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Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor

(neurotrophins/synaptic plasticity/gene targeting)

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ABSTRACT **Brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor (NGF) gene family, has been shown to influence the survival and differentiation of specific classes of neurons *in vitro* and *in vivo*. The possibility that neurotrophins are also involved in processes of neuronal plasticity has only recently begun to receive attention. To determine whether BDNF has a function in processes such as long-term potentiation (LTP), we produced a strain of mice with a deletion in the coding sequence of the BDNF gene. We then used hippocampal slices from these mice to investigate whether LTP was affected by this mutation. Homo- and heterozygous mutant mice showed significantly reduced LTP in the CA1 region of the hippocampus. The magnitude of the potentiation, as well as the percentage of cases in which LTP could be induced successfully, was clearly affected. According to the criteria tested, important pharmacological, anatomical, and morphological parameters in the hippocampus of these animals appear to be normal. These results suggest that BDNF might have a functional role in the expression of LTP in the hippocampus.**

Neurotrophic factors, in particular the members of the nerve growth factor (NGF) gene family, have so far been considered predominantly with regard to their function in regulating survival and differentiation of specific neuronal populations during embryonic development and the maintenance of characteristic neuronal function in adulthood (1–3). There is, however, evidence that neurotrophins might also be involved in neuronal plasticity (4–10). Long-term potentiation (LTP) is the most widely used paradigm to study cellular and molecular events underlying neuronal plasticity (11). We therefore used this paradigm in slices of the hippocampus from mice with targeted deletion of the brain-derived neurotrophic factor (BDNF) gene to test whether BDNF has a role in this important phenomenon of synaptic plasticity.

MATERIALS AND METHODS

In the gene-targeting construct, a 560-bp fragment from the BDNF protein-coding exon was replaced by the selection marker—a neomycin-resistance gene flanked by a glycerate kinase gene promoter and a polyadenylylation signal—thus deleting most of the mature BDNF coding sequence (Fig. 1). Embryonic stem cells (D3, 129Sv) containing the disrupted BDNF gene were injected into BALB/c mouse blastocysts for subsequent generation of chimeric mice. Chimeric males were crossed with NMRI females to produce heterozygotes. In keeping with previously published reports (12, 13), homozygous BDNF (−/−) mutant mice were retarded in growth and had reduced weight (down to only 25% of the wild type) from postnatal day 3 (P3) on. They displayed aberrant limb coordination and balance, showed a loss of neurons in the dorsal root ganglia, and usually died between 2 and 4 weeks after birth. Such abnormalities were never observed in heterozygous BDNF (+/−) mice.

Transverse hippocampal slices (400 μm thick) were prepared and maintained by standard procedures (medium, 124 mM NaCl/3 mM KCl/1.25 mM KH_2PO_4/2 mM $MgSO_4$/26 mM $NaHCO_3$/2.5 mM $CaCl_2$/10 mM glucose; temperature, 32 ± 0.2°C; submerged recording).

Monopolar tungsten electrodes in the CA3 Schaffer-collateral region were used for stimulation. Synaptic field potentials were elicited with a frequency of 0.1 Hz. Responses were recorded with glass electrodes placed in the apical dendritic region (stratum radiatum) of the CA1 pyramidal neurons. The slope of the excitatory postsynaptic potential (EPSP) was calculated and used as a measure for synaptic strength. LTP was induced with a tetanus of 3 × 30 pulses (100 Hz, 50-μs duration, 5-s interstimulus interval) with the strength of the test stimulus. Data were collected with a program written in LABVIEW (National Instruments, Austin, TX). Routinely, paired pulse facilitation was tested with 20-, 30- and 50-ms interstimulus interval. For the intracellular "pairing" procedure the postsynaptic cell was depolarized with current injection (1–2 nA for 100 ms), and during the depolarization an extracellular stimulus (50-μs duration) was applied to the Schaffer collaterals.

All measurements were carried out and analyzed in a strictly blind fashion—the distribution of the slices into the incubation chamber was done by a second investigator so that the investigator performing the measurements had no way of telling from which type of slice he was recording. Additionally, the genotype of all mice was analyzed only after the electrophysiological experiments and their evaluation. On each experimental day, two mice from the same litter with heterozygous parents were used.

