials (Kawai *et al*., 2002). The *in vitro* conditioned preparation has the advantage that it has intact eyes and statocysts, thus the *in vitro* conditioning reflects well the *in vivo* state and this preparation provides the good model to study the long lasting synaptic changes such as the process of synatogenesis. Since the time course of morphological changes to the type B photoreceptor has not yet been elucidated, the present study measures repeatedly the morphology of the type B cell for 1 hr after *in vitro* conditioning together with the conditioning specific physiological modifications.

MATERIALS AND METHODS

The methods were described in detail previously (Kawai *et al*., 2002), and are summarized below.

Animals

Hermissenda crassicornis were obtained from Sea Life Supply (Sand City, CA). Animals were maintained in 60 l artificial sea water, 'Sea Life' (Marine-Tech Co., Tokyo) aquaria (Aqua, Tokyo) under subdued yellow light (20 $\mu W/cm^2$) at 10°C on a 12-hr light: 12-hr dark cycle (on at 08:00) and fed dried clam.

Preparation

The circumesophageal ganglion was removed in artificial sea water (ASW) (430 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.4). Preparations were immobilized on a glass slide by the weight of stainless-steel pins whose ends were embedded in vaseline. The isolated circumesophageal ganglion was incubated with protease solution (sigma type VIII 1 mg/ml in ASW) to digest the surrounding connective tissue for 7 min at room temperature.

Electrophysiology

Intracellular recordings were made from the lateral type B cell. The type B cell was impaled with a 3 M KCl filled glass microelectrode of which the input resistance ranged from 25 to 35 MΩ. The glass microelectrode was connected by a chlorided silver wire to a high input impedance amplifier (7110A, Pelagic Electronics, Falmouth, MA). Voltage responses were monitored by a storage oscilloscope (DCS-7020, Kenwood, Tokyo) and recorded in a PC-AT compatible personal computer via an interface board (Digidata 1200B, AXON Instruments Co., Foster City, CA) controlled by pCLAMP (AXON Instruments Co., Foster City, CA). The slope input resistance was assessed from the liner regression of the I–V curve made by measuring the steady state voltage change due to constant current injection ranging from –0.2 nA to +0.2 nA with 0.1 nA step before and after *in vitro* conditioning.

Staining and morphological observation of type B cell

To evaluate morphology, the type B cell was stained with Alexa 488 (A-10436, Molecular Probe, Eugene, OR, 1% in water solution). The Alexa 488 was filled at the tip of electrode, the rest was filled with 3 M KCl. The resistance of the microelectrode was approximately 30 MΩ. After penetration into the cell, dye was injected iontophoretically by AC current pulse of 1 Hz, –0.5 nA in 50% duty cycle for 15 min. The morphology of the type B photoreceptor was observed with a confocal microscope (TCSNT, Leica, Heerbrugg, Switzerland) and evaluated from an overlay image scanning from the top to the bottom totally 6 times during an experiment, i.e. at the pre-conditioning occasion, 1 min, 5 min, 10 min, 30 min, and 60 min after the *in vitro* conditioning.

In vitro conditioning

The timing of a light flash from a tungsten halogen lump (HL-100, HOYA SCHOTT, Tokyo) was controlled by a solenoid mechanical shutter equipped in the lamp house and the light was guided to the preparation by a fiber optic bundle. The unattenuated light intensity at the preparation was 5.8 mW/cm^2 at 500 nm. The tactile stimulus was applied to the statocyst hair cell by a polished glass probe with a concave tip approximately 50 microns in diameter. This probe was connected to a piezo-driver (DPS-255, Dia Medical Co., Tokyo) that gave a maximal vibration of 19 μm in stroke operated at 33 Hz. After microelectrode penetration into the type B photoreceptor, cells of which the resting membrane potential did not exceed −40 mV after 10 min dark adaptation were discarded. Following another 10 min rest, 5 successive stimuli of 3 sec light (conditioning stimuli : CS) and 2 sec vibration (unconditioning stimuli : UCS) every 2 min were presented. Two groups of animals were evaluated: In the paired group (n=5), the 2 sec vibration was initiated 1 sec after the 3 sec light; thus the light and vibration overlapped. In the unpaired group (n=5), the 3 sec light and 2 sec vibration were separated by 60 sec; thus they did not overlap. In addition, an anisomycin group received paired training (overlapping stimuli) but the circumesophageal ganglion was bathed in anisomycin (CalBiochem-Novabiochem Co., San Diego, CA, 100 μM in ASW) containing ASW before the conditioning trials to examine the contribution of protein synthesis on morphological dynamics.

