NM1DA-Dependent Switch of proBDNF Actions on Developing GABAergic Synapses

Anais Langlois1,2,3, Diaba Diabira1,2,3, Nadine Ferrand1,2,3, Christophe Porcher1,2,3 and Jean-Luc Gaiarsa1,2,3

1 Institut National de la Santé et de la Recherche Médicale Unité 901, Marseille 13009, France, 2 Université de la Méditerranée, UMR S901 Aix-Marseille 2, Marseille 13009, France and 3 Institut de Neurobiologie de la Méditerranée, Marseille 13009, France.

The authors Christophe Porcher and Jean-Luc Gaiarsa contributed equally to this work.

Address correspondence to Jean-Luc Gaiarsa, Institut de Neurobiologie de la Méditerranée, Parc scientifique de Luminy, BP 13, 13273 Marseille Cedex 09, France. Email: gaiarsa@inmed.univ-mrs.fr.

The brain-derived neurotrophic factor (BDNF) has emerged as an important messenger for activity-dependent development of neuronal network. Recent findings have suggested that a significant proportion of BDNF can be secreted as a precursor (proBDNF) and cleaved by extracellular proteases to yield the mature form. While the actions of proBDNF on maturation and plasticity of excitatory synapses have been studied, the effect of the precursor on developing GABAergic synapses remains largely unknown. Here, we show that regulated secretion of proBDNF exerts a bidirectional control of GABAergic synaptic activity with NMDA receptors driving the polarity of the plasticity. While NMDA receptors are activated during ongoing synaptic activity, regulated Ca²⁺-dependent secretion of proBDNF signals via p75NTR to depress GABAergic synaptic activity, while in the absence of NMDA receptors activation, secreted proBDNF induces a p75NTR-dependent potentiation of GABAergic synaptic activity. These results revealed a new function for proBDNF-p75NTR signaling in synaptic plasticity and a novel mechanism by which synaptic activity can modulate the development of GABAergic synaptic connections.

Keywords: BDNF, GABA, proneurotrophin, p75 NTR, synaptic plasticity

Introduction

Appropriate wiring of neuronal networks depends on a tightly regulated sequence of events whereby neurogenesis and synaptogenesis are balanced by apoptosis and axonal pruning. Among the many factors essential in these developmental processes is the brain-derived neurotrophic factor (BDNF). BDNF is an activity-dependent secreted neurotrophin that mediates many aspects of brain development (Lu et al. 2005; Gottmann et al. 2009; Kuczewski et al. 2010). Studies of BDNF outcomes are challenging because of the complex interactions that take place between the different ligands and their receptors. Thus, like many peptide hormones or growth factors, BDNF is synthesized as a precursor (proBDNF) that is either converted intracellularly to mature BDNF (mBDNF) or secreted in an unprocessed form (Lee et al. 2001; Teng et al. 2005; Bergami et al. 2008; Nagappan et al. 2009; Yang, Siao, et al. 2009; Lee et al. 2001; Teng et al. 2005; Bergami et al. 2008; Nagappan et al. 2009; Yang, Siao, et al. 2009; Lee et al. 2001; Teng et al. 2005; Bergami et al. 2008; Nagappan et al. 2009; Yang, Siao, et al. 2009). ProBDNF and mBDNF are assumed to elicit biological functions that often vary in diverse directions (Lu et al. 2005). For instance, proBDNF signals via the tropomyosin-related Kinase receptor B (TrkB-R) to induce cell survival (Teng et al. 2005) or promote long-term potentiation (Korte et al. 1996; Patterson et al. 1996, 2001; Yang, Je, et al. 2009), while proBDNF binds to the p75 neurotrophin receptor (p75NTR) to induce apoptosis (Teng et al. 2005) or facilitate long-term depression (Woo et al. 2005; Rosch et al. 2005; Yang, Je, et al. 2009).

The contribution of mBDNF-TrkB signaling pathway to the development and maturation of GABAergic synapses is well established and documented (Lessmann and Brigadski 2009; Kuczewski et al. 2010). p75NTR and proBDNF expression are developmentally regulated with the highest levels in the first postnatal weeks of life, at a time of synapse formation and elimination (Yang, Siao, et al. 2009; Bartkowska et al. 2010). Whether and how proBDNF-p75NTR signaling affects developing GABAergic synapses is not known. The present study was aimed at determining the effects of proBDNF on developing GABAergic synapses. We show that regulated secretion of proBDNF can lead to either a long-lasting increase or decrease in GABAergic synaptic activity with NMDA receptors driving the switch from potentiation to depression. These results add new insights to the contribution of neurotrophins in activity-dependent refinement of synaptic connections.

Materials and Methods

Slices Preparation

Experiments were performed on hippocampal slices obtained from postnatal day-0 to -5 Wistar rats, as previously described (Kuczewski et al. 2008). All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Brains were removed and immersed into ice-cold (2-4 °C) artificial cerebrospinal fluid (ACSF) of the following composition (millimolar): NaCl, 126; KCl, 3.5; CaCl2, 2; MgCl2, 1.3; NaH2PO4, 1.2; NaHCO3, 25; and glucose, 11; pH 7.4, when equilibrated with 95% O2 and 5% CO2. Hippocampal slices (400 μm thick) were cut with a McIlwain tissue chopper and submerged in ACSF supplemented with NBQX (5μM) and D-AP5 (40μM) to reduce network-driven synaptic activity. In some experiments, slices were incubated with aprotinin, TrkB-IgG, TrkA-IgG, p75NTR function-blocking antibody, or TAT-pep5 for at least 3 h before recording. Slices were then transferred to a submerged recording chamber and perfused with ACSF (3 mL/min) at 34 °C.

