Role of Transforming Growth Factor–β in Long-Term Synaptic Facilitation in *Aplysia*

Fan Zhang, Shogo Endo, Leonard J. Cleary, Arnold Eskin, John H. Byrne*

The role of transforming growth factor–β (TGF-β) in long-term synaptic facilitation was examined in isolated *Aplysia* ganglia. Treatment with TGF-β1 induced long-term facilitation (24 and 48 hours), but not short-term (5 to 15 minutes) or intermediate-term (2 to 4 hours) facilitation. The long-term effects of TGF-β1 were not additive with those of serotonin. Moreover, serotonin-induced facilitation was blocked by an inhibitor of TGF-β. Thus, activation of TGF-β may be part of the cascade of events underlying long-term sensitization, consistent with the hypothesis that signaling molecules that participate in development also have roles in adult neuronal plasticity.

Long-term memory for sensitization of defensive withdrawal reflexes in *Aplysia* is correlated with facilitation of the synaptic connections between sensory and motor neurons (*1*) and with morphological changes of sensory neurons (*2*). Because transcription and translation are required for induction of the long-term forms of sensitization (*3*), synaptic facilitation (*4*), and morphological changes (*5*), a major focus of research on long-term memory is the identification and functional characterization of specific mRNAs that are regulated by treatments that induce long-term plasticity. The amount of mRNA for a tolloid/BMP-1 (bone morphogenetic protein)–like protein (apTBL-1) increases in sensory neurons of *Aplysia* after treatment with serotonin (5-hydroxytryptamine or 5-HT) for 1.5 hours or after long-term sensitization training (*6*). Both tolloid and BMP-1 are believed to function as secreted metalloproteases that activate the TGF-β family of proteins (*7*). The TGF-β proteins play an important role in neuronal development in both invertebrates and vertebrates (*8*). Although little is known about the role of TGF-β in synaptic plasticity, studies of a variety of growth factors indicate that neurotrophic factors are involved in synaptic modification (*9*). Thus, a TGF-β–like molecule or molecules in *Aplysia* (*10*) may also contribute to the formation of long-term facilitation.

To test whether TGF-β was capable of producing long-term facilitation, we first examined the long-term effects of TGF-β1 on synaptic connections between sensory and motor neurons in isolated pleural-pedal ganglia (*11*). After baseline amplitudes of excitatory postsynaptic potentials (EPSPs) were tested, TGF-β1 was applied at a final concentration of 100 ng/ml (*12*). EPSPs were tested 24 hours later, and TGF-β1 was washed out soon after. EPSPs were tested again at 48 hours. Treatment of ganglia with TGF-β1 for 24 hours increased the amplitudes of EPSPs [137 ± 9% (mean ± SEM) at 24 hours and 189 ± 24% at 48 hours], but no significant change occurred in the control group at 24 hours (95 ± 7%) or 48 hours (118 ± 17%) (Fig. 1). Several electrical properties of sensory and motor neurons were also tested, including the excitability of sensory neurons, the input resistance of motor neurons, and the spike threshold of motor neurons. No significant changes were observed in these parameters (*13*). Thus, TGF-β1 appeared to specifically affect the sensorimotor synapses.

Although TGF-β1 mimicked the effects of 5-HT on synaptic facilitation (*4, 14*), it did not mimic the effects of 5-HT on sensory neuron excitability (*15*). Therefore, the ability of TGF-β1 to mimic more rapid effects of 5-HT on synaptic strength was examined immediately (5 to 15 min), 2 hours, and 4 hours after the application of TGF-β1 (*16*). There was no significant effect of TGF-β1 at these time points (Fig. 2). As before, TGF-β1 did not affect the excitability of sensory neurons (*17*). These results further establish the specificity of action of TGF-β1.

Having demonstrated that TGF-β1 mimicked the long-term effects of 5-HT on synaptic connection, we next tested for an interaction between the two treatments. TGF-β1 [or only bovine serum albumin (BSA) in the control experiment] was applied to the bath immediately after 1.5 hours of treatment with 5-HT and was washed out after 24 hours. In the control group, 5-HT induced long-term facilitation at 24 hours (141 ± 22%) and 48 hours (189 ± 25%) (Fig. 3). In the group that received both 5-HT and TGF-β1, the EPSP amplitudes were increased 149 ± 12% at 24 hours and 223 ± 54% at 48 hours. There was no significant difference between the facilitation produced by 5-HT alone and that produced by the combination of 5-HT and TGF-β1. These nonadditive results suggest that 5-HT and TGF-β1 interact at some point on a signal pathway (*18*).