RESULTS

Wild-type (+/+) mice showed LTP in 87% of all cases (54 slices; 16 mice; age, P12–P67). Heterozygous BDNF-mutant (+/−) mice showed LTP in 27.7% of all slices (83 slices; 24 mice; age, P12–P67). In homozygous BDNF mutant (−/−) mice the results were age-dependent (Fig. 2*A*). These mice showed almost no LTP until P16 (1 successful LTP in 19 slices;

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Abbreviations: BDNF, brain-derived neurotrophic factor; EPSP, excitatory postsynaptic potential; LTP, long-term potentiation; NGF, nerve growth factor; AP-5, DL-2-amino-5-phosphonovalerate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; DNQX, 6,7-dinitroquinoxaline-2,3-dione; GABA, γ-aminobutyrate; NMDA, *N*-methyl-D-aspartate; P*n*, postnatal day *n*.

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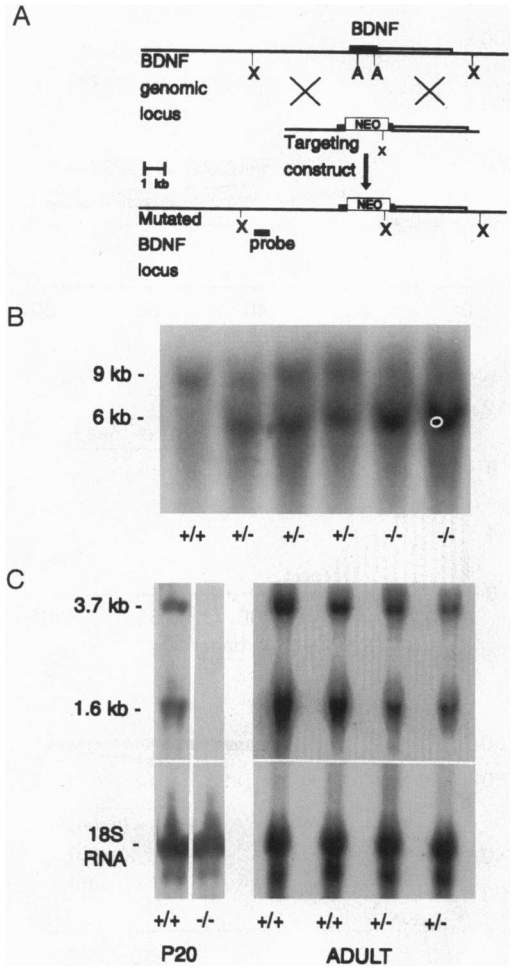

FIG. 1. Production of BDNF-deficient mice. (*A*) Mutation at the BDNF locus by homologous recombination. The final protein-coding exon of the BDNF gene is shown. The neomycin-resistance gene (NEO) was introduced between two *Apa* I (A) sites, resulting in the deletion of most of the BDNF protein-coding region. X, *Xba* I. (*B*) Genomic Southern blot of *Xba* I-digested DNA isolated from mouse tails in a litter derived from the crossing of two heterozygous mice. The diagnostic probe detects a wild-type band of 9 kb and a mutated band of 6 kb. (*C*) Northern blot analysis of total RNA (10 μg per lane) isolated from hippocampi of individual 20-day-old +/+ and −/− mice and adult +/+ and +/− mice. The blot was probed with a ^{32}P-labeled antisense RNA generated by *in vitro* transcription from the mouse BDNF protein-coding region. The hippocampi from +/− mice contain reduced levels of BDNF mRNA (<50% compared with wild type). No BDNF mRNA is detectable in the hippocampi of mutant −/− mice. The same blot was rehybridized with an 18S RNA probe as an internal control.

5 mice; age, P12–P16). From P17 to P28 the percentage of successful LTPs rose to 32.6% (46 slices; 12 mice).

