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proBDNF Negatively Regulates Neuronal Remodeling, Synaptic Transmission, and Synaptic Plasticity in Hippocampus

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SUMMARY

Experience-dependent plasticity shapes postnatal development of neural circuits, but the mechanisms that refine dendritic arbors, remodel spines, and impair synaptic activity are poorly understood. Mature brain-derived neurotrophic factor (BDNF) modulates neuronal morphology and synaptic plasticity, including long-term potentiation (LTP) via TrkB activation. BDNF is initially translated as proBDNF, which binds p75^{NTR}. In vitro, recombinant proBDNF modulates neuronal structure and alters hippocampal long-term plasticity, but the actions of endogenously expressed proBDNF are unclear. Therefore, we generated a cleavage-resistant *probdnf* knockin mouse. Our results demonstrate that proBDNF negatively regulates hippocampal dendritic complexity and spine density through p75^{NTR}. Hippocampal slices from *probdnf* mice exhibit depressed synaptic transmission, impaired LTP, and enhanced long-term depression (LTD) in area CA1. These results suggest that proBDNF acts in vivo as a biologically active factor that regulates hippocampal structure, synaptic transmission, and plasticity, effects that are distinct from those of mature BDNF.

INTRODUCTION

Cortical and hippocampal neurons have complex dendrites that are essential because they are the site for synaptic input, and reduced arborization is common in developmental disorders and neurodegenerative disease. However, mechanisms that regulate dendritic structure and function are incompletely understood. Cytoskeletal proteins (Kwon et al., 2011; Dietz et al., 2012; Iyer et al., 2012; Yanpallewar et al., 2012) and adhesion molecules (Hughes et al., 2007; Matthews et al., 2007; Lefebvre et al., 2012) clearly regulate dendritic and synaptic structure and function. However, less is known about the role of secreted ligands, although BDNF plays a critical role to modulate dendritic structure and synaptic function via TrkB (Yacoubian and Lo, 2000).

BDNF is a member of the neurotrophin family (Huang and Reichardt, 2001) and has robust effects on neuronal differentiation, synaptogenesis, and dendritic arborization, as well as synaptic transmission and plasticity (Reichardt, 2006; Bramham, 2008). The precursor of BDNF, proBDNF, is composed of an N-terminal prodomain and a C-terminal mature domain. proBDNF can be cleaved in secretory granules by proprotein convertases (Mowla et al., 1999). proBDNF can also be secreted and processed extracellularly by plasmin or by matrix metalloproteases to produce mature BDNF (Pang et al., 2004; Mizoguchi et al., 2011). Numerous studies suggest that binding of proBDNF to the p75 receptor (p75^{NTR}) and mature BDNF to the TrkB receptor have opposing effects on neuronal structure and synaptic plasticity (Woo et al., 2005; Cowansage et al., 2010; Teng et al., 2010). Thus, the relative levels of proBDNF and mature BDNF are likely to play important roles in modulating brain structure and function.

Whereas the actions of mature BDNF on hippocampal structure and synaptic plasticity are well defined (Minichiello, 2009; Orefice et al., 2013), the effects of proBDNF are less clear. Several studies suggest that proBDNF can be released from neurons. A report using hippocampal neurons from a knockin mouse expressing a C-terminal hemagglutinin (HA)-epitope-tagged BDNF (Yang et al., 2009b) used the HA tag to quantitatively detect proBDNF and mature BDNF, rather than relying on antibodies that recognize either proBDNF or mature BDNF. With this approach, it was shown that both proBDNF and mature

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BDNF were secreted upon depolarization (by increasing $[K+]_0$). A second report used electrical stimulation of hippocampal cultures and observed that proBDNF was the predominant secreted form after prolonged low-frequency stimulation (LFS; the frequency used to induce long-term depression [LTD]), whereas proBDNF and mature BDNF were released following prolonged high-frequency stimulation simulating theta rhythm (theta burst stimulation [TBS]; the frequency used to induce BDNF-dependent LTP; Nagappan et al., 2009). However, in a separate study using hippocampal neurons cultured with a $GABA_A$ receptor antagonist, mature BDNF was the predominant form (Matsumoto et al., 2008).

Effects of endogenously expressed proBDNF on hippocampal neurons have been inferred from studies using recombinant proBDNF protein. Treatment of cultured neurons with proBDNF elicits apoptosis and process retraction mediated by $p75^{NTR}$ (Teng et al., 2005; Je et al., 2012; Sun et al., 2012). In hippocampal area CA1, recombinant proBDNF enhanced LTD (Woo et al., 2005). In contrast, mature BDNF is required for maintenance of LTP induced by TBS (TBS-LTP; Kang et al., 1997; Korte et al., 1998; Chen et al., 1999). At neuromuscular synapses, recombinant proBDNF negatively regulates activity via $p75^{NTR}$ (Yang et al., 2009a).

Collectively, these studies suggest that proBDNF opposes the actions of mature BDNF on LTP. However, this is based on acute delivery of recombinant proBDNF, which fails to address whether proBDNF expressed by its endogenous promotor can elicit similar effects. Another issue that is unresolved is the relative levels of the two BDNF isoforms during postnatal hippocampal development. One study indicated that hippocampal proBDNF expression is highest in the second postnatal week, as quantitated using a tagged *bdnf* allele (Yang et al., 2009b). Like proBDNF, $p75^{NTR}$ levels are highest in early postnatal life and diminish in adulthood (Yang et al., 2009b). Therefore, the effects of endogenous proBDNF may be most relevant in early postnatal life. However, other studies suggest that mature BDNF is the predominant isoform at all ages (Rauskolb et al., 2010).

For these reasons, we generated a knockin mouse in which the proconvertase/furin cleavage site of BDNF was mutated and expressed under the control of endogenous BDNF promoter elements. Detection of the mutant allele was facilitated by inclusion of an HA epitope in the C terminus of proBDNF. To maintain embryo viability, only one *bdnf* allele was targeted (*probdnf-HA*/+), which results in overexpression of proBDNF and haploinsufficiency of the nontargeted *bdnf* allele. Therefore, *probdnf-HA*/+ mice were compared to BDNF haploinsufficient mice (*bdnf*+/−) to identify distinct, gain-of-function actions of proBDNF.

Here, we demonstrate localization of cleavage-resistant proBDNF is comparable to previous studies examining mature BDNF. We also show that proBDNF is released from cultured neurons and acts as a biologically active factor that alters dendritic arborization and spine density of hippocampal neurons in vivo. By comparing the structural defects of *probdnf-HA*/+ mice to *bdnf*+/− mice, we provide evidence that proBDNF reduces dendritic complexity in a $p75^{NTR}$-dependent manner, an effect that is distinct from *bdnf* haploinsuffiency.

Remarkably, endogenously expressed proBDNF results in greater deficits in basal synaptic transmission in area CA1 than *bdnf* haploinsufficiency, which is consistent with the observed defects in dendritic structure. In addition, *probdnf-HA*/+ mice exhibit impaired TBS-LTP and enhanced LTD, effects distinct from *bdnf* haploinsufficiency. These data support the hypothesis that proBDNF is an important endogenous modulator of neuronal structure and function.