One hypothesis to explain the interaction between 5-HT and TGF-β1 is that TGF-β mediates the effects of 5-HT. To test this hypothesis, we examined the effects of an inhibitor of TGF-β, recombinant human TGF-β soluble receptor II (TGF-β sRII) (*19*), on 5-HT–induced long-term facilitation. Continuous treatment with 5-HT for 1.5 hours produced long-term facilitation of the EPSP at both 24 hours (198 ± 44%) and 48 hours (233 ± 53%). Facilitation induced by 5-HT was significantly inhibited by TGF-β sRII (1 μg/ml) at 24 hours (108 ± 11%) and 48 hours (122 ± 16%) (Fig. 4A). TGF-β sRII also

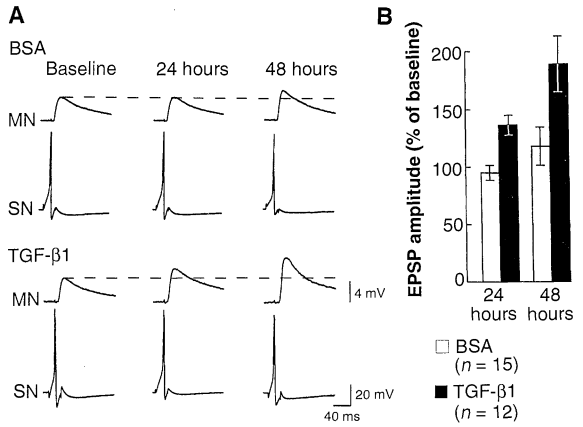

Fig. 1. Application of TGF-β1 (100 ng/ml) produced long-term enhancement of the connections between sensory and motor neurons. (**A**) Typical results illustrating action potentials elicited in the sensory neurons (SN, lower traces) and the monosynaptic EPSPs produced in the motor neurons (MN, upper traces) during the baseline, 24-hour, and 48-hour test periods. (**B**) Summary data at the 24- and 48-hour time points. Bars represent means ± SEM of the normalized amplitudes of the EPSPs. Two-way ANOVA with one repeat revealed a significant difference between the treatments of TGF-β1 and the control (BSA) ($F_{1,25} = 9.63$, $P < 0.005$) and a significant difference between the tests performed at 24 and 48 hours ($F_{1,25} = 11.11$, $P < 0.003$).

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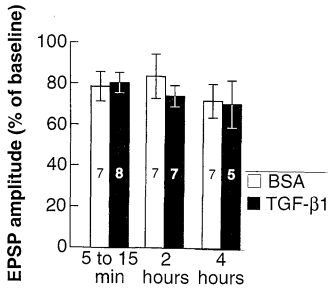

Fig. 2. TGF-β1 had neither short-term nor intermediate-term effects on synaptic strength. The effects of TGF-β1 on synaptic strength were examined immediately (5 to 15 min), 2 hours, and 4 hours after the application of TGF-β1. No significant difference was revealed by a *t* test at each of the time points.

