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Activation of p75^{NTR} by proBDNF facilitates hippocampal long-term depression

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Pro- and mature brain-derived neurotrophic factor (BDNF) activate two distinct receptors: p75 neurotrophin receptor (p75^{NTR}) and TrkB. Mature BDNF facilitates hippocampal synaptic potentiation through TrkB. Here we report that proBDNF, by activating p75^{NTR}, facilitates hippocampal long-term depression (LTD). Electron microscopy showed that p75^{NTR} localized in dendritic spines, in addition to afferent terminals, of CA1 neurons. Deletion of *p75*^{NTR} in mice selectively impaired the NMDA receptor–dependent LTD, without affecting other forms of synaptic plasticity. *p75*$^{NTR-/-}$ mice also showed a decrease in the expression of NR2B, an NMDA receptor subunit uniquely involved in LTD. Activation of p75^{NTR} by proBDNF enhanced NR2B-dependent LTD and NR2B-mediated synaptic currents. These results show a crucial role for proBDNF-p75^{NTR} signaling in LTD and its potential mechanism, and together with the finding that mature BDNF promotes synaptic potentiation, suggest a bidirectional regulation of synaptic plasticity by proBDNF and mature BDNF.

Persistent modifications of synapses, also known as long-term synaptic plasticity, are believed to be a fundamental mechanism for information storage and processing in the brain. These modifications include either strengthening or weakening of synaptic connections, respectively termed long-term potentiation (LTP) and long-term depression (LTD). An emerging concept is that synaptic plasticity is tightly controlled by neurotrophic factors, which themselves are produced and secreted in an activity-dependent manner[1,2]. Brain-derived neurotrophic factor (BDNF) is the best-studied neurotrophic protein that elicits diverse and profound effects on central synapses, including the regulation of hippocampal LTP[3–5].

Biological actions of BDNF are mediated by two separate receptors, the TrkB receptor tyrosine kinase and p75^{NTR}. Like other neurotrophins, BDNF is first synthesized as a precursor (proBDNF), which is proteolytically cleaved to form mature BDNF (mBDNF)[6–9]. Mature BDNF interacts preferentially with TrkB to activate its intrinsic tyrosine kinase activity, which in turn triggers multiple intracellular signaling pathways[10,11]. In contrast, pro-neurotrophins bind p75^{NTR} with high affinity[12]. Although p75^{NTR} lacks intrinsic enzymatic activity, it can trigger signaling transduction through its association with adaptor proteins distinct from Trk signaling cascades[10,11].

The finding that pro-neurotrophins serve as signaling molecules rather than inactive precursors has brought a new dimension of complexity to neurotrophin function[12–14]. Indeed, a number of recent studies showed that pro-neurotrophins, by activating p75^{NTR}, promote apoptosis in the peripheral nervous system[12,15–18]. This is in marked contrast to mature neurotrophins, which act to enhance neuronal survival. As a member of the tumor necrosis factor receptor family, p75^{NTR} encodes a type II death domain and is known to promote apoptosis, a function opposite to that of Trk receptors[16,19,20]. Thus, pro- and mature neurotrophins may elicit diametrically opposite cellular responses, through two distinct receptor-signaling systems.

Recently, studies have shown that endogenous proBDNF is secreted from neuronal cells[18,21] and accounts for a substantial proportion of total BDNF secreted extracellularly[8,22,23]. Although p75^{NTR}, a receptor for pro-neurotrophins, has been thought to be involved in cell death, the relative paucity of changes in cell death in brains of mice that lack full-length p75^{NTR} suggests that proBDNF-p75^{NTR} interaction may mediate neuronal functions other than apoptosis[24]. Recently, extracellular cleavage of proBDNF has been shown to be crucial for the expression of late-phase LTP in the hippocampus[25]. Because a major function of BDNF in the brain is to regulate hippocampal LTP, we asked whether proBDNF, if uncleaved, modulates synaptic plasticity through p75^{NTR}. In this study, we show that proBDNF, signaling through p75^{NTR}, selectively facilitates NMDA receptor–dependent LTD in the mammalian hippocampus. Furthermore, we show that this enhancement of LTD by proBDNF-p75^{NTR} is mediated through an upregulation of NR2B, an NMDA receptor subtype uniquely involved in LTD[26,27].

RESULTS

Normal hippocampal synaptic properties in *p75*$^{NTR-/-}$ mice

As an initial step toward characterizing *p75*$^{NTR-/-}$ mice, we examined basal synaptic function in hippocampal area CA1. We observed no difference in the input-output curves in *p75*$^{NTR+/+}$ and *p75*$^{NTR-/-}$

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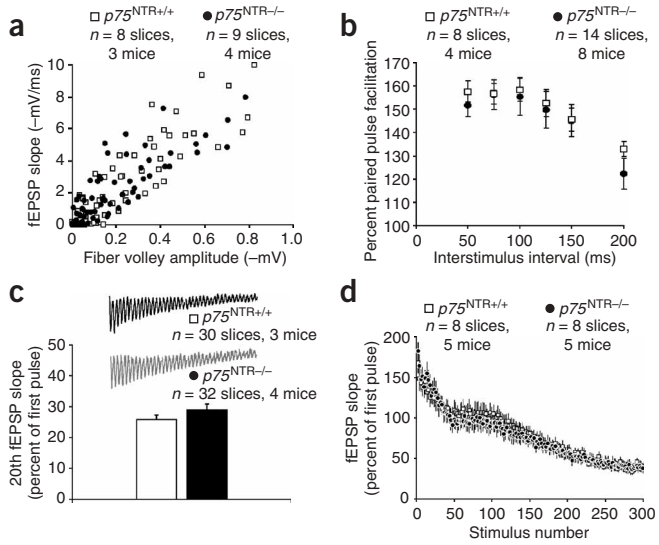

Figure 1 Intact presynaptic properties in CA1 synapses of $p75^{NTR-/-}$ mice. Field recordings were performed in hippocampal slices derived from $p75^{NTR+/+}$ and $p75^{NTR-/-}$ animals. (**a**) Normal input-output curves in $p75^{NTR-/-}$ slices. We plotted fEPSP slopes against their corresponding presynaptic fiber volley amplitude and found no difference in the distribution of values obtained from $p75^{NTR-/-}$ and age-matched controls. (**b**) Normal paired-pulse facilitation (PPF). We calculated PPF from the ratio of the second fEPSP slope to the first fEPSP and plotted it against different interstimulus intervals. There was no significant difference between $p75^{NTR-/-}$ and $p75^{NTR+/+}$ mice at all interstimulus intervals tested. (**c**) Normal synaptic responses to brief, HFS (100 Hz, 100 pulses) at $p75^{NTR-/-}$ synapses. Top, representative recordings of EPSP traces from $p75^{NTR+/+}$ (black) and $p75^{NTR-/-}$ (gray) hippocampi; bottom, summary results. (**d**) Normal synaptic responses to prolonged repetitive stimulation (14 Hz, 300 pulses) in $p75^{NTR-/-}$ mice, as compared with wild-type hippocampal slices. Time course of the effects of the stimulus trains is shown. Experiments in **c** and **d** were performed in the presence of AP5. Error bars, s.e.m.

