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The Effect of Brain-derived Neurotrophic Factor on Neuritogenesis and Synaptic Plasticity in *Aplysia* Neurons and the Hippocampal Cell Line HiB5

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Brain-derived neurotrophic factor (BDNF) plays a key role in the differentiation and neuritogenesis of developing neurons, and in the synaptic plasticity of mature neurons, in the mammalian nervous system. BDNF binds to the receptor tyrosine kinase TrkB and transmits neurotrophic signals by activating neuron-specific tyrosine phosphorylation pathways. However, the neurotrophic function of BDNF in *Aplysia* neurons is poorly understood. We examined the specific effect of BDNF on neurite outgrowth and synaptic plasticity in cultured *Aplysia* neurons and a multipotent rat hippocampal stem cell line (HiB5). Our study indicates that mammalian BDNF has no significant effect on the neuritogenesis, neurotransmitter release, excitability, and synaptic plasticity of cultured *Aplysia* neurons in our experimental conditions. In contrast, BDNF in combination with platelet-derived growth factor (PDGF) increases the length of the neurites and the number of spine-like structures in cells of HiB5.

Keywords: *Aplysia*; BDNF; Excitability; Long-Term Facilitation; Neuritogenesis; Neurotrophic Factors; Synaptic Depression; Synaptic Plasticity.

Introduction

The role of neurotrophic factors in neuronal development and synaptic plasticity has been extensively studied. Neurotrophic factors stimulate neuronal proliferation (Zurovec *et al.*, 2002), differentiation (Chao and Hempstead, 1995), survival (Kwon, 2002), neurite outgrowth and regeneration (Chen *et al.*, 2002; Munno *et al.*, 2000) both *in vivo* and *in vitro*. In many cases neurotrophic factors increase the number and length of neurites formed from the parent cell body of cultured neurons (Labelle and Leclere, 2000; Ozdinler and Erzurumlu, 2001; Srivatsan and Peretz, 1997; Takano *et al.*, 2002). Furthermore, they are critical for synapse formation during neurite outgrowth (Munno *et al.*, 2000) as well as for synaptic plasticity (for review, see Schinder and Poo, 2000). For example, presynaptic and postsynaptic secretion of brain-derived neurotrophic factor (BDNF) as a result of neuronal activity can modulate synaptic efficacy (Kang and Schuman, 1995; Kovalchuk *et al.*, 2002).

It is well known that many of the cellular and molecular mechanisms underlying neuronal development and synaptic plasticity are conserved between invertebrates and vertebrates. The physiological function of neurotrophic factors is also conserved (for review, see McKay *et al.*, 1999). In spite of the differences between species, vertebrate neurotrophic factors can influence invertebrate neuronal growth and synaptic plasticity. For instance, human transforming growth factor-beta1 (TGF-β1) induces long-term memory (Zhang *et al.*, 1997), reduces synaptic depression (Chin *et al.*, 2002), and increases long-term excitability (Chin *et al.*, 1999) in *Aplysia* neurons. However, the effect of other mammalian neurotrophic factors, including BDNF, that induce neuronal differentiation and long-term memory in mammals, has not been extensively studied in *Aplysia.*

In the present work we examined the effect of BDNF on primary cultures of *Aplysia* neurons and a rat multipotent hippocampal stem cell line (HiB5) (Kwon, 1997; Renfranz *et al.*, 1991). Application of BDNF had no significant effect on at least three aspects of *Aplysia* neurons:

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neuritogenesis, long-term synaptic plasticity, and short-term membrane excitability.

Materials and Methods

Primary cell cultures of *Aplysia* neurons Animals (*Aplysia kurodai*) weighing 70−120 g were purchased from a local supplier in Pusan and Yong-duk, Korea. Culture dishes and media were prepared as described previously (Lim *et al*., 1997; Schacher *et al*., 1983). For sensory neuron culture, homogenous *Aplysia* sensory neurons were removed from the ventral-caudal clusters of pleural ganglia with a segment (100−500 μm) of intact major axons, and plated on poly-L-lysine (Sigma, USA)-coated petri dishes. For sensory-motor co-culture, sensory neurons isolated from the pleural ganglia were co-cultured, as described previously, with the motor neuron LFS obtained from abdominal ganglia (Lee *et al*., 2001).