RESULTS

Evaluation of proBDNF Levels In Vivo

Quantitation of in vivo levels of proBDNF and mature BDNF proteins is technically challenging, given the subnanomolar concentration of BDNF isoforms as well as limitations in antibody specificity and sensitivity due to the very high sequence conservation of the BDNF mature domain. Prior studies have yielded conflicting results regarding the ratio of proBDNF to mature BDNF during development and adulthood (Yang et al., 2009b; Rauskolb et al., 2010; Dieni et al., 2012). To potentially resolve this issue, we used mice expressing two alleles of *bdnf* with a HA tag (*bdnf-HA/HA*; Yang et al., 2009b) to detect BDNF isoforms with HA-specific antibodies, which permits analysis of hippocampal lysates directly. Using this technique, we observe that the levels of BDNF isoforms are low at birth (postnatal day 0; P0) but rise rapidly to peak at P15–P21. proBDNF levels are highest at P15, with a reduction at P42 and at later ages (P90 and P210; Figure 1A, left). Mature BDNF protein levels are highest at P21 and remain readily detectable at later time points. In adulthood, mature BDNF is the most abundant isoform, consistent with other studies (Yang et al., 2009b; Rauskolb et al., 2010).

Evaluation of BDNF isoform protein expression during the first 2 postnatal weeks when overall peptide levels are low (using immunoprecipitation and western blot analysis) indicated that proBDNF was the most abundant isoform in the hippocampus at P0 (Figure 1A, right). proBDNF was readily detectable, although less abundant than mature BDNF at P15–P42 (Figure 1A, left). These results suggest that the effects of endogenous proBDNF protein would be most robust in early postnatal development (0–8 weeks), consistent with the higher levels of $p75^{NTR}$ in the hippocampus at this age (Woo et al., 2005; Yang et al., 2009b), particularly in CA1 pyramidal cell apical dendrites, postsynaptic to the Schaffer collateral axon terminals (Woo et al., 2005).

Generation of proBDNF-HA-Expressing Mice

To evaluate the physiological functions of proBDNF in vivo, we generated a *probdnf-HA* knockin mouse by replacing one *bdnf* allele with a *probdnf-HA* allele that contains a mutated proconvertase/furin cleavage site, leaving one endogenous allele to maintain viability (Figures 1B and 1C). A C-terminal HA epitope tag was added to facilitate detection of the introduced allele (*probdnf-HA*). This approach was undertaken because substitution of both *bdnf* alleles with a cleavage-resistant *probdnf* allele would likely result in high mortality, as mature BDNF is required for vascular development (Donovan et al., 2000). Also, a *probdnf* transgene would fail to recapitulate the complex transcriptional and translational regulation of the *bdnf*

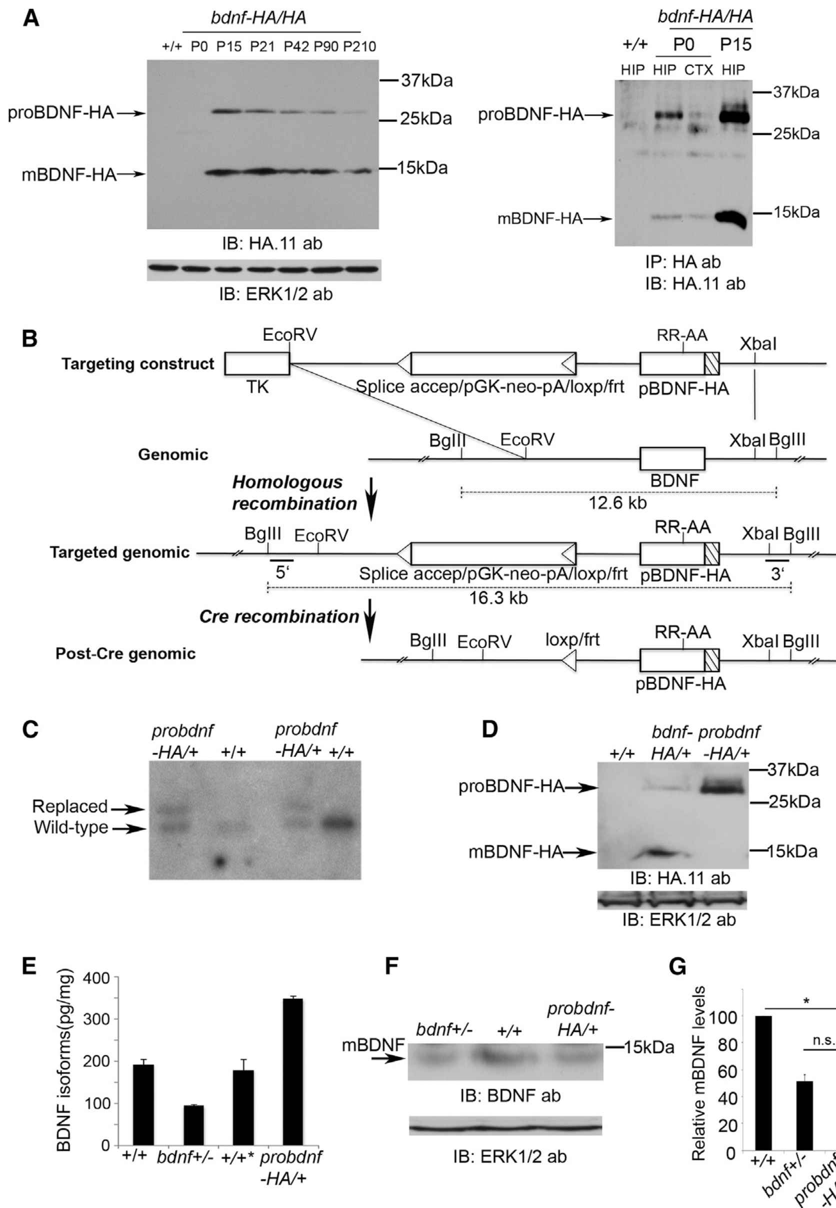

Figure 1. Generation of *probdnf-HA* Knockin Mice

(A) Developmental expression of proBDNF and mature BDNF. Left: hippocampi from mice of indicated ages expressing two alleles *of bdnf-HA* (*bdnf-HA/HA*) were lysed, and immunoblotting was performed using anti-HA. Immunoblots using anti-ERK1/2 were performed for a loading control. Right: immunoprecipitation/western blot analysis of hippocampi or cortices from wild-type (+/+) or *bdnf-HA/HA* mice of the indicated age. CTX, cortex; HIP, hippocampus; IB, immunoblotting; IP, immunoprecipitation.

(B) Strategy for generating the *probdnf-HA* knockin mice is shown schematically. TK, thymidine kinase gene.

(C) Southern blot analysis of embryonic stem cell line clones demonstrates that one endogenous *bdnf* allele was replaced by a *probdnf-HA* allele.

(D) Western blot analysis of proBDNF-HA expression in the hippocampi of *probdnf-HA*/+ mice at 4 months. Hippocampi were lysed, and immunoblotting was performed with anti-HA; anti-ERK1/2 was performed for a loading control.

(E) Quantitation of total BDNF isoforms (proBDNF + mature BDNF) utilizing ELISA from mice of indicated genotype at 5 months (n = 3 per genotye).

(F) Western blot analysis to determine the levels of mature BDNF in *probdnf-HA*/+ mice, *bdnf*+/− mice, and wild-type (+/+) mice at 5 months of age. Hippocampi were lysed, and immunoblotting was performed with anti-BDNF; anti-ERK1/2 was performed for a loading control.

(G) Quantitation of relative levels of mature BDNF from mice of indicated genotype. Hippocampal lysates (5 months) were analyzed by western blotting, and quantitation was performed using ImageJ (*p < 0.01; n = 3 per genotye). n.s., not significant.

