Induction of long-term potentiation and depression is reflected by corresponding changes in secretion of endogenous brain-derived neurotrophic factor

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Neurotrophins play an important role in modulating activity-dependent neuronal plasticity. In particular, threshold levels of brain-derived neurotrophic factor (BDNF) are required to induce long-term potentiation (LTP) in acute hippocampal slices. Conversely, the administration of exogenous BDNF prevents the induction of long-term depression (LTD) in the visual cortex. A long-standing missing link in the analysis of this modulatory role of BDNF was the determination of the time-course of endogenous BDNF secretion in the same organotypic preparation in which LTP and LTD are elicited. Here, we fulfilled this requirement in slices of perirhinal cortex. Classical theta-burst stimulation patterns evoking LTP lasting >180 min elicited a large increase in BDNF secretion that persisted 5–12 min beyond the stimulation period. Weaker theta-burst stimulation patterns leading only to the initial phase of LTP (≈35 min) were accompanied by a smaller increase in BDNF secretion lasting <1 min. Sequestration of BDNF by TrkB–IgG receptor bodies prevented LTP. Low-frequency stimulations leading to LTD were accompanied by reductions in BDNF secretion that never lasted beyond the duration of the stimulation.

Neurotrophins, in particular brain-derived neurotrophic factor (BDNF), play an important modulatory role in activity-dependent neuronal plasticity (1–5). The physiological relevance of this action is based on the observation that BDNF differentially modulates activity-dependent changes in synaptic strength, as reflected by long-term potentiation (LTP) and long-term depression (LTD), which are cellular paradigms for learning and memory (6). Hippocampal LTP is strongly impaired in both BDNF (7–9) and TrkB (10, 11) knock-out mice. Conversely, LTD in the visual cortex is facilitated by blocking anti-BDNF Abs or TrkB–IgG receptor bodies (12). It is of particular interest that in the hippocampus of BDNF knock-out mice (7) the extent of LTP reduction is the same in both homozygote and heterozygote animals, indicating that a minimal, critical concentration of BDNF has to be available to fulfill its modulatory functions. LTP could be rescued by adenoviral-mediated (re)expression of BDNF in the CA1 region (8) or administration of exogenous BDNF (9). Relatively detailed studies have been performed on the regulation of BDNF synthesis (13–17) and secretion (18–27). The studies concerned with the regulation of BDNF secretion have been conducted in reductionistic systems, such as synaptosomes (18) and dissociated neuronal cultures in which BDNF secretion was evoked by high potassium depolarization or exogenous neurotransmitter administration (19–24). Recent studies using primary cultures of hippocampal neurons were designed specifically to investigate the relationship between electrical stimulation parameters and BDNF secretion. However, to reach this goal either the overexpression of BDNF (25, 26) or long stimulation periods in nontranduced cultures (27) were necessary to permit the determination of BDNF secretion. These experiments were suitable to evaluate the basic mechanisms of BDNF secretion, but the results that were obtained may differ substantially from the situation encountered in integrated neuronal systems. Accordingly, in recent comprehensive reviews (1, 3, 5) addressing the modulatory role of neurotrophins in synaptic plasticity, the determination of endogenous BDNF secretion in parallel with LTP and LTD recording in the same organotypic neuronal system was emphasized as a missing link in this concept. Here, we have fulfilled this requirement in perirhinal (PRh) cortex slices.

Materials and Methods

Slice Preparation. The experiments were carried out in horizontal brain slices including PRh and lateral entorhinal cortex and hippocampus, obtained from juvenile (27- to 33-day-old) male Sprague–Dawley rats. Slices were prepared as described by Bilkey (28), stored at room temperature in artificial cerebrospinal fluid (126 mM NaCl/3 mM KCl/1.25 mM NaH_2PO_4/26 mM $NaHCO_3$/1 mM $MgSO_4$/2 mM $CaCl_2$/10 mM dextrose) gassed with 95% O_2 and 5% CO_2 at pH 7.4.