Neurite outgrowth assay Sensory neurons from the same ganglion were randomly divided into four groups and placed in dissociated cell culture. The groups were treated, respectively, with BDNF (100 ng/ml, Sigma, USA and Amgen, USA) plus BSA (1 μg/ml), hemolymph (50%), hemolymph (50%) plus BSA, or BSA alone, each dissolved in 50% isotonic L15 / 50% ASW (L15: Leibovitz L15 (Sigma, USA) with 400 mM NaCl, 27 mM $MgSO_4$, 27 mM $MgCl_2$, 11 mM $CaCl_2$, 10 mM KCl, and 2 mM $NaHCO_3$; ASW: 450 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 29 nM $MgCl_2$, 10 mM HEPES at pH 7.6). Each culture dish contained 5−15 sensory neurons in an area of 132.7 mm^2, distributed evenly without cell-cell contact. After the sensory neurons had grown for 2 d at 18°C in each medium, their morphology was examined with a phase-contrast microscope (Nikon DIAPHOT). Any neuron that sprouted neurites twice as long as the diameter of its cell body was regarded as a neuron with outgrown neurites, and we determined the number of such neurons in each plate. Values are presented as mean percentage ± SEM.

Recording membrane excitability Cultured sensory neurons were impaled with a single microelectrode (10−20 MΩ resistance) and held at their initial membrane potentials. After stabilization for 5−10 min, depolarizing current pulse steps ranging from 0.1 to 0.3 nA with a duration of 500 ms were pretested on each sensory neuron to determine the threshold of current that produced a single spike. Immediately after baseline recording, BDNF (10 ng/ml) (Sigma, USA and Amgen, USA) in BSA (100 ng/ml), 5-HT (10 μM), or BSA alone (100 ng/ml) was applied to the bath for 5 min or 24 h. After drug treatment, a 500 ms pulse was delivered to measure the number of spikes. The number of spikes during the 500 ms pulse represented membrane excitability. Only one sensory neuron was recorded from each culture plate. Values are presented as mean spike numbers ± SEM.

Synapse recording The excitatory postsynaptic potential (EPSP) was measured as described previously (Lee *et al*., 2001). The initial EPSP value was measured by extracellular stimulation of the sensory neuron. For depression recording, co-cultures were incubated for at least 2 h after the initial EPSP measurement. After BDNF (100 ng/ml) (Sigma, USA and Amgen, USA) with BSA (1 μg/ml) (or BSA alone) had been applied for 5 min, the sensory neuron was stimulated with 0.25 Hz stimuli for 80 s. For recording long-term synaptic transmission, the co-cultures received either five pulses of 5-HT (10 μM) for 5 min at 15 min intervals, or BDNF (100 ng/ml) with BSA (1 μg/ml) (or BSA alone) for 1 d before the second EPSP measurement. All values are presented as the mean percentage change in EPSP amplitude ± SEM before and after the applications.

BDNF assay in mammalian cells Cells of the neuronal stem cell line HiB5 were cultured in 24 well plates at a density of 2×10^4 / well and incubated for 2 d (80% confluence) in DMEM (Gibco-BRL, USA) supplemented with 0.11 g/L sodium pyruvate, 3.7 g/L $NaHCO_3$, 0.39 g/L HEPES, penicillin, streptomycin and 10% fetal bovine serum (Hyclone, USA) at 33°C, in a 5% CO_2 incubator. To induce differentiation they were transferred to the chemically defined medium N2 (P−) supplemented with 20 ng/ml PDGF (P+) or with 20 ng/ml PDGF and 50 ng/ml BDNF (P+B+), and incubated for 2 d at 39°C, the normal rat body temperature (Kwon, 1997; Renfranz *et al*., 1991). BDNFs were obtained from Sigma, Amgen, or UBI. After fixation with 2% par-aformaldehyde, HiB5 cells on coverslips were permeabilized in 0.5% NP-40 for 10 min, blocked with 5% normal serum for 30 min, and then incubated at 4°C overnight with anti PSD-95 antibody (dilution ratio, 1:400, UBI, USA) in 2% BSA-PBS, as described previously (Kwon *et al.*, 1997). Spine-like structures were visualized with anti-mouse secondary antibody conjugated with Cy3, and scanned with a confocal laser microscope (Carl Zeiss). Neurites longer then 200 μm length were measured using an image analysis program (Carl Zeiss).