Data are reported as mean ± SEM.

locus (Greenberg et al., 2009) and a bacterial artificial chromosome approach could result in multiple insertions. Because *probdnf-HA*/+ mice express one endogenous cleavable *bdnf* allele (Figure 1B), we analyzed mice haploinsufficient for BDNF (*bdnf*+/−; Lyons et al., 1999), enabling analysis of possible gain-of-function phenotypes due to increased proBDNF.

probdnf-HA/+ mice were viable and fertile. Moreover, *probdnf-HA*/+ mice exhibit weight gain that exceeds the weight gain (Figure S1) that is characteristic of *bdnf*+/− mice (Lyons et al., 1999). These results suggest that the weight gain observed in *probdnf-HA*/+ mice reflects haploinsufficiency of nontargeted *bdnf* and gain of function of proBDNF.

To verify that mutation of the proconvertase/furin site impairs cleavage and results in accumulation of intact proBDNF, we evaluated the expression of proBDNF and mature BDNF generated from the *probdnf-HA* allele by western blot analysis, using HA immunodetection of hippocampal lysates (Figure 1D). *bdnf-HA*/+ knockin mice in which the furin site was not mutated were analyzed as controls (Yang et al., 2009b). In *probdnf-HA*/+ mice, proBDNF-HA (~32.7 kDa) was detectable, but it was not processed to mature BDNF-HA (~14.2 kDa), confirming that the mutated sequence resulted in expression of intact proBDNF (Figure 1D). Hippocampal lysates from HA-epitope negative brains (+/+) were used as a control for antibody specificity. Lysates from *bdnf-HA*/+ brains documented that both proBDNF-HA and mature BDNF-HA were detectable in mice expressing cleavage-sensitive *bdnf-HA* alleles (Figure 1D).

To quantitatively evaluate BDNF isoforms, we performed ELISA analysis using reagents that detect the mature domain and thus quantitate both proBDNF and mature BDNF. Hippocampal lysates from *probdnf-HA*/+ mice and their wild-type littermates (+/+*), +/+ and *bdnf*+/− mice (5 months) were

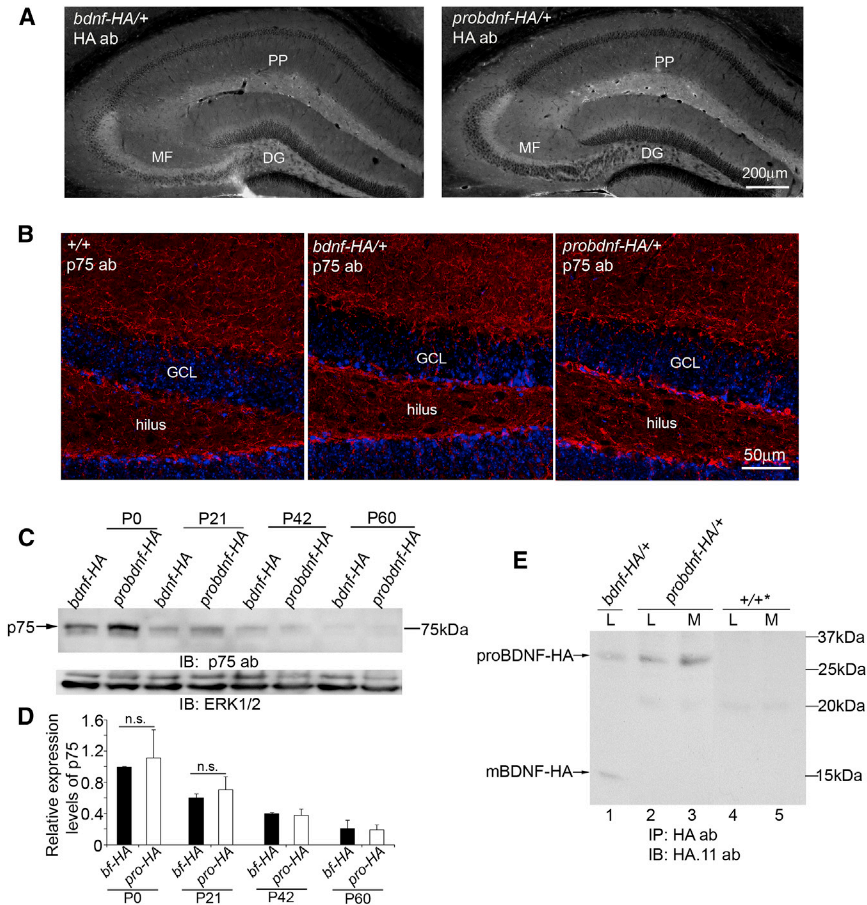

Figure 2. Detection of proBDNF-HA and p75^{NTR} and Secretion of proBDNF from Hippocampal Neurons of *probdnf-HA*/+ Mice

(A) Detection of HA immunoactivity from *probdnf-HA*/+ and *bdnf-HA*/+ brains using anti-HA and Cy3-conjugated streptavidin with image capture in black/white (white corresponds to immunoreactivity). Localization of proBDNF-HA in *probdnf-HA*/+ mice is comparable to *bdnf-HA*/+ mice, indicating that proBDNF-HA is transported normally in the mossy fibers in vivo. DG, dentate gyrus; MF, mossy fibers; PP, perforant pathway.

(B) p75^{NTR} localization in dentate gyrus of *probdnf-HA*/+, *bdnf-HA*/+, and wild-type (+/+) mice (7 weeks of age). Immunofluorescence was detected in dendritic processes of granule cells in all genotypes; cellular localization of p75^{NTR} in *probdnf-HA*/+ mice was comparable to the other genotypes. p75^{NTR} immunoreactivity is noted in red; DAPI is in blue. GCL, granule cell layer.

(C) p75^{NTR} expression in postnatal hippocampi from *probdnf-HA*/+ or *bdnf-HA*/+ mice of the indicated ages. Lysates were separated by SDS-PAGE, and immunoblotting was performed using anti-p75^{NTR}. Normalization was performed using anti-ERK1/2 detection.

(D) Quantification of p75^{NTR} expression level in postnatal mouse hippocampi at different ages indicated (three hippocampi per genotype). n.s., $p > 0.05$.

(E) Secretion of proBDNF-HA from hippocampal neurons cultured from *probdnf-HA*/+ and *bdnf-HA*/+ mice. Neurons were cultured for 7 days in depolarizing conditions. Media was collected and cell lysates prepared and immunoprecipitated with anti-HA. Mature BDNF-HA was not detected in *probdnf-HA*/+ lysates and media but was present in *bdnf-HA*/+ lysates. No immunoreactivity was detected in lysates or media in wild-type cultures (+/+*). L, cell lysates; M, cell medium.

Data are reported as mean ± SEM.

analyzed (Figure 1E). In *probdnf-HA*/+ hippocampal lysates, the levels of total BDNF isoforms were increased compared to wild-type mice, +/+* and +/+. As a control, the hippocampal lysates from *bdnf*+/− mice were also analyzed and showed an ~50% reduction in BDNF isoform levels as compared to +/+ mice (Figure 1E).

To determine the levels of mature BDNF in *probdnf-HA*/+ mice, we performed western blot analysis using an antibody specific for BDNF that detects mature BDNF. Hippocampal lysates from *probdnf-HA*/+ mice, *bdnf*+/− mice, and +/+ (5-month old; three mice per genotype) were analyzed. The levels of mature BDNF in *probdnf-HA*/+ mice were reduced as compared to +/+ mice (63.2% ± 6.9% *probdnf-HA*/+ as compared to +/+; $p = 0.0197$; Figures 1F and 1G). In *bdnf*+/− mice, mature BDNF levels were reduced (51.7% ± 4.7%; $p < 9.91$) compared to *bdnf*+/+ mice, but there was no significant difference between the levels of mature BDNF in *bdnf*+/− mice as compared to *probdnf-HA*/+ mice ($p = 0.29$; Figures 1F and 1G). These data strongly suggest that the phenotypes observed in *probdnf-HA*/+ mice are due to the gain of function of endogenously expressed proBDNF, rather than deficiency of mature BDNF, when compared to *bdnf*+/− mice.