Immunohistochemistry. Immunohistochemical analyses were performed in intact slices fixed in 4% paraformhaldeyde for 30 min at 4°C. Tissue sections were permeabilized with 1% Triton X-100 in PBS for 10 min and incubated with chicken anti-BDNF (Promega), mouse anti-neuronal nuclear antigen (NeuN; Chemicon), or mouse antiglial fibrillary acidic protein (Sigma) primary Abs diluted 1:20 for 1 h at room temperature. Samples were incubated with donkey anti-chicken Cy5-conjugated (The Jackson Laboratory), goat anti-rabbit FITC-conjugated (Sigma), or sheep anti-mouse FITC-conjugated (Sigma) secondary Abs diluted 1:20 for 1 h at room temperature. The slices were mounted in glycerol/PBS medium containing the antifading diazabicyclo[2.2.2]octane (2.5%, wt/vol). Immunoreactivity was evaluated by confocal analysis.

Electrophysiology. After recovering for at least 60 min, a single slice was transferred to a submersion recording chamber and perfused at the rate of 3 ml/min with artificial cerebrospinal fluid maintained at 34°C ± 0.2°C and saturated with 95% O_2 and 5% CO_2. Recordings started 60 min after the slice was placed into the chamber. Field potentials (FPs) evoked by horizontal pathway stimulation were recorded in PRh cortex with an extracellular microelectrode (glass micropipette filled with 0.25 M NaCl, 2–5 MΩ) placed in cortical layer II/III immediately

Abbreviations: BDNF, brain-derived neurotrophic factor; FP, field potential; LFS, low-frequency stimulation; LTD, long-term depression; LTP, long-term potentiation; NeuN, neuronal nuclear antigen; PRh, perirhinal; TBS, theta-burst stimulation; TTX, tetrodotoxin.

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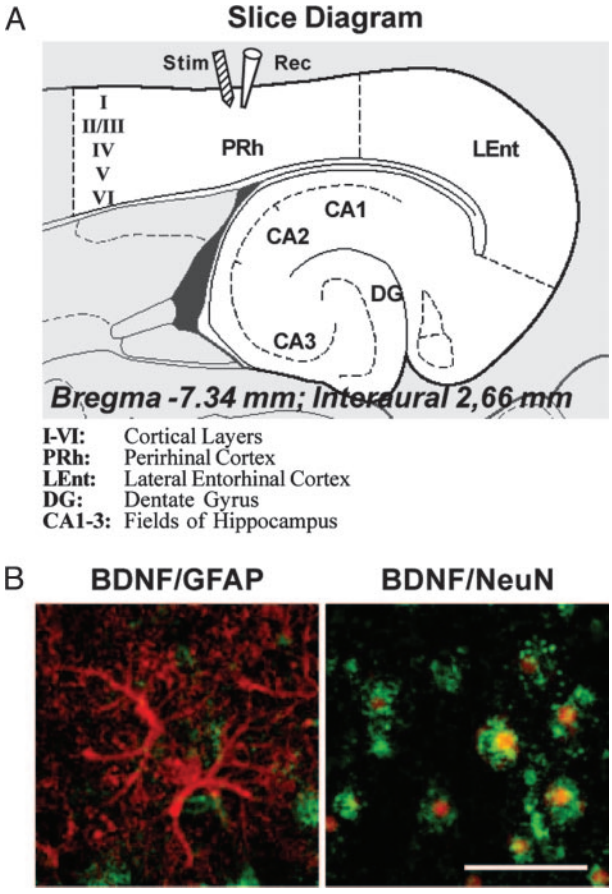

Fig. 1. BDNF expression in PRh cortex. (*A*) Schematic representation of the slice preparation that we used, including the PRh cortex, lateral entorhinal cortex, and hippocampus (white background), in which BDNF expression was revealed by immunohistochemistry using a polyclonal anti-BDNF Ab. (*B*) Double-labeling experiments show that, in a representative area of layer II/III, BDNF immunoreactivity (green) predominantly correlates with that of the neuronal nuclear marker NeuN (red) (BDNF/NeuN). Note that BDNF immunoreactivity shows discontinuous patchy features. BDNF immunoreactivity is not detectable in astrocytes (red), as shown in a representative area of layer I (BDNF/glial fibrillary acidic protein). (Scale bar indicates 50 μm.)

