Reduced Number of Functional Glutamatergic Synapses in Hippocampal Neurons Overexpressing Full-Length TrkB Receptors

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Brain-derived neurotrophic factor (BDNF) acutely modulates the efficacy of central glutamatergic synapses via activation of the receptor tyrosine kinase TrkB. On a longer time scale, recent evidence suggests an additional role of TrkB signaling in the formation of excitatory synaptic connections. Here, we have overexpressed full-length TrkB receptors (fl-TrkB) in hippocampal neurons, to investigate the contribution of BDNF signaling to the maturation of glutamatergic synapses. Using patch clamp recordings, we show a three-fold reduction in glutamatergic excitatory autaptic and synaptic current amplitudes in neurons overexpressing fl-TrkB, and application of saturating concentrations of BDNF and NT-4/5 completely reverses this effect. Compatible with these overexpression data, in untransfected neurons, scavenging of endogenous BDNF and NT-4/5 by TrkB-IgGs reduces excitatory autaptic current (EAC) amplitudes. By overexpression of truncated TrkB receptors (TrkB.T1, TrkB.T2) and a chimeric receptor containing only the intracellular domain of fl-TrkB, we show that intra- and extracellular domains of fl-TrkB are necessary to observe the EAC reduction. Labeling of presynaptic terminals with FM 4-64 revealed, that the reduced EAC amplitudes in fl-TrkB overexpressing neurons are accompanied by a two-fold reduction in synapse number. These results suggest, that ligand-independent signaling through fl-TrkB receptors can decrease glutamatergic synaptic strength, if sufficient amounts of BDNF or NT-4/5 are not available. J. Neurosci. Res. 66:327–336, 2001.

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To allow for the formation of synaptic connections of afferent axons with remote postsynaptic partners, specific neuronal cues are needed to orchestrate the coordinated development of pre- and postsynaptic specializations just when the afferent axons have reached their target area. The initial contact of pre- and postsynaptic partners in synapse formation is thought to be mediated by cell adhesion molecules such as cadherins or neuroligins/neurexins (see e.g., Fannon and Colman, 1996; Scheiffele et al., 2000). At glutamatergic presynaptic terminals, these initial sites of contact then recruit mobile pre-assembled transmitter vesicle modules in the axon, to form presynaptic boutons (Kraszewski et al., 1995; Ahmari et al., 2000), in apposition to postsynaptically clustered AMPA and NMDA receptors.

Interestingly, the BDNF receptor TrkB has recently been implicated in the regulation of postsynaptic AChR clustering at the neuromuscular junction (Gonzales et al., 1999; Wells et al., 1999), indicating a role of neurotrophins in synaptic maturation in the PNS. In the CNS, an involvement of TrkB in the development of glutamatergic synapses is suggested by the pronounced reduction in the number of synaptic contacts in the hippocampus of TrkB−/− mice (Martinez et al., 1998).

BDNF is a member of the protein family of neurotrophins and exerts most of its effects via binding to the TrkB receptor tyrosine kinase (fl-TrkB; Barbadic, 1994). Additional kinase deficient truncated TrkB receptor isoforms (TrkB.T1, TrkB.T2) can either mediate dominant negative effects on fl-TrkB receptor signaling or can play a role in ligand presentation (Bothwell, 1995). Expression of fl-TrkB in the hippocampus becomes detectable at embryonic day 16, and reaches adult levels at postnatal day five (P5). A strong augmentation of expression levels of BDNF and truncated TrkB receptors is seen around P5 and they further increase to nearly adult levels until P20 (Maisonpierre et al., 1990; Fryer et al., 1996). Thus, the time of most prominent synaptogenesis in the hippocampus between P5–P20 coincides with a continuous increase in the expression of BDNF and abundance of fl-TrkB receptors.
In accordance with these expression studies, acute application of BDNF and NT-4/5 has been well documented to modulate glutamatergic synaptic transmission on a fast time scale, with prevalent effects on the presynaptic efficacy of transmitter release (reviewed e.g., in Leßmann, 1998; Schuman, 1999).