Expression of proBDNF

BDNF protein expression is regionally restricted in the hippocampus, with high levels in dentate granule cells, particularly their mossy fiber projections (Conner et al., 1997; Scharfman et al., 2003; Yang et al., 2009b; Dieni et al., 2012). To determine if expression of the *probdnf-HA* allele alters the localization of hippocampal BDNF, HA-immunohistochemistry was performed in *probdnf-HA*/+ and *bdnf-HA*/+ sections from 5-week-old mice. HA immunoreactivity was prominent in granule cells, particularly in mossy fibers (Figure 2A), suggesting that the cellular localization of the mutant proBDNF-HA is comparable to mature BDNF.

To evaluate whether overexpression of proBDNF in the *probdnf-HA*/+ mouse altered the localization of p75^{NTR}, immunolocalization was performed. We observed no differences in localization of p75^{NTR} in *probdnf-HA*/+ mice, as compared to *bdnf-HA*/+ or wild-type mice (Figure 2B). We also compared p75^{NTR} expression in *bdnf-HA*/+ and *probdnf-HA*/+ mice; although there was a small increase in p75^{NTR} at P0 in *probdnf-HA*/+ mice, statistical analysis of three independent experiments showed that the difference was not significant ($p = 0.61$). P75^{NTR} levels were comparable at P21, P42, and P60 (Figures 2C and 2D).

To determine whether proBDNF-HA is released from hippocampal neurons, neurons from *probdnf-HA*/+ or wild-type (+/+) mice were cultured and matured in vitro. Following culture in depolarizing condition to induce release (Yang et al., 2009b), BDNF isoforms were collected from media and cell lysates using immunoprecipitation/western blot analysis with antibodies to HA. Only proBDNF-HA (~32.7 kDa) was detectable in cell lysates and media of hippocampal neurons from *probdnf-HA*/+ mice (Figure 2E, lanes 2 and 3). The absence of HA-tagged mature BDNF (~14.2 kDa) in cell lysates and media from cultures derived from *probdnf-HA*/+ mice suggests that the *probdnf-HA* allele is not efficiently processed to mature BDNF in the cell or following release. We also cultured hippocampal neurons from *bdnf-HA*/+ mice and confirmed that both proBDNF and mature BDNF were readily detectable in lysates (Figure 2E, lane 1). These results suggest that proBDNF-HA is released intact from cultured neurons.

proBDNF Expression Leads to Reduced Dendritic Arborization In Vivo

We observed no significant alterations in neuronal patterning using Nissl staining. To determine whether overexpression of proBDNF in *probdnf-HA*/+ mice altered neuronal morphology, we analyzed dendritic complexity in dentate granule cells, where hippocampal BDNF mRNA and protein levels are normally highest, and effects of mature BDNF on neuronal morphology have been described (Danzer et al., 2002; Gao et al., 2009). Two ages were compared: one age where proBDNF and $p75^{NTR}$ is highly expressed (1 month old) and an older age when $p75^{NTR}$ and proBDNF expression has declined in nontargeted mice (3.5 months old). Using Golgi staining, we analyzed individual granule cells to assess dendritic complexity (Chen et al., 2006), comparing *probdnf-HA*/+ mice, *bdnf*+/− mice to control for *bdnf* haploinsufficiency, *bdnf-HA*/+ mice to account for possible effects of the HA tag, as well as wild-type littermates (of *probdnf-HA*/+ mice: +/+*; of *bdnf*+/− mice: +/+).

At 1 month of age, Sholl analysis demonstrated that *probdnf-HA*/+ mice (Figure 3A, orange line) displayed decreased complexity of dendritic arbors at 80–180 μm distances from the soma, as compared to wild-type littermates (+/+*; Figure 3A). *Bdnf-HA*/+ mice did not exhibit differences when compared to wild-type, suggesting that the defect in *probdnf-HA*/+ is not due to the HA tag (Figure 3A). To determine whether the deficit in arborization in *probdnf-HA*/+ mice was a consequence of enhanced proBDNF or a lack of one nontargeted *bdnf* allele, we analyzed granule cells of *bdnf*+/− mice. Granule cells of *bdnf*+/− mice displayed decreased dendritic complexity compared to their wild-type littermates (Figure 3A). However, at distances ≥110 μm from the soma, granule cells of *probdnf-HA*/+ mice showed a greater decrease in complexity compared to *bdnf*+/− mice, suggesting that local proBDNF expression yielded a gain-of-function phenotype with defects in distal dendritic arborization (Figure 3A). Defects in dendritic complexity were even more pronounced in 3.5-month-old mice (Figure 3B; representative traces are shown in C), with *probdnf-HA*/+ mice displaying significantly reduced arborization ≥70 μm from the soma compared to wild-type (Figure 3B). Similar to the 1-month-old mice, reduced arborization ≥100 μm from the soma was greater in the granule cells from *probdnf-HA*/+ mice compared to *bdnf*+/− mice (Figure 3B). These results suggest that the reduced arborization due to proBDNF overexpression, initially established in early postnatal life, is progressive at later adult ages. To determine whether the effects of augmented levels of proBDNF-HA were mediated by $p75^{NTR}$, *probdnf-HA*/+ mice that were also deficient in $p75^{NTR}$ (*probdnf-HA*/+:*$p75^{NTR}$*−/−) were compared to control animals (+/+* or +/+:*$p75^{NTR}$*−/−) of 3.5 months of age, when the deficits in arborization are more prominent. Deficiency in $p75^{NTR}$ rescued the defects in dendritic complexity observed in *probdnf-HA*/+ mice (Figure 3D), confirming that alterations in dendritic morphology mediated by proBDNF were $p75^{NTR}$ dependent. These results suggest that proBDNF has adverse effects on dendritic development in vivo that are dependent upon $p75^{NTR}$ and cannot be explained simply by haploinsufficiency of *bdnf*.

To assess the impact of these effects on neuronal morphology on the entire hippocampus, we measured hippocampal volume of *probdnf-HA*/+ mice using MRI (Bath et al., 2009). Older mice were used (11 months old) to address the hypothesis that adverse effects of proBDNF accumulation over time would lead to a reduction in hippocampal volume. We observed a 14.4% decrease in hippocampal volume in the *probdnf-HA*/+ mice (Figure 3E; a representative MRI image is shown in Figure S2). There was no significant decrease in hippocampal volume when *bdnf*+/− mice were compared to wild-type littermates (+/+), suggesting that increased proBDNF levels in *probdnf-HA*/+ mice contributed to the reduction of hippocampal volume (Figure 3E).

proBDNF Expression Negatively Regulates Dendritic Spine Density

BDNF modulates the number of dendritic spines in hippocampal neurons in a TrkB-dependent manner (Minichiello, 2009; Orefice et al., 2013). To determine if proBDNF alters dendritic spine plasticity, we first evaluated dendritic spine density of granule cells and CA1 pyramidal cells of *probdnf-HA*/+ mice and their wild-type littermates (+/+*), as well *bdnf*+/− mice and their wild-type littermates (+/+; Figure 4A). We also examined *bdnf-HA*/+ mice to account for possible effects of the HA tag. We observed a significant reduction in dendritic spine density in granule cells of the dentate gyrus and CA1 pyramidal cells in 1-month-old *probdnf-HA*/+ mice compared to wild-type littermates (Figures 4B and 4C). The decrease in spine density in *probdnf-HA*/+ mice was greater than that observed in *bdnf*+/− mice (Figures 4B and 4C). There were no significant differences between control mice (+/+ and +/+*) and *bdnf-HA*/+ mice (Figures 4B and 4C). The data suggest that *probdnf-HA*/+ expression adversely influences dendritic spines as well as dendritic arbors. Collectively, the data also provide support for the conclusion that increased proBDNF has significant adverse effects on dendritic structure that cannot be explained by haploinsufficiency of *bdnf*.

