Transforming Growth Factor β1 Alters Synapsin Distribution and Modulates Synaptic Depression in *Aplysia*

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Transforming growth factor β1 (TGF-β1) induces long-term synaptic facilitation and long-term increases in excitability in *Aplysia*. Here we report that this growth factor has acute effects as well. Treatment of pleural–pedal ganglia with TGF-β1 for 5 min activated mitogen-activated protein kinase (MAPK) and stimulated the phosphorylation of synapsin in a MAPK-dependent manner. This phosphorylation appeared to modulate synapsin distribution in cultured sensory neurons. Control neurons exhibited a punctate distribution of synapsin along neurites, which appeared to represent high concentration aggregates of synapsin. TGF-β1-treated sensory neurons showed a significant reduction in the number of these puncta, an effect that was blocked by the MAP/ERK kinase inhibitor U0126. The functional consequence of TGF-β1 was tested by examining its effects on synaptic transmission at the sensorimotor synapse. Application of TGF-β1 reduced the magnitude of synaptic depression. This effect was dependent on MAPK, consistent with the hypothesis that TGF-β1 mobilizes synaptic vesicles through the phosphorylation of synapsin.

Key words: synapsin; Aplysia; *TGF-β1; synaptic depression; short-term plasticity; mobilization; phosphorylation; synaptic vesicles; MAPK*

Neurotrophins and cytokines are signaling molecules that play an important role in development. Recently, it has become clear that neurotrophins have modulatory effects on synaptic transmission and neuronal plasticity in mature organisms (for review, see Lu and Gottschalk, 2000). Interestingly, some of these modulatory effects are acute. For example, brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, reduces synaptic depression in a mitogen-activated protein kinase (MAPK)-dependent manner in rat hippocampal slices (Gottschalk et al., 1999). Because synaptic depression has been attributed to the depletion of the releasable pool of synaptic vesicles, the reduction in synaptic depression is likely to be caused by a presynaptic mechanism. Indeed, phosphorylation of synapsin I through a MAPK pathway appears to mediate the enhancement of neurotransmitter release induced by BDNF in rat and mouse synaptosomes (Jovanovic et al., 2000). Although acute modulatory effects of the neurotrophin family of growth factors have been examined extensively, little is known about acute modulatory effects of the transforming growth factor β (TGF-β) family of growth factors that play important roles in early development of the nervous system (Böttner et al., 2000). Because TGF-β1 mediates some forms of long-term neuronal plasticity in *Aplysia* (Zhang et al., 1997; Chin et al., 1999; Farr et al., 1999), we examined whether it had acute affects as well. In addition, we examined whether TGF-β1, like neurotrophins, might exert its acute effects via phosphorylation of synapsin.

We found that TGF-β1 activated MAPK and stimulated the phosphorylation of synapsin in *Aplysia* sensory neurons. In addition, TGF-β1 altered the distribution of synapsin in sensory neurons in a MAPK-dependent manner. Furthermore, TGF-β1 decreased low-frequency synaptic depression.

MATERIALS AND METHODS

MAPK phosphorylation assay. Aplysia californica (100–200 gm; Alacrity, Redondo Beach, CA, and Marinus, Long Beach, CA) were housed in cages within aquaria maintained at 15°C on a 12 hr light/dark cycle. Animals were anesthetized with isotonic $MgCl_2$, and paired pleural–pedal ganglia were removed and divided into control and experimental groups. Ganglia were treated in groups of three to provide enough protein for analysis. The pleural ganglia were desheathed to expose the ventral–caudal cluster of sensory neurons in a 1:1 solution of isotonic $MgCl_2$ and artificial seawater (ASW) containing (in mM): 460 NaCl, 10 KCl, 11.4 $CaCl_2$, 27 $MgCl_2$, and 10 HEPES. The ganglia "rested" in ASW for 2 hr at 15° C before recombinant human TGF-β1 (1 ng/ml; R & D Systems) or BSA control (Pentex) was applied for 5 min. The MEK inhibitor U0126 (20 μM; Promega) was applied for 1 hr before treatment with TGF-β1/BSA. Immediately after treatment, the pleural sensory clusters were excised and homogenized in ice-cold buffer con-

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taining (in mM): 10 EDTA, 20 Tris, pH 7.5, 1 Na orthovanadate, 1 DTT, 2 NaF, 2 NaPPi, 0.5 okadaic acid, 1 PMSF, 1% SDS, 1% protease inhibitor cocktail (Sigma, St. Louis, MO). After determination of protein content by Bradford assay, equal protein from each group was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with an antibody specific to the dually phosphorylated form of the ERK1/2 isoforms of MAPK (1:2500; Promega V8031) and then exposed to I^{125}-conjugated Protein A (1:1000). Immunoreactive bands were visualized by autoradiography.