Electrophysiological Recordings

Whole cell patch-clamp recordings of CA3 pyramidal neurons were performed with an Axopatch 200B amplifier (Axon Instruments). Microelectrodes (+4 MΩ) were filled with the following solution (mM): CsCl (110), K-glucuronate (30), N2-ethylhexyl-piperazine-N,N’,N’-tetra-acetic acid (HEPES) (10); ethylene glycol-bis (β-aminoethyl ether)-N,N’,N’,N’-tetra-acetic acid (EGTA) (1.1), CaCl2 (0.1), MgATP (4), and NaGTP (0.3). In some experiments, MK801 (1 mM) was added to the pipette solution. Spontaneous synaptic activity was monitored and stored with Axoscope 9.1 (Axon Instruments). Series resistance was monitored in response to a 5 mV pulse during the recordings. Spontaneous GABAergic receptor-mediated postsynaptic currents (sGABA-PSCs) were recorded in NBQX and D-AP5. The protocol to induce GABAergic synaptic plasticity consists in the washing out of the glutamatergic antagonist during 15 min (Kuczewski et al. 2008). In some experiments, the extracellular potassium concentration was increased to 5 mM only during the washout period, to increase the secretion of BDNF, which is required for the induction of the
GABAergic plasticity (Kuczewski et al. 2008). Because, activity recovery in 3.5 or 5 mM led to similar results (respectively, 38 ± 10 % (n = 18) and 30 ± 12 % (n = 8) increase in sGABA-PSCs frequency), the data were pooled. On average, the frequency and amplitude of sGABA-PSCs increased from 4.5 ± 0.4 to 5.7 ± 0.5 Hz (P = 0.0001) and 82 ± 12 to 100 ± 9 pA (P = 0.002) (n = 26, Fig. 1A-C). In some experiments, the identity of the spontaneous PSCs was confirmed by exogenous application of gabazin (5 μM) at the end of the recording session. sGABA-PSCs and series resistance (Rs) were analyzed off-line with Mini Analysis 6.0 software (Synaptosoft). To generate the average sGABA-PSCs, multiple overlapping events were discarded, and the remaining events were aligned on their rising phase. The effective plasticity was quantified as the mean frequency or amplitude sGABA-PSCs between the 10-min preceding activity recovery and the 20–30 min after activity recovery using the Kolmogorov-Smirnov (K-S test). We used paired Student’s t-test to evaluate difference between groups.

Immunohistochemistry
Transverse hippocampal cryosections of paraformaldehyde-fixed brain from P4 rats were processed as previously described (Fiorentino et al. 2009), using primary antibodies chicken anti-proBDNF (1:000, Chemicon MAB351) and rabbit anti-p75NTR (1:500; Abcam 8874). Cy3-conjugated secondary antibodies against chicken and rabbit IgG (1:1000; Invitrogen). Sections were washed and coverslips mounted using Vectashield (Vector). Immunoreactivity was visualized using laser scanning confocal microscope (Zeiss LSM 510 Meta) with 10× objective.

Phospho-CREB Activation
Immunostaining against the nonphosphorylated and phosphorylated forms of CREB was performed on hippocampal slices, which underwent a procedure similar to that used for the electrophysiological study. Hippocampal slices were transferred to the submerged recording chamber and perfused with NBQX and D-AP5 (3 mL/min, at 34 °C) for 10 min. In a first series of experiments, NBQX and D-AP5 were washed out for 15 min in the presence or absence of aprotinin. In another series, mBDNF (10 ng/mL) or CR-proBDNF (10 ng/mL) was applied for 15 min in the presence of NBQX and D-AP5. Control

Figure 1. Aprotinin uncovers activity-dependent long-lasting depression in GABAergic synaptic activity. (A) Upper traces: representative recordings of sGABA-PSCs illustrating the increase in events frequency after activity recovery in control ACSF. Average sGABA-PSCs are shown at a higher time scale. The graph illustrates the time course of sGABA-PSCs frequency modification in the same neuron. In this and following figures the frequency of sGABA-PSCs is expressed as percentage of control prerecovery value and plotted against time (bin = 30 s). (B) Upper traces: representative recordings illustrating the increase in sGABA-PSCs frequency after activity recovery in ACSF supplemented with aprotinin (3 μg/mL). The graph illustrates the time course of sGABA-PSCs frequency modification in the same neuron. (C) Average time course of sGABA-PSCs frequency before and after activity recovery in control ACSF (open symbol, n = 26) or with aprotinin (3 μg/mL, filled symbol, n = 17). (D) Percentage of frequency and amplitude changes 30 min after activity recovery in control ACSF (open symbol) or with aprotinin (filled symbol). Each symbol represents the result from one single cell.
experiments consist of slices maintained in NBQX and D-AP5 for 30 min. The slices were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer immediately after treatment. Cryostat-cut hippocampal sections (30 μm) were preincubated (1 h) in PBS-TritionX-100 (0.3%)-goat serum (3%) and co-incubated overnight at 4 °C with mouse anti-CREB (1:1000) and rabbit anti-phospho-CREB (pCREB, 1:1000) antibodies (Cell Signalling Technology Inc.). Immunoreactivity for pCREB and CREB were detected with an Alexa 488-coupled (A488) rabbit secondary antibody (1:500; FluorProbes) and a Cy3-coupled mouse secondary antibody (1:500; Jackson Immunoresearch Laboratories), respectively. Sections were washed and coverslips mounted using Vectashield (Vector) with the counterstained 4',6'-diamidino-2-phenylindole. Slices were imaged with a LSM 510 (Zeiss) confocal microscope using sequential dual channel recordings of A488 (pCREB) and Cy3 (CREB) fluorescence. The optical sections were digitized (1024 x 1024 pixels) and processed using Image J software. The pCREB to CREB intensity ratio was expressed as means ± SEM. The pCREB-A488 staining intensity versus the CREB-Cy3 staining intensity. Average data are presented as percentage of control slices. We used unpaired Student’s t-test to evaluate difference between groups.