adjacent to the rhinal fissure (Fig. 1*A*) and connected to a dc amplifier by an Ag/AgCl electrode. Constant-current square pulses (0.2 ms, 20–250 μA, 0.033 Hz) were applied by using a stimulus generator connected through a stimulus isolation unit to a concentric bipolar electrode (40–80 KΩ) positioned in layers II/III at $\approx$500 μm from the recording electrode in rostral direction. Stimulus intensity was adjusted to induce $\approx$50% of the maximal synaptic response. LTP was induced by a 100-Hz theta-burst stimulation (TBS), consisting of four sets of stimulations delivered 15 s apart, each one consisting of 10 bursts of five pulses at 100 Hz with interburst intervals of 150 ms (28). Two weaker TBSs were also used, including (*i*) 25-Hz TBS, differing only in the frequency at which the five pulses were delivered, and (*ii*) a 100-Hz TBS, consisting of two sets of stimulations at 100 Hz instead of four sets [TBS-100 Hz (two sets)]. LTD was induced by low-frequency stimulation (LFS) of either 3,000 stimuli at 5 Hz or 900 stimuli at 1 Hz. In an additional set of experiments, TrkB–IgG receptor bodies (1 μg/ml; Regeneron Pharmaceuticals, Tarrytown, NY) were added to cerebrospinal fluid during recovery of the slices and during the whole recording period until 12 min after a 100-Hz TBS. For all experiments, the values given are the mean $\pm$ SEM.

Analysis of Endogenous BDNF Secretion. BDNF was quantified in the slice perfusates by ELISA performed according to the methods described in ref. 21. Two mAbs (1 and 9) derived from immunized mice (29) were affinity-purified. Ab 1 was used as primary Ab, whereas Ab 9 was conjugated with horseradish peroxidase and used as secondary Ab. ELISA plates were coated overnight at 4°C with the Ab 1 in 50 mM sodium carbonate buffer (pH 9.7) and blocked overnight with ELISA buffer consisting of Hanks' buffer, 0.1% Triton X-100, and 2% BSA. Samples and standards of recombinant BDNF (0.5–1,000 pg/ml) were given into the plates together with the Ab 9 and incubated overnight at 4°C. After washing, bound secondary Ab was detected by incubation with TMB substrate for 30 min. The ELISA showed a sensitivity of 0.5 pg/ml BDNF. BDNF concentrations are expressed as percentage of initial basal secretion (mean of the values determined in the first three fractions collected before the beginning of the experiments). Values are given as mean $\pm$ SEM.

Results

BDNF Expression in PRh Cortex. We characterized the expression pattern of BDNF protein in brain slices including PRh cortex, lateral entorhinal cortex, and hippocampus (Fig. 1*A*), by immunohistochemistry using a polyclonal anti-BDNF Ab. BDNF-specific labeling varied markedly between the cortical layers I–VI of PRh cortex, with the most prominent labeling in layers II/III, V, and VI (Fig. 6, which is published as supporting information on the PNAS web site). Double immunofluorescence staining was analyzed at high magnifications showing different fields of layer II/III. The pattern of BDNF expression correlates with that of the neuronal nuclear protein NeuN (Fig. 1*B*), indicating a specific neuronal localization of BDNF. BDNF staining in neurons appears predominantly in a discontinuous, patchy pattern (Fig. 1*B*). There is no colocalization of glial fibrillary acidic protein and BDNF (Fig. 1*B*).

Characterization of Basal Secretion of Endogenous BDNF. In a second series of experiments, we evaluated in parallel synaptic transmission in PRh cortex and BDNF concentration in the slice-perfusion medium. Individual slices were placed in a submersion recording chamber and perfused at the rate of 3 ml/min. Electrophysiological recordings were carried out in layers II/III of PRh cortex, where BDNF staining was particularly intense (Fig. 6). Stimulation of horizontal fibers at 0.033 Hz evoked a negative FP, the amplitude of which did not change during the first 100 min of recording (Fig. 2*A*). In contrast, BDNF concentrations in the slice-perfusion medium, assayed by ELISA in 1-min fractions collected during the same experiments, decreased progressively to 92 $\pm$ 4% after 20 min and to 86 $\pm$ 3% after 100 min (Fig. 2*B*). A comparable rate of decrease in BDNF concentrations was observed in the absence of electrical stimulation (to 94 $\pm$ 3% after 20 min and to 82 $\pm$ 2% after 100 min; data not shown), indicating that endogenous BDNF secretion occurs spontaneously from the slice preparation. Basal release of endogenous BDNF, analyzed previously in hippocampal neurons (19, 21, 22, 30), results from combined "constitutive" and "regulated" secretion. The regulated component of this pathway is an activity-dependent process that includes both stimulatory and inhibitory signals (30). To evaluate whether and to what extent the regulated pathway plays an essential role in our slice preparations, the sodium channel blocker tetrodotoxin (TTX; 1 μM) was added to the perfusion medium. This treatment abolished the FPs (Fig. 2*C*) but caused only a slight reduction in basal BDNF secretion (Fig. 2*D*). Maximal decrease of secretion was observed during the first 10 min after TTX (to 87 $\pm$ 4%), then BDNF concentrations decreased more slowly, reaching the levels of control experiments at 100 min (to 84 $\pm$ 3%). These observations indicate that, under our experimental conditions,