synapses (**Fig. 1a**), suggesting that the deletion of $p75^{NTR}$ does not alter basal synaptic transmission. Similarly, paired-pulse facilitation at various interstimulus intervals, a commonly used method to infer changes in probability of neurotransmitter release, was virtually identical in both genotypes (**Fig. 1b**). We next examined the readily releasable pool of synaptic vesicles by applying a brief train of high-frequency stimulation (HFS; 100 Hz, 100 pulses) to the CA1 afferents in the presence of AP5, an NMDA receptor antagonist (50 μM). Field excitatory postsynaptic potential (fEPSP) slopes showed a continuous decline over time, indicative of a gradual depletion of docked vesicles, with no obvious differences between mutant and control slices (**Fig. 1c**). We also recorded synaptic responses to a prolonged train of repetitive stimulation (14 Hz, 300 pulses) in the presence of AP5 to determine the size of the reserve pool of vesicles[28]. Again we observed no difference between $p75^{NTR+/+}$ and $p75^{NTR-/-}$ slices (**Fig. 1d**). Thus deletion of $p75^{NTR}$ does not alter basic properties of CA1 synapses.

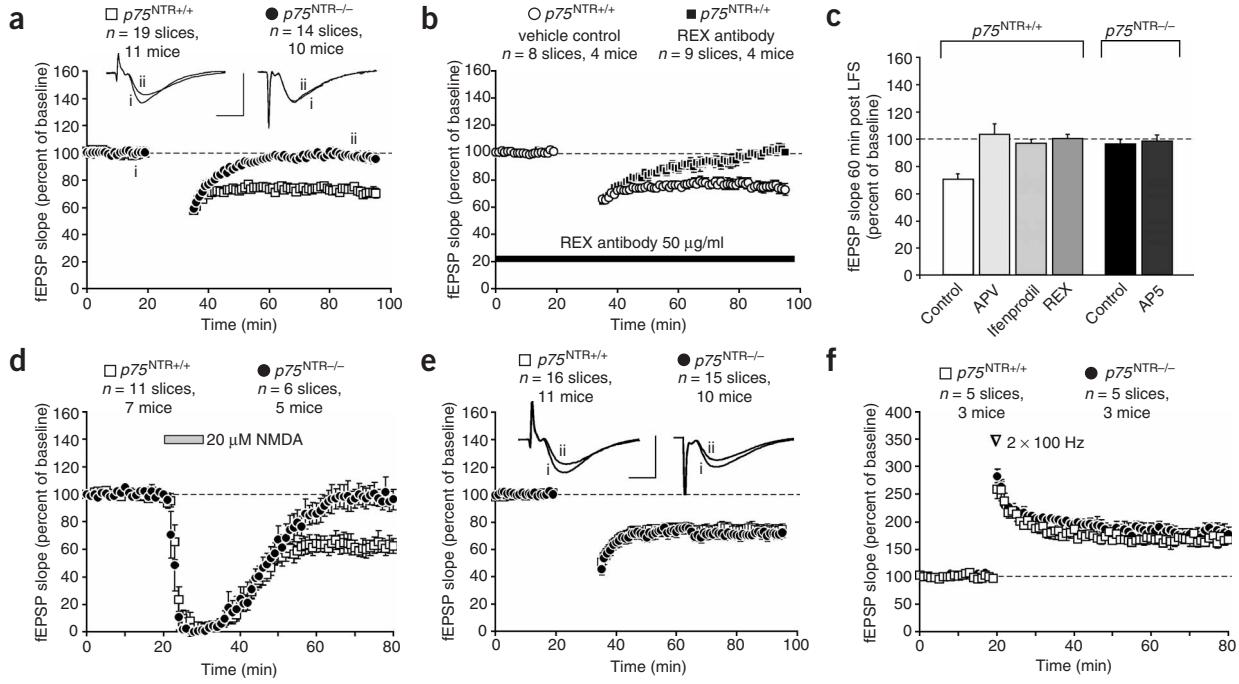

Figure 2 Selective deficit for NMDA receptor–dependent LTD in $p75^{NTR-/-}$ mice. (**a**) Lack of LTD induced by LFS in $p75^{NTR-/-}$ synapses. LFS (1 Hz, 15 min) was applied to the Schaeffer collaterals. Sample traces are shown at indicated time points labeled 'i' and 'ii'. Scale bars, 5 mV and 5 ms. (**b**) Blockade of LTD in $p75^{NTR+/+}$ mice by REX antibody. Hippocampal slices were incubated in REX during recovery and was also present throughout the experiment as indicated. (**c**) Summary bar graph depicting the lack of NMDA receptor–mediated LTD in $p75^{NTR-/-}$ animals. (**d**) Lack of LTD induced by NMDA perfusion in $p75^{NTR-/-}$ slices. NMDA was applied to slices, as indicated by the gray bar. (**e**) Intact NMDA receptor–independent LTD in $p75^{NTR-/-}$ slices. Paired stimulation (50 ms interval) was applied to the Schaeffer collaterals at low frequency (1 Hz, 15 min). (**f**) Normal LTP in $p75^{NTR-/-}$ slices. Two trains of HFS (100 Hz, 1 s) were applied. Error bars, s.e.m.