Elisa BDNF Immunodetection Assay
The BDNF sandwich ELISA Kit (Chemicon International, Temecula, CA, USA) was used to quantify the amount of BDNF in hippocampal slices which underwent a procedure similar to that used for the electrophysiological study. Immediately after the treatment, the slices were weighed, and snap frozen in liquid nitrogen and stored at -80 °C. For extraction of BDNF, 20-30 vol/wt of extraction buffer consisting of 100 mM Tris/HCl pH 7.0, containing 1 M NaCl, 4 mM EDTA, 2% Triton X-100 and the protease inhibitors 10 μg/mL aprotinin, 10 μg/mL leupeptin and 17 μg/mL phenyl-methylsulfonyl fluoride (PMSF). The homogenates were centrifuged (14,000 g for 20 s). Supernatants were collected and analyzed with a commercial two-antibody sandwich ELISA (BDNF Emax Immunoassay System; Chemicon) according to the protocol of the manufacturer. The total protein content of each supernatant was measured with a Bradford protein assay. The BDNF level was expressed in intact hippocampi. Analysis of the hippocampi was done for BDNF mRNA quantification by real-time PCR. BDNF mRNA was normalized to the concentration of GAPDH mRNA. (NBQX), Gabazin, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801) and D-2-amino-5-phosphohexamic acid (D-AP5) (Tocris Cookson); k252a, TAT-pep5 (Calbiochem); Nifedipine, aprotinin (Sigma); p75NTR antibody (ab1554, Millipore); cleavage-resistant (CR) proBDNF, mBDNF (Alomone labs); TrkA-IgG, TrkB-IgG (R&D system).

Results
Activity-Recovery Induces Plasticity of GABAergic Synaptic Activity
Spontaneous GABAergic receptor-mediated postsynaptic currents (sGABAergic_PSCs) were recorded from newborn rats CA3 pyramidal neurons in 5 μM NBQX and 40 μM D-AP5. After 10 min of stable GABAergic transmission, NBQX and D-AP5 were washed out for 15 min to recover global network activity. As already reported (Kuczewski et al. 2008), a transient activity recovery resulted in a long-lasting potentiation of GABAergic synaptic activity (LLD_GABA_A) consisting in a persistent increase in both frequency and amplitude of sGABAergic_PSCs (Fig. 1A). A summary of 26 experiments showed that the frequency and amplitude of sGABAergic_PSCs increased from 4.5 ± 0.4 to 5.7 ± 0.5 Hz (P = 0.0001) and 82 ± 12 to 100 ± 9 pA (P = 0.002), respectively (Fig. 1C,D). Recordings in the continuous presence of NBQX and D-AP5 showed no significant changes in sGABAergic_PSCs frequency or amplitude (from 3.7 ± 1.6 to 3.9 ± 1.8 Hz and 74 ± 11 to 68 ± 8 pA, n = 5, P = 0.1, for both). Consistent with our previous study demonstrating the contribution of the mBDNF-TrkB receptor signaling pathway in LLD_GABA_A induction (Kuczewski et al. 2008), LLD_GABA_A was mimicked by bath applied mBDNF (see below and Fig. 4C) and prevented by k252a (200 nM) added to the patch pipette solution (from 5.1 ± 0.9 to 5.4 ± 0.9 Hz and 90 ± 10 to 97 ± 9 pA, n = 10, P = 0.1 for both, data not shown).

To determine whether proBDNF is secreted in the developing hippocampus and to investigate its effect on developing GABAergic synapses, we repeated the same protocol in the continuous presence of the cell-impermeable serine protease inhibitor aprotinin (3 μg/mL), to prevent the extracellular conversion of proBDNF into mBDNF (Teng et al. 2005). In aprotinin, synaptic activity recovery unveiled a long-lasting depression of sGABAergic_PSCs frequency (LLD_GABA_A). A typical experiment is illustrated in Figure 1B. A summary of 17 similar experiments showed that sGABAergic_PSCs frequency was decreased by 31 ± 6% on average after activity-recovery in aprotinin (from 5.6 ± 0.4 to 4.1 ± 0.6 Hz, P = 0.0002, Fig. 1C,D). The kinetic properties of sGABAergic_PSCs were not affected (the rise time change from 1.29 ± 0.21 to 1.29 ± 0.07 (P = 0.2) and the decay time from 8.6 ± 0.9 to 9.1 ± 0.7 (P = 0.4). The effect on sGABAergic_PSCs amplitude was however more complex. An increase (n = 6), a decrease (n = 7), or no significant effect (n = 3) on sGABAergic_PSCs amplitude were observed (Fig. 1D). On average, a nonsignificant change of ± 13% in sGABAergic_PSCs amplitude was observed 30 min after activity recovery (n = 17, P = 0.2). It is worth noting that aprotinin had no effect per se on the frequency and amplitude of sGABAergic_PSCs (from 2.2 ± 0.9 to 2.3 ± 0.9 Hz (P = 0.4) and 56 ± 6 to 72 ± 19 pA (P = 0.1, n = 5), 30 min after aprotinin application). Thus, a transient recovery from activity deprivation with the cell-impermeable serine protease inhibitor aprotinin results in a long-lasting depression of sGABAergic_PSCs frequency (LLD_GABA_A).