Long-term effects of BDNF and NT-4/5 on the development of glutamatergic synapses in the hippocampus, however, are not yet fully resolved. Thus, chronic application of BDNF to cultured CNS neurons, has been shown to either enhance (Vicario-Abejon et al., 1998; Sherwood and Lo, 1999; Bolton et al., 2000) or to inhibit glutamatergic synaptic function (Rutherford et al., 1998), and these contradictory findings could result from different levels of endogenously expressed TrkB receptor isoforms in these different neuronal preparations.

To assure high level expression of specific TrkB receptor isoforms, we now have overexpressed the different TrkB receptors in cultured hippocampal neurons. We show that overexpression of non-liganded fl-TrkB reduces the synaptic strength of developing glutamatergic synapses. This effect is overcome by application of BDNF or NT-4/5, depending on intra- and extracellular domains of fl-TrkB, and is at least in part mediated by a reduction in the number of functional presynaptic terminals.

MATERIALS AND METHODS

Cell Culture

Dissociated postnatal rat hippocampal microcultures were prepared as described previously (Leßmann and Heumann, 1997), with minor modifications. To obtain the astrocytic microcysts, primary rat P0-P2 neocortical astrocytes were prepared and grown to confluence in plastic dishes containing Dulbecco’s modified Eagle medium + 10% fetal calf serum (DMEM/FCS, Invitrogen, Karlsruhe, Germany). These astrocytes were treated with trypsin (0.03%, 20 min at 37°C) and these contradictory findings could result from different levels of endogenously expressed TrkB receptor isoforms in these different neuronal preparations.

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Transfection

Expression plasmids under the control of a CMV promoter were generated for rat full-length TrkB, the respective truncated TrkB isoforms T1 and T2 (kindly provided by D. Middelmaas), and for a chimeric c-fms-TrkB receptor tyrosine kinase (Erdmann et al., 1998). All TrkB receptor constructs were cotransfected with a GFP expression plasmid (ratio: 3 [TrkB]:1 [GFP]) to allow identification of transfected neurons. Controls were transfected with 3 μg GFP plasmid only. To visualize TrkB receptor targeting in some experiments (Fig. 4), we constructed expression plasmids coding for N-terminal fl-TrkB, TrkB.T1, and TrkB.T2, respectively, fused at their C-termini to the coding sequence of GFP in the EGFP-N1 vector (Clontech, Heidelberg, Germany). Hippocampal microcultures were transfected (3 μg total DNA per 3.5 cm dish) with the different expression plasmids at 9–10 DIV, using the calcium-phosphate precipitation method as described previously (Haubensak et al., 1998; Kohrmann et al., 1999). In some experiments (Fig. 3) BDNF, NT-4/5 (50 and 100 ng/ml), NGF (100 ng/ml), or TrkB-IgGs (200 ng/ml) were added to the cultures 1 day after transfection. Human recombinant BDNF and NGF were from Alamone Labs (Israel), human recombinant NT-4/5 was kindly provided by Genentech, and TrkB-IgGs were a kind gift of R. Kolbeck.

Viability of neurons coexpressing the different types of TrkB receptors and GFP, was determined after staining with the blue fluorescent DNA dye Hoechst 33342. Cells were incubated with the dye (10 μg/ml in extracellular solution, 10 min, RT), and apoptotic cells were identified by DNA condensation and pyknotic nuclear morphology (as described previously by Edwards and Tolkovsky, 1994), using an inverted epifluorescence microscope.

Intact biological function of fl-TrkB receptors was checked by BDNF-induced fiber outgrowth of rat pheochromocytoma cells (PC12 cells) over-expressing fl-TrkB, fl-TrkB-GFP, and c-fms-TrkB, as described previously (Erdmann et al., 1998). According to previously described results (Haapasaalo et al., 1999), intact function of TrkB.T1 and TrkB.T2 and their GFP-tagged counterparts was judged by induction of filopodial growth in transfected COS7 cells.

Experimental Time Course

The first day after preparation corresponds to 1 DIV, and cells were transfected at 9–10 DIV. Neurotrophins or TrkB-IgGs were added once to each culture, 1 day after transfection (i.e., at 10–11 DIV). All electrophysiological recordings in transfected neurons were performed at 15–18 DIV. All FM staining experiments were performed at 14–18 DIV.