Synapsin phosphorylation assay. Pleural–pedal ganglia were isolated and desheathed as above. After overnight incubation in ASW containing 10 μCi/ml ^{32}P (specific activity of 4500 Ci/mmol) (Homayouni et al., 1995), ganglia were treated for 5 min with 1 ng/ml TGF-β1 or BSA. Ganglia were treated in groups of three to provide enough protein for analysis. Immediately after treatment, pleural ganglia were homogenized as above. Equal amounts of protein were subjected to immunoprecipitation in 5 vol of immunoprecipitation buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1% Triton X-100, and protease inhibitors), with 1:200 vol of antisera against *Aplysia* synapsin (anti-apSyn) and 1:10 vol of protein-A Sepharose. Immunoprecipitated material was resolved by SDS-PAGE and transferred to nitrocellulose. ^{32}P signal was quantified with a phosphorimager. The membranes were then immunoblotted with anti-apSyn antibody to quantify the amount of synapsin. The amount of phosphorylation was calculated as the ratio of the treated and control samples for each experiment, after normalization to protein content.

Immunocytochemistry. Culturing procedures followed those described by Rayport and Schacher (1986). Neurons were removed individually by microelectrodes with fine tips and plated on poly-L-lysine-coated glass coverslips in Petri dishes containing 50% hemolymph and 50% isotonic L15. After 5 d in culture, neurons were treated with TGF-β1 or BSA for 5 min and immediately fixed in 4% paraformaldehyde in PBS containing 30% sucrose. Cells were rinsed with PBS and incubated in 2% normal goat serum in PBS containing 0.1% Triton X-100 before incubation with anti-apSyn (1:200) overnight at 4°C. Cultures were rinsed with PBS and incubated with tetramethylrhodamine-conjugated goat anti-rabbit IgG. Immunofluorescence was viewed with confocal microscopy. A z-series of optical sections through the neurites (0.15 μm increments through ~10 μm) were taken with a Bio-Rad 1024 MP confocal microscope.

The individual performing the quantification of immunoreactivity did not know whether the sensory neuron had been treated with TGF-β1 or BSA. Stacked images of the arborizations of each cell were used for quantification. The extent of the arborization visible in the micrograph was traced using MetaMorph software to obtain a measure of the total neurite length. Aggregates, or puncta, of intense apSyn immunoreactivity were manually marked and automatically counted by the software.

Electrophysiology. Experiments were performed using a single sensory neuron cocultured with a single motor neuron. The motor neuron was impaled with an electrode filled with 3 M KAc (10 MΩ resistance), and resting potential and input resistance were measured. A single EPSP was evoked in the motor neuron by extracellular stimulation of the sensory neuron. The cocultures were then divided into groups such that the two groups consisted of cultures having similar distributions of EPSP amplitudes (BSA, 29.5 ± 4.4 mV; mean ± SEM; TGF-β1, 33.9 ± 7.5 mV). At least 2 hr later, 1 ng/ml TGF-β1 or BSA was applied for 5 min before the motor neuron was re-impaled for measurements. All treatments were performed in a blinded manner. The sensory neuron was stimulated with a 1 Hz stimulus for 20 sec. EPSPs were recorded in the motor neuron with resting membrane potential current clamped at −80 mV.

RESULTS

TGF-β1 induces synapsin phosphorylation

Because MAPK mediates neurotrophic factor-induced phosphorylation of synapsin (Jovanovic et al., 2000), we examined the ability of TGF-β1 to induce phosphorylation of *Aplysia* synapsin via MAPK. The *Aplysia* homolog of synapsin was recently cloned (Angers et al., 1999). *Aplysia* synapsin shows the same domain arrangement as other vertebrate and invertebrate synapsin I molecules and exhibits MAPK consensus phosphorylation sites. Although little is known about TGF-β1 signaling in neurons, recent results indicated that the MAPK cascade is engaged by TGF-β1. For example, TGF-β1 results in the transient activation of the MAPK pathway in chick ciliary ganglion neurons, which is necessary for the acute and sustained effects of TGF-β1 on the expression of calcium-activated potassium channels (Lhuillier and Dryer, 2000).