Activation of p75NTR and Intracellular Ca2+ Rise Are Required for LLD_GABA_A Induction
We next asked whether p75NTR activation contributes to LLD_GABA_A induction. We first performed immunostaining against proBDNF and p75NTR. Both proBDNF and p75NTR were distinguished in the CA1 and CA3 regions of the hippocampus and in the dentate gyrus of the newborn rats (Fig. 2A). The staining was prominent in pyramidal cell bodies and mild short processes extending from the somata to the stratum radiatum. The staining pattern was the same as that obtained by others (Yang, Xiao, et al. 2009). To test the contribution of p75NTR in LLD_GABA_A induction, hippocampal slices were incubated with...
Immunofluorescence detection of proBDNF and p75NTR in a 4-days-old rat hippocampi

...amplitude of sGABA-PSCs changed, respectively, from 3.7 ± 0.7 to 4.5 ± 1.2 Hz (n = 9, P = 0.2) and from 83 ± 8 to 101 ± 17 pA (n = 9, P = 0.06) in TAT-pep5-treated slices, and from 3.7 ± 0.8 to 4.9 ± 1.1 Hz (n = 7, P = 0.07) and from 87 ± 7 to 101 ± 14 pA (n = 7, P = 0.1) in p75NTR function-blocking antibody-treated slices. To determine the location of the p75NTR, TAT-pep5 (2μM) was added in the pipette solution. LLDGABA was not observed in TAT-pep5 loaded neurons (Fig. 2C, from 2.1 ± 0.7 to 2.5 ± 1 Hz (n = 7, P = 0.12) and from 75 ± 7 to 90 ± 12 pA (n = 7, P = 0.2). Although we cannot completely exclude a diffusion of TAT-pep5 from postsynaptic neuron to presynaptic terminals, these observations suggest that the activation of postsynaptic p75NTR is required for LLDGABA.

We next challenged this conclusion and tested the hypothesis that regulated secretion of BDNF may contribute to LLDGABA. Because, L-type Ca2+ channels are required for postsynaptic secretion of BDNF and are instrumental for the induction of BDNF-dependent forms of GABAergic synaptic plasticity (Gubellini et al. 2005; Mohajerani et al. 2007; Kuczewski et al. 2008; Sivakumaran et al. 2009), we investigated the effect of the L-type Ca2+ channel blocker nifedipine. Nifedipine (10 μM) prevented the induction of LLDGABA (from 2.1 ± 0.4 to 2.2 ± 0.5 Hz, P = 0.2, Fig. 3A; from 69 ± 17 to 87 ± 10 pA, P = 0.09, n = 5, not shown). To determine whether L-type Ca2+ channels activation is responsible for proBDNF secretion, we tested whether the cleavable-resistant form of proBDNF (CR-proBDNF, 10 ng/mL) could bypass the effect of nifedipine. CR-proBDNF applied during activity recovery rescued LLDGABA in nifedipine (Fig. 3A, from 4.3 ± 0.9 to 3.4 ± 0.7 Hz, P = 0.04; from 90 ± 27 to 110 ± 24 pA, P = 0.2, n = 6, not shown). The rescue of LLDGABA by bath applied CR-proBDNF was not observed in p75NTR function-blocking antibody-treated slices. The frequency and amplitude of sGABA-PSCs changed, respectively, from 1.8 ± 0.3 to 2.1 ± 0.4 Hz (P = 0.3) and 90 ± 10 to 110 ± 20 pA (P = 0.2, n = 5, not shown) after the application of CR-proBDNF in p75NTR antibody-treated slices. These results therefore show that a Ca2+-dependent secretion of proBDNF is required to trigger p75NTR-LLDGABA.

To deepen the relationship between Ca2+ and LLDGABA induction, the Ca2+ chelator BAPTA was added into the pipette solution. LLDGABA induction was prevented in BATA-loaded neurons (Fig. 3B, from 5.3 ± 0.5 to 5.9 ± 1.2 Hz, P = 0.2 and from 79 ± 9 to 82 ± 13 pA, P = 0.4, n = 9). Although the average change does not reach significance, a long-lasting potentiation of sGABA-PSCs frequency was observed in 4 of the 9 BAPTA-loaded neurons recorded (from 4.1 ± 1.3 to 7.4 ± 2.9 Hz). This observation suggests that blocking intracellular Ca2+-dependent process may uncover a potentiation of GABAergic synaptic activity (see below).

One possible interpretation of this result is that BAPTA prevents the Ca2+-dependent secretion of proBDNF leading to LLDGABA. Another interpretation is that LLDGABA induction requires a Ca2+-dependent, but L-type Ca2+ channel-independent, postsynaptic signaling cascade. To address this issue, we examine whether bath applied CR-proBDNF could rescue LLDGABA in BAPTA-loaded neurons. CR-proBDNF applied during the washout period had no significant effect on the frequency (Fig. 3B; from 4.5 ± 0.7 to 4.6 ± 0.8 Hz, P = 0.4, n = 6) and amplitude (from 58 ± 8 to 62 ± 8 pA, P = 0.2, n = 6, not shown) of sGABA-PSCs. Thus, in addition to the activation of downstream to p75NTR, a postsynaptic Ca2+-dependent signaling cascade is required to induce LLDGABA.
whether the activation of AMPA receptors is also required for the induction of LLD_\text{GABA-A}, the AMPA receptor antagonist NBQX was applied during the washout period. LLD_\text{GABA-A} was not observed in the presence of NBQX (5 \mu M) (Fig. 3C, from 3.3 \pm 0.5 to 3.7 \pm 0.7 Hz, P = 0.1, n = 8). However, CR-proBDNF (10 ng/mL) applied during activity recovery rescued LLD_\text{GABA-A} in NBQX (Fig. 3C, from 3.1 \pm 0.4 to 2.5 \pm 0.5 Hz, P = 0.007, from 103 \pm 9 to 109 \pm 13 \text{ pA}, P = 0.3, n = 6).

Overall, these experiments show that a transient recovery from activity deprivation in aprotinin induces a persistent depression of GABAergic synaptic activity by means of a cascade involving the activation of AMPA receptors, the secretion of proBDNF under the control of L-type Ca\textsuperscript{2+} channels and a postsynaptic Ca\textsuperscript{2+}-dependent and p75\textsuperscript{NTR}-dependent signaling cascade.