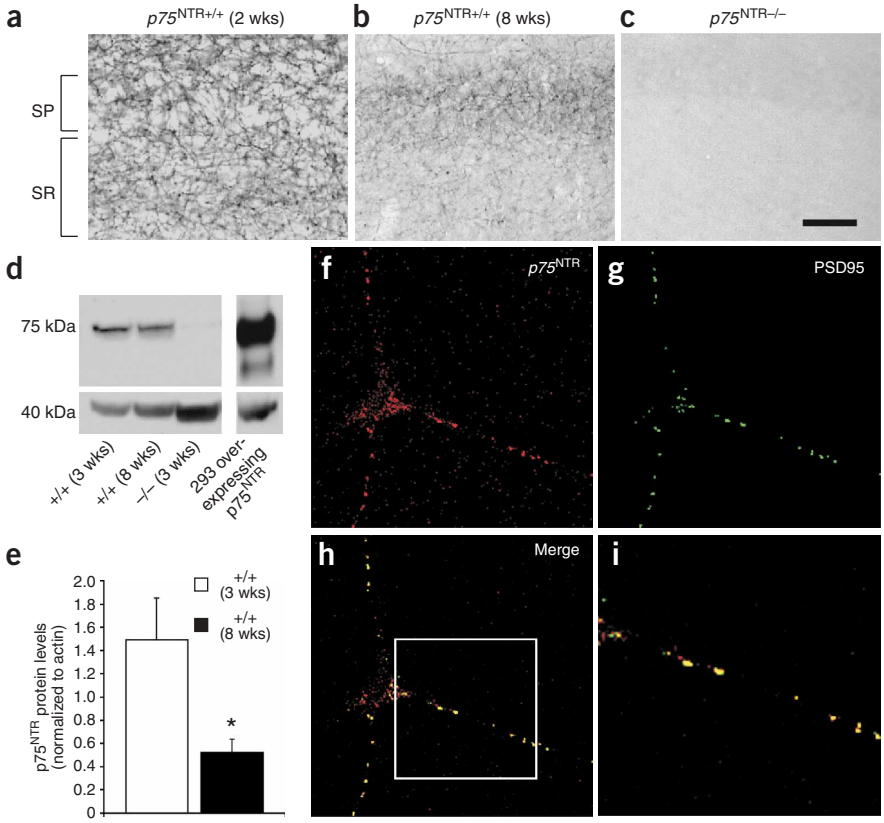

Figure 3 Localization of p75^{NTR} immunoreactivity in mouse hippocampus. (**a**,**b**) By light microscopy, we found p75^{NTR} immunoreactivity in fine, varicose fibers that were dense near stratum pyramidale (SP) and scattered throughout stratum radiatum (SR) of the hippocampal CA1 region. The number of p75^{NTR}-immunoreactive processes seemed greater in the hippocampi of 2-week-old than 8-week-old *p75*$^{NTR+/+}$ mice. (**c**) p75^{NTR} immunoreactivity was absent in the hippocampi of *p75*$^{NTR-/-}$ mice. All photographs from sections labeled with the R & D antibody. Scale bar, 100 μm. (**d**) Western blot showing the levels for p75^{NTR} protein. Measurements of protein levels were taken from hippocampal lysates prepared from young *p75*$^{NTR+/+}$ (3 weeks old), mature *p75*$^{NTR+/+}$ (8 weeks old) and *p75*$^{NTR-/-}$ (3 weeks old) mice. We observed an age-dependent increase in p75^{NTR} protein levels, with young mice having higher levels than the mature mice. p75^{NTR} protein was absent in *p75*$^{NTR-/-}$ mice. (**e**) Quantification of p75^{NTR} protein levels normalized to an actin control. $^*P < 0.05$. (**f–i**) Expression of p75^{NTR} in dendrites of hippocampal cultured neurons. Punctate staining of (**f**) p75^{NTR} and (**g**) PSD95 was observed in cultured hippocampal neurons (day 14) prepared from E16 mice and were often colocalized in the soma and dendrites (**h**,**i**). Error bars, s.e.m.

p75$^{NTR-/-}$ mice lack NMDA receptor–dependent LTD

To test the hypothesis that p75^{NTR} may have an opposing role to TrkB in LTP, we monitored the induction of LTD by applying a prolonged train of low-frequency stimulation (LFS; 1 Hz, 15 min) to hippocampal slices from juvenile *p75*$^{NTR+/+}$ and *p75*$^{NTR-/-}$ mice (3–4 weeks old). LFS applied to the Schaeffer collaterals elicited robust LTD in slices derived from *p75*$^{NTR+/+}$ mice (**Fig. 2a**). In marked contrast, slices from aged-matched *p75*$^{NTR-/-}$ mice did not show LTD. LFS induced an initial decrease in fEPSP slopes, which subsequently recovered back to baseline (**Fig. 2a**). Immediately after LFS, mean fEPSP slopes were 58 ± 2% and 65 ± 4% ($P > 0.5$), whereas those 60 min after LFS were 70 ± 4% and 95 ± 3% ($P < 0.05$) for *p75*$^{NTR+/+}$ and *p75*$^{NTR-/-}$ mice, respectively. To test whether p75^{NTR} has an indispensable role in LTD under genetically unmodified conditions, hippocampal slices from *p75*$^{NTR+/+}$ mice were perfused with REX function-blocking antibody, a reagent known to block *p75*^{NTR} function[29]. In slices that were incubated in REX antibody (50 μg/ml, 2 h), LFS did not induce LTD (**Fig. 2b**). In both genotypes, LFS-induced LTD was completely blocked by the general antagonist of NMDA receptor, AP5, suggesting that this form of LTD depends on the NMDA receptor (**Fig. 2c** and **Supplementary Fig. 1** online). Consistent with previous reports[26,27], application of ifenprodil (10 μM), an antagonist of NR2B involved in LTD, selectively blocked this form of LTD without affecting LTP in slices prepared from *p75*$^{NTR+/+}$ mice (**Fig. 2c** and **Supplementary Fig. 1**).

The NMDA receptor–dependent form of LTD can also be induced by bath application of NMDA. As in the case of LFS-induced LTD, LTD elicited by brief perfusion of NMDA was also impaired in juvenile *p75*$^{NTR-/-}$ mice (**Fig. 2d**). Application of NMDA (20 μM) for a period of 10 min induced LTD that lasted for the duration of the recording in *p75*$^{NTR+/+}$ slices, and this LTD was not observed in *p75*$^{NTR-/-}$ slices (**Fig. 2d**). In both genotypes, complete suppression of synaptic transmission occurred shortly after NMDA application. But fEPSP slopes slowly recovered to baseline values of 96 ± 7% in *p75*$^{NTR-/-}$ slices, whereas in *p75*$^{NTR+/+}$ slices the response remained depressed at a mean value of 62 ± 5% ($P < 0.01$). Coapplication of AP5 with NMDA blocked this form of LTD (data not shown).

In addition to NMDA receptor–dependent LTD, the young hippocampus expresses an NMDA receptor–independent form of LTD, which is induced by a twin-pulse LFS protocol[30,31]. Notably, the NMDA receptor–independent form of LTD was not altered in young juvenile *p75*$^{NTR-/-}$ mice. A 15-min train of twin pulses (50 ms apart) at 1 Hz elicited a robust and long-lasting depression in both genotypes (**Fig. 2e**). The mean fEPSP slope values recorded 60 min after the application of this protocol were 74 ± 4% and 72 ± 3% in wild-type and *p75*$^{NTR-/-}$ slices ($P > 0.2$), respectively. In agreement with previous studies[32], this twin-pulse LFS protocol induced a form of LTD that was independent of NMDA receptor activation. Perfusion of 50 μM AP5, which blocked the expression of LTD induced by 1 Hz, 15 min (**Fig. 2c**), did not affect the expression of LTD elicited by twin-pulse LFS stimulation (**Supplementary Fig. 1**).

Finally, we examined whether *p75*$^{NTR-/-}$ regulates NMDA receptor–dependent LTP. Application of tetanic stimulation (100 Hz, 1 s, 2 trains separated by 20 s) induced almost identical LTP in slices from *p75*$^{NTR+/+}$ and *p75*$^{NTR-/-}$ mice, suggesting that deletion of *p75*^{NTR} does not affect either induction or expression of LTP (**Fig. 2f**). Immediately after tetanus, the mean fEPSP slopes were 259 ± 17% and 283 ± 12% ($P > 0.5$) in *p75*$^{NTR+/+}$ and *p75*$^{NTR-/-}$ mice, respectively. At 60 min after HFS, fEPSP slopes remained potentiated above baseline with mean values of 167 ± 6% and 177 ± 14% ($P > 0.5$) for wild-type and *p75*$^{NTR-/-}$ mice, respectively. These results show a

