Transgenic Brain-Derived Neurotrophic Factor Modulates a Developing Cerebellar Inhibitory Synapse

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Abstract

Brain-derived neurotrophic factor (BDNF) has been shown to promote synapse formation and maturation in neurons of many brain regions, including inhibitory synapses. In the cerebellum, the Golgi cell-granule cell GABAergic synaptic responses undergo developmental transition from slow-decaying to fast-decaying kinetics, which parallels a developmental increase of GABA_A receptor $\alpha 6$ subunit expression in the cerebellar granule cells. In culture, BDNF accelerates the expression of GABA_A receptor $\alpha 6$ subunit expression in granule cells. Here we examined synaptic GABA_A response kinetics in *BDNF* transgenic mice. The mutant mouse, which carries a *BDNF* transgene driven by a β -actin promoter, overexpresses BDNF (two- to fivefold increase compared with wild types) in all brain regions. Recordings of the spontaneous GABA_A responses indicate that the decay time constant of the GABAergic responses decreases during early postnatal development; this transition is accelerated in the *BDNF* transgenic mouse. The amplitude of the spontaneous GABA_A responses was also larger in the transgenic mouse than in the wild-type mouse. However, the frequency of the spontaneous GABA_A responses were not different between the two groups. Our results suggest that BDNF may modulate GABAergic synapse maturation in the cerebellum.

Introduction

Brain-derived neurotrophic factor (BDNF), along with other members of the neurotrophin family, was first identified as one of the classical survival proteins in developing mammalian nervous system (Ghosh et al. 1994; Kubo et al. 1995; Nonomura et al. 1996). The expression of BDNF exhibits a unique temporal pattern. Whereas the expression levels of most other neurotrophic factors (e.g., nerve growth factor, neurotrophin 3) in many brain regions decrease during postnatal development, BDNF expression becomes prominent only after neurogenesis has occurred and stays at high levels in the mature central nervous system (CNS) (Leibrock et al. 1989; Maisonpierre et al. 1990; Rocamora et al. 1993). Concomitant with its expression pattern, BDNF was found to regulate events that occur during late stages of neuron development, such as dendritic growth and synaptic innervation (Gao et al. 1995; McAllister et al. 1995; Bartrup et al. 1997; Causing et al. 1997; Rutherford et al. 1998; Vicario-Abejon et al. 1998). BDNF has been shown to modulate GABAergic neurons and synapses. In culture, BDNF increases GABA content and glutamic acid decarboxylase (GAD) activity level in neostriatal (Mizuno et al. 1994; Ventimiglia et al. 1995) and neocortical neurons (Rutherford et al. 1997), and increases GABA uptake and soma size of cortical interneurons (Widmer and Hefti 1994). In *BDNF* transgenic mice, GAD65 and parvalbumin expression revealed accelerated emergence of the mature pattern of inhibitory presynaptic boutons in the visual cortex (Z.J. Huang, A. Kirkwood, V. Porciatti, T. Pizzorusso, M.F. Bear, L. Maffei, and S. Tonegawa, unpubl.). In addition to its modulatory effects on presynaptic GABAergic neurons, BDNF also accelerates the expression of postsynaptic GABA_A receptor subunits in cerebellar granule cell culture (Lin et al. 1998). However,

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these studies are more or less limited to biochemical and morphological characterization (but see Rutherford et al. 1998; Vicario-Abejon et al. 1998), largely due to a lack of electrophysiological assessment of GABAergic synapse development.

In the cerebellar cortex, the initial formation of inhibitory synapses between Golgi cells and granule cells occurs after granule cells migrate into the internal layer. The internal granule cells express a subset of GABA_A receptor subunits, and the receptor composition undergoes a developmental transition during the process of GABAergic synapse maturation (Laurie et al. 1992; Zheng et al. 1993; Fritschy et al. 1994; Thompson and Stephenson 1994). For example, the mRNA signal for the $\alpha 6$ subunit of the GABA_A receptor is not present until the end of the first postnatal week and is fully expressed only in granule cells exhibiting mature (i.e., fast-decaying; see Tia et al. 1996; L. Chen, S. Bao, X. Qiao, and R.F. Thompson, unpubl.) GABAergic synaptic responses (Zheng et al. 1993). Interestingly, application of BDNF accelerated $\alpha 6$ subunit expression in cultured cerebellar granule cells (Lin et al. 1998), suggesting that BDNF may modulate cerebellar GABAergic synapse maturation. In the current study, taking advantage of transgenic mice, we studied the effects of BDNF on developing cerebellar Golgi cell-granule cell synapses.