Results

Effects of BDNF on cultured *Aplysia* cells To analyze the potential role of BDNF in neurite outgrowth and morphological change in *Aplysia* neurons, sensory neurons were cultured from pleural ganglia of *Aplysia*. As shown in Fig. 1A, no distinct morphological change or additional neurite outgrowth was observed when neurons were cultured with BDNF, whereas most neurons cultured with hemolymph showed robust outgrowth of long, thin neurites from their major axons and cell bodies, as reported previously (Lim *et al*., 1997). To quantify neurite outgrowth, we determined the percentage of sensory neurons in each plate that showed neurite outgrowth. No major difference in neurite outgrowth was found between BDNF (8.96 ± 4.55%, n = 8 plates, total cell number = 84) and vehicle control (7.01 ± 5.53%, n = 8 plates, total cell number = 84). A higher (200 ng/ml) or lower (50 ng/ml) concentration of BDNF also had no effect on neurite out-

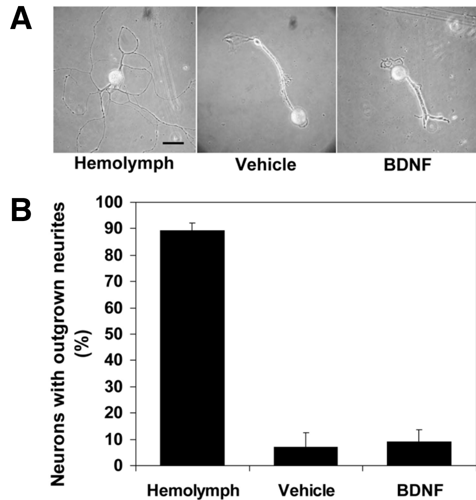

Fig. 1. The effect of BDNF on neuritogenesis in *Aplysia* sensory neurons. **A.** Phase-contrast micrographs of *Aplysia* sensory neurons in different media. In the presence of hemolymph, sensory neurons showed robust neuritogenesis. However, in medium containing BDNF or vehicle there was no additional neurite outgrowth from the growth cones. Scale bar = 50 μm. **B.** Percentage of *Aplysia* sensory neurons with neurites outgrown from the growth cone in media containing BDNF (n = 8 plates), vehicle alone (n = 8 plates), and hemolymph (n = 8 plates). Only hemolymph significantly increased neurite outgrowth ($F = 105.58$, $df = 2$, $p < 0.0001$ in a one-way ANOVA test).

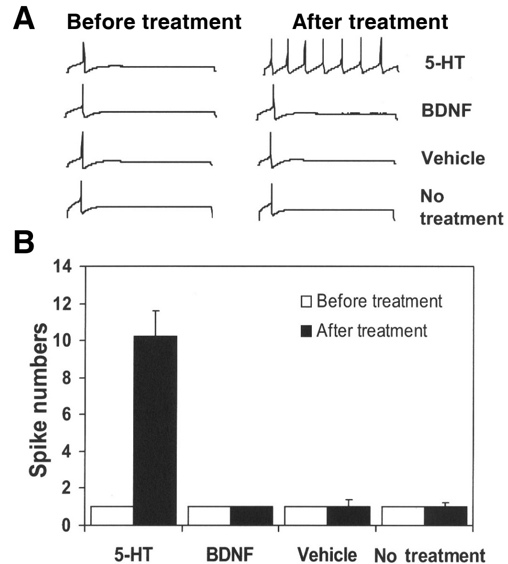

Fig. 2. The effect of BDNF on membrane excitability. **A.** Representative traces of action potentials during a 500-msec pulse of depolarization. **B.** Change in the evoked spike numbers. BDNF treatment had no effect on the excitability of cultured *Aplysia* sensory neurons ($n = 3$, $p > 0.1$; two-tailed paired t-test) whereas 5-HT increased spike numbers ($n = 5$, $p < 0.003$; two-tailed paired t-test). Vehicle treatment, or no treatment at all, had no affect (vehicle, n = 4; no treatment control, n = 6).

growth (data not shown). In contrast, hemolymph greatly stimulated neurite outgrowth (89.13 ± 3.14%, n = 8 plates, total counted cell number = 107). Treatment with BDNF in addition to hemolymph did not further enhance neurite outgrowth (data not shown). There was no inhibitory effect of vehicle on neurite outgrowth when added along with hemolymph (data not shown). Together, these results indicate that application of BDNF does not induce neurite outgrowth in *Aplysia* sensory neurons.