TrkB Immunocytochemistry

Fl-TrkB overexpressing neurons were fixed (4% formaldehyde in PBS) and permeabilized (0.1% Triton X-100) using standard procedures (Haubensak et al., 1998). Polyclonal rabbit pan-TrkB antibody (Ab-1, Calbiochem, Darmstadt, Germany; 1:200) was incubated for 24 hr at 4°C, followed by incubation (RT, 1 hr) with biotin-conjugated anti-rabbit secondary antibody (DAKO Diagnostika, Hamburg, Germany; 1:100), and with extravidin-TRITC (Sigma-Aldrich, Munchen, Germany; 1:200), respectively.

Electrophysiology and FM Staining

Whole cell patch clamp recordings were obtained from transfected hippocampal neurons between 9–18 DIV. The standard extracellular solution (ECS) contained (in mM): NaCl 108, KCl 3, HEPES 15, CaCl₂ 2, MgCl₂ 1, glucose 10; pH adjusted to 7.3. The patch pipette solution contained (in mM):
K-glucuronate 80, NaCl 5, HEPES 15, EGTA 10, CaCl2 0.2, MgCl2 1, ATP 2, GTP 1, phosphocreatine 5, creatinephosphokinase (50 U/ml), pH 7.3. Cells were voltage clamped at −60 mV using an EPC-7 patch clamp amplifier (HEKA, Lambrecht, Germany). Before digitization (at 5 kHz with a DigiData 1200 interface, Axon Instruments, Inc., Union City, CA), currents were filtered at 3 kHz and stored on a PC using clampEx software (Axon Inc.).

Staining of presynaptic terminals with FM 4-64 was performed according to standard procedures (see e.g., Henkel and Betz, 1995). Briefly, presynaptic terminals were labeled by a 1 min challenge of cells (14–18 DIV) with 10 μM FM 4-64 in high K+ (50 mM) ECS, followed by extensive washing in 0 Ca2+ ECS.

**Fluorescence Imaging**

FM 4-64 stained cells were inspected with an inverted epi-fluorescence microscope (Olympus IX 70). GFP fluorescence was detected using a narrow emission bandpass GFP filter set (AHF Analysentechnik, Germany). The red FM 4-64 and TRITC fluorescence, was detected with a custom built filter set (excitation: 530–550 nm; dichroic mirror: 570 nm; emission: 590–650 nm). Pictures were captured with a digital CCD camera (Sensys1401E, Visitron, Puchheim, Germany) operated with MetaView software (Universal Imaging, Downingtown, PA). In all figures, error bars = SEM. Significance of differences was tested using the two-tailed Student’s t-test.

**RESULTS**

**Development of Glutamatergic Autaptic Connections**

To investigate synaptic strength of developing glutamatergic synapses, patch-clamp recordings were obtained from postnatal rat hippocampal neurons in microcultures (Fig. 1A; 3–10 neurons per astrocyte island), between 9–18 days in vitro (DIV). Glutamate receptor mediated excitatory autaptic currents (EACs; autapse = synapse of a neuron onto itself) were elicited by short depolarizing current injections (to +20 mV, 2 msec; the corresponding action currents are truncated) and recorded at a holding potential of −60 mV. As shown previously (Leßmann and Heumann, 1997), under our recording conditions, EACs are mediated predominantly (>95% of peak current amplitude) by AMPA receptors. At 9–11 DIV, roughly 70% of the neurons have received excitatory autaptic input with current amplitudes of 293 ± 89 pA (mean ± SEM; Fig.1), further increasing to 566 ± 148 pA after 15–18 DIV. Only 10.3% (n = 211) of the neurons recorded from were GABAergic, and were excluded from the analysis.

**Overexpression of fl-TrkB Reduces Glutamatergic Synaptic Strength**

Hippocampal microcultures were transfected at 9–10 DIV (when glutamatergic synaptogenesis has already started, see above) with a cDNA coding for fl-TrkB, together with a GFP plasmid to enable identification of transfected cells. Immunofluorescent detection of fl-TrkB (see Fig. 4D) revealed a roughly 15-fold overexpression (judged by fluorescence intensity) of TrkB receptors in transfected vs. untransfected neurons, and successful coexpression of the fluorescent marker and TrkB was observed.
in 88 ± 4% of transfected neurons (n = 161 cells). As controls, sister cultures were transfected with GFP alone or were left untransfected.