Materials and Methods

ANIMALS

BDNF transgenic mouse was generated (at Regeneron Pharmaceuticals Inc.) with germ-line insertion of a human *BDNF* transgene (Croll et al. 1999) driven by a β -actin promoter. Heterozygous transgenic mice (C57BL/6 $\times$ DBA/2) were used to breed homozygous, heterozygous, and wild-type littermates (at a University of Southern California vivarium). Both homozygotes and heterozygotes, were pooled as transgenics, whereas wild types and some age-matched C57BL/6 inbreds were used as controls. As both homozygote and heterozygote exhibit normal phenotype, the experiments were conducted blindly with experimenters unaware of the genotypes of the mice. Genomic Southern blot analysis was used to identify the *BDNF* transgene.

HISTOLOGY

Transgenic and age-matched wild-type mice were deeply anesthetized with Halothane, and

transcardially perfused with 0.9% saline and 10% buffered formalin. The brains were removed and postfixed in 10% formalin for at least 24 hr. Then the brains were sliced with a sliding microtome. Parasagittal sections (40 μ m) were mounted onto gelatin-coated slides, stained with cresyl violet, and observed with light microscope.

IN SITU HYBRIDIZATION AND NORTHERN BLOTS

The procedures for in situ hybridization and Northern blots were the same as in Qiao et al. (1996). Briefly, brains from both wild-type and mutant adult mice were excised after decapitation, frozen on dry ice, sliced on a cryostat at 16 μ m, and mounted on subbed slides. The mutant and wild-type brain sections were mounted on the same slides and processed identically for optimal comparison. The sections were fixed with 4% p-formaldehyde in 0.1 M PBS for 30 min and pretreated with 0.25% acetic anhydride and 0.1 M triethanolamine for 10 min. Then, the tissue sections were incubated overnight at 50°C with ³⁵S-labeled riboprobe (5 $\times$ 10⁶ cpm/ml) prepared from a pGEM-4Z plasmid containing a 460-bp insert of rat BDNF cDNA clone (Phillips et al. 1990). The hybridization solution also contains 50% formamide, 10% dextran sulfate, 300 mM NaCl, 0.5 mg/ml yeast RNA, 10 μ M dithiothreitol, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.0). After hybridization, the sections were rinsed in 4 $\times$ SSC, digested with 20 μ g/ml RNase A at 37°C for 30 min, and washed through descending concentrations of 1 $\times$ SSC to 0.1 $\times$ SSC at 60–70°C. Finally, the slides were dehydrated in ethanol, dried, and exposed to X-ray film (Kodak X-Omat, Rochester, NY) for 3–5 days.

For Northern blots, total RNA was extracted from mice cerebella with RNA/DNA protein isolation TRI Reagent (Molecular Research Center, Cincinnati, OH). Briefly, the cerebellum of each animal was homogenized in TRI Reagent solution, suspended in a 1:5 volume of chloroform, and centrifuged at 12,000g for 15 min. The RNA contained in the aqueous phase was transferred and precipitated with isopropanol. The samples were then centrifuged at 12,000g for 15 min to pellet the RNA. After being washed with 75% cold ethanol, the pellet was dissolved in RNase-free water. The total RNA (20 μ g) was separated by standard methods on a formaldehyde-denaturing agarose gel. The RNA was then blotted to neutral nylon membranes (Max-Strength Nytran, Schleicher & Schuell,

Keene, NH) and hybridized to random prime ^{32}P -labeled *BDNF* cDNA probe ($\sim 3 \times 10^6$ cpm/ml; Strategene, La Jolla, CA). The membrane was then washed in graded SSC, dried, and exposed to PhosphorImager plates (Molecular Dynamics, Eugene, OR). Relative abundance of mRNA was quantified by reading the plates. For accurate quantification, the same blot was stripped off and hybridized to ^{32}P -labeled β -actin probe. The ratio of *BDNF*/ β -actin optical densities was then used for comparison.

BRAIN SLICES

The mouse was decapitated, the cerebellum removed and placed in ice-cold artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4 , 2.5 mM CaCl_2 , 1.3 mM MgCl_2 , 26 mM NaHCO_3 , 10 mM glucose, buffered with 5% CO_2 and 95% O_2). The cerebellar vermis was sliced sagittally (400 μm) with a vibrotome. The slices were incubated in ACSF for at least 30 min before being transferred into a submersion recording chamber. Incubations and all subsequent experiments were done at room temperature.