Endogenous proBDNF Negatively Regulates Schaffer Collateral Transmission

To evaluate basal synaptic transmission, the Schaffer collateral input was tested because the effects of mature BDNF and of

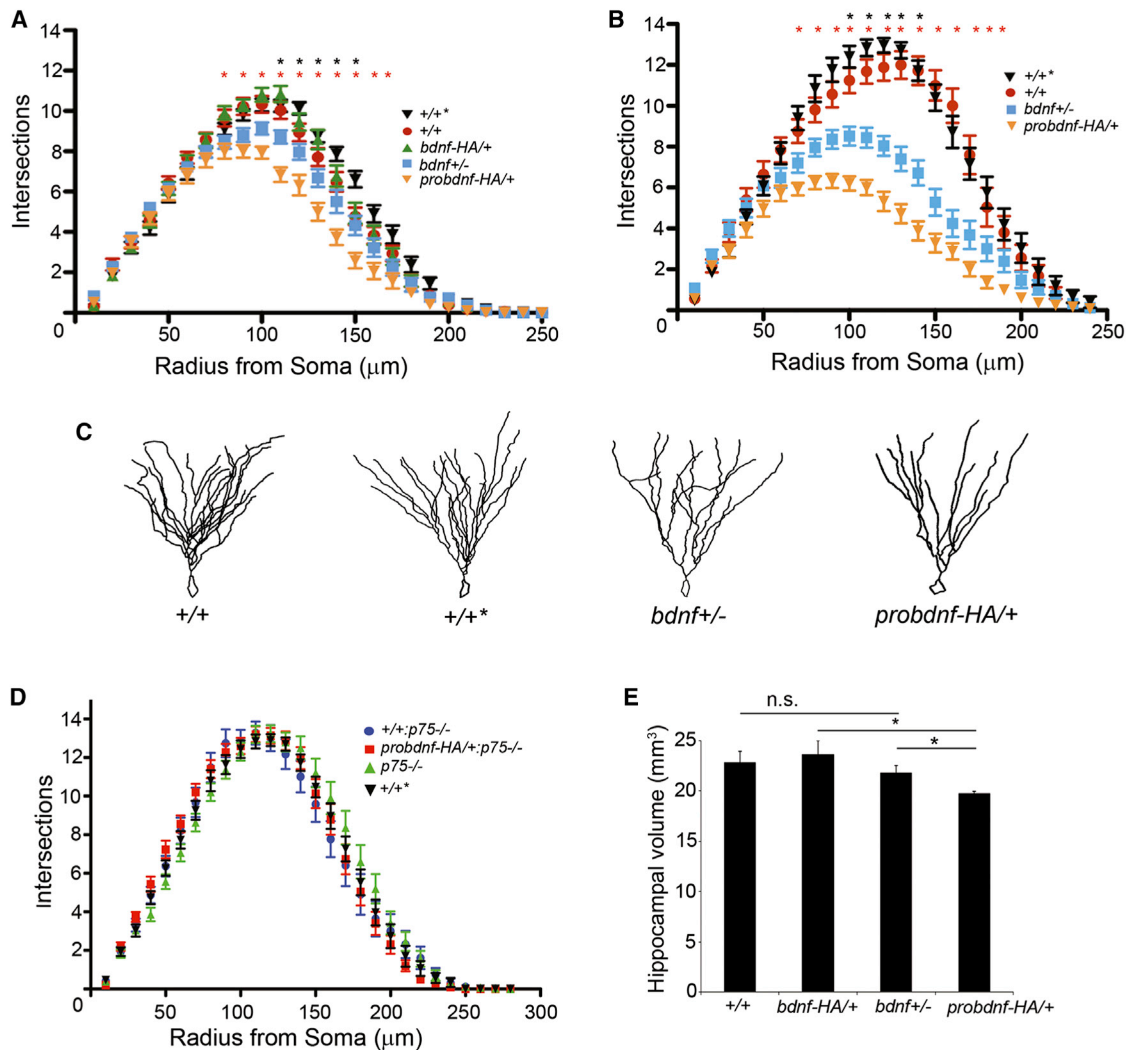

Figure 3. Altered Hippocampal Anatomy in *probdnf-HA*/+ Mice

(A and B) Sholl analysis of dentate granule cells from P30 (A) or P105 (B) mice. Forty neurons from four or five animals were analyzed per genotype. Results are presented as mean ± SEM. Red asterisks indicate significant differences between *probdnf-HA*/+ mice and controls; black asterisks indicate significant differences between *probdnf-HA*/+ mice and *bdnf*+/− mice ($p < 0.01$).

(C) Representative traces of Golgi-stained granule cells from control mice (+/+), wild-type littermates of *probdnf-HA*/+ mice (+/+*), *bdnf*+/− mice, and *probdnf-HA*/+ mice at P105.

(D) Sholl analysis of granule cells from P105 mice. To confirm the reduction of dendritic arborization in proBDNF-expressing mice was mediated by $p75^{NTR}$ receptors, Golgi-stained dentate granule neurons from *probdnf-HA*/+:$p75^{NTR}-/-$, +/+:$p75^{NTR}-/-$, $p75^{NTR}-/-$, and wild-type littermates of *probdnf-HA*/+ (+/+*) were traced. The deletion of $p75^{NTR}$ rescued the reduction of dendritic arborization in *probdnf-HA*/+ mice.

(E) Reduced hippocampal volume in *probdnf-HA* knockin mice. Total hippocampal volume was quantitated by Cavalieri analysis of MRI images of brains from 11-month wild-type mice (+/+; n = 3), *bdnf*+/− (n = 5), *bdnf-HA*/+ (n = 4), and *probdnf-HA*/+ mice (n = 5). Asterisk indicates $p < 0.01$ by Student's t test. n.s., p = 0.12. Data are reported as mean ± SEM.

recombinant proBDNF have been well studied at this synapse (Korte et al., 1995; Patterson et al., 1996; Pang and Lu, 2004; Woo et al., 2005). In addition to *probdnf-HA*/+ and wild-type littermates (+/+*) and *bdnf*+/− and wild-type littermates (+/+), we also evaluated *bdnf-HA*+ mice to determine if there were detectable physiological effects of the HA tag, which was not the case (discussed further below). Methods to measure field excitatory postsynaptic potentials (fEPSPs) are shown in Figure S3.

fEPSPs showed a greater impairment in *probdnf-HA*/+ mice than other genotypes (Figures 5A and 5B). When slopes were plotted as a function of the fiber volley amplitude, *probdnf-HA*/+ mice were most severely affected (two-way repeated measures ANOVA [RMANOVA]; $p < 0.01$; post hoc tests, $p < 0.05$; Figure 5B1). When plotted as a function of stimulus strength, fEPSPs of *probdnf-HA*/+ mice were also smaller (two-way RMANOVA; $p = 0.02$, post hoc test, $p < 0.05$; Figure 5B2). *bdnf*+/− mice were not different than wild-type