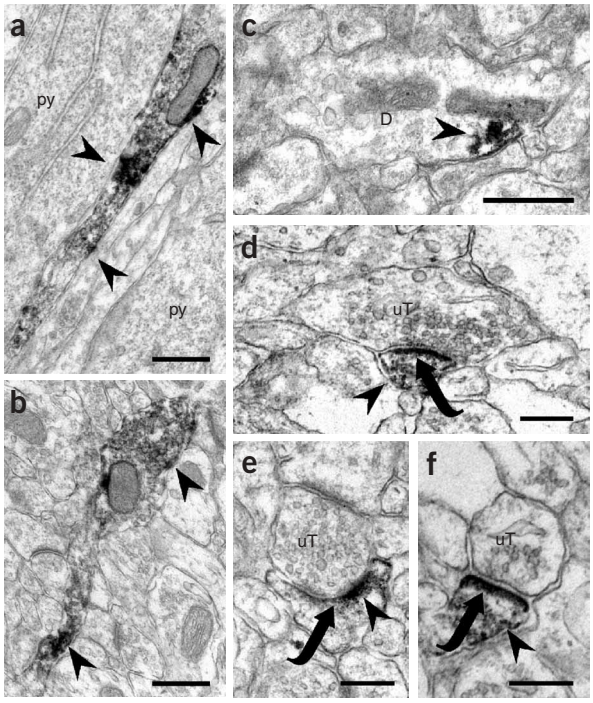

Figure 4 Electron microscopic localization of p75^{NTR} immunoreactivity in CA1 region of the *p75*$^{NTR+/+}$ mouse. (**a**) Within stratum pyramidale, we found p75^{NTR} immunoreactivity (arrowhead) in an axon terminal that contained numerous small synaptic vesicles and abuted a pyramidal cell perikaryon (py). (**b**) A p75^{NTR}-immunoreactive axon terminal with a preterminal axon was found in the neuropil of stratum radiatum. (**c**) An aggregate of p75NTR immunoreactivity (arrowhead) was found near the plasma membrane of a dendritic shaft (D). (**d–f**) Within stratum radiatum, p75^{NTR} immunoreactivity was detected in dendritic spines that received contacts (curved arrows) from unlabeled terminals (uT). (**e**) p75^{NTR} immunoreactivity was denser near the synapse. Scale bars for **a–c**, 500 nm. Scale bars for **d–f**, 250 nm.

selective role for p75^{NTR} in one form of hippocampal synaptic plasticity: the NMDA receptor–dependent form of LTD.

Expression of p75^{NTR} in the mouse hippocampus

To assess whether p75^{NTR} could directly mediate these effects, we sought to localize p75^{NTR} immunoreactivity in the mouse hippocampus, using an antibody that detects the extracellular domain of p75^{NTR}. Light microscopic analysis of sections showed p75^{NTR} immunoreactivity in very fine varicose fibers throughout the hippocampus (**Fig. 3a**,**b**), which was absent in the *p75*$^{NTR-/-}$ mouse (**Fig. 3c**). Within the CA1 region, p75^{NTR}-immunoreactive fibers were concentrated near stratum pyramidale and were more evenly dispersed in strata oriens and radiatum. Side-by-side comparison showed that sections of hippocampi from young juvenile mice (postnatal day (P) 14; **Fig. 3a**) seemed to express greater p75^{NTR} immunoreactivity than did older juvenile mice (P60; **Fig. 3b**). Similar results were obtained using an antibody against the intracellular domain of p75^{NTR} (data not shown). Western blot analysis confirmed that p75^{NTR} expression levels are inversely related to age (**Fig. 3d**). Quantitative analysis indicated that younger mice (P21) had significantly higher p75^{NTR} receptor levels than did older mice (P60; **Fig. 3e**). These results are consistent with the observation that robust LTD is induced more readily in younger juvenile hippocampus[33,34]. To determine the subcellular localization of p75^{NTR}, we performed double staining on mouse hippocampal neurons derived from embryonic day 16 (E16) wild-type mice, using antibodies against p75^{NTR} and a dendritic spine marker PSD95. After 14 d *in vitro*, we observed p75^{NTR} immunoreactivity as punctate staining around the cell body as well as dendrites (**Fig. 3f**), with distribution very similar to that of PSD95 immunoreactivity (**Fig. 3g**). The majority of p75^{NTR} immunoreactivity was colocalized with PSD95 immunoreactivity in the dendrites of hippocampal neurons, suggesting their localization in spines (**Fig. 3h**,**i**).

To firmly establish that p75^{NTR} is localized at CA1 synapses, we performed immunoelectron microscopy in stratum radiatum of the hippocampal CA1 region. In agreement with a previous study in adult rats[35], we observed p75^{NTR} immunoreactivity in axons and axon terminals (**Fig. 4a**,**b**). Axon terminals (0.4–0.6 μm in diameter) contained numerous small synaptic vesicles and often abutted pyramidal cell perikarya (**Fig. 4a**). But many CA1 pyramidal neurons also expressed p75^{NTR} immunoreactivity. We observed the immunoreactive profiles in dendritic shafts (**Fig. 4c**), spines (**Fig. 4d–f**) and glial profiles (data not shown). Within dendritic profiles, p75^{NTR} immunoreactivity was usually aggregated near the plasma membrane, and was denser in the spine head near the synaptic specialization. Additional examples of p75^{NTR}-labeled dendritic spines are shown in **Supplementary Figure 2**. Labeling of axon, terminal and spine profiles was absent in the *p75*$^{NTR-/-}$ mice. Taken together, our immunoelectron microscopy experiments raise the possibility that activation of p75^{NTR} on the CA1

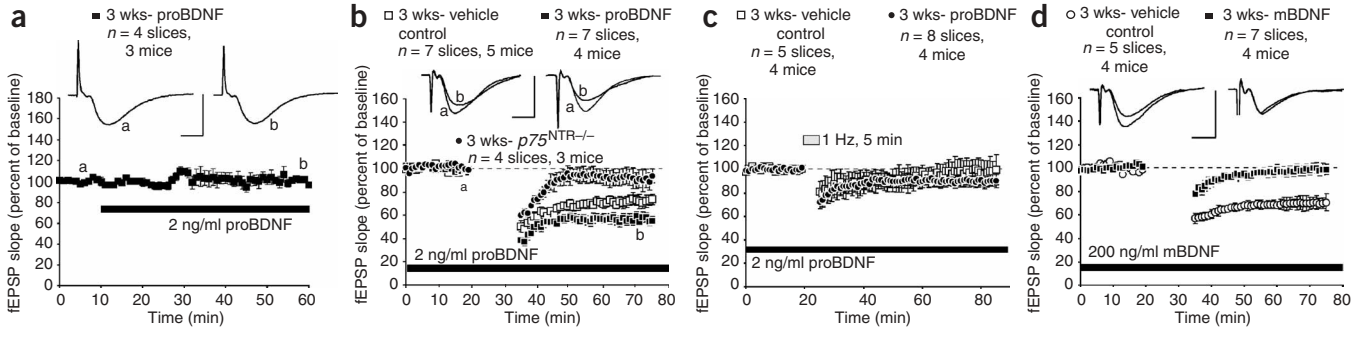

Figure 5 Enhancement of NMDA receptor–dependent LTD by proBDNF. (**a**) Lack of effect on basal transmission. Application of proBDNF, a ligand for p75^{NTR}, did not induce synaptic depression. (**b**) Effect of proBDNF on the expression of NMDA receptor–dependent LTD. Hippocampal slices were incubated with purified, cleavage-resistant proBDNF for at least 1 h before recording. proBDNF enhanced LTD in young *p75*$^{NTR+/+}$ hippocampal slices as compared with vehicle control, but not in *p75*$^{NTR-/-}$ slices. (**c**) Pairing proBDNF with a weak LFS protocol did not induce LTD. (**d**) Opposing actions of mBDNF on LTD. Incubation of mBDNF inhibited the expression of LTD. Error bars, s.e.m.