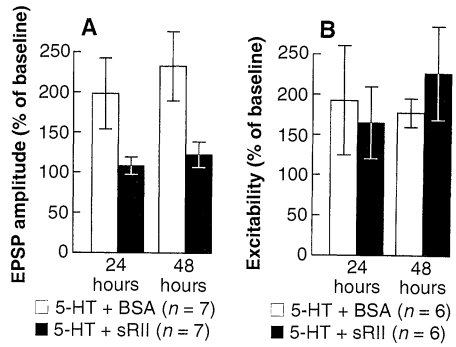

Fig. 4. TGF-β sRII, a TGF-β inhibitor, blocked 5-HT–induced long-term facilitation but not long-term enhancement of excitability. In the control group (5-HT + BSA), 5 μM 5-HT was applied for 1.5 hours in the presence of BSA alone. In the 5-HT + TGF-β sRII group, 5-HT was applied in the presence of TGF-β sRII as well as BSA. BSA and TGF-β sRII were applied 1 hour before the application of 5-HT and washed out after 24 hours. (**A**) Summary data showing that 5-HT–induced facilitation was inhibited by TGF-β sRII ($F_{1,6}$ = 9.57, $P < 0.03$). In this set of experiments, the left and right ganglia from the same animal were always paired, one used for TGF-β sRII treatment and the other used as control. Therefore, a two-way ANOVA with two repeats was performed. (**B**) Summary data showing that 5-HT–induced long-term enhancement of excitability was not significantly affected by TGF-β sRII ($F_{1,5}$ = 0.79) in the same experiments as above.

inhibited long-term facilitation produced by electrical stimulation of peripheral nerves (*20*). A separate experiment showed that application of TGF-β sRII alone had no significant effects on synaptic efficacy (105 ± 7% at 24 hours and 109 ± 5% at 48 hours, n = 8), compared with the control group (120 ± 6% at 24 hours and 127 ± 13% at 48 hours, n = 8) [two-way analysis of variance (ANOVA) with one repeat, $F_{1,14}$ = 3.39]. In addition, the effects of TGF-β sRII on 5-HT–induced long-term enhancement of excitability were examined. No significant difference was observed between the 5-HT group (192 ± 68% at 24 hours and 177 ± 18% at 48 hours) and the 5-HT plus TGF-β sRII group (165 ± 45% at 24 hours and 226 ± 58% at 48 hours) (Fig. 4B), which is consistent with the observation that TGF-β1 did not affect excitability. These results further support the hypothesis that 5-HT and TGF-β induce long-term facilitation through a common pathway. More provocatively, they suggest that TGF-β is necessary for long-term facilitation induced by 5-HT and by nerve stimulation.

Our results provide evidence for the role of TGF-β in learning and memory. In *Aplysia*, 5-HT is released as a consequence of behavioral training (*21*). Thus, the ability of TGF-β sRII to inhibit the long-term facilitation produced by 5-HT indicates that TGF-β has a behavioral role. TGF-β did not mimic all effects of behavioral training or 5-HT, however. For example, TGF-β1 had neither a short-term effect on the synaptic connection nor a long-term effect on excitability of the sensory neuron, whereas sensitization training and 5-HT produce both of these effects (*1*, *15*, *22*). Thus, 5-HT treatment or sensitization training have some effects that do not appear to be mediated by TGF-β (*23*).

Although little is known about the signal mechanisms downstream of the TGF-β receptor, a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family may be involved (*24*). MAPKKK phosphorylates and activates MAPK through the phosphorylation and activation of MAPKK (*25*). Injection of an antibody to MAPK into sensory neurons blocks long-term facilitation in *Aplysia* (*26*). Because TGF-β1 had neither short-term nor intermediate-term effects on the synaptic connection, the long-term effect of TGF-β1 may be mediated through transcriptional and translational events. Transcription and translation are involved in the growth and remodeling of sensory neurons (*5*), which suggests that TGF-β may play a role in neurite rearrangement. Together with the results of several recent studies (*8*, *9*), our results suggest that growth factors play an important role in adult neural plasticity as well as in early development, and raise questions regarding the number of growth factors involved in neural plasticity and the coordination of their activities during development, learning, and memory.