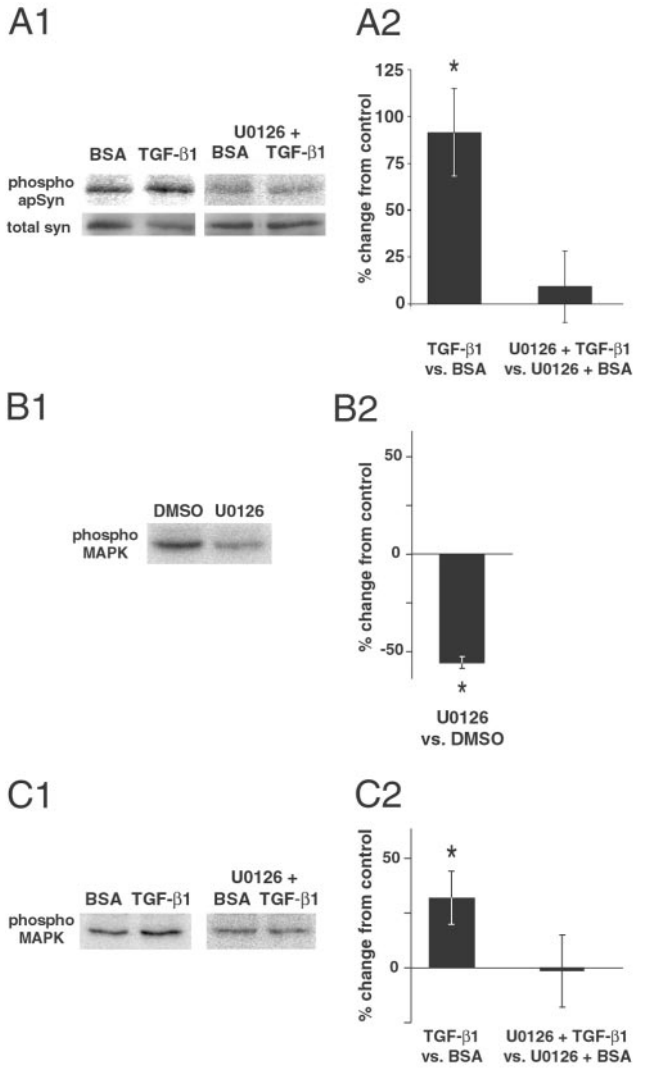

Fig. 1. TGF-β1 activated MAPK and induced synapsin phosphorylation. *A1*, Autoradiographs (*top panels*) show levels of phosphorylated synapsin (*phospho apSyn*) in ganglia treated with *BSA* (control), *TGF-β1*, *U0126 + BSA*, or *U0126 + TGF-β1*. Western blots (*bottom panels*) of the same membranes show total protein levels. For quantification of effects of TGF-β1 on synapsin phosphorylation, phosphorylation signal was normalized to total synapsin determined by Western blot. *A2*, Summary data showing that TGF-β1 increased synapsin phosphorylation by 91%. Treatment with U0126 blocked the increase in synapsin phosphorylation by TGF-β1. *B*, *C*, Western blots show levels of dually phosphorylated MAPK (*phospho MAPK*) in sensory neuron clusters after specified treatments. Equal amounts of protein were loaded from control and experimental extracts and probed with the antibody to dually phosphorylated, active MAPK. *B1*, *B2*, U0126 alone (20 μM, 1 hr) decreased basal levels of dually phosphorylated MAPK by 56%. *C1*, *C2*, TGF-β1 increased levels of dually phosphorylated, active MAPK by 37%. U0126 blocked the ability of TGF-β1 to increase levels of dually phosphorylated MAPK over levels found in U0126 + BSA controls. $^*p < 0.05$.

Treatment of pleural–pedal ganglia with 1 ng/ml TGF-β1 for 5 min increased synapsin phosphorylation by 91 ± 23% (mean ± SEM; $n = 4$; $t_3 = 3.9$; $p < 0.05$) compared with BSA control (Fig. 1*A1*,*A2*). The amount of phosphorylation was calculated as the ratio of the treated and control samples for each experiment,

after normalization to protein content. After autoradiography, Western blot analysis was performed using an antibody specific to *Aplysia* synapsin to quantify the amounts of total protein. Overall, no significant difference was found in the protein levels of synapsin between BSA- and TGF-β1-treated samples (data not shown). The TGF-β1-induced increase in phosphorylation was blocked by preincubation with 20 μM U0126, a specific (Davies et al., 2000) inhibitor of MEK (9 ± 19% change in phosphorylation compared with U0126 + BSA control; $n = 5$; $t_4 = 0.48$; $p = 0.65$) (Fig. 1*A1*,*A2*). We verified that U0126 inhibited MEK in *Aplysia*. U0126 decreased basal MAPK phosphorylation in sensory neurons ($n = 3$; $t_2 = 4.41$; $p < 0.05$) (Fig. 1*B1*,*B2*). These results indicate that TGF-β1 induces synapsin phosphorylation through a MAPK-dependent pathway.