Five to 8 days after transfection (i.e., after 15–18 DIV) large GFP expressing neurons showing pyramidal cell like morphology were recorded from (Fig. 1A). As shown in Fig. 1C, fl-TrkB overexpressing neurons revealed a highly significant (P < 0.0001) three-fold reduction of EAC amplitudes (189 ± 32 pA; mean ± SEM; 198 cells; 18 experiments) compared to GFP transfected controls (606 ± 83 pA; 96 cells; 9 experiments).

To test for a possible presynaptic contribution to this reduction in EAC amplitudes, we analyzed paired-pulse facilitation (PPF; interstimulus interval: 50 msec; extracellular solution: 2 mM Ca²⁺, 1 mM Mg²⁺), which is an indicator for the efficiency of transmitter release (Zucker, 1989). On average (Fig. 2), fl-TrkB overexpressing neurons showed modest PPF (2. EAC/1. EAC amplitude: 1.12 ± 0.10; n = 42 cells), whereas GFP expressing control cells showed paired-pulse depression (0.89 ± 0.04; n = 38 cells; significantly different from fl-TrkB with P < 0.05). Hence, the presynaptic probability of glutamate release is reduced in fl-TrkB overexpressing neurons.

The time to peak (TTP; 0–100%) of autaptic responses and the time constant of EAC decay (τ) for fl-TrkB overexpressing neurons (TTP: 3.8 ± 0.6 msec; τ: 9.3 ± 2.7 msec; compare Fig. 1B) were not significantly different from GFP expressing and untransfected control cells, respectively.

**BDNF and NT-4/5 Reverse the fl-TrkB-Induced EAC Reduction**

To determine the influence of ligand binding, BDNF and NT-4/5 were applied (1 day after transfection) to fl-TrkB overexpressing and to GFP expressing control cultures, respectively. In TrkB overexpressing cells, BDNF and NT-4/5 antagonized the TrkB induced EAC reduction in a dose-dependent manner (Fig. 3A, left panel), whereas NGF (that cannot bind to TrkB) was ineffective in this assay. In GFP expressing control cells, treatment with BDNF (Fig. 3A, right panel) did not significantly change EAC amplitudes. Interestingly, treatment of GFP expressing control cells with TrkB-IgGs (200 ng/ml; to scavenge endogenously secreted BDNF) induced a similar reduction in EAC amplitudes as observed for fl-TrkB overexpressing neurons in the absence of exogenously added ligands (see above). Thus, it is tempting to speculate that low concentrations of BDNF (or NT-4/5) relative to the expression level of fl-TrkB results in a fraction of non-liganded fl-TrkB receptors in a given cell determines whether the development of glutamatergic synapses is promoted.

To investigate whether the BDNF-induced antagonism of EAC reduction is a consequence of enhanced neuronal survival, we determined the percentage of viable neurons in cultures overexpressing fl-TrkB (n = 155 cells; 3 independent experiments), compared to GFP only expressing controls (n = 189 cells). In both treatment groups, >98% of transfected neurons were viable, both, in the absence or presence of added BDNF. This suggests that chronic treatment with BDNF does not change the percentage of living transfected neurons in our experiments. Furthermore, in all experiments, only cells with fast inactivating Na⁺ currents >500 pA were included for analysis.
assure that healthy neurons were selected for autaptic recordings.

EAC Reduction Depends on Intra- and Extracellular Domains of fl-TrkB

To determine whether the intracellular domain of fl-TrkB is necessary for the effect, we transfected neurons with either of the two tyrosine kinase deficient TrkB splice variants, TrkB.T1 and TrkB.T2 (Middlemas et al., 1991). Likewise, to check whether the extracellular domain of fl-TrkB is dispensable for the effect, we overexpressed a chimeric receptor consisting of the extracellular domain of the mouse CSF-1 receptor (c-fms) and the intracellular domain of fl-TrkB (c-fms-TrkB; Erdmann et al., 1998). Neither of these TrkB receptor variants revealed a significant ($P > 0.1$) reduction in EAC amplitudes compared to GFP expressing control cells (Fig. 3B). Accordingly, EAC amplitudes for these groups of cells (T1, T2, Chimera) were significantly different from those of fl-TrkB overexpressing neurons ($P < 0.01$), strongly discouraging any unspecific effects on EAC amplitudes due to the overexpression paradigm as such.