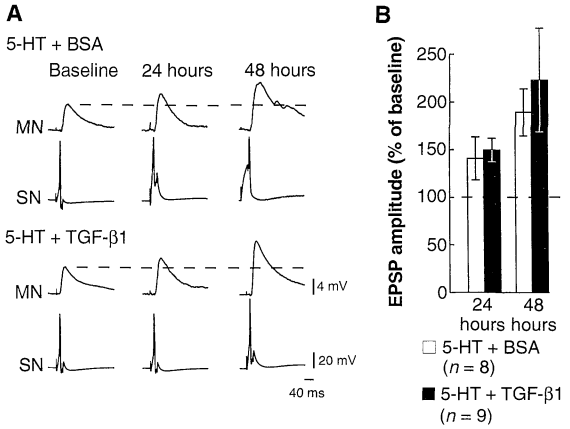

Fig. 3. Long-term enhancement produced by TGF-β1 was not additive with long-term facilitation induced by application of 2 μM 5-HT for 1.5 hours. TGF-β1 (or only BSA in the control experiment) was applied immediately after the 1.5-hour application of 5-HT. (**A**) Typical results illustrating that 5-HT alone induced long-term facilitation, and that this facilitation was not further enhanced when TGF-β1 was applied after the treatment with 5-HT. (**B**) Summary data at the 24- and 48-hour time points. No significant differences between the effects produced by 5-HT and those produced by 5-HT plus TGF-β1 were observed (ANOVA, $F_{1,15}$ = 1.09).

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10. A TGF-β–like molecule was found in *Aplysia* hemolymph with an antibody to human TGF-β1 (S. Endo *et al.*, unpublished data).
11. Intracellular recordings were made from tail sensory and motor neurons in isolated pleural-pedal ganglia [F. Zhang, J. R. Goldsmith, J. H. Byrne, *J. Neurophysiol.* **72**, 778 (1994)]. The experiments were performed at 15°C. After baseline tests of the sensori-

motor EPSP, cells surrounding the sensory neuron and the motor neuron were labeled with Fast Green [D. V. Buonomano and J. H. Byrne, *Science* **249**, 420 (1990)]. The ganglia were incubated in L15 culture medium (Sigma) at 15°C. The culture medium contained 430 mM NaCl, 27 mM $MgSO_4$, 11 mM $CaCl_2$, 27 mM $MgCl_2$, 10 mM KCl, streptomycin sulfate (0.10 g/liter), penicillin-G (0.12 g/liter), and Hepes (7.15 g/liter). The pH of the culture medium was adjusted to 7.65. At 24 and 48 hours, the same sensory and motor neurons were reimpaled and EPSPs were reexamined. In each test period, three EPSPs were elicited by injecting brief suprathreshold depolarizing currents into a sensory neuron at an interstimulus interval (ISI) of 10 s. The mean values at 24 and 48 hours were normalized to that of the baseline test. All experiments were performed in a blind fashion. The experimenter did not know the composition of the solutions that were applied until after the sequence of experiments was completed.