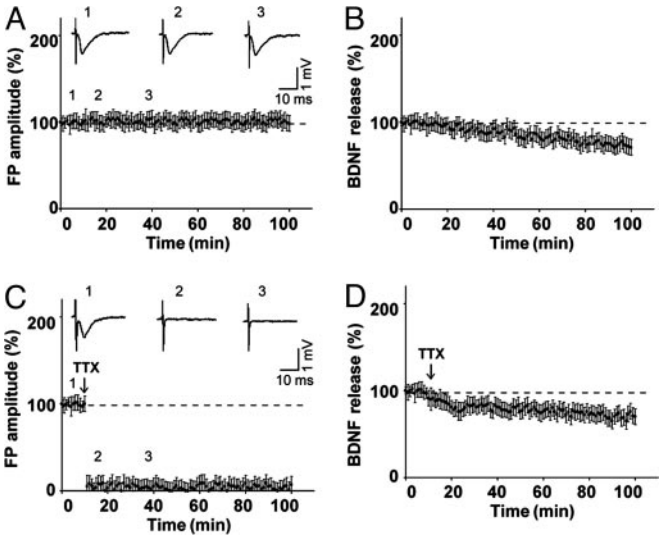

Fig. 2. Parallel analysis of FPs in PRh cortex and endogenous BDNF secretion in the perfusion medium during basal synaptic stimulation. (*A*) Individual slices were placed in a submersion-recording chamber and perfused at 3 ml/min. Electrophysiological recordings were carried out by an extracellular microelectrode (Rec) placed in layers II/III of PRh cortex (see Fig. 1*A*). Constant-current square pulses applied to horizontal afferent fibers using a concentric bipolar electrode (Stim) evoked negative FPs of which the amplitude did not change during the 100 min of recording ($n = 6$). (*B*) BDNF concentrations in the slice-perfusion medium were assayed by ELISA in 1-min fractions collected during the experiments described in *A*. Note that BDNF concentrations decreased progressively during the experiments (to 92 ± 4% after 20 min and to 86 ± 3% after 100 min). The 100% BDNF concentrations in the individual experiments were as follows: 135, 24, 34, 68, 24, and 19 pg/ml. (*C*) Electrophysiological recordings, as described in *A*, were performed in the absence (0–10 min) and in the presence (10–100 min) of the sodium channel blocker TTX (1 μM) added (arrow) to the perfusing medium. Note that TTX treatment abolishes the FPs ($n = 6$). (*D*) BDNF secretion during the experiments reported in *C*. BDNF secretion decreased initially (to 87 ± 4% after 20 min, i.e., 10 min after TTX application), reaching similar values as under control conditions after 100 min (to 84 ± 3%). The 100% BDNF concentrations in the individual experiments were as follows: 79, 35, 19, 56, 22, and 47 pg/ml.

only a small proportion of endogenous BDNF secretion results from spontaneous neuronal activity.

Increase of Endogenous BDNF Secretion During LTP. We investigated whether endogenous BDNF secretion is responsive to high-frequency stimulation leading to LTP in the PRh cortex. A 100-Hz TBS (for details, see *Materials and Methods*) rapidly induced an increase in FP amplitude (Fig. 3*A*) that lasted for >180 min. BDNF secretion increased instantaneously after starting the 100-Hz TBS and, remarkably, remained elevated for 5–12 min beyond the end of the stimulation (Fig. 3*B*). We interfered with the function of BDNF by using TrkB–IgG receptor bodies. The slices were incubated with TrkB–IgG (1 μg/ml) for 1 h before their transfer to the recording chamber. TrkB–IgG was maintained in the perfusion medium until 12 min after a 100-Hz TBS. The sequestration of BDNF by TrkB–IgG prevented LTP (Fig. 3*C*). Moreover, to evaluate whether the observed pattern of BDNF secretion is needed for LTP maintenance, a stimulation procedure inducing only the initial phase of LTP was used. A 100-Hz TBS (two sets), consisting of two sets of stimulations instead of four, induced LTP of smaller amplitude lasting ≈35 min (Fig. 4*A*) and caused a relatively small increase in BDNF secretion, restricted to a single (1-min) fraction (Fig. 4*B*). Furthermore, we evaluated whether BDNF secretion is different when a TBS not leading to LTP is applied. A 25-Hz TBS (differing only in frequency from 100-Hz TBS) did not cause any change in synaptic strength (Fig. 4*C*) and induced only a short-lasting (1-min) increase of BDNF secretion (Fig. 4*D*).

