ABSTRACT: Transforming growth factor-β̂ (TGF-β̂s) are widely expressed and play roles as multifunctional growth factors and regulators of key events in development, disease, and repair. However, it is not known whether TGF-β affects the plasticity of hippocampal neurons. As a first step to address this issue, we examined whether TGF-β̂2 modulated the electrophysiological and biochemical properties of cultured hippocampal neurons. We found that prolonged 24 h treatment with TGF-β̂2 induced facilitation of evoked postsynaptic currents (ePSCs). This facilitation was associated with a decrease in short-term synaptic depression of ePSCs and increases in both the amplitude and frequency of spontaneous miniature postsynaptic currents (mPSCs). The long-term changes of ePSCs and mPSCs may be associated with cAMP response element-binding protein (CREB), which has been previously implicated in long-term potentiation. Immunofluorescence techniques and Western blot analysis both revealed that TGF-β̂2 enhanced the phosphorylation of CREB. Together, these results suggest that TGF-β̂2 may play a role in the cascade of events underlying long-term synaptic facilitation in hippocampus, and that CREB may be an important mediator of these effects.

KEY WORDS: transforming growth factor-β̂; CREB; phosphorylation; hippocampus; synapse plasticity; ePSC

Transforming growth factor-β̂s (TGF-β̂s) are widely expressed, and serve as multifunctional growth factors and regulators of key events in development, disease, and repair (Massague et al., 2000; Javelaud and Mauviel, 2004). Five isoforms of TGF-β have been identified (TGF-β̂ 1–5) that are 65–80% homologous. Hippocampal neurons are immunoreactive for TGF-β̂2 and 3 (Flanders et al., 1998). Recently, novel roles for TGF-β in neuronal plasticity in vertebrates and invertebrates have emerged. For example, in cultured Aplysia neurons, intermediate- (6 h) or long-term (24–48 h) application of TGF-β̂1 induces long-term changes in excitability and facilitates sensory-motor neuron synapses (Zhang et al., 1997; Chin et al., 1999, 2002; Farr et al., 1999), and short-term (5 min) application regulates the distribution of synapsin.

and modulates short-term synaptic depression (Chin et al., 2006). In acutely dissociated cholinergic basal forebrain neurons obtained from young Sprague-Dawley rats, brief application of TGF-β̂2 acutely inhibits K+ evoked calcium influx (Williams et al., 2002). Activin, a member of the TGF-β family, affects the electrophysiological properties of hippocampal neurons, but results are conflicting. One study reports that activin impairs the induction of long-term potentiation (LTP) (Ikegaya et al., 1997), whereas another study reports that it facilitates the maintenance of LTP (Inokuchi et al., 2003). However, the effect of TGF-β̂ itself on synaptic plasticity in hippocampal neurons has not been investigated. In order to investigate whether TGF-β̂2 modulates the electrophysiological and biochemical properties of cultured hippocampal neurons, we analyzed the effect of application of TGF-β̂2 on cultured hippocampal autapses.