Correct delivery of overexpressed receptors to the cell surface was checked by transfection of hippocampal neurons with GFP-tagged versions of truncated and fl-TrkB receptors, respectively. As shown in Figure 4, a rim of plasma membrane resident GFP fluorescence was evident in all cells expressing fl-TrkB-GFP or TrkB.T1-GFP. Identical results were obtained for TrkB.T2-GFP (not shown). Likewise, as shown by TrkB immunocytochemistry, also coexpression of fl-TrkB and GFP resulted in correct cell surface expression of the receptor (Fig. 4D). Finally, as determined by fiber outgrowth assays in fl-TrkB expressing PC12 cells (Fig. 4E), and by induction of filopodial growth in COS7 cells (by truncated TrkB receptors, data not shown), all TrkB constructs used in our study showed intact biological function of cell surface expressed receptors.

Taken together, these data suggest that intact intra- and extracellular domains of signaling competent fl-TrkB are necessary to mediate the reduction in EAC amplitudes.

Pre- Or Postsynaptic Overexpression of fl-TrkB Reduces Synaptic Strength

To directly compare possible pre- versus postsynaptic components of the fl-TrkB-induced reduction in synaptic current amplitudes, we performed pair recordings, with only one of the two neurons overexpressing fl-TrkB (Fig. 5). Synaptic pairs with a fl-TrkB overexpressing neuron showed a roughly two-fold reduction in autaptic and synaptic current amplitudes in all connections with fl-TrkB overexpressed in the pre- or the postsynaptic cell (Fig. 5A,C; connections a,b,d), as compared to the internal control pathway (connection c: significantly different from a,b,d with $P < 0.05$; $n = 17$ pairs). In contrast, there was no significant difference in current amplitudes between the different connections in GFP only expressing control cells (Fig. 5B,C; gray bars; $P > 0.30$, $n = 18$ pairs). These results suggest, that overexpression of fl-TrkB in either the pre- or the postsynaptic neuron is sufficient to induce the reduction of glutamatergic synaptic current amplitudes.

Two-Fold Reduction in the Number of Functional Synaptic Boutons by fl-TrkB

To explore, whether overexpressing fl-TrkB affects the number of functional synaptic contacts, we labeled synaptic terminals by activity-dependent uptake of the red fluorescent presynaptic marker FM 4-64 (Betz et al., 1996). Only single neuron microcultures (at 14–18 DIV) were analyzed here, to assure that all detected presynaptic terminals originate from the transfected neuron, thus allowing to quantify absolute numbers of synaptic terminals/neuron. For quantification of FM puncta, an intensity threshold was set to two-fold the background noise, and the number and average intensity of
the displayed FM puncta (0.5–2.0 μm in diameter) above this threshold were quantified. As determined by post-hoc immunocytochemistry, >90% of these FM puncta were colocalized with presynaptic marker proteins (data not shown). Likewise, >90% of the FM puncta could be destained within 2 min of stimulation with 50 mM K+ (data not shown).

On average, fl-TrkB overexpressing neurons had 186 ± 25 (i.e., 51% of control) functional autaptic terminals (n = 22 cells, 6 independent experiments; Fig. 6), compared to 364 ± 39 boutons in only GFP expressing controls (31 cells, 6 experiments; significantly different with P < 0.001). The mean fluorescence intensity of the FM-puncta was not significantly different between the two groups of cells, indicating similar levels of dye uptake under both conditions.

Taken together, these data suggest that the reduction in EAC amplitudes in fl-TrkB overexpressing neurons is at least in part due to a smaller number of functional presynaptic terminals.