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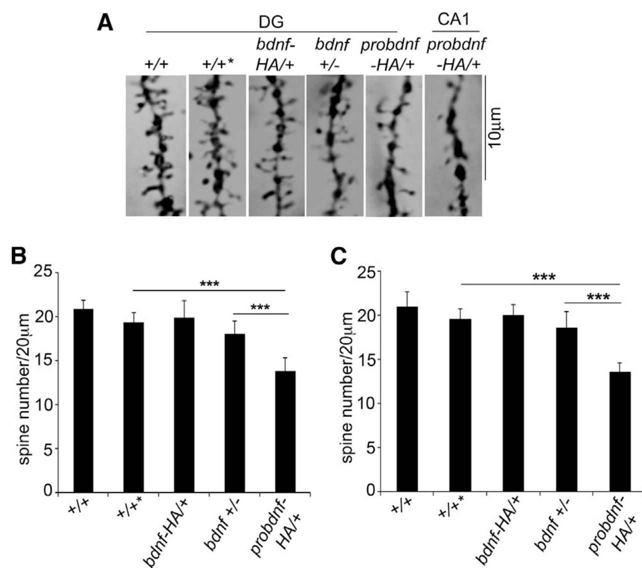

Figure 4. proBDNF Negatively Regulates Spine Formation

(A) Representative images of Golgi-stained dendritic spines of granule neurons (DG) or pyramidal neurons in region CA1 from wild-type mice (+/+), wild-type littermates of *probdnf-HA*/+ mice (+/+*), *bdnf*+/−, *bdnf-HA*/+, and *probdnf-HA*/+ mice.

(B and C) Reduction of dendritic spine density in *probdnf-HA*/+ knockin mice at 1 month. Brain sections from mice of the indicated genotypes were subjected to Golgi staining. Dendritic spines of 15 neurons/mouse of three or four mice per genotype were counted at 100×. Total spine number along a 20-μm-long dendrite was measured. *probdnf-HA*/+ mice had fewer spines compared to *bdnf*+/− mice in CA1 (B) and the DG (C). Differences were significant (Student's t tests, ***p < 0.0001).

Data are reported as mean ± SEM.

(two-way RMANOVA; p = 0.07, post hoc test, p > 0.05; Figure 5A3), similar to previous studies (Pozzo-Miller et al., 1999; Lessmann et al., 2011). These results suggest that overexpression of proBDNF impairs fEPSPs, an effect not observed by haploinsufficiency of *bdnf*.

Other characteristics of fEPSPs besides slope (amplitude, half-duration, or area of fEPSPs; latency to the fEPSP peak) were not significantly different among the groups (Figure S4A; Table S1). There also was no significant effect of genotype on fiber volley incidence or amplitude, latency to the volley peak, or paired-pulse facilitation (Figure S4B). In summary, overexpression of proBDNF severely impaired basal transmission at the Schaffer collateral synapse. The effect was specific because there was no detectable effect on latency or fiber volley. In contrast, BDNF haploinsufficiency did not decrease basal transmission, as reported previously (Korte et al., 1995; Lessmann et al., 2011), although Patterson et al. (1996) found deficits.

Endogenous proBDNF Negatively Regulates Synaptic Plasticity

We next evaluated posttetanic potentiation (PTP) and long-term potentiation (LTP) using TBS (Figure 6A). PTP was reduced in *probdnf-HA*/+ mice (90.4% ± 8.7%) compared to *bdnf*+/− mice (121.5% ± 10.8%) and control mice (+/+: 147.0% ± 13.2%; +/+*: 144.2% ± 11.9%; one-way ANOVA; p < 0.01; Figure 6A). Although PTP in *bdnf*+/− mice appeared to be less than PTP in controls (Figure 6A), the difference was not significant (post hoc test, p > 0.05). In contrast, PTP in *probdnf-HA*/+ mice was significantly less than PTP in *bdnf*+/− mice and wild-type littermates (post hoc tests, p < 0.05).

fEPSP slopes in control mice showed LTP (+/+: 132.0% ± 0.8%; +/+*: 133.6% ± 2.5%; Figure 6A), whereas delayed depression in fEPSPs occurred in slices from *probdnf-HA*/+ mice (73.2% ± 2.2%; Figure 6A). LTP was not maintained in *bdnf*+/− mice, with fEPSPs returning to baseline after TBS (98.0% ± 2.2%; Figure 6A) consistent with prior studies (Patterson et al., 1996). The effect of genotype on LTP was significant (one-way ANOVA; p < 0.01), with *probdnf-HA*/+ mice showing the smallest fEPSP slopes 60 min after TBS, compared to all other groups (post hoc t tests, p < 0.05). To exclude the effects caused by the HA tag, we compared *bdnf-HA*/+ mice with controls (+/+ and +/+*), and found that there were no significant differences in fEPSPs, PTP, or LTP (+/+ and +/+*; Figure S5). Therefore, *probdnf-HA*/+ mice and *bdnf*+/− mice had deficits in TBS-LTP maintenance, and *probdnf-HA*/+ mice also showed delayed depression of fEPSPs.

Previous studies suggest that mature BDNF and TrkB receptors are only required for LTP following TBS (Kang et al., 1997; Korte et al., 1998; Chen et al., 1999). Thus, we asked if the abnormalities in LTP in *probdnf-HA*/+ mice were specific to TBS. LTP following BDNF-independent, high-frequency stimulation (HFS)-induced LTP was similar across genotypes (+/+*: 142.5% ± 4.3%; *probdnf-HA*/+ mice: 142.3% ± 4.2%; Student's t test, p = 0.96; Figures 6B2 and 6B3). PTP was not significantly different either (+/+*: 146.7% ± 5.5%; *probdnf-HA*/+: 134.7% ± 6.8%; Student's t test, p = 0.19; Figures 6B2 and 6B3). Therefore, *probdnf-HA*/+ mice had defects in TBS-LTP, but not HFS-LTP, suggesting the effects of proBDNF were specific.

Because recombinant proBDNF facilitates LTD following LFS (Woo et al., 2005), we used similar procedures to ask if LTD was altered in *probdnf-HA*/+ mice (Figure 6C). *probdnf-HA*/+ mice had greater STD compared to their wild-type littermates (+/+*: 90.0% ± 5.4%; *probdnf-HA*/+: 67.4% ± 7.2%; Student's t test, p = 0.01; Figure 6C). *probdnf-HA*/+ mice also had greater LTD than their littermates (+/+*: 87.5% ± 8.3%; *probdnf-HA*/+: 59.6% ± 8.4%; Student's t test, p = 0.03; Figure 6C), suggesting that, like recombinant proBDNF, endogenous proBDNF facilitates Schaffer collateral LTD.

DISCUSSION

Here, we evaluated the actions of proBDNF in vivo using knockin mice that express one allele of cleavage-resistant *probdnf* under the control of its endogenous promoters. As a consequence, this mouse expresses one nontargeted *bdnf* allele, which can be cleaved (*bdnf*+/−). Thus, comparisons were made to *bdnf*+/− mice to detect specific gain-of-function phenotypes of proBDNF. From these comparisons, we observed that *probdnf* overexpression resulted in specific effects, including: (1) reduced dendritic arbors and spine density, (2) decreased hippocampal volume, (3) impairment in synaptic transmission and LTP following TBS, and (4) enhanced LTD following LFS. The results suggest that proBDNF has potent