**Suppression of the mBDNF-TrkB Signaling Does Not Account for LLD_\text{GABA-A} Induction**

Activation of p75\textsuperscript{NTR} has been reported to cause axonal degeneration (Singh et al. 2008) or cell death (Song et al. 2010), at least in part by suppressing Trk receptor-dependent signaling pathways. Because, LLD_\text{GABA-A} induction requires the activation of TrkB receptors by endogenous BDNF (Kuczewski et al. 2008), we thought to address whether the mBDNF-TrkB receptor signaling is impaired in the presence of aprotinin. We first investigated whether aprotinin may have affected the production of BDNF using qRT-PCR and ELISA analysis. We found no significant differences in the BDNF mRNA (n = 4 intact hippocampi for each condition, P = 0.3) and protein levels (n = 4 slices for each condition, P = 0.7) between control and aprotinin-treated slices (Fig. 4A,B).

We next tested the effect of mBDNF on sGABA\textsubscript{A}-PSCs. Bath applied mBDNF (10 ng/mL) induced a persistent enhancement of sGABA\textsubscript{A}-PSCs frequency and amplitude in the absence (from 3.4 \pm 0.9 to 4.3 \pm 1.1 Hz, P = 0.005 and from 68 \pm 5 to 90 \pm 8 \text{ pA}, n = 8, P = 0.04) and the presence (from 3.4 \pm 1.2 to 4.4 \pm 1.5 Hz, 66 \pm 9 to 85 \pm 8 \text{ pA}, n = 7, P = 0.04) of aprotinin (Fig. 4C). We also tested whether exogenous mBDNF (10 ng/mL) could rescue the deficit of LLD_\text{GABA-A} in aprotinin. In control experiments, sGABA\textsubscript{A}-PSCs frequency and amplitude decreased by, respectively, 25 \pm 5\% and 10 \pm 12\% (n = 5), 30 min after activity recovery in aprotinin (Fig. 4D). When, mBDNF was applied during the recovery period in aprotinin, the frequency and amplitude of sGABA\textsubscript{A}-PSCs increased by, respectively, 13 \pm 17\% and 35 \pm 14\% (n = 6, Fig. 4D). Thus, mBDNF applied during the recovery period in aprotinin led to a significant enhancement of sGABA\textsubscript{A}-PSCs frequency and amplitude when compared with activity recovery in aprotinin alone (P = 0.001, unpaired t-test). These results therefore show that the mBDNF-TrkB signaling is not affected by aprotinin.

To confirm this observation, we used the phosphorylated form of the cAMP response element–binding protein (CREB) to monitor TrkB receptor activation by endogenous BDNF (Fiorentino et al. 2009). To normalize the results obtained from different slices, the pCREB to CREB ratio was quantified. Recovery of synaptic activity in control ACSF significantly increased the pCREB/CREB ratio, an effect prevented by TrkB-IgG, but not by TrkA-IgG (Fig. 5). The increase in pCREB/CREB ratio was not observed when activity recovered with aprotinin (Fig. 5). Aprotinin, however, did not abolish the response to exogenous mBDNF (Fig. 5), thus supporting the
family, k252a, does not uncover \( \text{LD}_{\text{GABA}} \) (Kuczewski et al. 2008), these data show that the activation of \( p75^{\text{NTR}} \), but not the inactivation of the mBDNF-TrkB signaling, is required for \( \text{LD}_{\text{GABA}} \) induction in apritin.

**ProBDNF-p75^{NTR} Signaling Potentiates GABAergic Synaptic Activity in the Absence of NMDA Receptor Activation**

Since most forms of synaptic plasticity require the activation of NMDA receptors, we investigated the effect of D-AP5 on \( \text{LD}_{\text{GABA}} \) induction. We found that activity recovery in D-AP5 and apritin led to a long-lasting increase in sGABA-A-PSCs frequency and amplitude. The Figure 6A illustrates a typical experiment. A summary of 17 similar experiments showed a significant increase in both frequency (from 2.8 ± 0.9 to 4.3 ± 1.5 Hz, \( n = 17 \), \( P = 0.01 \)) and amplitude (from 83 ± 10 to 120 ± 21 pA, \( n = 17 \), \( P = 0.02 \)) of sGABA-A-PSCs (Fig. 6B,C), with no changes in rise time (from 1.32 ± 0.09 to 1.35 ± 0.1, \( P = 0.4 \)) and decay time (from 8.5 ± 0.4 to 9.2 ± 0.6, \( P = 0.2 \)).

To determine whether postsynaptic NMDA receptors are involved, the NMDA channel blockers MK801 was added into the pipette solution. On average, the frequency and amplitude of sGABA-A-PSCs increased respectively from 4.9 ± 1.2 to 5.4 ± 1.1 Hz (\( P = 0.3 \)) and from 69 ± 18 to 79 ± 20 Hz (\( P = 0.4 \), \( n = 9 \), data not shown). However, even though the modification was not significant on average, a long lasting potentiation of sGABA-A-PSCs frequency in 5 of the 9 MK801-loaded neurons (from 3.5 ± 1.3 to 5.3 ± 1.6 Hz, \( P = 0.01 \), \( n = 5 \)). This result shows that the activation of postsynaptic NMDA receptors is required to induce \( \text{LD}_{\text{GABA}} \) and suggests that blockade of these receptors could uncover a long lasting potentiation of GABAergic synaptic activity.

The enhancement of GABAergic activity induced by activity-recovery in the presence of D-AP5 was not observed in slices preincubated with TAT-pep5 (Fig. 6D, from 3.3 ± 0.7 to 3.3 ± 0.8 Hz and 85 ± 11 to 96 ± 16 pA, \( n = 7 \), \( P = 0.1 \)) or with the \( p75^{NTR} \) function-blocking antibody (Fig. 6D, from 2.8 ± 0.6 to 3.1 ± 0.6 Hz, \( P = 0.06 \) and 85 ± 14 to 87 ± 10 pA, \( P = 0.2 \), \( n = 6 \)).