Image processing

The morphology of the terminal branch in the type B cell was examined using a confocal microscope. The images obtained by confocal microscopy were processed by image analysis software (Scion-image beta 4, Scion. Co, Frederick, MD). Each image involved 256×256 pixel scanning from the top of the branch to the bottom in 0.49 μm steps along the z axis. Each pixel was represented as a brightness index value between 0 and 255. The maximum intensity projection (MIP) was defined by the maximum brightness index along the z axis which yielded the largest cross-sectional image of the type B photoreceptor terminal. A binary image was obtained from the MIP image using a threshold operation; the threshold was adjusted visually for each preparation and maintained constant through the series of images. Though the binary image tended to depict a larger area than the MIP image, we analyzed binary images because it was difficult to distinguish the border of fine structure of the type B photoreceptor terminal from the background in the MIP image. Terminal length was previously shown to be a morphological change correlated with classical conditioning (Kawai *et al*., 2002); thus dynamical changes in terminal length over time were measured from the binary images. The ratio of the terminal length was calculated from images of post- to pre-conditioning.

Statistical analyses

All the statistical tests were performed *t*-test with Origin 5.0 (Microcal, Northampton, MA). The difference between pre and post conditioning was tested by a two population paired *t*-test. A two population independent *t*-test was done for Unpaired group versus Paired group.

RESULTS

Morphology

A series of images after *in vitro* conditioning for both a paired and an unpaired preparation is demonstrated in Fig. 1. The original MIP images made prior to conditioning are shown at the top and the binary images produced by the threshold operation are shown below the MIP image. Binary images made at various time points after conditioning are shown below the pre-conditioning images. Fig. 1 shows that 'focusing' at the terminal branch is detectable five minutes after paired conditioning. The cross-sectional area of the image at the terminal branch is slightly decreased along both the x and y axis (centro-lateral direction). This shrinkage is obvious at 10 min and is maintained for the rest of the 60 min observation period. In contrast to the dynamical change observed in the paired treatment group, images of the unpaired group exhibit no changes during the whole course of the experiment.

The change in terminal length ratio over time for both groups of animals is shown in Table 1. The terminal length ratio of the unpaired group remained constant at the pre-conditioning level throughout the experiment. There was no statistical significance in the terminal length ratio of the unpaired group at any point. In the paired group, the ratio was not statistically different from the pre-conditioning level 1 min after conditioning, however, it was significantly decreased at 5 min, obvious at 10 min, and persisted at a constant level for 60 min. Also, the difference between paired and unpaired terminal length was statistically significant, as shown in Table 1.