ELECTROPHYSIOLOGY

Spontaneous GABA_A currents of granule cells were recorded with whole-cell configuration, and the blind techniques (Blanton et al. 1989) were used to form tight seals. The impedance of the patch pipettes ranges from 3 to 6 M Ω when filled with internal solution (140 mM CsCl, 2 mM MgCl_2 , 5 mM EGTA, 10 mM HEPES, 4 mM Na_2ATP , 0.3 mM Na_3GTP at pH 7.3). Granule cells were identified by their typical low whole-cell capacitance (~ 5 pF) and high input resistance (>1 G Ω) (D'Angelo et al. 1993). CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 10 μM) and D-APV [D(-)-2-amino-5-phosphonopentanoic acid, 100 μM] were included in ACSF to eliminate glutamatergic synaptic currents. After the whole-cell configuration was achieved, 3 min were allowed for chloride to diffuse into the cells. Spontaneous GABA_A synaptic currents were then continuously recorded ($V_m = -80$ mV) for 5 min. Spontaneous GABA_A -mediated events were identified with custom-made programs by criteria adopted from Raastad et al. (1996). Briefly, a current deflection is considered an inhibitory postsynaptic current (IPSC) event if it fits well with a α function, and exhibits an amplitude four times larger than the standard deviation of baseline

noise, 20%–80% rise time is shorter than 1 msec, and 80%–20% decay time longer than 5 msec. All IPSC events were later visually verified by experimenters. Decay time constant of the GABA_A -mediated synaptic currents was calculated by the average of all events in the 5-min recording period. Response frequency and average amplitude for each cell were also measured with custom-made programs. Data are presented as average $\pm$ S.E.M.

Results

In adult wild-type mouse, *BDNF* is highly expressed in the hippocampus, neocortex, and the cerebellum (Leibrock et al. 1989; Hofer et al. 1990; Maisonpierre et al. 1990; Rocamora et al. 1993). In *BDNF* transgenic mice, the *BDNF* mRNA levels, as shown in the autoradiographs (Fig. 1), are significantly elevated throughout the whole brain. In the cerebellum, dense signal is mainly located in the granule cell layer. Densitometry of the autoradiographs revealed two- to fivefold increase of *BDNF* mRNA level in the adult cerebellum of transgenic mouse (data not shown). Unlike endogenous *BDNF* expression in the wild-type mouse, which starts postnatally (Maisonpierre et al. 1990), a high level of *BDNF* mRNA was seen in the transgenic mouse brain as early as embryonic day 18 (Croll et al. 1999). We compared cerebellar *BDNF* expression in transgenic and wild-type mice during postnatal development using Northern blot analysis of total cerebellar RNA hybridized with a *BDNF* cDNA probe (Qiao et al. 1996). As shown in Figure 2, the expression levels of endogenous *BDNF* in transgenic mice are similar to those in wild types, which are not evident at postnatal day 9 (P9), low at P13, and relatively high at P17. However, in

Figure 1: Expression patterns of *BDNF* mRNA in adult brains of wild-type (WT) and *BDNF* transgenic (TG) mice.

Figure 2: Northern blot analysis of BDNF mRNA levels in the cerebellum of wild-type (WT) and BDNF transgenic (TG) mice at postnatal day 9 (P9), day 13 (P13), and day 17 (P17).

transgenic mice, the *BDNF* transgene is highly expressed at all ages examined (P9, P13, P17, and >P28).

Despite the high level of transgenic BDNF expression, the mutant mouse exhibits no sign of abnormal brain development. In the cerebellar cortex, the laminar structures develop normally (Fig. 3). The numbers of external and internal granule cells are comparable between transgenic and wild-type mice examined at four ages, P9, P13, P17, and P21, suggesting that the excessive BDNF does not affect the proliferation, migration, and survival of the cerebellar granule cells.