Autapses were derived from low-density cultures of hippocampal neurons obtained from 1- to 3-day postnatal Sprague-Dawley rat hippocampi, as described previously (Zarei and Dani, 1994). Cells were plated at densities of 20K–40K cells/ml on microisolads (Bekkers and Stevens, 1991) and cultured in neurobasal medium supplemented with B27 (Life Technologies, Gaithersburg, MD) for 7–21 days. Cultures were treated with TGF-β̂2 (50 ng/ml) or bovine serum albumin (BSA) alone (for controls) for 24 h in a CO2 incubator in a “blind” manner before recording. This concentration was chosen, because similar concentrations have been used by others examining the effects of TGF-β̂2 on microglia (Schilling et al., 2001). Following the treatment period, whole cell patch clamp was performed. Patch electrodes (TW 150F-4, WPI, Sarasota, FL) had resistances of 2–6 MΩ and contained the following (in mM): 121.5 K-gluconate, 17.5 KCl, 9 NaCl, 1 MgCl2, 10 HEPES, 0.2 EGTA, 2 Mg-ATP, 0.5 Li-GTP, adjusted to pH 7.2 with KOH (Brody and Yue, 2000). Neurons were clamped at ~60 mV with a patch clamp amplifier (Axopatch 200A, Axon Instruments, Union City, CA) and a voltage step (2 ms) from the holding potential to +20 mV was applied to elicit an evoked postsynaptic current (ePSC) using Clampex in pClamp 8 (Axon Instruments). To elicit short-term synaptic depression,
five trains (ITI, 10 s) of four pulses (ISI, 50 ms) were delivered (Fig. 1A1). For each experiment, the average amplitudes of ePSCs elicited by four voltage pulses from the five trains were determined. The traces illustrated are the averages from the 21 and 42 individual experiments in BSA and TGF-β2, respectively. (A2) Summary data for the amplitude of the first of the four ePSCs in (A1). The amplitude with TGF-β2 treatment is significantly larger than that with BSA treatment (P < 0.05, Student’s t-test). (A3) Summary of depression of ePSCs in the presence of TGF-β2 or BSA. For each train of four ePSCs, the amplitudes were normalized to the first ePSC amplitude. For the second through the fourth ePSC, the difference between the TGF-β2 and BSA amplitudes is significant (P < 0.05, repeated measures ANOVA). (B1) Representative examples of mPSCs in the presence of TGF-β2 or BSA. (B2) Amplitude distributions of mPSCs recorded in the presence of BSA or TGF-β2. The difference between the two distributions is significant (P < 0.05, Kolmogorov–Smirnov test). (B3) Frequency distributions of mPSCs recorded in the presence of BSA or TGF-β2. The difference between the two distributions is significant (P < 0.0001, Kolmogorov–Smirnov test). In Figures 1–2, data points represent mean ± SEM.

Twenty four hours incubation with TGF-β1 has previously been used to induce long-term synaptic facilitation in Aplysia (Zhang et al., 1997). We found that a similar 24-h treatment of TGF-β2 affects both synaptic strength and short-term synaptic depression. Specifically, the treatment facilitated the amplitude of the first ePSC induced by a series of four pulses [Figs. 1A1, A2, 2.99 ± 0.31 nA in BSA (n = 21) and 4.13 ± 0.23 nA in TGF-β2 (n = 42)] (T[61] = 2.95, P = 0.005, Student’s t-test). We hypothesized that the increased amplitude of the first ePSC would be associated with increased synaptic depression by the se-
ries of four pulses, because the larger first ePSC would lead to greater depletion of the pool of available transmitter. However, unexpectedly, the short-term synaptic depression produced by the train of four pulses was reduced by treatment with TGF-β2 (Fig. 1A3, TGF-β2 effect, \( F_{1,2} = 4.27, P = 0.04 \); trial effect from 2nd to 4th, \( F_{1,2} = 131.57, P < 0.0001 \); interaction effect between TGF-β2 and pulse trial, \( F_{1,2} = 7.10, P = 0.001 \), 2nd versus 4th). Because the effect on amplitude of the mPSCs was most pronounced for the large-amplitude events (Fig. 1B2), the increased amplitude of the initial ePSC was associated with an increase in both the amplitude and frequency of mPSCs [Figs. 1B1, B2, BSA (n = 6) and TGF-β2 (n = 8), \( P = 0.04 \), Kolmogorov–Smirnov test] and the frequency of mPSCs [Figs. 1B1, B3, BSA (n = 6) and TGF-β2 (n = 8), \( P < 0.0001 \), Kolmogorov–Smirnov test]. Interestingly, the effect on amplitude of the mPSCs was most pronounced for the large-amplitude events (Fig. 1B2). Close inspection of mPSCs indicated that the individual large-amplitude events were single events, rather than summations of multiple events of smaller amplitude.