In contrast to the pairing specific contraction in terminal

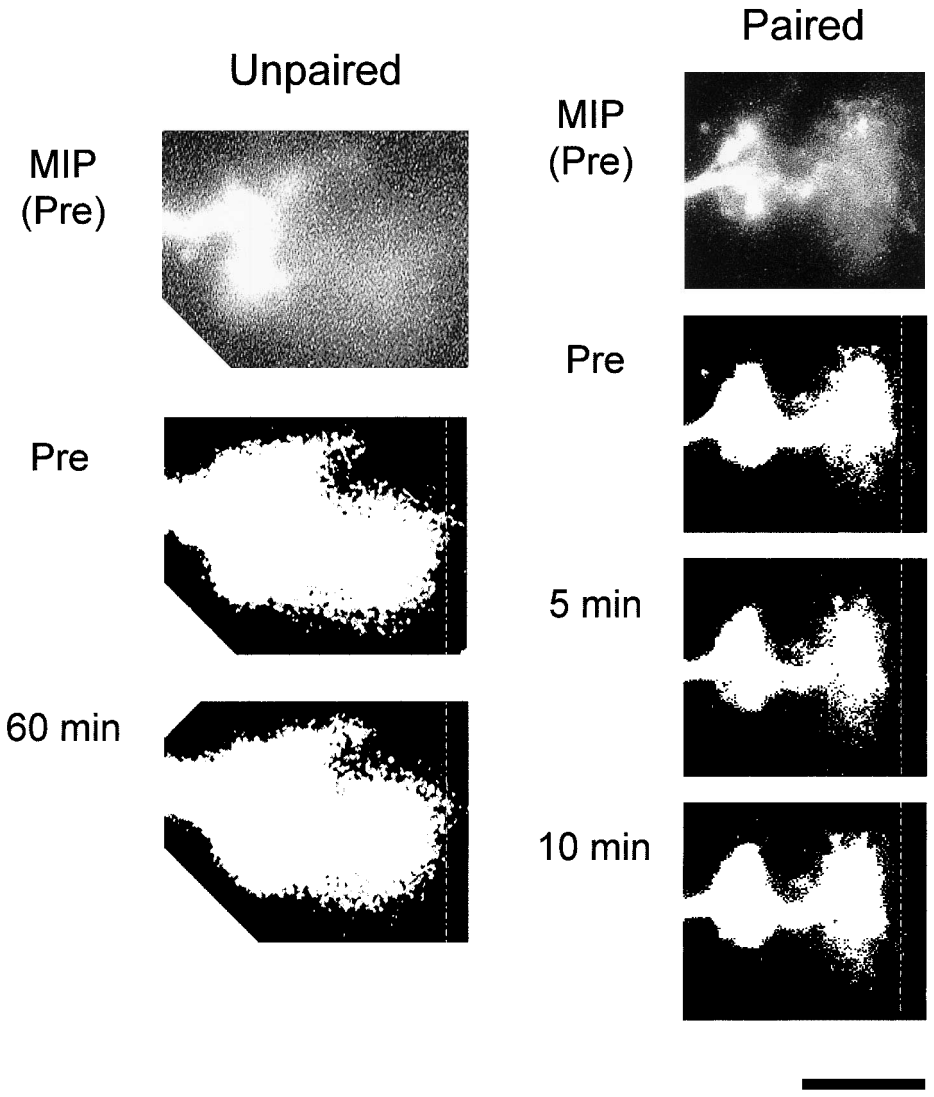

Fig. 1. Temporal images of the type B photoreceptor axon terminal. The top of each column shows the MIP (maximum intensity projection) image prior to conditioning. The remaining panels are binary images. No marked change is observed from the unpaired group in the left column. On the other hand, in images from the paired group, the terminal starts contracting at 5–10 min after conditioning and retains this shape during the rest of observation. Dashed line indicates the pre-conditioning level of terminal length. Scale bar 40 μm.

Table 1. Statistics of the temporal input resistance and terminal length ratio. The comparison between pre and post conditioning were tested by two population paired *t*-test. Two population independent *t*-test was done for Unpaired group versus Paired group. S.D.: significantly different, N.S.: no significance

Time After Conditioning	Terminal Length Ratio			Input Resistance		
	Paired (V.S. Pre, n=5)	Unpaired (V.S. Pre, n=5)	Paired (n=5) V.S. Unpaired (n=5)	Paired (V.S. Pre, n=5)	Unpaired (V.S. Pre, n=5)	Paired (n=5) V.S. Unpaired (n=5)
1 min	t=1.991, P=0.117, N.S.	t=–0.931, P=0.404, N.S.	t=1.853, P=0.101, N.S.	t=–2.994, P=0.040, S.D.	t=2.064, P=0.108, N.S.	t=2.697, P=0.027, S.D.
5 min	t=6.708, P=0.003, S.D.	t=–1.077, P=0.341, N.S.	t=–3.500, P=0.008, S.D.	t=–3.075, P=0.037, S.D.	t=2.023, P=0.113, N.S.	t=2.857, P=0.021, S.D.
10 min	t=5.076, P=0.007, S.D.	t=–0.826, P=0.456, N.S.	t=3.793, P=0.005, S.D.	t=–14.136, P=0.0001, S.D.	t=0.302, P=0.765, N.S.	t=–7.026, P=0.0001, S.D.
30 min	t=4.020, P=0.016, S.D.	t=–0.023, P=0.983, N.S.	t=2.904, P=0.020, S.D.	t=–3.232, P=0.032, S.D.	t=–0.310, P=0.772, N.S.	t=–2.644, P=0.030, S.D.
60 min	t=5.108, P=0.007, S.D.	t=0.040, P=0.970, N.S.	t=2.779, P=0.024, S.D.	t=–4.829, P=0.008, S.D.	t=0.674, P=0.537, N.S.	t=–4.235, P=0.003, S.D.