To study Golgi cell-granule cell inhibitory synapses, cerebellar granule cells were recorded with whole-cell configuration, a blind method (Blanton et al. 1989) was used to form tight seals. After forming whole cell, a series of voltage steps were applied and cells that did not exhibit any voltage-activated currents (most likely glial cells) were discarded. Granule cells were identified by their small whole-cell capacitance (~ 5 pF) and large input resistance (>1 G Ω). Spontaneous GABA_A receptor-mediated synaptic currents were recorded in the presence of 10 μ M CNQX and 100 μ M D-APV, and

were completely blocked by 30 μ M picrotoxin, a GABA_A receptor antagonist. A total of 255 cerebellar granule cells were recorded from *BDNF* transgenic mice and wild-type controls at four ages, P9 (wild type, $n = 44$; transgenic, $n = 34$), P13 (wild type, $n = 32$; transgenic, $n = 52$), P17 (wild type, $n = 20$; transgenic, $n = 37$), and P21 (wild type, $n = 19$; transgenic, $n = 19$). The spontaneous GABA_A responses were averaged for each cell, and the decay phase of the average response was fit to a biexponential algorithm. To facilitate comparison, an amplitude-weighted average of the two decay time constants was calculated for each cell. A clear developmental decline of the decay time constant (Tia et al. 1996; L. Chen, S. Bao, X. Qiao, and R.F. Thompson, unpubl.) was observed in both transgenic and control mice (Fig. 4A). This process appeared to be accelerated in the transgenic mouse, as evidenced by a significant difference in the average decay time constant at P9 (34 transgenic cells, 44 control cells; one-way ANOVA, $F [1, 76] = 3.97$, $P > 0.05$). Figure 4B shows spontaneous GABA_A receptor-mediated synaptic responses recorded from P9 transgenic and wild-type granule cells.

A previous study (Vicario-Abejon et al. 1998) showed that prolonged treatment of cultured hippocampal neurons with exogenous BDNF caused increase in spontaneous IPSC amplitude. We compared the average IPSC amplitudes recorded from transgenic and wild-type granule cells and found that the BDNF transgene increased IPSC amplitude in the P9 group (Fig. 5A, $F [1, 76] = 4.05$, $P \leq 0.05$). The IPSC amplitude distribution is shifted in transgenic mice compared with that in wild-type mice (Fig. 5B).

BDNF has been shown to induce a variety of presynaptic modifications, such as increasing GABA content and GAD activity in cultured GABAergic

Figure 3: Laminar structures of the cerebellar cortex of wild-type and BDNF transgenic mice. The cerebellum of the transgenic mouse exhibits normal laminar structures. (EGL) external granule cell layer; (PC) Purkinje cell layer; (IGL) internal granule cell layer. Scale bar, 100 μ m.

Figure 4: (A) Average decay time constant of the spontaneous IPSCs recorded from cerebellar granule cells. The IPSC decay time constants at both wild-type and the transgenic synapses exhibit developmental decrease from P9 to P21. At P9, IPSC exhibited significantly faster decay at transgenic synapses (open bars) than at wild-type synapses (black bars). (*) $P \leq 0.05$. (B) Examples of spontaneous IPSCs recorded from P9 cerebellar granule cells of wild-type and BDNF transgenic mice. Note the faster decay of IPSCs recorded from transgenic granule cells than those recorded from wild-type cells. (C) Examples of average IPSCs of the same wild-type and transgenic cells as in B.

neurons (Mizuno et al. 1994; Ventimiglia et al. 1995; Rutherford et al. 1997), promoting axonal growth and branching in cultured hippocampal inhibitory neurons (Vicario-Abejon et al. 1998), and accelerating emergence of the mature pattern of inhibitory presynaptic boutons in the visual cortex of *BDNF* transgenic mice (Z.J. Huang, A. Kirkwood, V. Porciatti, T. Pizzorusso, M.F. Bear, L. Maffei, and S. Tonegawa, unpubl.) Granule cell IPSCs are tetrodotoxin sensitive, and therefore are caused by Golgi cell discharges (Tia et al. 1996). In the present study, we did not observe significant differences in IPSC frequency between transgenic and wild-type mice at all four age groups tested. For example, at P9, the average IPSC frequency is 7.4 ± 1.2 for wild type mice (44 cells) and 9.6 ± 1.3

(34 cells) for *BDNF* transgenic mice (see Fig. 6, one-way ANOVA, $F[1,76] = 3.90$, $P > 0.1$).

Discussion

BDNF has been shown to modulate developing inhibitory synapse in both cell cultures and in transgenic mice. During early postnatal development, the inhibitory cerebellar Golgi cell-granule cell synapse undergoes developmental transitions in receptor composition (Laurie et al. 1992; Zheng et al. 1993; Fritschy et al. 1994; Thompson and

Figure 5: (A) Average amplitude of the IPSCs recorded from cerebellar granule cells. (*) $P \leq 0.05$. (B) Distributions of average IPSC amplitudes of P9 cerebellar granule cells. The difference between the transgenic (black bars) and the wild-type (gray bars) distributions is plotted as an inset.