TGF-β1 activates MAPK in sensory neurons

Because MAPK activity appeared to be necessary for TGF-β1-induced synapsin phosphorylation, we investigated whether TGF-β1 activated MAPK in sensory neurons. Although many species contain ERK1 and ERK2 isoforms of MAPK, only one immunoreactive band (43 kDa) was identified by Western blot analysis in the *Aplysia* CNS (Michael et al., 1998). In confirmation of these results, the antibody to dually phosphorylated, activated ERK1/2 recognized a single band of 43 kDa in sensory neuron extracts (Fig. 1*B1*,*C1*). Furthermore, TGF-β1 (1 ng/ml for 5 min) increased the levels of dually phosphorylated MAPK compared with BSA-treated controls ($n = 7$; $t_6 = 2.66$; $p < 0.05$) (Fig. 1*C1*,1*C2*). The increase in active MAPK by TGF-β1 was completely blocked in the presence of 20 μM U0126 ($n = 4$; $t_3 = 1.05$; $p = 0.37$) (Fig. 1*C1*,1*C2*). These results demonstrate that TGF-β1 activates MAPK and supports the hypothesis that TGF-β1 leads to synapsin phosphorylation via MAPK.

TGF-β1 alters the distribution of synapsin in sensory neurons

Phosphorylation of synapsin alters its binding properties to both synaptic vesicles and cytoskeletal elements. After high-frequency stimulation of the frog neuromuscular junction, 30% of synapsin dissociated from synaptic vesicles (Torri Tarelli et al., 1992). Recently, this result was confirmed in cultured hippocampal neurons by the finding that synaptic activity induced the dispersion of synapsin in a phosphorylation state-dependent manner (Chi et al., 2001). Moreover, in *Aplysia* sensory neurons, the neuromodulatory transmitter serotonin (5-HT) induced the dispersion of synapsin (Angers et al., 2000). Thus, the distribution of synapsin was examined after treatment with BSA or TGF-β1 for 5 min. In control cultures, synapsin immunoreactivity was present in a punctate pattern distributed along neurites ($n = 6$) (Fig. 2*A*, *left panels*). However, in TGF-β1-treated neurons, ~54% fewer puncta could be detected ($n = 6$) (Fig. 2*A*, *right panels*, *B*). This effect was statistically significant ($t_{10} = 2.38$; $p < 0.05$). To test the hypothesis that MAPK mediates the effect of TGF-β1 on the dispersion of synapsin, sensory neurons were incubated with 20 μM U0126 for 1 hr before treatment with either TGF-β1 or BSA. The MEK inhibitor blocked the ability of TGF-β1 to decrease the number of puncta ($n = 10$ for U0126 + BSA; $n = 9$ for U0126 + TGF-β1; $t_{17} = 0.6$; $p = 0.56$) (Fig. 2*A*, *bottom panels*, *C*).

TGF-β1 modulates synaptic depression

The interactions of synapsin with vesicle membranes, actin, and other cytoskeleton proteins are believed to tether synaptic vesicles into a "reserve pool" of vesicles. It is thought that the abolition of these interactions by phosphorylation of synapsin