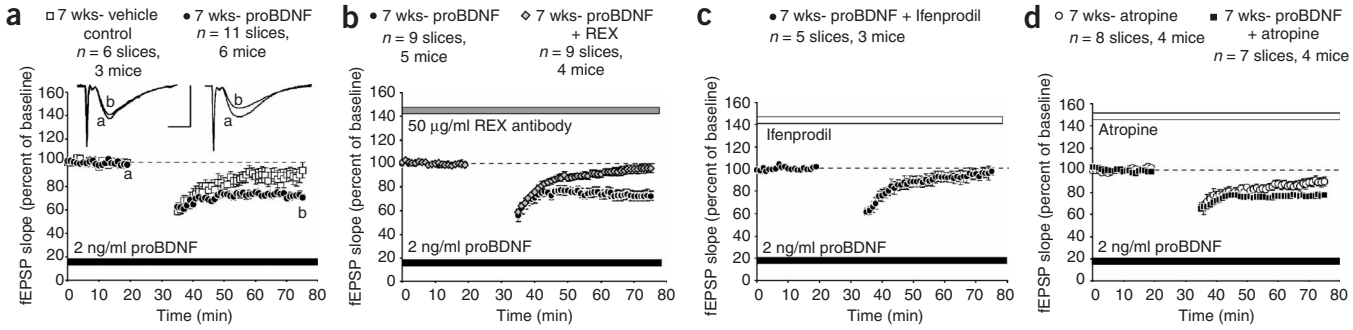

Figure 6 Enhancement of hippocampal LTD by proBNDF is mediated by p75^{NTR} and NR2B. (**a**) Pronounced enhancement of LTD by proBDNF in mature mice (7–8 weeks old). Hippocampal slices derived from older mice that normally expressed attenuated levels of LTD showed robust LTD in the presence of proBDNF. (**b**,**c**) Inhibition of proBDNF-mediated enhancement of LTD by blocking p75^{NTR} or NR2B function. (**b**) Incubation of hippocampal slices in REX (50 μg/ml) blocked the effects of proBDNF. (**c**) Ifenprodil blocked the enhancement of LTD normally observed with proBDNF. (**d**) Facilitation of LTD by proBDNF is not dependent on the cholinergic system. Atropine (5 μM) did not affect LTD or the enhancement of LTD by proBDNF. Error bars, s.e.m.

pyramidal-cell dendrites or their afferents contributes to the expression of NMDA receptor–dependent LTD.

Facilitation of NMDA receptor–dependent LTD by proBDNF

Because proBDNF is a high-affinity ligand for p75^{NTR} that can be secreted from hippocampal neurons[22], we tested whether activation of p75^{NTR} by proBDNF regulates NMDA-dependent LTD. Perfusion of a cleavage-resistant form of proBDNF (2 ng/ml) to hippocampal slices had no effect on basal synaptic transmission recorded over an extended period of time (**Fig. 5a**). Next, we treated the hippocampal slices from *p75*$^{NTR+/+}$ mice with proBDNF for more than 1 h before inducing LTD. In young juvenile slices that already showed pronounced LTD, treatment with proBDNF, but not vehicle, resulted in a small but notable increase in LTD (**Fig. 5b**). The mean values of fEPSP slopes 60 min after the application of LFS for vehicle- and proBDNF- treated slices were 70 ± 7%, and 56 ± 3%, respectively. Moreover, application of proBDNF to slices from *p75*$^{NTR-/-}$ mice elicited little effect, suggesting that proBDNF facilitated LTD through activation of p75^{NTR} (**Fig. 5b**; fEPSP slope at 60 min after LFS, 96 ± 8%). Application of proBDNF-containing perfusates collected at the end of several hippocampal slice experiments, but not control perfusates, to cultured neuromuscular synapses resulted in a marked reduction in synaptic transmission. This suggests that proBDNF in the slice-recording chamber remains intact and biologically active for many hours (data not shown). To determine whether proBDNF reduced the threshold of LTD induction, we stimulated hippocampal slices using a weaker stimulation protocol (1 Hz, 5 min) in the presence of proBDNF. This protocol did not induce LTD in vehicle-treated slices, and treatment with proBDNF had little effect (**Fig. 5c**; fEPSP slope at 60 min in control and proBDNF-treated slices were 100 ± 13% and 90 ± 5%, respectively). In contrast to proBDNF, incubation of hippocampal slices with mBDNF attenuated LTD expression (**Fig. 5d**). This is consistent with a previous study that examined the modulation of hippocampal LTD by mBDNF[36].

In older juvenile slices in which LTD is attenuated, incubation of slices in proBDNF elicited a stronger facilitatory effect on LTD than it did in young juvenile slices (**Fig. 6a**). The mean values of fEPSPs 60 min after LFS were 94 ± 7% and 71 ± 2% for slices that received vehicle control and those that received proBDNF, respectively. Moreover, pretreatment with the p75^{NTR}-blocking REX antibody completely prevented the effect of proBDNF on LFS-induced LTD (**Fig. 6b**), indicating the effect of proBDNF is mediated by p75^{NTR}. In slices treated with proBDNF and the NR2B antagonist ifenprodil