Decrease of Endogenous BDNF Secretion During LTD. The last series of experiments were designed to evaluate whether BDNF secretion also changes in response to LTD-inducing stimulation in PRh cortex. LFS consisting of 3,000 stimuli at 5 Hz induced LTD (Fig. 5*A*) lasting >180 min. Notably, LFS also immediately induced a consistent reduction in BDNF secretion, which lasted during LFS and then returned to basal levels despite the persistence of synaptic depression (Fig. 5*B*). A comparable pattern of BDNF secretion was observed when LTD lasting ≈40 min was induced by 900 stimuli at 1 Hz (Fig. 4 *C* and *D*).

Discussion

In this study, we addressed a long-standing missing link in the analysis of the modulatory role of BDNF in activity-dependent synaptic plasticity, namely, the determination of endogenous BDNF secretion during induction and maintenance of LTP and LTD in an integrated organotypic neuronal system.

To date the analysis of BDNF secretion in the context of activity-dependent synaptic plasticity has been focused on LTP, evaluated in "bits and pieces" in reductionistic systems in which BDNF was either overexpressed (25, 26) or endogenous BDNF secretion was determined in cultures of high densities kept for a relative short time (72 h) precluding full differentiation, by using

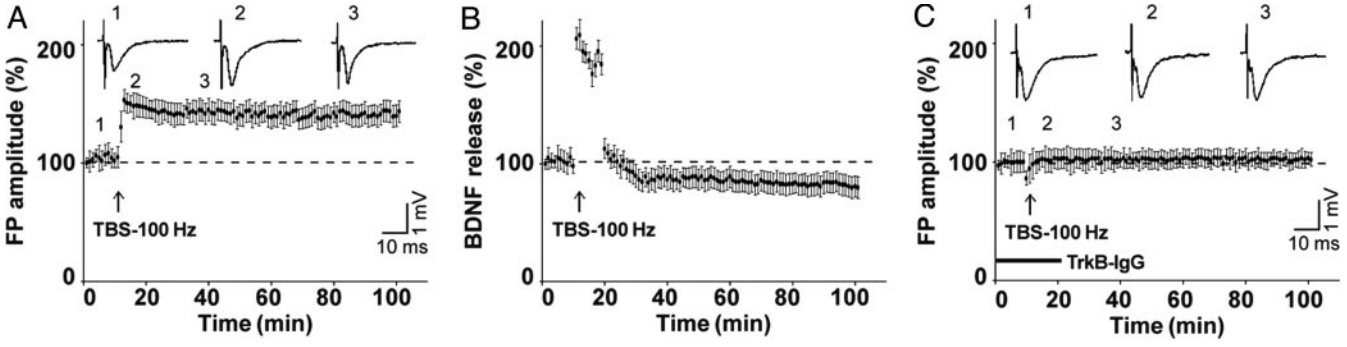

Fig. 3. Parallel analysis of FPs in PRh cortex and endogenous BDNF secretion during basal stimulation and LTP-inducing TBS. (*A*) LTP was induced by 100-Hz TBS. This stimulation induced an increase in FP amplitude that persisted for >180 min ($n = 6$). (*B*) Time course of BDNF secretion during the experiments shown in *A*. BDNF secretion rapidly increased after 100-Hz TBS, and the high levels persisted for 5–12 min before declining to basal values. The 100% BDNF concentrations in the individual experiments were as follows: 166, 16, 18, 73, 25, and 39 pg/ml. (*C*) LTP induction by 100-Hz TBS was prevented by the presence of TrkB–IgG receptor bodies (1 μg/ml) ($n = 5$).