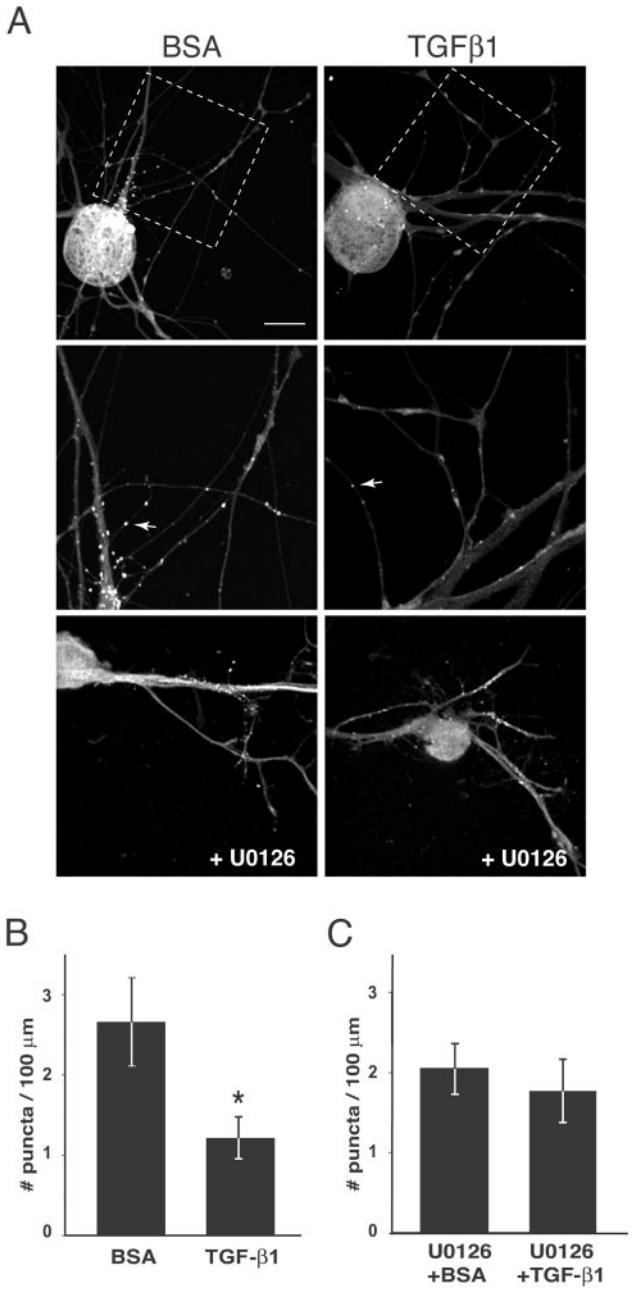

Fig. 2. TGF-β1 induced dispersion of synapsin in cultured sensory neurons. *A*, Confocal images of cultured sensory neurons immunostained for synapsin. Control neuron (*top left panel*) shows punctate pattern of staining. TGF-β1-treated neuron (*top right panel*) exhibits fewer detectable puncta. The area defined by *dashed lines* is seen in higher magnification in the *middle panels*. *Arrows* denote puncta. There is no difference in staining when neurons are treated with U0126 before BSA (*bottom left panel*) or TGF-β1 (*bottom right panel*). Scale bar: 80 μm (40 μm for *middle panels*). *B*, *C*, Summary graphs showing the reduction in the number of synapsin puncta per 100 μm neurite length in TGF-β1-treated neurons compared with BSA controls (*B*) and blockade of the effect of TGF-β1 by U0126 (*C*). MetaMorph software was used to measure total neurite length and count the number of puncta. $^*p < 0.05$; Student's *t* test.

(Jovanovic et al., 1996; Matsubara et al., 1996; Hosaka et al., 1999) results in the release of synapsin-bound vesicles from the reserve pool, allowing vesicle mobilization to the readily releasable pool of vesicles (Turner et al., 1999). Augmentation of the readily releasable pool of vesicles may then lead to an increase in

synaptic efficacy. The results presented above suggest that TGF-β1 treatment may alter the distribution of synapsin within the terminal, thereby altering the availability of synaptic vesicles in the reserve pool for mobilization to the readily releasable pool. If so, TGF-β1-treated synapses would be less susceptible to depression. Thus, to examine whether the effects of TGF-β1 on the phosphorylation state and distribution of synapsin have a physiological consequence, we studied synaptic depression evoked by low-frequency stimuli. To challenge the release machinery, EPSPs were elicited at an interstimulus interval of 1 sec, a rate that leads to significant homosynaptic depression (Byrne, 1982) and partially depletes the synaptic vesicle pools (Gingrich and Byrne, 1985; Armitage and Siegelbaum, 1998; Royer et al., 2000).

TGF-β1 (1 ng/ml for 5 min) reduced the magnitude of synaptic depression (Fig. 3*B1*,*B2*). The amplitude of the EPSP at steady state was ~50% greater in TGF-β1-treated cocultures ($n = 5$) compared with controls ($n = 6$; $F_{(1,17)} = 6.77$; $p < 0.05$; repeated measures ANOVA on EPSPs 3–20). A repeated measures ANOVA was also used to compare the amplitudes of pretreatment EPSPs with the first EPSP in the train in BSA- and TGF-β1-treated groups. There was no significant between-group difference in the amplitudes of EPSPs in the BSA- or TGF-β1-treated groups ($F_{(1,9)} = 0.03$; $p = 0.88$). Although there was significant reduction of the EPSPs in both the BSA- and TGF-β1-treated groups ($F_{(1,9)} = 11.8$; $p < 0.01$), which is presumably caused by rundown over the 2 hr pretest phase, there was no significant interaction effect between treatment (BSA vs TGF-β1) and time (pretest vs first EPSP of train; $F_{(1,9)} = 4.58$; $p = 0.06$). Consequently, it is unlikely that the effect of TGF-β1 on the reversal of synaptic depression during a train of stimuli (Fig. 3*A*) is caused by a shift in baseline synaptic transmission. These results are consistent with previous findings that TGF-β1 affects long-term, but not short-term, synaptic efficacy tested with a single EPSP (Zhang et al., 1997). These results also indicate that TGF-β1 alters the steady-state level of transmitter release, which could be dependent on the mobilization of vesicles from the reserve pool to the readily releasable pool of transmitter (Turner et al., 1999; Thomson, 2000), an effect that would be expressed when the synapse is challenged with a train of action potentials.