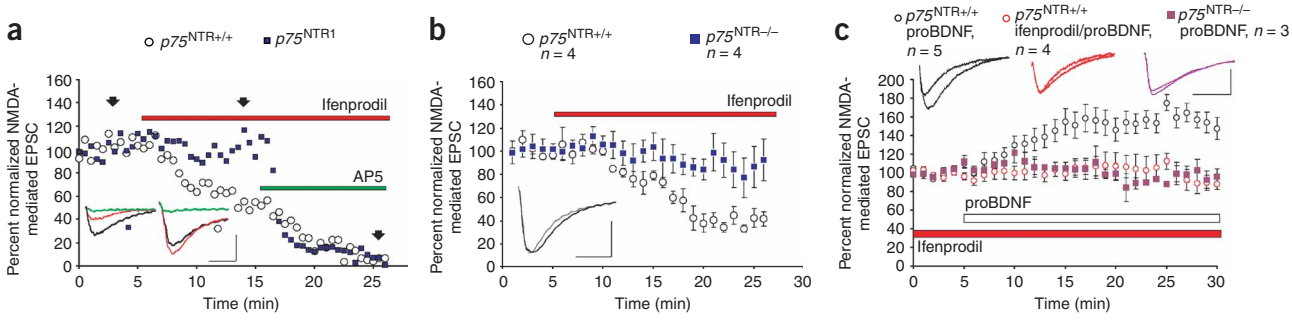

Figure 7 Relationship between p75^{NTR} and NR2B at hippocampal CA1 synapse. (**a**) Reduced expression of NR2B-mediated synaptic currents in *p75*$^{NTR-/-}$ mice. Evoked whole-cell responses were recorded from CA1 pyramidal neurons in the presence of AHPA receptor antagonist DNQX and GABA receptor antagonist bicuculline. Application of ifenprodil selectively decreased NMDA-mediated synaptic currents recorded from *p75*$^{NTR+/+}$ but not in *p75*$^{NTR-/-}$ hippocampal slices. Arrowheads indicate timepoints of sample NMDA-mediated current traces during baseline (black), after ifenprodil application (red) and after final AP5 application (green), which verified that evoked synaptic currents were mediated by NMDA receptors. (**b**) Summary plot depicting the sensitivity of NMDA currents to ifenprodil is dependent on p75^{NTR}. Normalized averaged traces depicted a slower decay rate of NMDA receptor–mediated EPSC in *p75*$^{NTR+/+}$ than *p75*$^{NTR-/-}$ mice. (**c**) Recruitment of NR2B by proBDNF-p75^{NTR}. Application of proBDNF rapidly enhanced NMDA receptor–mediated currents in slices derived from *p75*$^{NTR+/+}$ but not in *p75*$^{NTR-/-}$ animals. In the presence of ifenprodil, the potentiation of NMDA receptor currents by proBDNF was inhibited. Scale bars, 50 pA and 40 ms. Error bars, s.e.m.

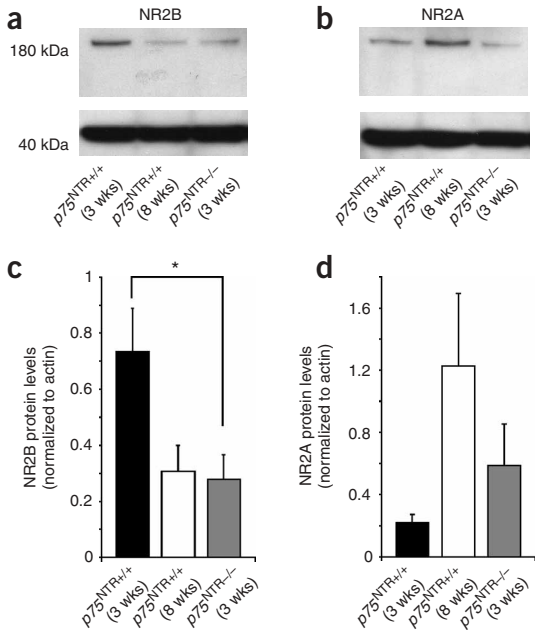

Figure 8 Attenuated levels of NR2B expression in $p75^{NTR-/-}$ mice. (**a**,**b**) Western blot showing the levels for NR2B and NR2A protein. NR2B levels were substantially higher in hippocampal tissues taken from young animals than in older animals. Furthermore, NR2B probed in young $p75^{NTR-/-}$ tissue samples showed significantly lower levels ($P < 0.05$). (**c**,**d**) Quantification of protein levels of NR2B and NR2A normalized to an actin control. Error bars, s.e.m.

(10 μM), LFS no longer induced LTD (**Fig. 6c**), suggesting that proBDNF regulates NR2B-mediated LTD. Because a previous report showed that application of carbachol facilitates LTD[37], we tested whether proBDNF enhances LTD by regulating cholinergic inputs to the hippocampus. In the presence of the muscuranic receptor antagonist atropine (5 μM), proBDNF still facilitated LTD (**Fig. 6d**). Thus, it is less likely that proBDNF enhanced LTD by regulating cholinergic inputs. Finally, proBDNF selectively regulated LTD without affecting LTP. When hippocampal slices were incubated with proBDNF and subsequently subjected to two trains of HFS, LTP was readily induced and was not different from slices that were only incubated with the vehicle control (**Supplementary Fig. 3**). In addition, proBDNF also had no effect on late-phase LTP, induced either with long theta burst stimulation (**Supplementary Fig. 3**) or four trains of tetanic stimulation (4× 100 Hz, 1 s; data not shown). These results suggest that the proBDNF selectively facilitates hippocampal LTD through the activation of $p75^{NTR}$.

Regulation of NR2B expression by proBDNF-$p75^{NTR}$

An emerging concept in the field of hippocampal plasticity is that LTP and LTD are differentially controlled by two different subtypes of NMDA receptors: NR2A and NR2B, respectively[26,27]. Given that the NMDA-dependent LTD, but not LTP, was selectively impaired in the $p75^{NTR-/-}$ mice, we tested whether proBDNF-$p75^{NTR}$ regulated specific subtypes of NMDA receptors by performing whole-cell voltage-clamp recordings of CA1 pyramidal neurons. Resting membrane potential and input resistance of CA1 neurons were not different between $p75^{NTR-/-}$ and $p75^{NTR+/+}$ mice (data not shown). We stimulated Schaffer collaterals in the presence of AMPA and GABA receptor antagonists to induce synaptic NMDA currents. We observed a marked difference in the subunit composition of NMDA receptors between the two genotypes. Application of ifenprodil at a concentration that blocked LTD expression reduced isolated synaptic NMDA currents by more than 50% in slices derived from $p75^{NTR+/+}$ young juvenile mice (**Fig. 7a**,**b**). In contrast, application of ifenprodil had little or no effect on synaptic NMDA currents in age-matched $p75^{NTR-/-}$ slices (**Fig. 7a**,**b**). When we applied a high concentration of AP5, a general antagonist for all subtypes of NMDA receptors, the NMDA-mediated excitatory postsynaptic currents (EPSCs) were eliminated in both $p75^{NTR+/+}$ and $p75^{NTR-/-}$ slices (**Fig. 7a**). Thus, although the NR2B-containing NMDA receptor accounts for a substantial proportion of total NMDA channels at the $p75^{NTR+/+}$ CA1 synapses, which is made evident by the ifenprodil sensitivity and by the slower decay rate of the NMDA receptor–mediated EPSC (**Fig. 7b**), $p75^{NTR-/-}$ mice primarily express NMDA receptors that contain subunits other than NR2B.

To investigate whether proBDNF directly regulates NR2B-mediated synaptic currents, we applied proBDNF to hippocampal slices. Such application markedly potentiated the NMDA currents at the CA1 synapses (**Fig. 7c**). The mean amplitude of the NMDA current after proBDNF treatment was 147 ± 12% when normalized to the baseline. In two extended recordings, the amplitude of the NMDA currents returned to near baseline levels after application of ifenprodil (data not shown). When the slices were pretreated with ifenprodil, application of proBDNF no longer potentiated the NMDA currents (**Fig. 7c**). Similarly, proBDNF did not enhance NMDA currents when applied to slices derived from $p75^{NTR-/-}$ mice. These results suggest that proBDNF through $p75^{NTR}$ selectively potentiates the NR2B component of the NMDA currents.