Growth factors usually bind to receptors and directly or indirectly activate signal pathways, leading to changes in neuronal excitability (Purcell and Carew, 2001). We tested whether BDNF enhances excitability in cultured *Aplysia* sensory neurons. Figure 2 shows that, unlike 5-HT, it did not increase spike numbers (5-HT, 10.2 ± 1.4, n = 5; vehicle, 1.0 ± 0.4, n = 4; BDNF, 1.0 ± 0.0, n = 3; no treatment, 1.0 ± 0.2, n = 6). A higher concentration of BDNF (100 ng/ml) also did not enhance excitability (n = 2). Similarly, 24 h treatment with BDNF had no effect (n = 2). These results demonstrate that BDNF does not increase the membrane excitability of *Aplysia* sensory neurons.

To determine whether BDNF, like other neurotrophic factors (Chin *et al*., 2002; Kang and Schuman, 1995; Zhang *et al*., 1997), affects the synaptic plasticity of mature synapses, we measured the magnitude of synaptic depression, and recorded synaptic potential changes in response to BDNF in co-cultured *Aplysia* sensory-motor neurons. As shown in Fig. 3, there was no significant difference between the magnitude of synaptic depression in BDNF-treated cells and those receiving vehicle (BDNF, n = 4; vehicle, n = 3). BDNF also induced no significant change in long-term synaptic transmission, unlike 5-HT (Fig. 4; 5-HT, 182.2 ± 28.3%, n = 3; vehicle, −17.4 ± 10.7%, n = 3; BDNF, −37.6 ± 7.2%, n = 9; no treatment, −20.8 ± 16.7%, n = 3). Evidently BDNF by itself cannot induce long-term synaptic facilitation. Taken together, our results show that BDNF does not affect synaptic plasticity in *Aplysia* sensory-motor co-cultures.

Effects of BDNF on cultured mammalian cells To analyze the effect of BDNF on neurite outgrowth and morphology of mammalian neurons, we utilized a multipotent hippocampal stem cell line (HiB5). HiB5 was isolated from embryonic rat hippocampus on day 16 when the precursors of pyramidal cells start to divide; it was immortalized by the temperature sensitive SV40 large T antigen. HiB5 cells grow at the permissive temperature of 33°C and when incubated in defined growth factor-free medium at 39°C they stop growing and initiate differentiation (Kwon, 1997a; Ranfranz, 1991). The addition of PDGF-B stimulates neuronal differentiation (Kwon, 2002). Most of the PDGF-treated cells extend processes that are immunostained for markers intimately associated with

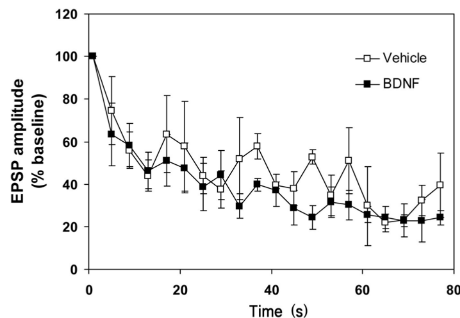

Fig. 3. Synaptic depression induced by low-frequency stimuli after BDNF treatment in *Aplysia* sensory-motor synapses. EPSP amplitudes are normalized to the first EPSP in the train. BDNF treatment (■, n = 4) had no effect on synaptic depression compared with vehicle (□, n = 2) (F = 1.81, df = 1, p > 0.1 in a one-way ANOVA test).

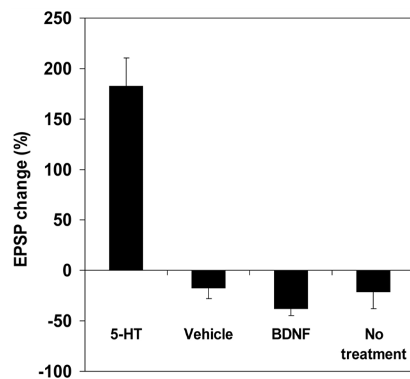

Fig. 4. Percentage change in EPSP amplitude after BDNF treatment in sensory-motor co-cultured *Aplysia* neurons. BDNF treatment did not influence synaptic strength whereas 5-HT induced long-term synaptic facilitation. There was a slight decrease in synaptic strength after treatment with vehicle, BDNF, or no treatment (5-HT, n = 3; vehicle, n = 2; BDNF, n = 9; no treatment control, n = 3) (F = 47.2, df = 3, p < 0.0001 by one-way ANOVA test).