We next examined whether MAPK played a role in the effect of TGF-β1 on synaptic depression. Preincubation with U0126 for 1 hr blocked the ability of TGF-β1 to modulate the steady-state level of synaptic depression ($n = 5$ for BSA; $n = 4$ for TGF-β1; $F_{(1,17)} = 0.82$; $p = 0.39$; repeated measures ANOVA on EPSPs 3–20) (Fig. 3*C1*,*C2*). In addition, treatment with U0126 alone, which decreases the activity of endogenous MAPK (Fig. 1*C*), affected the properties of synaptic depression. The amplitude of the second EPSP of the train in U0126 + BSA-treated neurons was significantly lower than in BSA-treated neurons, indicating that inhibition of endogenous MAPK resulted in more pronounced synaptic depression ($t_9 = 3.49$; $p < 0.01$) (Fig. 3*C2*, *inset*).

TGF-β1 did not appear to have any obvious effects on the biophysical properties of the motor neuron. There was no difference in resting potential (BSA, before, −64 ± 1.1 mV, after, −62.3 ± 1.7 mV, $t_{10} = -0.83$, $p = 0.43$; TGF-β1, before, −62.8 ± 1.0 mV, after, −60.4 ± 1.7 mV, $t_8 = -1.21$, $p = 0.26$) or input resistance (BSA, before, 11.0 ± 0.4 MΩ, after, 10.8 ± 0.5 MΩ, $t_{10} = 0.21$; $p = 0.21$; TGF-β1, before, 14.3 ± 1.8 MΩ, after, 14.3 ± 1.6 MΩ, $t_8 = -0.03$, $p = 0.97$) of the postsynaptic cell after treatment.