The electrophysiological effects of TGF-β2 observed following 24-h treatment with TGF-β2 could be due to an acute effect of TGF-β2 on the properties of hippocampal neurons. To test this possibility, ePSCs, synaptic depression, and the amplitude and frequency of mPSCs were measured before and during a 30-min treatment with TGF-β2. TGF-β2 had no obvious effect on any of these measures (data not shown). In addition, we treated cultures for 2–8 h. This intermediate-duration treatment with TGF-β2 did not change any of the measured electrophysiological properties (data not shown). These results indicate that the changes observed at 24 h after the beginning of treatment are slow to develop. TGF-β isoforms have been shown to activate extracellular-regulated kinase isoforms of MAP kinase, which can lead to phosphorylation and activation of cAMP response element-binding protein (CREB) (Impey et al., 1998; Lhuillier and Dryer, 2000). The transcription factor CREB has been implicated in many examples of changes in long-term synaptic strength and long-term memory (Josselyn et al., 2001; Schmitz et al., 2003). Consequently, we examined whether TGF-β2 could induce the phosphorylation of CREB. Although the electrophysiological effects of TGF-β2 were only observed after prolonged treatment, we hypothesized that transcriptional events necessary for these long-term effects are initiated earlier. Indeed, CREB phosphorylation occurs ∼5–10 min after stimulation of hippocampal neurons by depolarization (Wu et al., 2001). Therefore, we treated cultured hippocampal neurons with 50 ng/ml of TGF-β2 for 5 or 30 min and then probed CREB phosphorylation using immunofluorescence techniques and Western blot analysis. Cultures were processed for immunofluorescence staining as described previously (Chin et al., 2002), using a primary antibody against phospho-CREB (UBI, Lake Placid, NY, in 1:250 dilution) and a Cy3-conjugated secondary antibody (Jackson Lab, West Grove, PA, in 1:250 dilution). Immunofluorescence was detected with confocal microscopy. Neurons were randomly chosen for analysis by capturing images of the first 20–25 neurons observed during scanning of cover slips from each experimental condition. A z-series of optical sections through the cell body (0.2 μm increments through ∼2 μm) was taken with a Bio-Rad 1024 MP confocal microscope, and the middle section through the nuclei was analyzed using Metamorph Offline software (Universal Imaging Corporation).

Immunoreactivity to phospho-CREB was specifically localized in the nucleus (Fig. 2A). The 5-min treatment with TGF-β2 (Fig. 2A2) did not increase the level of phospho-CREB (Fig. 2B, 102% ± 4.3%, n = 4), compared to the BSA-treated control group (Figs. 2A1, B). However, the 30-min treatment with TGF-β2 (Figs. 2A4, B) significantly increased the level of phospho-CREB by 35% ± 7.6% (t4 = 3.954, P = 0.017).

These immunofluorescence results were confirmed with Western blot analysis. Both phosphospecific and total CREB antibodies recognize a doublet of bands near 43 kDa on immunoblots; therefore, we measured the sum of both bands by densitometry using ImageQuant 5.0 software. For each treated group, the degree of CREB phosphorylation was normalized to the amount of total CREB and displayed as a percent relative to the control group that was treated with BSA for the same amount of time. No significant difference was found either in the level of total CREB in all treatments or in the degree of CREB phosphorylation between samples that underwent 5 min of TGF-β2-treatment vs. 5 min of BSA treatment (Figs. 2C, D). However, consistent with the immunofluorescence results, 30-min treatment with TGF-β2 significantly increased the phosphorylation of CREB by 51% compared to the 30-min BSA control (Fig. 2C and D, t4 = 3.879, P = 0.018). Moreover, there was no significant difference in the increase of pCREB1 in 30-min TGF-β2 treatment between immunofluorescence results and Western blot analysis results (t5 = −1.079, P = 0.316).