In addition, the magnitude of the potentiation was different between wild-type and mutant mice [mean EPSP slope as percentage of baseline 60 min after the tetanus was 189.8 ± 11.1% (n = 47) for the +/+ mice, 141.8 ± 9.9% (n = 23) for the +/− mice, and 135.3 ± 5.4% (n = 16) for the −/− mice] even if only successful LTPs were included (Fig. 2*B*). The difference between +/+ mice and +/− or −/− mice is significant ($P < 0.01$, two-tailed t test), whereas the difference between +/− and −/− mice is not significant ($P > 0.05$). It is noteworthy that +/− and −/− animals show a slow decline of their already small LTP over time (e.g., see the last 40 min of recording in Fig. 2*B*). This might indicate that in those mice in which LTP can still be induced, a later phase of LTP is significantly affected.

Fig. 2 *C–F* show the frequency distributions of synaptic enhancements by a tetanus for the wild-type and mutant mice. These distributions again show that in +/+ mice the enhancement in most cases is >150%, whereas the enhancement for +/− and −/− mice is substantially different from 100% only in a few cases. Fig 2*F* shows the cumulative distribution of synaptic enhancements 30–60 min after a tetanus. There is a clear shift from +/+ to −/− mice in terms of the amount of synaptic strengthening, whereas +/− and −/− mice (after P16) show almost no difference in their synaptic strength after a tetanus. Fig. 2*F* also helps to clarify the seemingly paradoxical result from Fig. 2*A* that −/− mice (after P16) are slightly less impaired in terms of LTP than +/− mice. Fig. 2*F* shows that the curves for +/− and −/− mice are practically superimposed. However, at the 120% level (which we arbitrarily chose as the level for successful LTP) the curve for the +/− mice is slightly shifted to the left relative to the curve for the −/−, which explains why according to our criterion the −/− mice show a slightly higher incidence of LTP than the +/− mice. The general impression from Fig. 2*F* that the curves for +/− and −/− mice are similar is corroborated by statistical analysis which shows no significant difference between the data from +/− and −/− mice ($P > 0.05$, two-tailed t test).

A number of control experiments in BDNF-deficient mice ensured that the failure to induce LTP was not merely due to impaired synaptic transmission. According to our tests, synaptic transmission in mutant (+/− and −/−) mice was indistinguishable from that in wild-type mice in four ways: (*i*) equivalent synaptic responses were evoked with similar stimulus strength; (*ii*) the area under the field EPSP produced by a single stimulus was equal; (*iii*) paired-pulse facilitation was normal in both wild-type and mutant animals (Table 1); and (*iv*) even when the induction of LTP failed, all slices showed posttetanic potentiation when the tetanus was applied (e.g., see Fig. 3).

To ensure that the pharmacological properties of neurons from mutant animals are normal, we tested the functionality of *N*-methyl-D-aspartate (NMDA), γ-aminobutyrate (GABA), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. (*i*) AP-5 (50 μM), an NMDA antagonist, blocked the induction of LTP in all mouse types completely (as can be seen for a −/− mouse in Fig. 3*A*). After washout of AP-5 it was possible to induce LTP in 4 of 5 experiments (+/+ mice), in 2 of 11 experiments (+/− mice), and in 3 of 9 experiments (−/− mice). These results indicate that when LTP was inducible (Fig. 3*A*) in +/− or −/− mice, it was a pharmacologically "normal" LTP (as it was blocked by AP-5). When LTP could not be induced (Fig. 3*B*), it was not due to a lack of NMDA responses (see time point b in Fig. 3*B*). (*ii*) Application of bicuculline (10 μM) and picrotoxin (10 μM) showed that GABAergic inhibition was functional also in mutant animals (Fig. 3). (*iii*) In the presence of the AMPA antagonist DNQX (10 μM, in artificial cerebrospinal fluid with 1 mM Mg^{2+}) slices from mutant mice showed an NMDA component of the EPSP comparable to the wild-type case (reduction of the signal by 77 ± 19% in three +/+ mice, 71 ± 21% in five +/− mice, and 74 ± 25% in 4 −/− mice; see also Fig. 3). This NMDA component of the field EPSP was reduced to zero when AP-5 was applied (together with DNQX; see Fig. 3 for −/− mice).