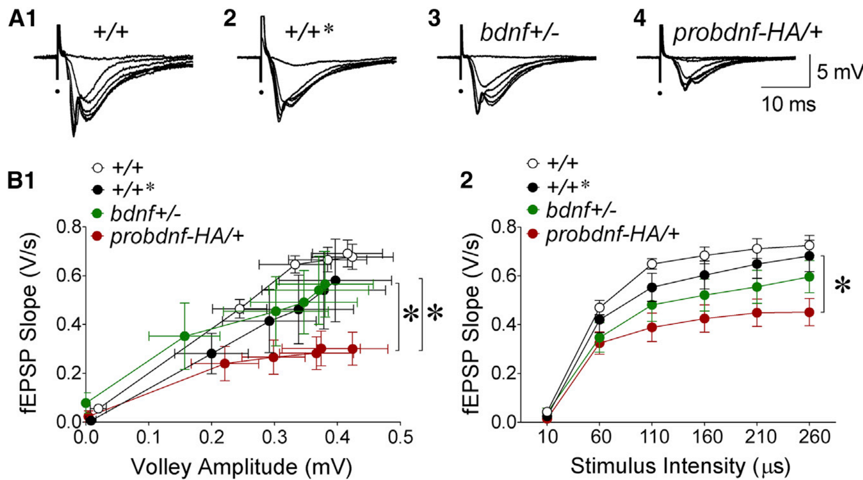

Figure 5. Basal Transmission Is Adversely Affected in *probdnf-HA*/+ Mice

(A) fEPSPs elicited at several stimulus strengths are superimposed. Representative responses to stimulation are shown for +/+ mice (wild-type littermates of *bdnf*+/− mice (14 slices, seven mice), +/+* (wild-type littermates of *probdnf-HA*/+ mice; 21 slices, 13 mice), *bdnf*+/− mice (ten slices, five mice), and *probdnf-HA*/+ mice (22 slices, 15 mice).

(B) fEPSP slope is plotted in relation to fiber volley amplitude (1). *probdnf-HA*/+ mice were more severely affected than +/+* mice (analysis of covariance; $p = 0.04$) and *bdnf*+/− mice ($p = 0.02$). fEPSP slope is plotted in relation to stimulus strength, reflected by the duration of a constant current (100 μA) stimulus (2). *probdnf-HA*/+ mice were more severely affected than littermate controls (+/+*; two-way RMANOVA, $p < 0.05$). *probdnf-HA*/+ mice were not significantly different from *bdnf*+/− mice (two-way RMANOVA, $p > 0.05$) but were significantly impaired relative to *bdnf*+/− mice at the maximal stimulus (Table S1). Data are reported as mean ± SEM.

effects that are likely to play a role in the development of hippocampal circuitry in early postnatal life when proBDNF and p75^{NTR} are normally highest. They also suggest that proBDNF could influence hippocampal-dependent functions later in life because proBDNF and p75^{NTR} levels are lower but are still sufficient to modulate synaptic transmission and plasticity, as demonstrated by the electrophysiological data provided here.

proBDNF Regulates Hippocampal Structure

Substitution of one cleavage-resistant *bdnf* allele for an endogenous *bdnf* allele permitted evaluation of proBDNF function under the complex control of its endogenous promoters, providing a more physiologically relevant approach to study proBDNF than previously possible. Neuronal localization and secretion of proBDNF were comparable to that observed in hippocampi and cultured neurons of mice with cleavable *bdnf* alleles, suggesting that amino acid substitutions that impair proconvertase-mediated cleavage per se do not alter intracellular sorting and trafficking of proBDNF. No significant changes in p75^{NTR} levels were observed in *probdnf-HA*/+ mice in adulthood, suggesting that compensatory changes in response to accumulated proBDNF were not striking.

In *probdnf-HA*/+ mice, a prominent phenotype was observed in granule cells: a reduction in dendritic arborization. This reduction was more severe than that observed with the loss of a single *bdnf* allele and required p75^{NTR} expression, as mice deficient in p75^{NTR} but expressing proBDNF did not display this phenotype. These results define in vivo actions of proBDNF that are mediated specifically by p75^{NTR}. Ultrastructural studies suggest that p75^{NTR} is expressed on CA1 pyramidal cell dendrites at the synaptic cleft (Woo et al., 2005). Therefore, locally elevated levels of proBDNF may lead to reduced dendritic arbors by a direct effect of p75^{NTR} on hippocampal dendrites. Increased proBDNF also reduced dendritic spine density of granule cells, to a degree that exceeded that observed in mice with *bdnf* haploinsufficiency.

The mechanisms by which proBDNF reduces dendritic arborization and spine density are not known, but proneurotrophin-induced activation of p75^{NTR} mediates acute growth cone collapse via inactivation of Rac and phosphorylation of fascin leading to destabilization of actin filaments (Deinhardt et al., 2011). Other studies have implicated activation of RhoA by proBDNF (Sun et al., 2012). Fascin, Rac, and RhoA are expressed in the juvenile hippocampus, but further studies will be required to determine if these pathways are activated in vivo. P75^{NTR} also modulates spine density, as p75^{NTR} deficiency increases spine density in hippocampal neurons, whereas p75^{NTR} overexpression has the opposite effect (Zagrebelsky et al., 2005). These effects have been attributed to an interaction of p75^{NTR} with galectin-1 (Plachta et al., 2007); however, the studies here suggest that proBDNF is an endogenous ligand that negatively regulates dendritic spines in vivo via p75^{NTR}.

The Role of proBDNF in Hippocampal Synaptic Transmission

In contrast to the well-established mechanisms by which mature BDNF modulates LTP at the Schaffer collateral synapse (Korte et al., 1995, 1998; Patterson et al., 1996; Chen et al., 1999; Pang and Lu, 2004; Lu et al., 2008), the effects of endogenously expressed proBDNF have not been established. Prior studies indicate that recombinant proBDNF facilitates LTD in hippocampal slices by activation of p75^{NTR} (Woo et al., 2005) and exogenous proBDNF induces synaptic depression at the neuromuscular synapse (Yang et al., 2009a). Other studies have shown that p75^{NTR} modulates LTD, although the relevant ligands were not identified (Rösch et al., 2005). Here, we suggest that there are potent effects of endogenously expressed proBDNF on synaptic transmission and plasticity. Mice expressing cleavage-resistant proBDNF exhibited a significant impairment in Schaffer collateral transmission with very low-frequency stimulation (to reduce the likelihood of axonal proBDNF or mature BDNF release). Because release was unlikely, the impairment in basal synaptic transmission is presumably due to reduced dendritic complexity and dendritic spines in cleavage-resistant, proBDNF-HA-overexpressing mice. *bdnf* haploinsufficiency

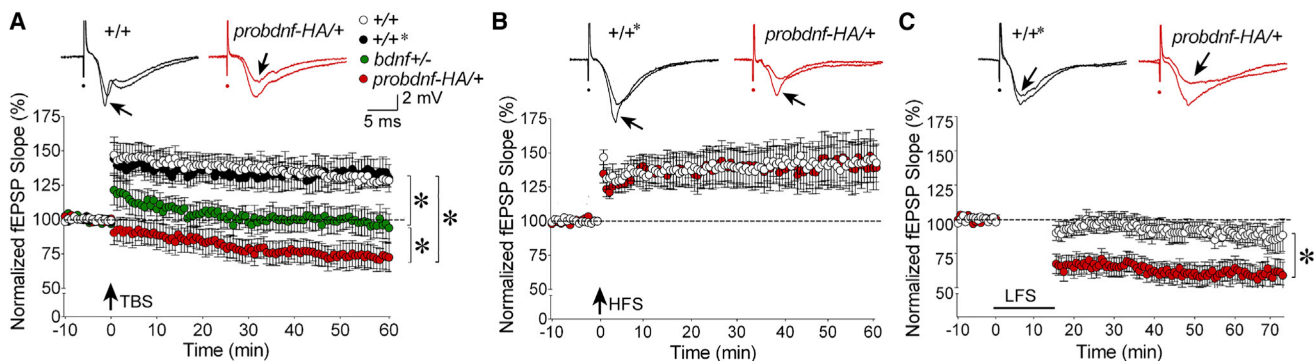

Figure 6. TBS-LTP Is Impaired, HFS-LTP Is Unaffected, and LTD Is Enhanced in *probdnf-HA*/+ Mice

(A) Top: representative fEPSPs, pre- (no arrow) and 60 min post- (arrow) TBS. Bottom: TBS-LTP was most impaired in *probdnf-HA*/+ mice. There were significant differences ($p < 0.05$, large asterisks), except for +/+ versus +/+* genotypes.