We next examined the changes in the expression of NMDA receptor subtypes by immunohistochemistry and western blot analysis. Immunostaining showed that in the CA1 area, the NR2B staining of soma and apical dendrites seemed to be reduced in sections derived from young juvenile $p75^{NTR-/-}$ mice as compared with slices from $p75^{NTR+/+}$ mice of the same age (**Supplementary Fig. 4**). Older juvenile $p75^{NTR+/+}$ mice also seemed to show lower levels of NR2B immunoreactivity (**Supplementary Fig. 4**). Western blots confirmed this developmental decrease of NR2B, paralleling the developmental decrease in LTD expression (**Fig. 8a**). As a comparison, we also probed NR2A expression and observed that it was higher in younger juvenile (3-week-old) than in older juvenile (8-week-old) mice (**Fig. 8b**). Quantitative analysis showed the level of NR2B in $p75^{NTR-/-}$ hippocampus was significantly lower than that in wild-type hippocampus (**Fig. 8c**). But there was no difference in the level of NR2A protein between $p75^{NTR+/+}$ and $p75^{NTR-/-}$ mice (**Fig. 8d**; $P > 0.5$). These results suggest that $p75^{NTR}$ mutation selectively alters the expression of NR2B-containing NMDA receptors in hippocampal CA1.

DISCUSSION

Recent studies suggest that pro-neurotrophins serve as signaling molecules rather than inactive precursors. Several studies have indicated that proBDNF, rather than mBDNF, is the major form secreted from CNS neurons[8,22,23]. But whether proBDNF has any biological function in synaptic plasticity remains unknown. The fact that $p75^{NTR-/-}$ mice have impairments in several learning and memory tasks[38,39] and findings in a recent study showing a lack of LTD[40] suggest that proBDNF may have a role in hippocampal synaptic plasticity. Here we provide evidence that proBDNF, signaling through $p75^{NTR}$, is important for NMDA receptor–dependent LTD in the hippocampus.

Our electrophysiology experiments showed that deletion of *p75*^{NTR} in mice selectively impaired the NMDA receptor–dependent form of LTD, without affecting other forms of synaptic plasticity tested. As p75^{NTR} is known to interact with Nogo66, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein, pro–nerve growth factor (proNGF) and proBDNF[41], it is important to identify the endogenous ligand that activates p75^{NTR} under physiological conditions. Here, application of the p75^{NTR}-blocking antibody REX virtually abolished LTD in wild-type mice, probably by inhibiting the interaction between endogenous proBDNF with p75^{NTR}. Moreover, application of proBDNF to hippocampal slices enhanced LTD. Such potentiating effects were absent in *p75*$^{NTR-/-}$ mice, and were prevented by the REX antibody. Thus, we have identified proBDNF as an endogenous ligand that activates p75^{NTR} to promote LTD.

Growing evidence now supports the notion that LTP and LTD in the hippocampus are mediated by the NR2A and NR2B subunits of NMDA receptor, respectively[26,27]. We have shown that deletion of p75^{NTR} selectively impaired, and proBDNF selectively enhanced, the NMDA receptor–dependent form of LTD. Neither p75^{NTR} mutation nor proBDNF affected the NMDA receptor–dependent LTP. In parallel, the *p75*$^{NTR-/-}$ mice showed not only a selective decrease in NR2B (but not NR2A) expression in the hippocampus, but also a marked reduction in NR2B-mediated currents at the CA1 synapse. Notably, application of proBDNF potentiated the NR2B-mediated currents at CA1 synapses, suggesting that activation of p75^{NTR} by proBDNF increased NR2B at the synapses. The enhancement of LTD by proBDNF was prevented by the NR2B antagonist ifenprodil. These results provide strong support for the idea that endogenously secreted proBDNF, by acting on p75^{NTR}, promotes NMDA-dependent LTD by enhancing the expression of NR2B at CA1 synapses. To fully establish a causal link, however, it must still be shown that expression of NR2B in the hippocampus rescues the LTD impairment in *p75*$^{NTR-/-}$ slices.

Exactly how proBDNF signals through p75^{NTR} to regulate NR2B remains to be established. Immunostaining and western blotting experiments showed a substantial reduction in NR2B, but not NR2A, throughout the hippocampus of the *p75*$^{NTR-/-}$ mice, suggesting that proBDNF-p75^{NTR} controls the expression of NR2B. This could possibly be achieved through activation of c-Jun kinase[42] or NF-κB[43], which are both expressed in hippocampal neurons. On the other hand, application of exogenous proBDNF enhanced NR2B-mediated synaptic currents in a p75^{NTR}-dependent manner. Because the increase in NR2B currents occurred fairly rapidly after proBDNF application, this effect is less likely to be the result of p75^{NTR}-mediated transcription and translation. It is possible that proBDNF facilitates the membrane insertion, trafficking into synapses or the channel properties of NR2B, all of which could, in theory, enhance LTD. Further investigation is necessary to distinguish these possibilities.

Another finding that sheds light on the mechanisms of proBDNF regulation of LTD is the localization of p75^{NTR} in CA1 synapses. It has long been believed that p75^{NTR} protein in the hippocampus is primarily derived from basal forebrain cholinergic afferents[44,45]. It is possible that proBDNF is acting at p75^{NTR} on the cholinergic afferents to induce the release of acetylcholine, which would also facilitate LTD[37]. But we have shown that atropine, a cholinergic antagonist, did not significantly alter the effect of proBDNF on LTD. Rather than an indirect mechanism, the enhancement of LTD may be the result of proBDNF directly binding to p75^{NTR} expressed by CA1 hippocampal neurons. In support of this notion, immunohistochemistry at light and electron microscopic levels showed robust p75^{NTR} expression in CA1 dendritic spines in addition to the afferent terminals in the hippocampus. Moreover, double staining conducted on cultured hippocampal neurons showed that p75^{NTR} is colocalized with PSD95, a marker for postsynaptic density often localized in dendritic spines. Collectively, these results raise the possibility that proBDNF regulates hippocampal LTD by acting directly on p75^{NTR} in the dendritic spines of CA1 neurons.

Our study has several important implications. First, we have identified a new and specific function for proBDNF-p75^{NTR} signaling in synaptic plasticity, which is in marked contrast to the conventional view of pro-neurotrophins and p75^{NTR} in regulating neuronal apoptosis. Second, we have provided some insights into the mechanisms by which proBDNF controls hippocampal LTD. Our results suggest that proBDNF is an endogenous ligand for p75^{NTR} receptors, which are localized on CA1 dendritic shafts and spines. Moreover, proBDNF promotes hippocampal LTD by regulating the expression of the NR2B subunit. Finally, our study suggests a bidirectional control of hippocampal plasticity by BDNF. Our recent work showed that tissue plasminogen activator, by activating the extracellular protease plasmin, converts the precursor proBDNF to the mBDNF, and such conversion is required for late-phase LTP in the hippocampus[25]. We now show that proBDNF, if not cleaved, enhances hippocampal LTD. These results support a provocative 'yin-yang' hypothesis[46]: uncleaved proBDNF enhances LTD through p75^{NTR}, whereas activation of TrkB by mBDNF facilitates LTP and also inhibits LTD. Thus, proteolysis of the precursor form of BDNF becomes an important control mechanism for the direction of hippocampal plasticity.

METHODS

Mice. *p75*$^{NTR-/-}$ mice, as characterized previously[47], were backcrossed onto a C57BL/6 background for more than five generations. All animal procedures conformed to the US National Institutes of Health animal welfare guidelines.