To exclude the possibility that the failure to induce LTP was due to impaired fiber function [resulting, for instance, in a lack of cooperativity (14)], we also performed intracellular recordings and used paired pre- and postsynaptic stimulation to induce changes in synaptic efficacy. This procedure has the advantage that no changes in the frequency of fiber stimulation are necessary and hence changes in biophysical or morphological properties of the presynaptic afferents are less likely to affect this form of synaptic plasticity. The results of these experiments showed no qualitative difference to the extracel-

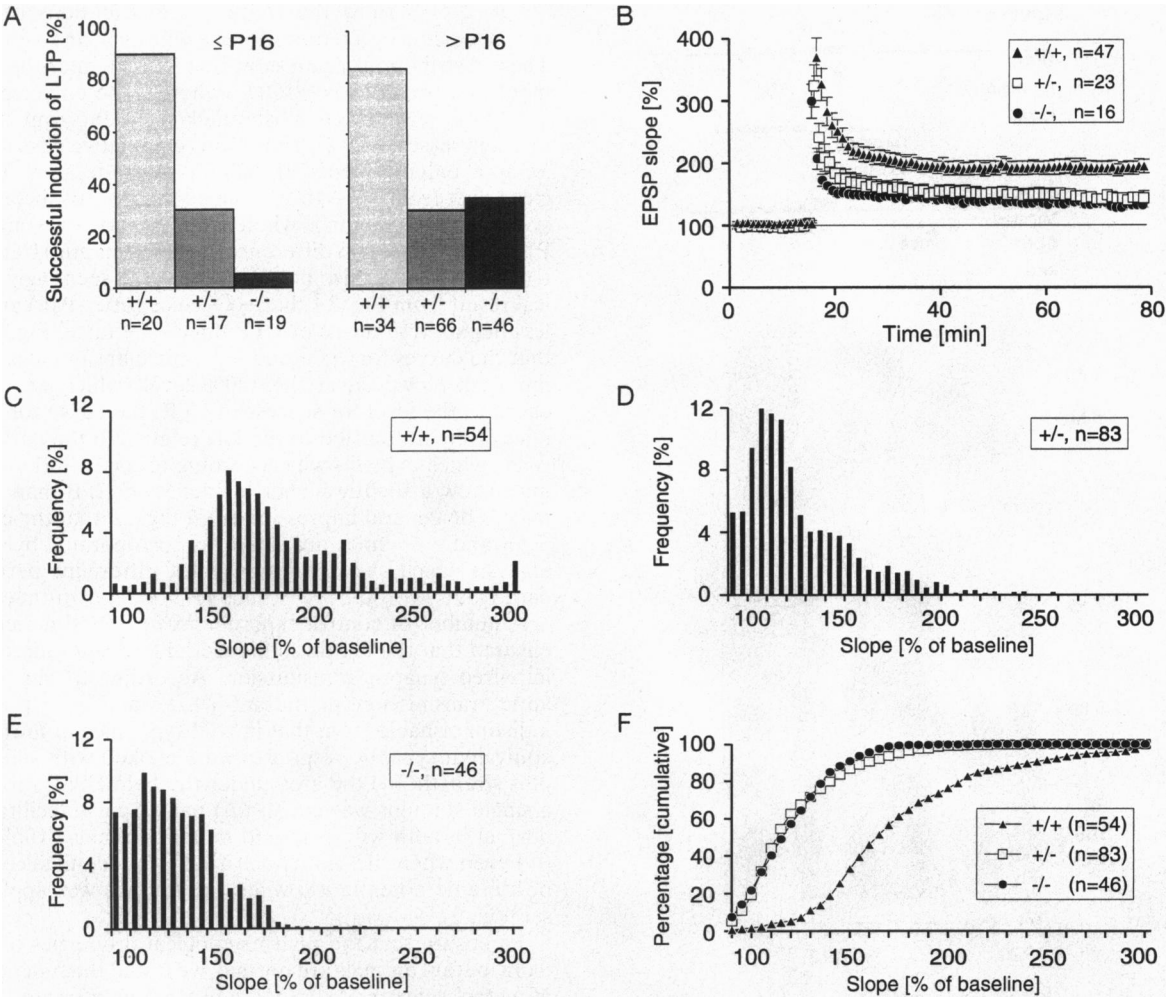

FIG. 2. LTP in hippocampal slices from BDNF-deficient and wild-type mice. (*A*) Percentage of successful LTP inductions for +/+, +/−, and −/− mice at or before P16 or older than P16. LTP was judged successful if 60 min after the tetanus the slope of the field EPSP was >120% of the baseline value. (*B*) LTP in hippocampal slices from +/+, +/−, and −/− mice; data plotted are only from experiments in which LTP could be induced. Symbols represent average responses (mean over six responses). Error bars show SEM; n, number of slices. (*C–E*) Frequency distributions of the potentiation magnitudes for the different BDNF genotypes. For +/+ mice the median of the frequency distribution is 165% (*C*). For +/− and −/− mutant mice, most potentiations are close to 100% (*D* and *E*), indicating that for these mice, potentiation generally was unsuccessful. (*F*) Cumulative percentage distribution of the posttetanic field EPSP slopes, expressed as percentage of pretetanus values. All data points recorded 30–60 min after induction of LTP are included. The ordinate gives the fraction of the data points that exhibit a posttetanic field EPSP slope less or equal to the value shown on the abscissa. The curves very quickly reach 100% for +/− and −/− mice, indicating that very few slices show a substantial potentiation. In contrast +/+ slices reach 100% only later—i.e., many of them show strong potentiation.