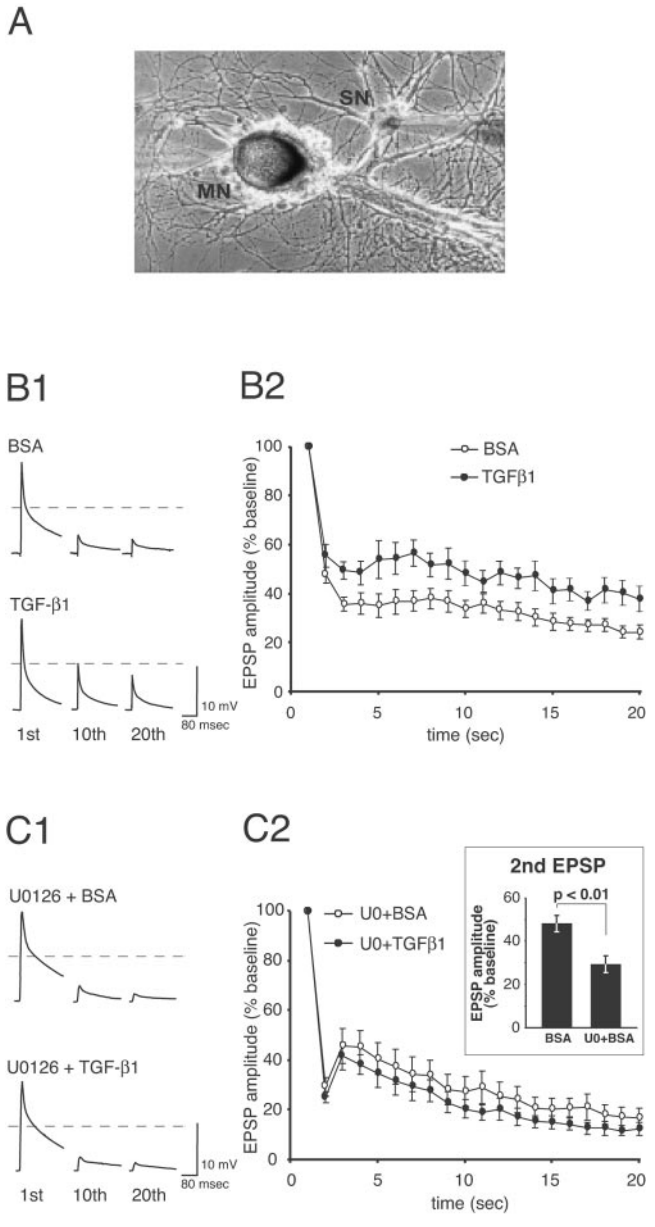

Fig. 3. TGF-β1, via MAPK, reduces synaptic depression induced by low-frequency stimuli. *A*, Phase-contrast image of cocultured sensory neuron (*SN*) and motor neuron (*MN*) from which recordings were made. The SN was stimulated (20 sec duration, 1 Hz) with an extracellular electrode (*right*), and EPSPs were measured in the MN with an intracellular electrode (*left*). *B1*, Examples of EPSPs recorded from cocultures treated with BSA or TGF-β1. For clarity, only the 1st, 10th, and 20th EPSPs of the train are shown. EPSPs of the TGF-β1-treated neurons were scaled by a factor of 89% to account for the initial difference in amplitude in these examples. The *dashed line* was drawn arbitrarily at half-maximal amplitude for comparison purposes. *B2*, Group data. EPSP amplitudes are normalized to the first EPSP in the train. TGF-β1 (●) reduced synaptic depression compared with BSA controls (○) ($p < 0.05$; repeated measures two-way ANOVA, with an interaction between the number of stimulations and treatment, $p < 0.02$). *C1*, Examples of EPSPs recorded from cocultures treated with *U0126 + BSA* or *U0126 + TGF-β1*. EPSPs of the U0126 + TGF-β1-treated group were scaled by a factor of 109% to those of the U0126 + BSA-treated group to account for the initial difference in amplitude in these examples. *C2*, U0126 (●) blocks the ability of TGF-β1 to modulate depression compared with control (○). *Inset*, Comparison of *BSA* and *U0+BSA* groups demonstrates that basal MAPK activity affects the amplitude of the second EPSP.

DISCUSSION

Although growth factors are traditionally thought of as being essential for the growth and differentiation of neuronal populations, several recent studies indicate that they play a critical role in the acute modulation of synaptic transmission (for review, see Schinder and Poo, 2000). At least one mechanism by which growth factors can alter synaptic transmission is through the modulation of synapsin (Jovanovic et al., 1996, 2000). The involvement of synapsin in the maintenance of a reserve pool of synaptic vesicles in nerve terminals has been suggested by several functional and morphological studies (Torri Tarelli et al., 1992; Turner et al., 1999; Thomson, 2000; Chi et al., 2001), providing further support for the hypothesis that synapsin modulation may impact neurotransmitter release. The results presented here are consistent with these findings and suggest a new role for TGF-β1 in synaptic plasticity.

Modulation of synapsin by phosphorylation

The observation that TGF-β1 disrupts the normal punctate distribution of synapsin supports the hypothesis that TGF-β1-mediated phosphorylation of synapsin has a functional consequence in terms of synapsin localization. The punctate staining pattern may represent high-concentration aggregates of synapsin such as might be expected when many synapsin molecules are bound to the reserve pool of synaptic vesicles by interactions with synaptic vesicles or cytoskeletal elements. The reduction in detectable puncta after TGF-β1 treatment may represent the phosphorylation of synapsin and subsequent dispersion of synapsin and synapsin-bound vesicles from high-concentration aggregates. Phosphorylation by MAPK controls interactions of synapsin with the cytoskeleton (Matsubara et al., 1996; Jovanovic et al., 2000). Thus, phosphorylation of synapsin by MAPK may lead to alteration of synapsin distribution by terminating the interaction between synapsin and the cytoskeleton, allowing the dispersion of synapsin.