Stephenson 1994) and synaptic response kinetics (Tia et al. 1996; L. Chen, S. Bao, X. Qiao, and R.F. Thompson, unpubl.), providing a suitable system for the studying effects of neurotrophins on synapse development. Taking advantage of a transgenic mutant mouse carrying a β -actin promoter-driven human *BDNF* transgene, we show that BDNF overexpression causes decrease in IPSC decay time constant and increase in IPSC amplitude in cerebellar granule cells at early developmental stage (i.e., P9). Because reduction in decay time constant normally occurs during the maturation process at the cerebellar Golgi cell-granule cell synapse, the decreased IPSC decay time constant may be a result of accelerated synapse maturation. BDNF is involved in many aspects of neuronal development. For example, evidence indicates that, in the cerebellum, BDNF is important for neuronal survival (Segal et al. 1992; Gao et al. 1995; Kubo et al. 1995; Minichiello and Klein 1996; Nonomura et al. 1996; Schwartz et al. 1997), differentiation (Segal et al. 1992; Minichiello and Klein 1996; Schwartz et al. 1997; Lin et al. 1998), axonal outgrowth (Segal et al. 1995) and neurite extension (Gao et al. 1995; Schwartz et al. 1997).

Although the transgenic BDNF is expressed at high levels at all ages examined (P9, P13, P17, and >P28), the electrophysiological differences between the two genotypes were only observed at P9. Developmental alterations in expression patterns of TrkB and endogenous BDNF may confer this narrow time window in which the inhibitory synapse is sensitive to BDNF. For example, levels of TrkB receptor mRNAs (especially the kinase-

containing splicing variants) in cerebellar granule cells peak in the first postnatal week and decrease later (Segal et al. 1995). The levels of TrkB receptors may determine the responsiveness of cerebellar granule cells to BDNF. In addition, endogenous BDNF expression becomes evident at P13 and may saturate TrkB receptors (levels of which have been reduced from the peak in the first postnatal week) on the granule cells.

The accelerated synapse maturation and increased spontaneous IPSC amplitude in the present study are in general agreement with results from culture neurons (Vicario-Abejon et al. 1998). However, the effects observed in *BDNF* transgenic mice are much less robust than those in cultures. This may be attributed to differences in cell types and experimental conditions. It is possible that cerebellar granule cells and/or Golgi cells are simply less responsive to BDNF than hippocampal neurons, or that a certain level of endogenous BDNF (might not be present in the culture) undercuts the effects of exogenous BDNF.

BDNF affects both presynaptic and postsynaptic neurons of GABAergic synapses. On the presynaptic side, it enhances GABA content of inhibitory neurons (Mizuno et al. 1994; Ventimiglia et al. 1995; Rutherford et al. 1997) and promotes growth and/or branching of axons and presynaptic boutons (Vicario-Abejon et al. 1998; Z.J. Huang, A. Kirkwood, V. Porciatti, T. Pizzorusso, M.F. Bear, L. Maffei, and S. Tonegawa, unpubl.). On the postsynaptic side, BDNF modulates GABA_A receptor expression (Lin et al. 1998) and shifts spontaneous IPSC amplitude distribution (Vicario-Abejon et al. 1998). In the cerebellar Golgi cell-granule cell synapse, the IPSC decay kinetics are determined by postsynaptic receptor desensitization (Jones and Westbrook 1995) and the developmental decline of the IPSC decay time constant is correlated with the expression of the GABA_A Receptor $\alpha 6$ subunit (Zheng et al. 1993; Tia et al. 1996). Thus, the accelerated transition of IPSC kinetics observed in the present study was likely to be caused by BDNF on granule cells. We did not observe a significant effect of transgenic *BDNF* on IPSC frequencies (as seen in Vicario-Abejon et al. 1998), but there was considerable variation among the cells. Further biochemical analysis of the pre- and postsynaptic neurons (i.e., GABA_A receptor expression in granule cells, and GABA content and GAD expression in Golgi cells, respectively) may help understand how transgenic BDNF modulates the cerebellar GABAergic synapse.

Figure 6: Distributions of spontaneous IPSC frequencies of P9 cerebellar granule cells. No difference was found between transgenic (open bars) and wild-type (black bars) IPSC frequencies.

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