The induction of GABAergic synaptic potentiation was also prevented by intracellular infusion of TAT-pep5 (2 \( \mu \)M) (Fig. 6E, from 2.4 ± 0.8 to 2.2 ± 0.6 Hz, \( P = 0.3 \) and 81 ± 9 to 85 ± 8 pA, \( P = 0.2 \), \( n = 10 \)). This form of plasticity will be referred to hereafter as \( p75^{NTR-LLPGABA} \).

To determine whether regulated secretion of BDNF is required for \( p75^{NTR-LLPGABA} \) induction, we investigated the effect of the L-type \( Ca^{2+} \) channel blocker nifedipine. Nifedipine prevented the induction of \( p75^{NTR-LLPGABA} \) (Fig. 7A, from 3.1 ± 0.8 to 3.2 ± 0.9 Hz, \( P = 0.3 \), from 77 ± 14 to 84 ± 14 pA, \( P = 0.4 \), \( n = 7 \)). Moreover, CR-proBDNF applied during activity recovery in D-AP5 and nifedipine rescued the long-lasting potentiation of sGABA-A-PSCs (Fig. 7A, from 1.9 ± 0.5 to 2.4 ± 0.4 Hz, from 73 ± 5 to 94 ± 9 pA, \( P = 0.04 \), \( n = 8 \), an effect prevented by the \( p75^{NTR} \) function-blocking antibody (from 2.6 ± 0.1 to 2.3 ± 0.6 Hz, from 94 ± 14 to 115 ± 20 pA, \( P = 0.1 \), \( n = 8 \), data not shown). Thus, regulated secretion of proBDNF is required to induce \( p75^{NTR-LLPGABA} \). Next, we investigated whether the induction of \( p75^{NTR-LLPGABA} \) requires a postsynaptic \( Ca^{2+} \)-dependent signaling cascade by loading the postsynaptic neuron with BAPTA. A long-lasting potentiation of sGABA-A-PSCs frequency (Fig. 7B, from 2.0 ± 0.7 to

**Figure 4.** The mBDNF-TrkB receptor signaling is not impaired in the presence of apritin. (A,B) Mean native BDNF mRNA (A, 4 intact hippocampi for each condition) and protein (B, 4 slices for each condition) levels in control ACSF or apritin-treated tissue. (C) Average time course of the effect of mBDNF (10 ng/mL) on sGABA-A-PSCs frequency in the absence (control, open symbol, \( n = 8 \)) or the presence (aprotinin, filled symbol, \( n = 7 \)) of apritin. (D) Average time course of sGABA-A-PSCs frequency before and after activity recovery in apritin in the absence (control, open symbol, \( n = 5 \)) or the presence of mBDNF (washout in mBDNF, filled symbol, \( n = 6 \)) during the recovery period.

Conclusion that the mBDNF-TrkB signaling is not affected by apritin.

Overall, with the observation that the membrane permeable inhibitor of protein tyrosine kinase coupled to Trk receptor
2.5 ± 0.6 Hz, \( P = 0.05, n = 6 \) and amplitude (from 41 ± 4 to 56 ± 7 pA, \( P = 0.02, n = 6 \), data not shown) were however observed in BAPTA-loaded neurons following activity recovery in aprotinin and D-AP5.

As stated above, aprotinin had no effect per se on the frequency and amplitude of sGABAergic-PSCs recorded in the presence of D-AP5 and NBQX (from 2.2 ± 0.9 to 2.3 ± 0.9 Hz, \( P = 0.4 \) and 56 ± 6 to 72 ± 19 pA, \( P = 0.1, n = 5 \)), indicating that p75NTR-LLPGABA-A induction required the activation of AMPA receptors during the recovery of synaptic activity. We therefore asked whether bath applied CR-proBDNF could induce p75NTR-LLPGABA-A in the presence of NBQX and D-AP5. Bath applied CR-proBDNF (10 ng/mL) induced a long-lasting potentiation of GABAergic synaptic activity (Fig. 7C, from 3.1 ± 0.7 to 4.2 ± 0.8 Hz, \( P = 0.004 \), from 83 ± 13 to 109 ± 813 pA, \( P = 0.001, n = 14 \)). We used pCREB to determine whether TrkB-Rs were activated in these conditions and found that neither activity recovery in aprotinin and D-AP5 nor bath applied CR-proBDNF in NBQX and D-AP5 increases the pCREB/CREB ratio (Fig. 5).

Overall, these data show that regulated secretion of proBDNF can signal through p75NTR to either depress or enhance GABAergic synaptic activity (p75NTR-LLDGABA-A and p75NTR-LLPGABA-A), with NMDA receptors driving the polarity of the plasticity.

### Discussion

The major conclusion of the present study is that, in the presence of the serine protease inhibitor aprotinin, spontaneous glutamatergic activity can lead to a p75NTR-dependent long-lasting potentiation or depression of GABAergic synaptic activity in the newborn rat hippocampus. Both forms of synaptic plasticity required a Ca\(^{2+}\)-dependent secretion of proBDNF that plays an instructive role through the activation of postsynaptic p75NTR. The present study also shows that the NMDA receptors drive the switch from potentiation to depression in GABAergic activity (Fig. 8).