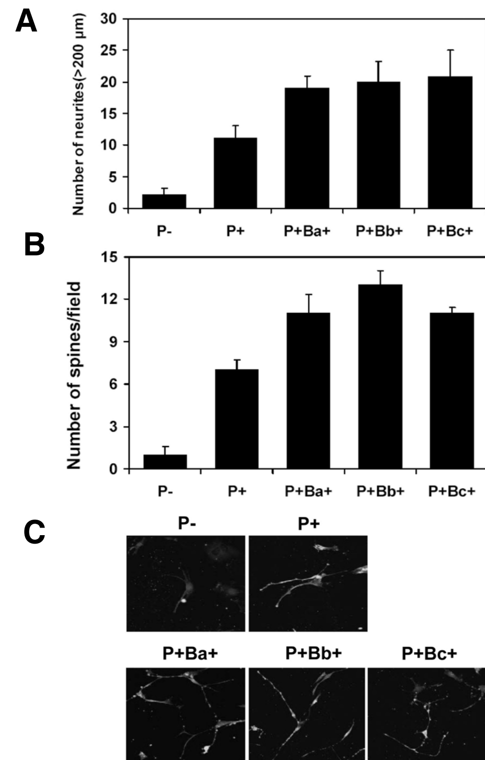

Fig. 5. BDNF increases the length of neurites and the number of spine-like structures in PDGF-primed HiB5 cells. **A.** Numbers of neurites greater than 200 µm in length in differentiating HiB5 cells in the absence (P−) or presence of PDGF-B (P+) or PDGF-B and BDNF (P+B+). Ba, Bb, and Bc indicate BDNF from Amgen, Sigma, and UBI, respectively. Each bar represents the mean ± SEM (n = 4). At least 40 fields were examined for each experimental point. **B.** Numbers of spine-like structures in differentiating HiB5 cells. Each bar represents the mean ± SEM (n = 4). At least 20 fields were examined for each experimental point. **C.** Laser confocal images of HiB5 cells with spine-like structures immunostained with anti-PSD-95 antibodies (green). HiB5 cells were incubated for two days in the presence of PDGF-B alone or PDGF-B and BDNF. Scale bar = 100 µm.

neuronal differentiation, such as neurofilament (NF) and acetylated tubulin (data not shown). When we supplemented differentiating HiB5 cells with BDNF in addition to PDGF-B, the number of neurites exceeding 200 µm in length increased 1.6 to 2 fold compared to PDGF-B alone (Fig. 5A; P-, 2.0 ± 1.2, n = 4; P+, 11.0 ± 2.0, n = 4; P+Ba+, 19.0 ± 2.0, n = 4; P+Bb+, 20.0 ± 3.2, n = 4; P+Bc+, 20.8 ± 4.3, n = 4). To investigate the formation of spine-like structures in response to BDNF, we immunostained differentiating HiB5 cells with the fluorescent dye Cy 3 to detect postsynaptic density protein-95 in the postsynaptic dendritic spines. As shown in Figs. 5B and 5C, addition of BDNF increased the number of spine-like structures (P-, 1.0 ± 0.6, n = 4; P+, 7.0 ± 0.7, n = 4; P+Ba+, 11.0 ± 1.4, n = 4; P+Bb+, 12.8 ± 1.0, n = 4; P+Bc+, 11.0 ± 0.4, n = 4). Thus addition of BDNF to PDGF-primed cells increases both the length of neurites and the number of spine-like structures.

Discussion

In this study, we investigated the physiological effects of exogenous human BDNF on *Aplysia* neurons and rat HiB5 cells. We observed no dramatic effect of BDNF on the neurite outgrowth, membrane excitability, or synaptic plasticity of *Aplysia* neurons. However, it did increase neurite outgrowth and numbers of spine-like structures in rat HiB5 cells.

Many groups have reported that exogenous application

of BDNF affects neurite outgrowth in many types of neurons of the vertebrate central nervous system. It generally increases axonal or dendritic branching and growth *in vivo* (Lom and Cohen-Cory, 1999) and *in vitro* (Labelle and Leclerc, 2000). Consistent with these reports, we also observed that BDNF increased the length of neurites and the number of spine-like structures in a rat hippocampal cell line HiB5. However, BDNF does not have a positive effect on neurite outgrowth in all neuronal cell types. For example, it inhibited dendritic growth in cortical neurons (McAllister *et al*., 1997).

In the case of *Aplysia* bag cell neurons, the effect of BDNF on neurite outgrowth was found to be substrate-dependent: outgrowth was slightly reduced on poly-L-lysine whereas it was unaffected on collagen (Gruenbaum and Carew, 1999). In view of the different effect of BDNF on different neuronal cell types, we explored the role of BDNF in neurite outgrowth in *Aplysia* sensory neurons. In our experiments BDNF produced no dramatic change in the neurite outgrowth of *Aplysia* sensory neurons, which is consistent with previous reports (*Soc. Neurosci. Abstr.* Vol. 23, Ranpura *et al*., 1997).