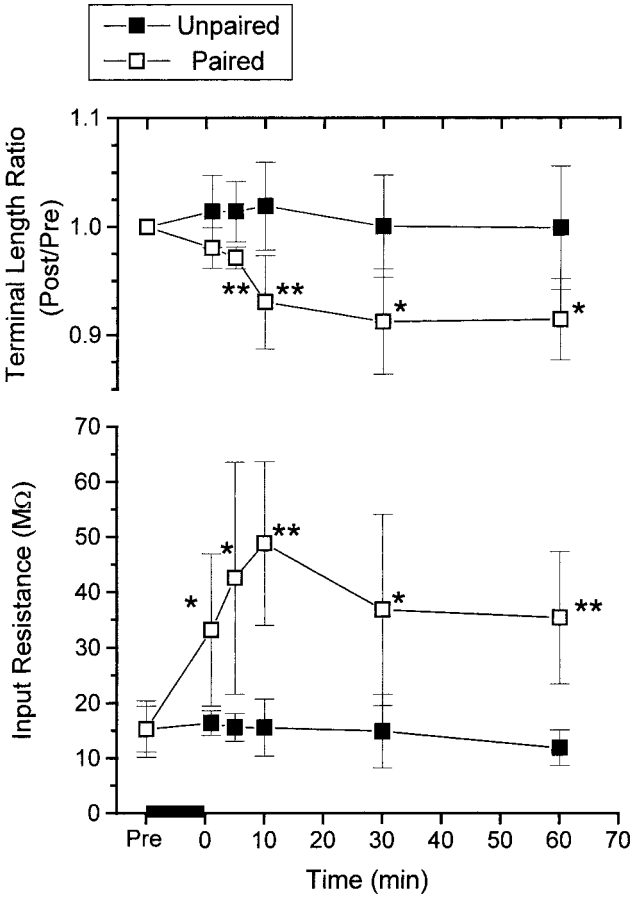

Fig. 2. Temporal comparison of terminal length ratio (upper column) and input resistance (lower column). Data are represented as mean±SD. Significant decrease (P<0.01) of the terminal length ratio of the paired group (–□–) is detected 5–10 min after *in vitro* conditioning as indicated by a bold bar on the abscissa, and this decrease is held until 60 min. Input resistance in the paired group continuously and linearly increases to 50 MΩ by 10 min after conditioning, then decreases to 35 MΩ at 30 min, and maintains this value until 60 min. The unpaired group (–■–) exhibits no significant difference during the observation. The dynamics of the input resistance and the terminal length ratio are in parallel after 30 min after conditioning. * P<0.05, ** P<0.01

length seen in ASW, we could not detect any significant morphological changes with 5 paired conditioning trails in 5 out of 5 preparations (data not shown) bathed in the protein synthesis inhibitor anisomycin. This finding suggested that the terminal focusing requires protein synthesis.

Input resistance

According to the hypothesis (Alkon, 1987) after the classical conditioning K^+ channel is inactivated due to phosphorylation of the GTP-binding protein, calexcitin caused by various protein kinases and this enhances the B cell excitability to yield the increase of input resistance. This process is instantaneous occurring even by one paired *in vitro* conditioning (Matzel and Rogers, 1993) but the enhancement of neuronal excitability is not long-lasting. While the terminal contraction is assumed to be a long-lasting process to develop memory formation, we examined the temporal changes in input resistance and terminal length ratio after *in vitro* conditioning.

Input resistance of the unpaired group never changed during the course of the experiment, while that of the paired group increased linearly to 50 MΩ until 10 min after conditioning, then decreased to 35 MΩ by 30 min after conditioning. This value was more than two times higher than the pre-conditioning level and was maintained for the duration of the recording period, at least 60 min. These increments in input resistance were significantly different from both pre-conditioning level and from that of unpaired animals as shown in Table 1.

Fig. 2 demonstrates the dynamics of the input resistance and the terminal length ratio. For paired, but not unpaired groups, the former measure increased while the latter measure significantly reduced during the first 10 min after conditioning. Both measures remained at a constant level between 30 min and 60 min after conditioning.

DISCUSSION

The modification at the type B photoreceptor caused by

in vitro conditioning was studied with special attention to the dynamics of physiological changes (input resistance) and morphological changes (terminal length ratio). We previously demonstrated that five paired presentations of light and vestibular stimulation to the isolated nervous system of *Hermissenda* resulted in an increase in excitability of the identified neuron, the type B photoreceptor, as indicated by an increase in input resistance. Further morphological observations revealed that the terminal branch of the type B photoreceptor contracted along the centro-lateral axis within an hr after conditioning (Kawai *et al*., 2002). Although we do not have any evidence that the conditioning specific contraction at the terminal branch of the B cell are involved in genetic modification, the functional remodeling at the identified neuron, the type B photoreceptor occurred faster than those ever reported.