Fig. 4. Overexpressed TrkB receptors are targeted to the plasma membrane. Hippocampal neurons (A–D) were transfected (at 8 DIV), with fl-TrkB-GFP (A), with TrkB.T1-GFP (B), were cotransfected with fl-TrkB and GFP (D), or were left untransfected (C). A,B: Living cells were monitored for GFP fluorescence at 14 DIV. The arrowheads indicate plasma membrane expression of both receptors, evident from the rim of fluorescence on the cell surface. The inset shows an example of plasma membrane expression of TrkB.T1 in the dendrite of a transfected neuron. C: Absence of membrane-associated fluorescence in a living untransfected hippocampal neuron at 14 DIV (exposure time 4 times longer than in A,B). D: TrkB-immunocytochemistry of a fl-TrkB and GFP cotransfected neuron reveals membrane targeting (see arrow heads) of untagged fl-TrkB receptors. E: fl-TrkB-GFP expressing PC12 cell at 4 DIV, after addition of 100 ng/ml BDNF for 72 hr. The BDNF-induced fiber outgrowth reveals intact signaling of membrane targeted (see arrow heads) fl-TrkB in transfected cells.
Finally, we investigated by Sholl analysis (Sholl, 1953) of GFP fluorescence images, whether the fl-TrkB-induced reduction in synapse number could be due to changed dendritic morphology. Thus, the number of dendritic intersections with overlaying concentric rings of increasing diameter (i.e., 20, 40, 60, 80 μm) were determined in GFP-expressing (n = 8), and fl-TrkB/GFP coexpressing neurons (n = 22), but neither the number of primary dendrites (GFP: 5 ± 1; TrkB/GFP: 5 ± 1), nor the number of dendritic branches (e.g., at 60 μm; GFP: 14 ± 2; TrkB/GFP: 13 ± 2) were changed. Likewise, the total dendritic length (GFP: 2,109 ± 186 μm, n = 6 cells;
TrkB/GFP: 1,469 ± 125 μm, n = 5 cells) in these two groups of cells was not significantly altered (P > 0.05). These results are inconsistent with the possibility that altered dendritic morphology could account for the fl-TrkB induced synapse reduction.

**DISCUSSION**

Our results show, that overexpression of fl-TrkB in the absence of exogenously added ligand induces a three-fold reduction in EAC amplitudes, and a two-fold decrease in the number of active presynaptic terminals. As shown in Figure 3B, this effect is specific for fl-TrkB and cannot be observed with any other TrkB receptor isoform, which argues against an unspecific effect due to overexpression of any plasma membrane receptor in our cells. Because application of exogenously added BDNF and NT-4/5 revert the EAC reduction in fl-TrkB overexpressing neurons, our results suggest that the balance between the expression level of fl-TrkB and ligand availability can regulate synaptic development in hippocampal neurons. This interpretation is corroborated by the observed synapse reduction in TrkB-IgG treated control cultures.

The lack of EAC reduction by overexpressing the truncated receptors TrkB.T1 and TrkB.T2 and the chimeric c-fms-TrkB receptor, respectively, reveals that extracellular and intracellular domains of overexpressed fl-TrkB receptors are necessary to observe the effect. In addition, the correct cell surface expression and the intact biological function of the overexpressed fl-TrkB receptors (Fig. 4) suggest, that the observed effects on synaptic efficacy are mediated by signaling competent fl-TrkB receptors, rather than e.g., by scavenging of endogenously released BDNF via signaling-incompetent overexpressed TrkB receptors.

Our results, that chronic treatment with either BDNF or NT-4/5 enhances EACs in fl-TrkB overexpressing neurons are consistent with the finding, that TrkB receptor ligands can principally induce synaptic maturation in hippocampal neurons (Sherwood and Lo, 1999; Bolton et al., 2000). Likewise, the observed reduced synaptic strength in untransfected neurons after chronic treatment with TrkB-IgGs to scavenge endogenous BDNF, is also in line with previously published results in hippocampal neurons (Bolton et al., 2000). In accordance with one group (Vicario-Abejon et al., 1998; results with E18 neurons therein) but at variance with the results from Sherwood and Lo (1999), we did not observe an effect of exogenously applied BDNF on synaptic strength of our control cells, not overexpressing fl-TrkB (Fig. 3). This could result from different levels of endogenous BDNF in these diverse hippocampal cultures, which determines whether exogenously supplied BDNF can provoke additional effects. Taken together, these heterogeneous results imply, that TrkB-mediated long-term modulation of glutamatergic synapses is sensitive to details of the neuronal preparation, which could be due to different expression levels of either BDNF or of the different TrkB receptor isoforms.