12. Human TGF-β (TGF-β1, R&D Systems) was used in the experiments. Lyophilized recombinant human TGF-β1 samples were reconstituted with BSA solution. The final concentration of BSA was adjusted to 10 μg/ml in all the experiments. In control experiments, only BSA solution was used. In pilot experiments, no effects were observed when TGF-β1 was used at 10 ng/ml. The relatively high dose of TGF-β1 required to induce long-term facilitation could be attributable to a difference in potency of the species-specific forms. However, the concentration of TGF-β1 in human serum is >30 ng/ml [I. E. Eder *et al.*, *J. Urol.* **156**, 953 (1996)]. There are currently no known data from *Aplysia* or other systems regarding the effective duration of TGF-β application. Therefore, TGF-β1 was applied for 24 hours to provide sufficient time to induce long-term facilitation.
13. Excitability of a sensory neuron (separate from the one used for the EPSP test) was measured as the number of action potentials elicited during a constant-current pulse (1 s, 2 nA). In the TGF-β1 group, the number of action potentials was 144 ± 22% of baseline at 24 hours and 124 ± 20% at 48 hours (n = 15). These values were not significantly different ($F_{1,27}$ = 0.35) from the control group (115 ± 16% at 24 hours and 127 ± 20% at 48 hours, n = 14). The input resistance of motor neurons was measured by injecting a hyperpolarizing current pulse (2 nA). There was no significant difference ($F_{1,25}$ = 0.09) between the input resistance of the control group (102 ± 9% of baseline at 24 hours and 89 ± 8% at 48 hours) and the TGF-β1 group (98 ± 6% at 24 hours and 89 ± 7% at 48 hours). The threshold of a motor neuron was measured by injecting a graded depolarizing current until an action potential was elicited. No significant difference ($F_{1,26}$ = 0.26) in the threshold was observed between the control group (99 ± 2% at 24 hours and 100 ± 2% at 48 hours) and the TGF-β1 group (101 ± 3% at 24 hours and 101 ± 3% at 48 hours).
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16. The same sensory and motor neurons were impaled three times (baseline, after 2 hours, and after 4 hours). TGF-β1 or BSA was applied immediately after the baseline test. Then, the connections were tested three times in each period, with an ISI of 5 min. The average of the three values in each period was normalized to the baseline value.
17. F. Zhang, S. Endo, L. J. Cleary, A. Eskin, J. H. Byrne, data not shown.
18. The nonadditive effect is probably not the result of a ceiling, because more facilitation was seen at 48 hours than at 24 hours. In addition, the sensorimotor synapses can be increased to 300 to 400% of baseline [M. Stopfer and T. J. Carew, *J. Neurosci.* **16**, 4933 (1996); F. Zhang and J. H. Byrne, unpublished data], which is more than the extent of facilitation reported here. Moreover, the peak amplitudes of the EPSPs did not approach either the reversal potential of the EPSP or the spike threshold, which indicated that a simple biophysical limitation on the amplitude of the EPSP was not reached.
19. TGF-β sRII (R&D Systems) is a polypeptide containing the extracellular domain of human TGF-β receptor type II [H. Y. Lin, X. F. Wang, E. Ng-Eaton, R. A. Weinberg, H. F. Lodish, *Cell* **68**, 775 (1992)]. This is a small soluble molecule that is capable of binding TGF-β1, TGF-β3, and TGF-β5 with sufficient affinity to act as an inhibitor with a half-maximal effective dose (ED_{50}) of ~30 ng/ml [M. L.-S. Tsang *et al.*, *Cytokine* **7**, 389 (1995)]. This approach was preferred to an attempt with antibodies to TGF-β1, because immunoglobulin does not penetrate well through the neuropil of the isolated ganglion preparation.
20. F. Zhang and J. H. Byrne, data not shown. Nerves were stimulated as described [F. Zhang *et al.*, in (*11*)].
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23. Several other second messenger cascades and regulatory proteins have been implicated in mediating aspects of the cellular changes associated with long-term sensitization [see (*22*) for reviews]. These agents could be components of the TGF-β pathway or could act in parallel to it. In addition, cleavage and activation of procollagen by apTBL-1, in a manner analogous to BMP-1 [E. Kessler, K. Takahara, L. Biniaminov, M. Brusel, D. S. Greenspan, *Science* **271**, 360 (1996)], could contribute to sensitization.
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Isolation of a Common Receptor for Coxsackie B Viruses and Adenoviruses 2 and 5

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A complementary DNA clone has been isolated that encodes a coxsackievirus and adenovirus receptor (CAR). When transfected with CAR complementary DNA, nonpermissive hamster cells became susceptible to coxsackie B virus attachment and infection. Furthermore, consistent with previous studies demonstrating that adenovirus infection depends on attachment of a viral fiber to the target cell, CAR-transfected hamster cells bound adenovirus in a fiber-dependent fashion and showed a 100-fold increase in susceptibility to virus-mediated gene transfer. Identification of CAR as a receptor for these two unrelated and structurally distinct viral pathogens is important for understanding viral pathogenesis and has implications for therapeutic gene delivery with adenovirus vectors.

Adenoviruses and coxsackieviruses are common human pathogens. Adenoviruses are nonenveloped DNA viruses that cause respiratory and gastrointestinal infections (*1*) as well as infections of the heart (*2*). Adenoviruses have also been adapted for use as vectors for vaccination and gene therapy (*3*). Coxsackie B viruses are nonenveloped RNA viruses belonging to the picornavirus family. They cause meningoencephalitis (*4*), are implicated in acute pancreatitis (*5*) and as triggering agents in childhood-onset diabetes (*6*), and are the viruses most frequently identified in acute infections of the heart (*7*).

Coxsackievirus B3 forms a detergent-stable complex with a 46-kD HeLa cell surface protein (*8*). We have used a monoclonal antibody (mAb) raised against this complex (*9*) to isolate and clone a 46-kD protein that mediates attachment and infection by coxsackie B viruses. The same receptor also functions in adenovirus attachment and adenovirus-mediated gene transfer.

The coxsackievirus and adenovirus receptor (CAR) protein was purified from HeLa cell lysates by immunoaffinity chromatography (*10*) with mAb RmcB (*9*), the

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