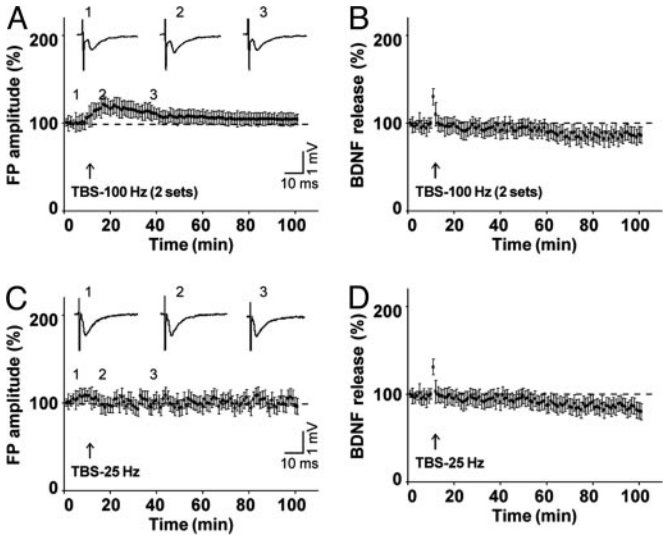

Fig. 4. Parallel analysis of FPs in PRh cortex and endogenous BDNF secretion during basal and weak TBSs. (*A*) A 100-Hz TBS (two sets) consisting of two instead of four sets of stimulations, induced only the initial phase of LTP (n = 8). (*B*) Time course of BDNF secretion during the experiments reported in *A*. A 100-Hz TBS (two sets) caused a small increase in BDNF secretion. The 100% BDNF concentrations in the individual experiments were as follows: 37, 29, 79, 122, 189, 20, 48, and 21 pg/ml. (*C*) The 25-Hz TBS failed to induce LTP. The 25-Hz TBS differs from 100-Hz TBS only in that the frequency was delivered at 25 Hz instead of 100 Hz. In our experiments, this reduced frequency always failed to induce LTP (n = 6). (*D*) Time course of BDNF secretion during the experiments reported in *C*. The 25-Hz TBS caused a small increase in BDNF secretion that was restricted to a single (1-min) collected fraction. The 100% BDNF concentrations in the individual experiments were as follows: 61, 19, 22, 82, 224, and 41 pg/ml.

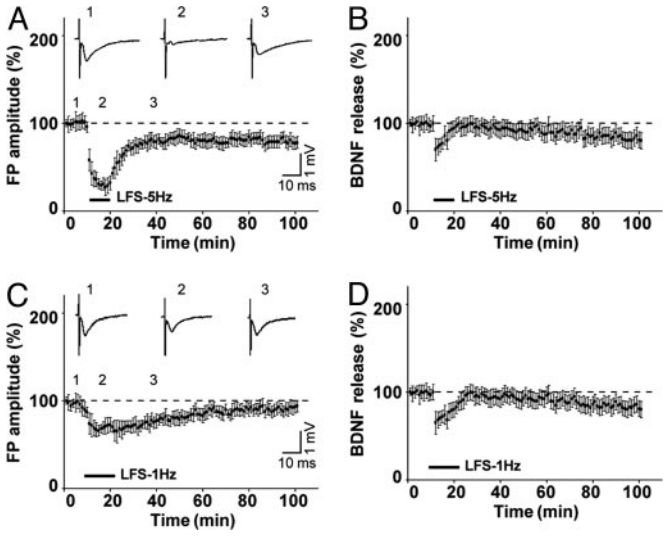

Fig. 5. Parallel analysis of FPs in PRh cortex and endogenous BDNF secretion in the perfusion medium, during basal and LTD-inducing stimulations. (*A*) LTD induced by a LFS of 3,000 stimuli at 5 Hz. LTD persisted for >180 min (n = 6). (*B*) Time course of BDNF secretion during the experiments shown in *A*. LFS immediately induced a reduction in BDNF secretion. Maximum reduction in BDNF secretion occurred within the first min of LFS and lasted for several minutes before returning to basal levels. The 100% BDNF concentrations in the individual experiments were as follows: 73, 39, 95, 45, 224, and 73 pg/ml. (*C*) LFS consisting of 900 stimuli at 1 Hz induced a LTD lasting ≈40 min (n = 6). (*D*) Time course of BDNF secretion during the experiments shown in *C*. The 100% BDNF concentrations in the individual experiments were as follows: 51, 69, 221, 172, 124, and 91 pg/ml.