The present experiment showed that input resistance increased linearly during the first 10 min after the paired *in vitro* conditioning, then decreased for the next 20 min and finally held constant at this level, which was more than two times higher than that of the pre conditioning level, for another 30 min. The transient increase in input resistance was observed previously by Matzel and Rogers (Matzel and Rogers, 1993). One trial *in vitro* conditioning with light and mechanical stimulation to statocyst hair cells resulted in a transient increase in input resistance by 30% in comparison with pre-conditioning levels at 1 min after training and this initial rise disappeared during the next 4 min. This transient increase was blocked when the type B photoreceptor was injected with EGTA prior to pairing suggesting that this increase is Ca^{2+}-dependent. Further they showed the input resistance increased more than 30% for at least 10 min after 9 trials of paired *in vitro* conditioning and this increase was enhanced more if the membrane potential was held at the resting level, –60 mV. Ramirez *et al*. showed that two training trials produced a short-term increase in excitability that dissipated within 45 min, whereas nine trials produced a persistent (at least 90-min) increase in excitability. Following training in the protein synthesis inhibitor anisomycin, a short-term (5- to 45-min) but not persistent (90- min) increase in excitability in the B photoreceptors was observed (Ramirez *et al*., 1998). They argued that short-term retention in *Hermissenda* is protein synthesis independent but that new protein synthesis is initiated during or shortly after the training event. Our present findings indicated that 5 training trials produced a persistent change in excitability and the terminal branch contraction involved modulation of protein synthesis dependent processes. With 5 paired training trails, the first 10 min is the maximum excitability period, during which input resistance increases and the conformational change (focusing) at the axon terminal branch proceeds. Subsequent to this period is the maintenance period when the terminal branch length ratio remains constant. Matzel and Rogers argued that the rise in resistance was dependent on presynaptic stimulation occurring in conjunction with the rise in postsynaptic Ca^{2+}, which was liberated from intracellular stores in response to light (Matzel and Rogers, 1993). This cumulative Ca^{2+} rise may trigger the potentiation cascade such as activation of protein kinase C, phosphorylation of GTP-binding protein 'calexcitin' (Nelson *et al*., 1996) and then consolidation of memory. In support of this hypothesis, potassium channels were inactivated by phosphorylation with protein kinase C specific to the paired conditioned B photoreceptor (Etcheberrigaray *et al*., 1992). Activation of calexcitin resulted in modulation of the ryanodine receptor (Nelson *et al*., 1999) and the ryanodine receptor in the type B cell is necessary for the cellular changes underlying associative memory storage (Blackwell and Alkon, 1999).

Recent studies demonstrated rapid morphological changes at the spine of a rat hippocampal neuron induced by LTP (Engert and Bonhoeffer, 1999). In other studies on rat hippocampal neurons contrasting morphological changes were reported: application of caffeine resulted in elongation (Korkotian and Segal, 1999), while application of glutamate resulted in shrinkage (Segal *et al*., 2000). The morphological change observed in the present study was even faster than these findings in the rat hippocampal studies suggestive to be the fastest process involved in the paired conditioning.

To change cellular morphology, the cytoskeleton would be affected by some intracellular molecular complex. Culture studies indicate that actin polymerization plays a role in changing cellular shape (Hatada *et al*., 2000; Korkotian and Segal, 2001). From fibroblast and neuronal culture studies, another possibility is raised that one of the G-proteins (Rho-GTPases) contributes to reorganize cell shape, especially on cytoskeletal components (Hall, 1998; Maekawa *et al*., 1999; Luo, 2000). Thus, activation of the conditioning specific calcium sensor G-protein, calexcitin may contribute to contraction at the terminal branch of the B cell.

In conclusion this is the first report to demonstrate the dynamical conformational change at the identified neuron, the type B photoreceptor cell after *in vitro* conditioning. This study revealed that five paired presentations of light and vibration stimuli resulted in immediate enhancement of neuronal excitability of the type B photoreceptor in *Hermissenda*. The resistance peaked at 10 min after conditioning and decreased to a resistance of twice the pre-conditioning level for the rest of the experiment. The morphology at the B cell terminal started to contract 5 min after conditioning and kept this shape for the rest of the experiment. Morphological modulation with 5 paired training trials did not occur in anisomycin containing ASW. The dynamics in physiology and morphology were completely parallel 30 min after conditioning. These findings suggested that *in vitro* conditioning induced contraction was followed by the enhancement of the type B cell excitability and was dependent on protein synthesis.

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