lular recordings (LTP in five of seven slices from three +/+ mice, in three of eight slices from three +/− mice, and in one of three slices from two −/− mice older than P16, and zero of three slices from two −/− mice younger than P17.

Golgi and Nissl stains revealed a qualitatively normal hippocampal anatomy in all mouse types with regard to cell shape, cell size, dendritic branching, and spine density (Fig. 4 *A* and *B*; see also refs. 12 and 13). To verify that also the morphology of hippocampal pyramidal neurons was normal, we filled these neurons intracellularly with neurobiotin. No apparent difference in cell morphology could be detected among the different mouse types within the limit of light microscopy (for a Golgi stain, see Fig. 4*C*).

Table 1. Paired pulse facilitation is not affected in BDNF-mutant mice

Genotype	Paired pulse facilitation,* %	No. of slices
+/+	211 ± 27	26
+/−	208 ± 24	38
−/−	204 ± 28	35

*Ratio of second EPSP slope to first EPSP slope. Interstimulus intervals for the data presented were 30 ms.

DISCUSSION

The idea that neurotrophins may play an essential role in neuronal plasticity was until recently based on several observations: (*i*) The synthesis of neurotrophins, in particular BDNF and NGF, is regulated by neuronal activity (15–18). Most importantly, neurotrophin mRNA levels are upregulated *in vitro* and *in vivo* by stimulation parameters which are known to induce hippocampal LTP (19–21). (*ii*) NGF, which is most likely representative of all neurotrophins, can be released in an activity-dependent manner (22) particularly from hippocampal dendrites (23). (*iii*) Neurotrophins can enhance the release of conventional transmitter substances (10, 24–27). These three points qualify neurotrophins as retrograde messengers, which due to their very specific Trk receptors could have a much more distinct and limited action than other postulated retrograde messengers (28, 29).