long-lasting electrical stimulations (27). Here, we determined the secretion of endogenous BDNF in the perfusion fluid in the same slice preparation from which we recorded initiation and maintenance of LTP. It has to be taken into account that concentrations of endogenous BDNF in the perfusion fluid do not only reflect changes occurring at the site of synaptic modifications. Under the given experimental conditions, changes in endogenous BDNF secretion are underestimated because the stimulation-mediated BDNF secretion is restricted to the surroundings of the stimulating electrodes, whereas the whole slice contributes to the measured BDNF concentrations. Moreover, BDNF concentrations in the perfusion medium represent a balance resulting from BDNF secretion and BDNF removal by internalization after binding to TrkB receptors. Ideally, a most accurate quantification of activity-dependent BDNF secretion at the sites of synaptic modifications could be envisaged by developing a detection system corresponding to the local quantification of biogenic amines by amperometry (31).

The most remarkable observation in our study is that stimulations inducing LTP lasting >180 min trigger a rapid, large increase in BDNF secretion that persists for 5–12 min (Fig. 3*B*). In contrast, stimulation procedures that induce only the initial phase of LTP (Fig. 4*A*) lead to a very short-lasting (1 min) increase in BDNF secretion of much smaller magnitude (Fig. 4*B*). Moreover, LTP was prevented by BDNF sequestration by TrkB–IgG (Fig. 3*C*). These observations suggest that BDNF is required for LTP occurrence, and that prolonged duration of BDNF secretion at high levels is needed for LTP maintenance. Signaling pathways via TrkB receptor activation resulting in translational (32) and transcriptional (33) regulations may be involved in this process. We also found that stimulation parameters failing to induce LTP were accompanied by a small and very short-lasting increase in BDNF secretion (Fig. 4*D*). This pattern of secretion might be different from the pattern that occurs when only the initial phase of LTP was induced, but the difference could not be revealed within the resolution limits of our system.

As to the mechanisms responsible for the prolonged pattern of BDNF secretion, it can be hypothesized that only stimulations inducing LTP lasting >180 min trigger the secretion of threshold BDNF concentrations that are necessary to activate autoregulatory mechanisms such as BDNF-mediated BDNF secretion via TrkB receptors (21, 34). This process may be initiated by critical cytosolic calcium concentrations needed for self-sustained BDNF secretion. This interpretation is supported by previous experiments in our laboratory, showing that BDNF secretion in hippocampal cultures is rapidly induced by BDNF by means of a mechanism involving TrkB-mediated IP3 formation (23) and subsequent calcium release from intracellular stores (21, 23). This positive feedback might persist until counterregulatory mechanisms come into play, such as internalization of TrkB receptors and/or a reduction of BDNF secretion induced by nitric oxide→protein kinase G signaling (30).

The observation that LTD is accompanied by a reduction in BDNF concentrations below the basal secretion levels (Fig. 5) was to some extent surprising. However, previous studies have demonstrated that in the visual cortex the suppression of BDNF function by anti-BDNF Abs or TrkB–IgG receptor bodies facilitates LTD induction (12). Conversely, LTD is markedly decreased by exogenous BDNF in both rat hippocampal and visual cortex slices (35–37). These observations suggest the existence of mechanisms by which the amount of BDNF available at synaptic sites can be reduced to facilitate LTD induction. Here, we provide evidence that such mechanisms exist and are regulated by stimulation parameters leading to LTD in PRh cortex. We found that a stimulation (5 Hz) inducing LTD lasting >180 min (Fig. 5*A*)

decreased BDNF secretion during the period of stimulation (Fig. 5*B*). As in the case of LTP, the changes in BDNF concentrations determined underestimate the effect at the site of stimulation, and could reflect a complete down-regulation of spontaneous BDNF secretion in the stimulated region. A similar decrease in BDNF secretion (Fig. 5*D*) was observed when a 1-Hz stimulation inducing LTD lasting ≈40 min (Fig. 5*C*) was applied (38). Unlike for LTP, the maintenance of LTD does not seem to require a more prolonged change in BDNF secretion. The observed reduction cannot be explained fully by silencing spontaneous neuronal activity because 1 μM TTX induced only a minor reduction in basal BDNF secretion (Fig. 2*D*).

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