TGF-β induces short-term (Chin et al., 2002; Williams et al., 2002) and long-term (Zhang et al., 1997; Chin et al., 1999, 2006; Farr et al., 1999) modulation of neuronal properties. In the present study, TGF-β2 did not appear to have any acute effects on PSCs measured with our protocol. This lack of effect was not due to an intrinsic lag in the signal transduction cascade of TGF-β2, because TGF-β2 led to a fairly rapid phosphorylation of CREB (Fig. 2). Thus, either TGF-β2 does not have acute effects on the electrophysiological properties of cultured hippocampal neurons, or any effects were not detected by our screening protocol. However, prolonged (24-h) application of TGF-β2 to hippocampal neurons increased both ePSCs and mPSCs in hippocampal neurons (Figs. 1A, B). The increase in the amplitude of ePSCs produced by TGF-β2 is consistent with the results of Zhang et al. (1997), who found that treatment with TGF-β1 induced long-term (24 and 48 h), but not acute (5–15 min) or intermediate-term (2–4 h) facilitation of sensorimotor synaptic connections in Aplysia. Although the mechanisms underlying the increase in the frequency and amplitude of the mPSCs were not investigated in

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this study, a change in the frequency of mPSCs is commonly attributed to modulation of the amount of spontaneously released transmitter from the presynaptic terminal, whereas an increase in the amplitude of mPSCs could be due to an increase in the amount of transmitter per vesicle and/or an increased sensitivity of the postsynaptic membrane to transmitter. The TGF-β2-induced increased frequency of mPSCs could be due to an elevated level of intracellular Ca$^{2+}$ in the presynaptic terminal. Expression of a ryanodine receptor-Ca$^{2+}$ channel is increased in response to 15–24-h treatment with TGF-β in mink lung epithelial cells (Giannini et al., 1992). This result is similar to the time course of our electrophysiological observations. An increase in the number of ryanodine receptors in the endoplasmic reticulum (ER) of the presynaptic terminal could lead to an increased steady-state efflux of Ca$^{2+}$ from the ER, an increase in the presynaptic Ca$^{2+}$ level and consequently increased spontaneous transmitter release.

In the Drosophila CNS, presynaptic expression of an activated TGF-β receptor and postsynaptic expression of TGF-β ligand increases the amplitude of mPSCs (Baines, 2004). Because the TGF-β receptor is expressed presynaptically, these results suggest that the increased amplitude of the mPSC is due to an increase in the amount of transmitter per vesicle. A similar presynaptic action of TGF-β2 at hippocampal synapses could explain our observed increase in amplitude of the mPSC. TGF-βs reportedly modulate K$^+$ channels in microglia.
Our finding that long-term application of TGF-β2 results in phosphorylation of CREB suggests that CREB may activate gene transcription necessary for the long-term effects of TGF-β2. Interestingly, expression of active CREB in rat hippocampus increases silent synapses and enhances LTP (Marie et al., 2005). In addition, recent findings from transcription repressor MeCP2-knockout mice suggest that short-term synaptic depression and the frequency of mEPSCs can be regulated by controlling gene transcription (Nelson et al., 2006). These data raise the intriguing possibility that TGF-β2-modulated short-term synaptic depression and mEPSCs could be controlled at the level of CREB-dependent gene transcription. In addition, it is possible that TGF-β may engage other transcription factors as well (Massague et al., 2000). Future studies will explore the causal link between CREB activation and the expression of the long-term effects of TGF-β2 on synaptic transmission.

Our results provide the first evidence that TGF-β2 modulates synaptic plasticity and leads to phosphorylation of CREB in hippocampus. It will be important to examine the signaling cascades and expression mechanisms that mediate this effect further, and to examine the extent to which TGF-β2 is involved in other forms of hippocampal synaptic plasticity, such as LTP and LTD, and whether it is engaged in memory processing by the hippocampus.

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