Tyrosine kinases modulate short-term membrane excitability in *Aplysia* sensory neurons (Purcell and Carew, 2001), and BDNF activates receptor tyrosine kinase. Also, neurotrophic factors such as BDNF and TGF-β1 affect the reduction of synaptic depression induced by high-frequency stimulation. Application of TGF-β1 reduced the magnitude of synaptic depression in co-cultured *Aplysia* sensory-motor synapses (Chin *et al*., 2002) and BDNF greatly attenuated synaptic fatigue induced by high frequency stimulation, termed tetanic stimulation, at CA1 synapses (Gottschalk *et al*., 1999). In contrast with these short-term effects, BDNF did not increase the excitability of sensory neurons or affect the synaptic depression of sensory-to-motor synapses in our study.

Many reports point to the involvement of BDNF in long-term synaptic plasticity. Thus, application of BDNF produced a dramatic and sustained (2 to 3 h) enhancement of synaptic strength at the Schaffer collateral-CA1 synapses (Kang and Schuman, 1995). Activation of TrkB, the BDNF-specific receptor tyrosine kinase, and of its signaling pathway, is necessary for the formation of theta-burst or pairing-induced L-LTP (Kang *et al*., 1997). In addition, a TrkB-like protein has been detected in *Aplysia* neurons and exposure of sensory-to-motor synapses to TrkB-Fc fusion protein, an endogenous BDNF scavenger, reduced 5-HT induced long-term synaptic facilitation (*Soc. Neurosci. Abstr.* Vol. 25, Giustetto *et al*., 1999). These results show that TrkB signaling is necessary for 5-HT induced long-term facilitation.

However, it is not clear whether BDNF by itself is sufficient for long-term facilitation in *Aplysia* sensory-to-motor synapses. Our data indicate that BDNF treatment is not sufficient for inducing long-term synaptic changes of *Aplysia* sensory-motor synapses. On the other hand there is a previous report that BDNF treatment alone produced a slight enhancement of long-term synaptic strength in *Aplysia* pleural-pedal connections (*Soc. Neurosci. Abstr.* Vol. 22, McKay and Carew, 1996). The discrepancy between these observations may be due to differences in experimental conditions. For example, additional effects of interneuron and glial cells could be involved in the pleural-pedal system.

Why did BDNF application have such a modest effect on the neurite outgrowth, membrane excitability, and synaptic plasticity of *Aplysia* neurons? Firstly, it is possible that human BDNF cannot effectively activate the TrkB-like receptor or components of its signaling cascade in *Aplysia* neurons. Consistent with this view, *Lymnaea* and *Drosophila melanogaster* lack homologues of neurotrophins, although they have homologues of Trk receptors (Heerssen and Segal, 2002; Jaaro *et al*., 2001). Thus, neurotrophic factors other than BDNF may be the ligands of the TrkB-like receptor in *Aplysia*. Secondly, the mode of application of BDNF may be critical for its efficacy. A previous report showed that penetration of BDNF into hippocampal slices is dramatically influenced by the perfusion rate, and that hippocampal slices perfused with BDNF at a very slow flow rate failed to show synaptic potentiation (Kang *et al*., 1996). Thus, there may be some particular mode of application required for physiological function. Finally, a recent report showed that BDNF, or activation of endogenous tyrosine kinase, combined with a single pulse of 5-HT-- which normally produces only short-term facilitation -- enhanced long-term facilitation by activating mitogen-activated protein kinase (MAPK) (Purcell *et al*., 2003). Thus BDNF may provide additional signals, possibly via MAPK, that promote the change from short-term to long-term facilitation.

In summary, application of BDNF alone had no dramatic effect on neuritogenesis, short-term membrane excitability and synaptic depression, or on long-term synaptic plasticity, of cultured *Aplysia* sensory neurons, in contrast to its effect on the hippocampal cell line HiB5. The exact reason for the insignificant effect on *Aplysia* under our experimental conditions remains to be determined. Because we did not employ *Aplysia* homologues of BDNF and the TrkB receptor, our experimental approach has inherent limitations. Hence in order to obtain a clearer picture of the role of BDNF in *Aplysia*, cloning of the *Aplysia* homologues of BDNF and the TrkB receptor should be undertaken.

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