**ProBDNF-p75 Signaling Induces a Bidirectional Control of GABAergic Synaptic Activity in the Developing Rat Hippocampus**

BDNF is synthesized as precursor that undergoes proteolytic cleavage to generate the mature form. Whether proBDNF is secreted has been debated (Matsumoto et al. 2008; Yang, Siao, et al. 2009). In the present study, we provided evidences suggesting that a significant proportion of BDNF is secreted as a precursor in the newborn rat hippocampus and signals via p75NTR to induce a bidirectional control of GABAergic synaptic activity. Thus, we found that activity recovery with the cell-impermeable serine-protease inhibitor aprotinin—which among different protease inhibits plasmin activity (Teng et al.
2005)—can either depress or enhance the level of GABAergic synaptic activity. Both forms of plasticity rely on proBDNF-p75NTR signaling because they were prevented by blockers of the p75NTR signaling and rescued by bath applied CR-proBDNF, under conditions that prevented the regulated postsynaptic secretion of BDNF (i.e., with nifedipine or NBQX; Lessmann et al. 2003; Kuczewski et al. 2008; Matsuda et al. 2009). Furthermore, the experiments showing that bath applied CR-proBDNF leads to a p75NTR-potentiation or -depression of GABAergic synaptic activity when applied, respectively, in the absence (Fig. 3A,C) or the presence (Fig. 7A,C) of the NMDA receptor antagonist D-AP5, clearly exemplified the

Figure 6. Blockade of NMDA receptors reverse the polarity of GABAergic synaptic plasticity. (A) Right traces show representative recordings illustrating the increase in sGABA-PSCs frequency after activity recovery in aprotinin and D-AP5. Average sGABA-PSCs are shown at a higher time scale. The graph illustrates the time course of sGABA-PSCs frequency modification in the same neuron. (B) Average time course of sGABA-PSCs frequency before and after activity recovery in aprotinin (control, open symbol, n = 6) or aprotinin and D-AP5 (40 μM, filled symbol, n = 17) during the recovery period. (C) Average frequency and amplitude changes 30 min after activity recovery in aprotinin (open symbol) or aprotinin and D-AP5 (filled symbol). (D) Average time course of sGABA-PSCs frequency before and after activity recovery in the presence of aprotinin and D-AP5 in TAT-pep5 (open symbol, n = 7) or p75NTR antibody (filled symbol, n = 6) treated slices. (E) Average time course of sGABA-PSCs frequency before and after activity recovery in TAT-pep5 loaded neurons (n = 10).
bidirectional action of proBDNF-p75NTR signaling on GABAergic synaptic activity. Thus, activity-dependent activation of NMDA receptors can switch the action of the proBDNF-p75NTR pathway on GABAergic synaptic activity from potentiation (p75NTR-LLPGABA-A) to depression (p75NTR-LLDGABA-A).

Regulated Secretion of BDNF Is Required to Induce GABAergic Synaptic Plasticity
BDNF can be secreted from both the axon and dendrite in a regulated (Ca^{2+}-dependent) and constitutive (Ca^{2+}-independent) manner (Lessmann and Brigadski 2009). Previous studies have reported that dendritic release of BDNF is prevented by L-type Ca^{2+} channel blockers (Kolarow et al. 2007; Matsuda et al. 2009), while axonal BDNF release is prevented by N-type Ca^{2+} channel blockers (Balkowiec and Katz 2002; Wang et al. 2002; Matsuda et al. 2009). In the present study, the L-type Ca^{2+} channel blocker nifedipine prevents the induction of both p75NTR-LLDGABA-A and p75NTR-LLPGABA-A, an effect rescued by exogenous application of CR-proBDNF. Along with the finding that GABAergic interneurons do not produce neurotrophins themselves (Ernfors et al. 1990; Gorba and Wahle 1999), this result suggests that a dendritic secretion of proBDNF from the CA3 pyramidal neurons is required to induce p75NTR-LLDGABA-A and p75NTR-LLPGABA-A (Fig. 8). Even if L-type Ca^{2+} channels are unlikely to be activated in the recorded neuron (the potential was clamped at −70 mV), L-type Ca^{2+} channel located on the neighboring cells are activated during activity recovery and will contribute to the secretion of BDNF leading to GABAergic synaptic plasticity (Kuczewski et al. 2008). We further show that the AMPA receptor antagonist NBQX prevents the induction of both forms of plasticity and that exogenous application of CR-proBDNF rescued the inhibitory effect of NBQX. These observations suggest that the activation of AMPA receptors during activity recovery is required to trigger the secretion of proBDNF and subsequent GABAergic synaptic plasticity. In agreement with this hypothesis, using ELISA BDNF immunodetection, we have previously shown that BDNF secretion is upregulated during the recovery of AMPA receptors mediated synaptic activity in the developing rat hippocampus (Kuczewski et al. 2008). Moreover, a dendritic AMPA receptor-dependent secretion of BDNF-GFP has been observed following presynaptic tetanic stimulation of glutamatergic terminals in neuronal cultures (Hartmann et al. 2001). Overall, these observations support the notion that a regulated dendritic secretion of proBDNF plays an instructive role in the induction of p75NTR-LLDGABA-A and p75NTR-LLPGABA-A in the developing rat hippocampus (Fig. 8).

Receptors Location and Underlying Mechanisms
Pre- and postsynaptic TrkB receptor activation has been reported to up or down regulate the function of GABA receptors, to increase or decrease the number or activity of GABAergic terminals and to modulate the excitability of GABAergic interneurons (Gottmann et al. 2009). The effects of p75NTR activation on the GABAergic circuitry have been less documented (Gascon et al. 2005; Salama-Cohen et al. 2006; Lin et al. 2007). In the present study, TAT-pep5 added in the recording solution prevents the induction of both p75NTR-LLDGABA-A and p75NTR-LLPGABA-A. Although we cannot completely exclude retrograde diffusion of TAT-pep5 from the postsynaptic neuron to the presynaptic terminals, this result suggests that the p75NTR are located on the CA3 pyramidal neurons. This conclusion is supported by previous studies showing that GABAergic interneurons are not equipped with