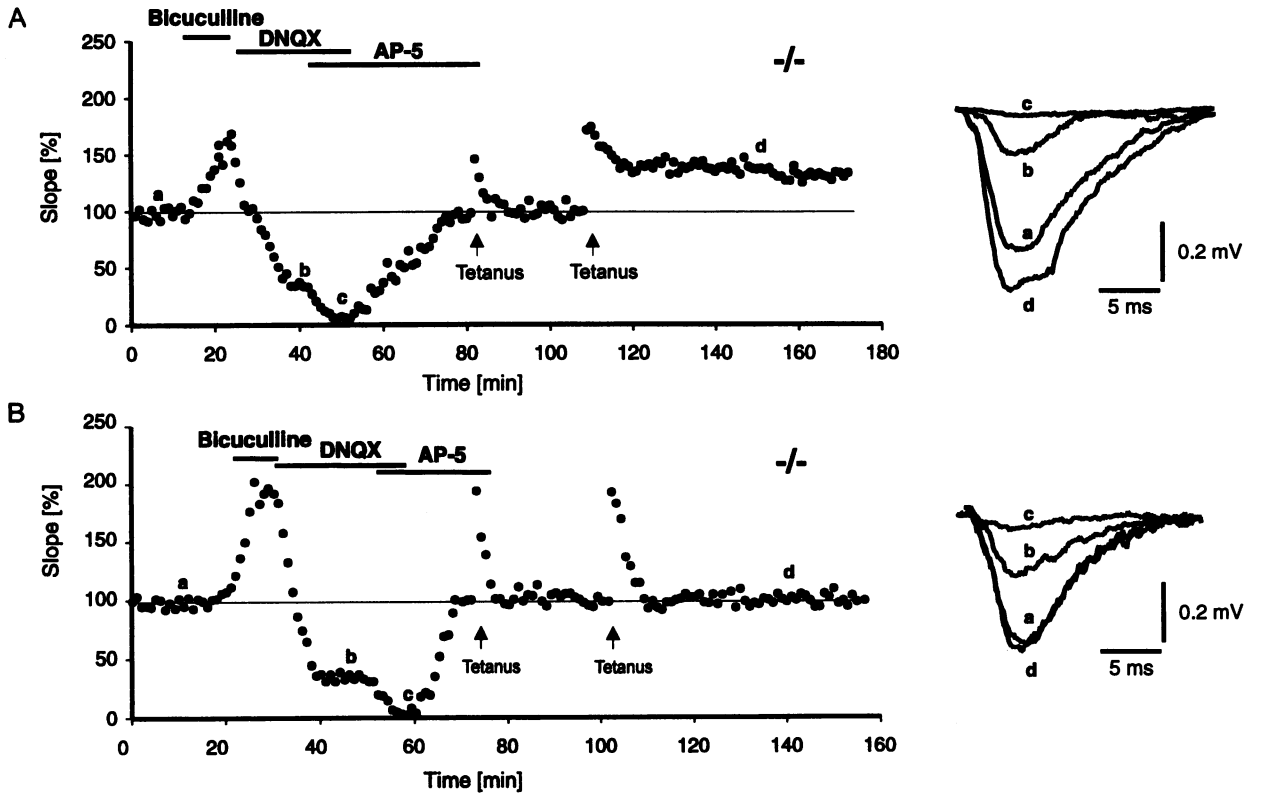

FIG. 3. Pharmacological experiments with −/− mice. The graphs show the time course of change in the field EPSP slope for a slice treated with bicuculline, 6,7-dinitroquinoxaline-2,3-dione (DNQX), and DL-2-amino-5-phosphonovalerate (AP-5). An experiment with successful induction of LTP (*A*) and a case of an unsuccessful induction of LTP (*B*) are shown. The clear response at time point b shows that the failure to induce LTP with the second tetanus cannot be attributed to a lack of NMDA responses. Sample field EPSP traces in response to stimulation of the Schaffer collateral—commissural pathway are displayed to the right: a, baseline trace in normal artificial cerebrospinal fluid; b, after the addition of DNQX (10 μM, with 1 mM Mg^{2+}); c, DNQX plus AP-5 (50 μM, with 1 mM Mg^{2+}); d, after a tetanus in normal artificial cerebrospinal fluid after AP-5 has been washed out. Lowercase letters next to the curve in the main graph indicate the time points at which the sample responses were taken. Values are average responses with a mean over six trials.

Our results showing a strong impairment of LTP in BDNF-deficient mice lends further support to the idea that neurotrophins play an important role in synaptic plasticity. Although it is surprising that +/− mice show the same amount of impairment as their −/− siblings, this observation is paralleled by other findings in the peripheral nervous system. A similar "threshold" effect on the survival of slowly adapting cutaneous mechanoreceptors has been observed in heterozygous mice carrying mutations of the neurotrophin 3 gene (G. Lewin, personal communication). This indicates that also in other cases there is a critical dosage for neurotrophins. In the case of LTP it appears that >50% of the wild-type BDNF level is needed for a fully functional system.