There is evidence that in cultured *Aplysia* neurons the presence of an appropriate postsynaptic partner can differentially influence both synapse formation and modulation (Glanzman et al., 1989, 1990, 1991). Thus, it is possible that the results of these experiments may reflect developmental effects of TGF-β1 rather than an effect on mature synapses. However, it is clear from images of isolated sensory neurons that varicosities, morphological swellings that indicate presynaptic specializations, exist in these cultures (Fig. 2*A*). In addition, varicosities of isolated sensory neurons exhibit intense immunoreactivity for glutamate (Levenson et al., 2000), indicating that the neurotransmitter is localized in varicosities even in the absence of a postsynaptic neuron. Because synapsin immunoreactivity is also localized to varicosities, it appears that vesicles and synapsin coexist in isolated sensory neurons. Thus, it seems likely that synapsin may be regulated similarly in isolated sensory neurons as well as in cocultured presynaptic and postsynaptic neurons. Indeed, modulation of synapsin puncta, by the neuromodulator 5-HT, occurs similarly in isolated sensory neurons as well as in sensory neurons cocultured with a postsynaptic neuron (Angers et al., 2000). Thus, it does not seem likely that the effect of TGF-β1 on synapsin puncta in isolated sensory neurons is caused by a developmental effect on neurons that would not be pertinent to mature synapses.

Synapsin modulation and synaptic depression

The finding that TGF-β1 reduces synaptic depression via a MAPK-dependent mechanism, in conjunction with the findings that TGF-β1 leads to the phosphorylation of synapsin and alters its neuritic distribution, suggests that the reduction in synaptic depression is mediated by the phosphorylation of synapsin by MAPK. These actions of TGF-β1 are reminiscent of recently described actions of BDNF in synaptic transmission. In addition to phosphorylating synapsin and increasing neurotransmitter release (Jovanovic et al., 2000), BDNF reduces synaptic fatigue in a MAPK-dependent manner in rat hippocampal slices (Gottschalk et al., 1999). Because synaptic fatigue has been attributed to the depletion of the releasable pool of synaptic vesicles (Zucker, 1989; Larkman et al., 1991; Dobrunz and Stevens, 1997), the reduction in synaptic fatigue induced by BDNF appears to be caused by a presynaptic mechanism, possibly by increased mobilization of vesicles via the modulation of synapsin.

MAPK modulation of synaptic depression

In these experiments, there was a difference in the kinetics of synaptic depression between control cultures (cultures receiving only BSA) and cultures treated with the MEK inhibitor U0126 (Fig. 3*C2*, *inset*). One possible explanation for the transient decrease in the amplitude of the second EPSP of the train in the presence of U0126 is that there are at least two mechanisms for mobilization depending on the requirement for calcium (Rosenmund and Stevens, 1996; Kuromi and Kidokoro, 2000). Ca^{2+}-dependent (activity-dependent) mobilization is recruited after the second stimulus, whereas basal MAPK activity may be important for Ca^{2+}-independent vesicle mobilization at the beginning of the stimulus train. In the presence of U0126, Ca^{2+}-independent mobilization may be impaired because of the lack of MAPK activity, and thus EPSP amplitude decreases sharply, below the level of mobilization that is supported by Ca^{2+}-dependent mobilization. After the second stimulus, Ca^{2+}-dependent mobilization may be recruited, and the subsequent rebound in EPSP amplitude may reflect the mobilization of vesicles into the readily releasable pool.

Because basal MAPK activity affects synaptic depression, it might be expected then that inhibition of MAPK-induced phosphorylation of synapsin may lead to increased numbers of synapsin puncta. However, although the regulation of synapsin function by increased phosphorylation has been studied over the last several years, very little information is known regarding how synapsin is regulated when phosphorylation is decreased below basal levels. In our study, no significant change in the number of synapsin puncta was observed in the presence of the inhibitor of MAPK activity (Fig. 2*A*,*C*). In fact, there was a trend for a slight decrease in synapsin puncta (Fig. 2, compare *left bars* in *B* and *C*). Indeed, under some circumstances, this trend becomes statistically significant, as observed in another study (Fioravante et al., 2001). Therefore, basal phosphorylation of synapsin may play an additional, thus far uncharacterized, role in the regulation of synapsin. In addition, there may be numerous MAPK substrate proteins affected by modulatory factors, and the net effect on puncta and transmitter release could be caused by a complex interaction among them.

TGF-β1 has been shown previously to induce long-term synaptic facilitation (Zhang et al., 1997). The present work provides the first evidence that TGF-β1 signaling produces acute effects on synaptic transmission and that at least one target of TGF-β1 is synapsin. Because many neurons physiologically fire in bursts of action potentials, modulation of synapsin and thus transmitter release by TGF-β1 may have profound effects on the efficacy of synaptic communication. Furthermore, these results, together

with the recent findings that synapsin phosphorylation by MAPK is correlated with BDNF-induced enhancement of neurotransmitter release in rat and mouse synaptosomes (Jovanovic et al., 2000) and that BDNF reduces synaptic fatigue via a MAPK-dependent mechanism (Gottschalk et al., 1999), provide strong evidence for the importance of MAPK activity in the acute modulation of neurotransmitter release.

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