Electrophysiological recording. We prepared transverse hippocampal slices (400 μm) from *p75*$^{NTR-/-}$ and age-matched wild-type mice that were young (3–4 weeks old) or mature (8–10 weeks old). We maintained slices in an interface chamber exposed to a humidified atmosphere of 95% O_2 and 5% CO_2. After a minimum recovery period of at least 2 h, we recorded fEPSPs using an Axoclamp-2B amplifier (Axon Instruments) with an artificial cerebral spinal fluid (ACSF; composition was as previously described[25])–filled glass microelectrode (1–3 MΩ) positioned in the stratum radiatum of hippocampal area CA1. We applied tetanic stimulation or LFS after stable baseline was established. Stimulus intensity was adjusted to evoke fEPSP approximately 40% of the maximum. We induced LTP by two trains of HFS (100 Hz, 1 s, separated by 20 s), whereas we induced LTD by either single (for NMDA receptor–dependent LTD) or twin pulses (for NMDA receptor–independent LTD, 50 ms interstimulus interval) applied at a rate of 1 Hz for 15 min.

We made whole-cell recordings from CA1 pyramidal neurons of hippocampal slices using the blind patch method. We filled recording electrodes (5–8 MΩ) with intracellular solution containing (in mM): 130 K-gluconate, 10 HEPES, 2 Mg_2ATP, 0.5 Na_3GTP, 11 EGTA, 10 phosphocreatine and 5 QX-314. We evoked EPSCs at a rate of 0.05 Hz by a placing a stimulating electrode in the stratum radiatum. We monitored access resistance throughout experiments, and it ranged from 10–25 MΩ. We discarded data when access resistance changed by >20% during an experiment. To isolate NMDA-mediated EPSCs, we voltage clamped neurons at –50 mV in the presence of 20 μM DNQX and 10 μM bicuculline methiodide. We recorded signals using an Axopatch 200B, filtered them at 5 kHz and digitized them at 10 kHz.

We initially prepared NMDA (Tocris) and the antagonists atropine (Sigma), ifenprodil (Sigma), AP5 (Sigma), NMDA (Sigma), DNQX (Sigma) and bicuculline (Sigma) as concentrated stock solutions and diluted them with ACSF to the specified concentrations before each experiment. We added 1% BSA to proBDNF[25] before perfusion. The vehicle control consisted of 1% BSA and the proteinase inhibitors. We incubated hippocampal slices with either mBDNF or proBDNF for at least 1 h before experiments.

p75^{NTR} immunohistochemistry and electron microscopy. We deeply anesthetized mice ($n = 5$) and perfused them through the ascending aorta sequentially with 1,000 units/ml heparin in normal saline followed by 40 ml of a mixture of 3.75% acrolein and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). We removed brains and postfixed them for 30 min, and then cut them into 40 μm sections using a Vibratome and collected them in phosphate buffer. We transferred free-floating sections to 1% sodium borohydride for 30 min and washed them extensively. We then then transferred them to 0.1 M Tris-saline (TS, pH 7.6) and incubated them sequentially in: (i) 0.5% BSA, 30 min; (ii) p75-specific antibody (Promega, 1:1,000; R&D Systems, 1: 400) in 0.1% BSA and 0.25% Triton X-100, 48 h; (iii) biotinylated rabbit-specific goat antibody or goat-specific rabbit antibody (Vector Laboratories) at 1:400 in 0.1% BSA, 30 min; (iv) avidin-biotin complex (VectaStain Elite kit, 30 min); (v) diaminobenzidine and H_2O_2, 8 min. Finally, we mounted sections onto slides previously coated with 1% gelatin, dried them and dehydrated them through an alcohol series into xylenes, and put them on coverslips using DPX (Aldrich). We took photographs using a Nikon Eclipse 80i microscope and a Photometrix digital camera.

For electron microscopy, we prepared sections as previously described[48]. We counterstained ultrathin hippocampal sections containing CA1 with uranyl acetate and Reyonold lead citrate. We examined final preparations using a Tecnai Biotwin electron microscope (FEI). We identified hippocampal processes as neuronal using previously described criteria[49]. In particular, dendritic profiles contained regular microtubular arrays and were usually postsynaptic to axon terminal profiles. Terminal profiles had minimal diameters greater than 0.2 μm, contained numerous small synaptic vesicles and often contacted other neuronal profiles.

Cleavage-resistant proBDNF. We purified the histidine-tagged, cleavage-resistant proBDNF as previously described[25]. We introduced point mutations into the proconvertase cleavage site of proBDNF to convert the Arg-Arg (amino acid positions 129,130) to Ala-Ala. *In vitro* experiments showed that this proBDNF is resistant to cleavage by plasmin. We dialyzed the protein against ACSF, and stored it in small aliquots at −80°C until use.

p75^{NTR} and PSD95 immunocytochemistry in cultured neurons. We cultured mouse hippocampal neurons as previously described[50]. Briefly, we dissected hippocampi from embryonic day 16 (E16) mice, dissociated them in tyrosine solution and plated them on coverslips coated with polylysine and laminin at 5,000 cells/coverslip. We grew cells in serum-free medium Neurobasal plus B27 (Life Technologies) at 37°C, 5% CO_2 incubator. After 14 d *in vitro*, we fixed the cells in 4% paraformaldehyde, permeabilized them with 0.1% Triton X-100 and incubated them with a p75^{NTR}-specific rabbit antibody (Promega, 1:1,000 dilution), PSD95-specific mouse antibodies (1:300, Cell Signaling) in 3% BSA, followed by incubation with and Alex Fluor 488 (green for PSD95)– and Alex Fluor 546 (red for p75^{NTR})–conjugated secondary antibodies (Molecular Probes). We obtained images with a Zeiss confocal laser-scanning microscope using a 60× and a 100× (NA 1.40) objective.

NMDA receptor immunohistochemistry. We anesthetized mice and transcardially perfused them with saline, then fixed them with 4% paraformaldehyde in PBS. We removed intact brains, post-fixed them in 4% paraformaldehyde for an additional 2 h, and placed them in 30% sucrose cryoprotectant solution for 48 h. Then we embedded the brains in OCT medium and stored them at −80°C. We then sectioned frozen brains coronally at 40 μm using a Leica cryotome. For immunolabeling experiments, we blocked the prepared sections in normal serum, incubated them with NR2B-specific antibody (Chemicon) overnight at 4°C and then incubated them with biotinylated secondary antibody. Next we processed sections using an immunoperoxidase-based Vectastain system.

Western blot. We separated proteins from homogenized hippocampal tissues electrophoresis, and transferred them to PVDF membranes. After blocking using 5% BSA, we then incubated membranes with a rabbit polyclonal antibody against one of the following: NR2B (Chemicon 1:500); 9992 p75^{NTR}-specific antibody (generated in the laboratory of B.L.H.; 1:1,000); or β-actin (ABCAM; 1:10,000) followed by a peroxidase-labeled rabbit-specific goat IgG. We processed blots and developed them using a chemilumininescent substrate (ECL plus). We scanned developed blots at high resolution and analyzed them using ImageJ software gel analyzer. We used band intensity normalized to an actin loading control to assess the protein levels.

Statistical analysis. We used a Student *t*-test (two groups) or an analysis of variance (ANOVA) with a Tukey-Kramer post-test (three or more groups) for statistical comparisons of mean fEPSP slopes, with a significance level of $P < 0.05$. All values shown are mean ± s.e.m., with n = number of slices.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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