As much as it was unexpected to find equal impairment of LTP in +/− mice and −/− mice, this fact also makes a much stronger case for the direct involvement of BDNF in the expression of LTP. Since the +/− mice, in contrast to their −/− siblings, do not show any apparent phenotype, it seems

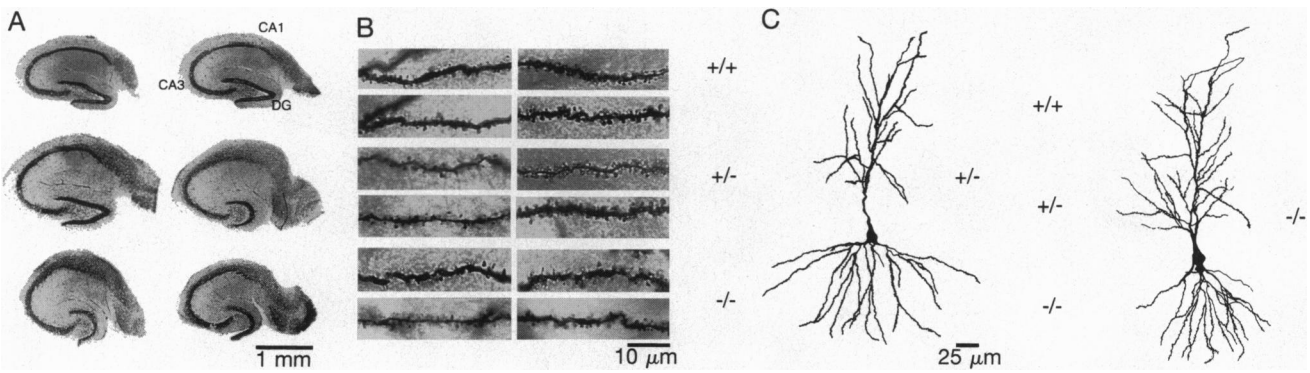

FIG. 4. Anatomy and morphology of the hippocampus and of hippocampal neurons. (*A*) Nissl stains of the hippocampus for all genotypes (+/+, +/−, and −/−). DG, dentate gyrus. (*B*) Dendritic segments from Golgi-stained CA1 pyramidal neurons in the hippocampus for all genotypes. Considerable differences in spine densities were observed within the different genotypes (left column, low densities; right column, high densities). No significant difference in spine density could be observed between the different genotypes ($P > 0.05$, two-tailed *t*-test). (*C*) Golgi-stained CA1 pyramidal neurons from BDNF-deficient mice. No abnormalities in terms of soma size, cell shape, and dendritic branching could be observed. Nissl and Golgi stains were done according to standard procedures. Note that due to limitations of the Golgi staining technique, reconstructions are likely to be incomplete.

unlikely that gross abnormalities would be responsible for the observed effects. Nevertheless, it is still possible—although in our view less likely—that the lack of BDNF during development specifically alters the molecular machinery necessary to produce LTP. Such a more subtle effect cannot be ruled out with the present experiments.

Our results also show that BDNF is certainly not the only important factor in LTP expression; in some cases LTP is still inducible even without any BDNF (e.g., see Fig. 3*A*). Therefore, although BDNF seems to play a critical role in the expression of LTP, it does not seem to be essential under all circumstances. This could be explained by the fact that a retrograde messenger is not called into action under all circumstances. Experiments using quantal analysis have shown that the locus of expression of LTP depends on the initial setting of the synapses (30). When, for instance, the initial setting of presynaptic release is high, synaptic strengthening is achieved postsynaptically (30). Under these circumstances a retrograde messenger is not involved in the generation of LTP, and thus its occasional occurrence in BDNF-deficient animals could easily be explained.

Our data show that animals with a defect in the BDNF gene show a substantially impaired expression of LTP. Since no obvious physiological, anatomical, or morphological abnormality can account for this result, we propose a functional role for BDNF in the expression of LTP in the hippocampus.

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