Because BDNF and NT-4/5 are known to modulate axonal as well as dendritic outgrowth in central neurons (McAllister et al., 1999), it could be argued, that the inhibition of synaptic development in our fl-TrkB overexpressing neurons results from reduced neurite outgrowth in these cells. As shown by the Sholl analysis, however, we did not detect any obvious reduction in dendritic growth in fl-TrkB overexpressing neurons compared to GFP controls (see also Fig. 1 and 6). This was an expected finding, because TrkB-induced changes in dendritic morphology upon overexpression of fl-TrkB depend on exogenously added ligand (Yacoubian and Lo, 2000), which was not added in the relevant experiments here (see Figs. 1,2,5,6). Thus, our data are more compatible with reduced synaptic maturation in fl-TrkB overexpressing neurons, rather than with altered dendritic morphology.

**Pre- Versus Postsynaptic fl-TrkB-Induced Synaptic Changes**

Our synaptic pair recordings indicate, that overexpression of fl-TrkB in either the pre- or the postsynaptic neuron is similarly effective in reducing synaptic current amplitudes. The observed decrease in the number of FM 4-64 labeled presynaptic terminals, however, suggest a presynaptic locus of expression of the fl-TrkB-induced synapse reduction, and the enhanced level of PPF suggests reduced probability of transmitter release at the remaining terminals.

These results are compatible with the BDNF-dependent regulation of the development of presynaptic specializations, as has been reported for PNS and CNS synapses (Causing et al., 1997; Martinez et al., 1998). The starting level in our model is different, however, in that non-ligated fl-TrkB seems to have a negative effect on synaptic maturation, rather than being without effect. This could indicate, that the balance between fl-TrkB expression and BDNF availability is the actual information that is transmitted in BDNF-induced presynaptic differentiation.

At the neuromuscular junction, postsynaptic fl-TrkB receptors mediate by an as yet unknown molecular mechanism, ACh receptor clustering in the postsynaptic membrane (Gonzales et al., 1999; Wells et al., 1999), thus enabling enhanced postsynaptic current amplitudes. This suggests, that also postsynaptic TrkB signaling contributes to synaptic maturation, at least at nerve muscle synapses. It remains to be shown, whether similar postsynaptic mechanisms also apply for hippocampal neurons, as is indicated by the reduction in EPSC amplitudes upon exclusively postsynaptic overexpression of fl-TrkB (Fig. 5).

Although often used to discriminate between pre- and postsynaptic modifications, we did not analyze miniature autaptic currents here, because it is not possible to distinguish them from synaptic miniature currents originating from non-TrkB overexpressing cells in our cultures. This unknown degree of contamination with synaptic miniature currents prevented direct correlation with evoked autaptic currents.
Possible Molecular Mechanisms for fl-TrkB-Induced Synapse Reduction

Our results suggest that non-liganded fl-TrkB receptors exist in our cultures, either upon overexpressing fl-TrkB without adding exogenous ligand, or by scavenging endogenous BDNF from untransfected cells (see Fig. 3A). Under both conditions, BDNF might stimulate only a fraction of the present fl-TrkB receptors, and the down-stream signaling would depend on the balance between liganded and non-liganded fl-TrkB receptors.

At the molecular level, this ratio could be detected by interaction of non-liganded fl-TrkB receptors with either plasma membrane resident or with intracellular binding partners. Interestingly, recent studies have identified several intracellular proteins that bind to TrkB independent of ligand stimulation, including the adaptor protein SNT (Peng et al., 1995), the tyrosine kinase c-Ab1 (Yano et al., 2000,) and the cytoplasmic motor protein dynein (Yano et al., 2001). It remains to be shown whether one of these binding partners contributes to the fl-TrkB-induced synapse reduction.

In addition to these classical TrkB binding partners, several lines of evidence suggest a functional interaction of fl-TrkB with cell adhesion molecules (Tannahill et al., 1995), including N-cadherin (Zhou et al., 1997) and the neural cell adhesion molecule (NCAM; Muller et al., 1995), which are known to be involved in the fl-TrkB-induced inhibition of synaptic development observed in our study.

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