(B) Top: representative fEPSPs, pre- (no arrow) and 60 min post- (arrow) HFS. Bottom: differences in HFS-LTP between *probdnf-HA*/+ mice (red) and wild-type littermates (+/+*; white) were not significant.

(C) Top: representative fEPSPs, pre- (no arrow) and 60 min post- (arrow) LFS. Bottom: LTD was greater in *probdnf-HA*/+ mice compared to +/+* ($p < 0.05$). Data are reported as mean ± SEM.

could also be a factor but cannot explain the result completely, because overexpression of proBDNF resulted in greater—and different—deficits than *bdnf* haploinsufficiency.

probdnf-HA/+ mice showed increased LTD following LFS, consistent with the enhancement in LTD previously found using recombinant proBDNF (Woo et al., 2005). Interestingly, *probdnf-HA*/+ mice exhibited a delayed depression after TBS—which is surprising because superfusion of recombinant proBDNF has not led to this response to TBS (Woo et al., 2005). These results suggest that there may be differences between the neuronal release of endogenously expressed proBDNF and superfusion or developmental changes in arborization due to overexpression of proBDNF. In comparison, *bdnf*+/− mice exhibited a lack of TBS-LTP maintenance, consistent with previous studies (Korte et al., 1998). A mechanism that may contribute to the more-significant phenotype in *probdnf-HA*/+ as compared to *bdnf*+/− mice after TBS is the greater reduction in dendritic arbors and spines in *probdnf-HA*/+ mice. Fewer spines would be likely to reduce the effects of glutamate-induced postsynaptic depolarization. This explanation is consistent with accumulating evidence that spine remodeling is coupled to functional synaptic plasticity (Yuste and Bonhoeffer, 2001).

Another possible contribution to the defect in TBS-LTP in *probdnf-HA*/+ mice is an impairment in proBDNF cleavage following secretion. In hippocampal neurons, tissue plasminogen activator (tPA), plasmin and proBDNF are copackaged in dense core vesicles (Lochner et al., 2008), and it has been postulated that plasmin cleaves proBDNF to form a pool of postsynaptic mature BDNF to stabilize LTP (Pang et al., 2004; Pang and Lu, 2004). Using fluorogenic probes, local conversion of proBDNF to mature BDNF has been detected near axonal processes (Je et al., 2012). *probdnf-HA*/+ mice, with a reduced susceptibility to cleavage, may show impairment in LTP stabilization following TBS. Increased LTD could result from increased proBDNF, consistent with the effects of proBDNF on hippocampal slices to facilitate LTD (Woo et al., 2005).

The response to TBS in *probdnf-HA*/+ mice relative to *bdnf*+/− mice deserves additional comment because *probdnf-HA*/+ mice exhibited a small STD rather than PTP. Although impaired PTP/STD would suggest impaired presynaptic function, paired-pulse facilitation, which is mediated by presynaptic mechanisms, was unaffected. Therefore, we suggest that the response of *probdnf-HA*/+ mice to TBS reflects the sum of *bdnf* haploinsuffiency and excess proBDNF. *bdnf* haploinsufficiency would lead to PTP, but not LTP, after TBS, and excess proBDNF would facilitate STD and LTD after LFS (Woo et al., 2005).

In conclusion, the results demonstrate a major negative role for endogenously expressed proBDNF in hippocampal neuronal morphology and synaptic plasticity. The expression patterns of proBDNF and $p75^{NTR}$ within the first weeks of postnatal development in the mouse suggest that $p75^{NTR}$ activation by proBDNF plays an important role in modulating hippocampal circuits during this critical developmental time frame. Thus, this ligand-receptor system may be a key regulator in shaping neural circuitry and synaptic plasticity, effects that may be maintained through adulthood.

EXPERIMENTAL PROCEDURES

Generation of *probdnf-HA* Knockin Mice

probdnf-HA knockin mice were generated by substituting one allele of the murine *bdnf* gene with the murine *bdnf* gene in which the furin cleavage site (RVRR) was mutated to RVAA, and a HA-epitope tag was added to the C terminus (Figure 1B; Supplemental Experimental Procedures).

Immunoprecipitation and Western Blot Analysis

Tissues were lysed in lysis buffer and resolved by SDS-PAGE or subjected to immunoprecipitation and western blot analysis as previously published (Yang et al., 2009b).

Perfusion and Preparation of Sections for Immunofluorescence Staining

Mice were anesthetized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were postfixed overnight at +4°C, cryoprotected, and 30 μm sections were obtained. HA immunodetection was

performed as previously described (Yang et al., 2009b; Supplemental Experimental Procedures). Details of $p75^{NTR}$ immunodetection are included in the Supplemental Experimental Procedures. Animal experiments were performed according to The Research Animal Resource Center of Weill Cornell Medical College and national regulations.

Release of proBDNF from Cultured Hippocampal Neurons

Hippocampi were dissected from mice, and dissociated neurons were cultured as described previously (Yang et al., 2009b; Supplemental Experimental Procedures). The harvested medium and cell lysates were subjected to immunoprecipitation and western blot analysis as described in the Supplemental Experimental Procedures.

Measurement of Hippocampal Volume with Nuclear MRI

Animals were anesthetized and transcardially perfused with 0.9% saline, 0.1% sodium nitrite, and 5% gadolinium-DTPA (Magnevist; Berlex Laboratories) and then 4% paraformaldehyde solution and 5% Magnevist in PBS. Brains were stored in 0.1 M PBS containing 5% Magnevist for 3–7 days prior to imaging with a 3.0 T MRI system (GE Medical Systems) equipped with 50 mT/m gradients operating at 150 mT/m per ms. All other parameters are as described in Bath et al. (2009) and in the Supplemental Experimental Procedures.

Rapid Golgi Impregnation and Analysis

Golgi impregnation used the Golgi-Cox method and labeled neurons were analyzed in a blinded manner, for Sholl analysis as described previously (Chen et al., 2006; see also the Supplemental Experimental Procedures).

Electrophysiological Procedures

Horizontal hippocampal slices (400 μm thick) were cut in ice cold artificial cerebral spinal fluid using standard procedures (Skucas et al., 2011; Supplemental Experimental Procedures). LTP/LTD were induced with half-maximal stimuli. The protocol for TBS was ten trains of four pulses at 100 Hz (200 ms apart); for HFS, there were four bursts of 100 pulses at 100 Hz (20 s apart). LTD was induced using 900 stimuli at 1 Hz (LFS).

Data Analysis

fEPSP analysis is shown in Figure S3. For LTP/LTD, fEPSP slopes were normalized to the baseline mean. PTP and STD were defined by the first response after the stimulus train used to induce LTP or LTD. LTP/LTD amplitude was defined by the mean of fEPSPs recorded 50–60 min following induction.

Statistics

Data are reported as mean ± SEM, and significance was $p < 0.05$ (see also the Supplemental Experimental Procedures).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.040.

AUTHOR CONTRIBUTIONS

C.-J.S. and W.M. generated the *probdnf-HA* mouse; J.Y., Q.M., T.M, D.J., R.C., K.G.B., and D.B. performed experiments analyzing the proBDNF mouse; L.C.H.-H., J.J.L., and H.E.S. carried out the electrophysiological experiments; and H.E.S., B.L.H., L.C.H.-H., F.S.L., and J.Y. designed the experiments and wrote the manuscript.

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