Figure 7. Activity recovery in aprotinin and D-AP5 leads to a 75NTR-dependent enhancement of GABAergic synaptic activity. (A) Average time course of sGABA-PSCE frequency before and after activity recovery with aprotinin, D-AP5 and nifedipine in the absence (control, open symbol, n = 6) or the presence of CR-proBDNF (washout in CR-proBDNF, filled symbol, n = 8) during the recovery period. (B) Average time course of sGABA-PSCE frequency before and after activity recovery in the presence of aprotinin and D-AP5 in BAPTA-loaded neurons (n = 6). (C) Average time course of the effect of CR-proBDNF (10 ng/mL) on sGABA-PSCE frequency.
postsynaptic p75NTR in the adult hippocampus (Dougherty and Milner 1999; Holm et al. 2009). We also showed that both activity recovery and exogenous application of CR-proBDNF failed to induce LLDGABA-A in BAPTA-loaded cells. This observation suggests that, in addition to the L type-dependent secretion of proBDNF, a postsynaptic Ca²⁺-dependent signaling is required to induce LLDGABA-A. We can only speculate on 3 possible relationships between postsynaptic Ca²⁺ activation and postsynaptic [Ca²⁺]: either p75NTR activation induces a rise in [Ca²⁺], leading to LLDGABA-A, or a basal level of [Ca²⁺], is required to allow p75NTR-dependent induction of LLDGABA-A, or a concomitant activation of postsynaptic p75NTR and Ca²⁺ influx through NMDA channels triggers LLDGABA-A. Based on the observation that postsynaptic loading of BAPTA and MK801 led to same outcomes on GABAergic synaptic activity, we may suggest that Ca²⁺ entering through postsynaptic NMDA receptors is required for the induction of LLDGABA-A (Fig. 8). Moreover, a potentiation of GABAergic synaptic activity was observed in about 50% of the BAPTA- or MK801-loaded neurons, an effect consistent with the action of D-AP5. This observation may suggest that postsynaptic NMDA receptors and subsequent Ca²⁺ influx through these channels may regulate the direction of the GABAergic plasticity, although we cannot completely exclude the contribution of NMDA receptors located on other sites (either presynaptic or postsynaptic on neighboring pyramidal cells) in this process.

The downstream signaling pathways involved as well as the mechanisms underlying the expression of GABAergic synaptic plasticity are at present still to be determined. Activation of p75NTR has been reported to cause axonal degeneration (Singh et al. 2008) or cell death (Song et al. 2010), at least in part by suppressing Trk receptor-dependent signaling pathways. However, the observations that mBDNF, applied during the recovery period, could potentiate GABAergic synaptic activity (Fig. 4D), and phosphorylate CREB (Fig. 5) in aprotinin shows that antagonistic interactions between p75NTR and TrkB-Rs are not required for LLDGABA-A induction. p75NTR form multimeric complexes with different proteins and coreceptors providing a wide range of biological actions depending on the cellular context (Lu et al. 2005). For instance, both promoting (Brann et al. 1999; Gascon et al. 2005) and inhibiting (Yamashita et al. 2002; Zagrebelsky et al. 2005) effects of p75NTR activation have been reported on neurite outgrowth. The modifications of GABAergic synaptic activity observed in the present study could have resulted from modifications in the firing rate of GABAergic interneurons, in the number of functional synaptic connections, or in the probability of GABA release. To date, p75NTR activation has been reported to enhance the dendritic complexity of cultured GABAergic neurons derived from the subventricular zone (Gascon et al. 2005), to increase the number of GABAergic terminals in hippocampal cultures (Salama-Cohen et al. 2006) and to promote the expression of GABAergic neuronal phenotype in the basal forebrain (Lin et al. 2007). Interestingly, in the former study, p75NTR were not located on GABAergic interneurons but on cholinergic neurons, suggesting a noncell autonomous regulation of GABAergic neuron development (Lin et al. 2007). p75NTR activation has also been reported to result in long-lasting depression of synaptic strength coupled with synapse elimination at the neuromuscular junction (Yang, Je, et al. 2009) and CA1-Schaffer collaterals junction (Egashira et al. 2010). Further studies will be required to determine whether similar phenomenon occurs at the GABAergic terminals in the developing rat hippocampus.

**Conclusion**

The present study shows that regulated secretion of proBDNF can exert a bidirectional control of GABAergic synaptic activity in the developing hippocampus. The formation of neuronal circuit is dependent upon spontaneous or sensory-driven synaptic activities determining, for instance, which connections are maintained and which are eliminated. Regulated secretion of BDNF contributes to activity-dependent neuronal network wiring. The general thought is that BDNF signals via TrkB receptors to strengthen synaptic connections and

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**Figure 8.** Paracrine actions of proBDNF on GABAergic synaptic activity. Schematic representation of the steps leading to the p75NTR-dependent LLPGABA-A and LLDGABA-A. CA3 pyramidal cells release proBDNF in response to the activation of L-type Ca²⁺ channel during ongoing activation of AMPA receptors. Under control conditions, proBDNF is cleaved by extracellular protease (plasmin) to yield mBDNF leading to TrkB-dependent LLPGABA-A (Kuczewski et al. 2008). When the extracellular protease is inhibited, proBDNF accumulates in the extracellular space, activates postsynaptic p75NTR and leads to NMDA-dependent LLDGABA-A or NMDA-independent LLPGABA-A. It is worth noting that our results do not exclude the possibility that a fraction of mBDNF can be secreted during synaptic activity.
promote network development. Because, the extracellular conversion of proBDNF can be modulated physiological conditions depending on the level and/or pattern of synaptic activity generated by the neuronal network (Nagappan et al. 2009), our results suggest that p75NTR activation may also fine-tune GABAergic connectivity under physiological conditions, underscoring the importance of determining the mBDNF to proBDNF ratio in different in vivo situations.

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