Cerebellar Brain-Derived Neurotrophic Factor–TrkB Defect Associated with Impairment of Eyeblink Conditioning in *Stargazer* Mutant Mice

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In the spontaneous ataxic mutant mouse *stargazer*, there is a selective reduction of brain-derived neurotrophic factor (BDNF) mRNA expression in the cerebellum. BDNF protein levels in the cerebellum are reduced by 70%. Despite normal levels of full-length and truncated TrkB receptor, constitutive and neurotrophin-4/5-induced tyrosine phosphorylation was significantly reduced in several signal transduction molecules, including phospholipase-C γ 1, erk1, and erk2. Morphological examination revealed an increased number of external granule

cells at postnatal day 15 and the presence of abnormal neurons resembling immature granule cells in the adult. These abnormalities are associated with a severe impairment in the acquisition of classical eyeblink conditioning, indicating cerebellar malfunction. Our data suggest that normal BDNF expression and TrkB signal transduction in the cerebellum are necessary for learning and plasticity in this model.

Key words: mutant mice; neurotrophic factor; BDNF; TrkB; signal transduction; eyeblink conditioning; learning; cerebellum

Neurotrophic factors are important regulators of the formation and function of the nervous system during development and are required for maintenance in the adult brain. The family of the neurotrophins includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and the neurotrophins (NTs) 3, 4/5, and 6. Although most of the neurotrophins are highly expressed in immature regions of the nervous system during development, the regulation of BDNF expression is most prominent in brain regions in which neurogenesis has already occurred (Maisonpierre et al., 1990). The BDNF level generally increases as these regions mature and remains the most abundant neurotrophin in the adult brain (Leibrock et al., 1989; Maisonpierre et al., 1990). In mouse and rat, the histogenesis and morphogenesis of cerebellum primarily occur within the first postnatal month (Jacobson, 1991; Altman and Bayer, 1997). Correspondingly, BDNF mRNA expression in the cerebellum is not apparent until postnatal day 15 (P15) but then rises quickly and peaks at approximately P20 in rat (Rocamora et al., 1993) and mouse (Qiao et al., 1996), coinciding with granule cell migration and maturation.

The cerebellum plays an important role in movement and posture. Malformation and lesions of the cerebellum disrupt motor coordination and impair balance. In addition to the classical role in the fine motor control, increasing evidence has shown that the cerebellum is also involved in a variety of nonmotor cognitive functions, including sensory discrimination (Gao et al.,

1996), attention (Allen et al., 1997), and learning and memory (Thompson, 1986; Andreasen et al., 1995). Classical eyeblink conditioning is a form of sensorimotor learning that depends crucially on the cerebellum (Thompson, 1990; Yeo, 1991). More recent studies in several spontaneous and transgenic mutant mice have shown the importance of both the cerebellar cortex and interpositus nucleus in this learning paradigm (Chen et al., 1995, 1996; Shibuki et al., 1996; Kim and Thompson, 1997). Because the neural circuitry involved in this form of learning has been well characterized (Thompson, 1986, 1990), the eyeblink conditioning paradigm allows us to screen for the cellular and molecular substrates, which are required for cerebellar function and plasticity.

Our knowledge of BDNF function *in vivo* primarily comes from studies of BDNF knock-out transgenic mice (Ernfors et al., 1994; Conover et al., 1995; Liu et al., 1995). However, the high early postnatal mortality and the universal deletion of the gene make it difficult to determine the exact role of BDNF on a specific neuronal type in the brain, especially at late developmental stages. We have reported previously a specific and pronounced deficit in BDNF mRNA expression in the cerebellum of the *stargazer* (*stg*) mouse (Qiao et al., 1996), a spontaneous mutant with ataxia and generalized spike-wave seizures. Other brain areas of this mouse express normal levels of BDNF, and mRNA levels for NGF and NT-3 were normal throughout the brain, including the cerebellum. We took advantage of this unique mutant mouse model and its region-specific defect of BDNF expression to study the role of BDNF in cerebellar development and function.

MATERIALS AND METHODS

Animals. Wild-type (C57BL/6J; +/+) and mutant (C3B6Fe+; *stg/stg*) mice were obtained from the breeding colonies of The Jackson Laboratory (Bar Harbor, ME) and maintained in the University of Southern

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California Gerontology Center vivarium on a 12 hr light/dark cycle with food and water available *ad libitum*. Heterozygous males (+/*stg*) and homozygous females (*stg/stg*) were mated to produce *stg* mutant mice. Because we have not found any significant phenotype difference so far between +/+ and +/*stg*, nonmutant littermates (+/–) including both genotypes were used in developmental and behavioral studies as wild-type controls.

BDNF enzyme immunoassay. For BDNF enzyme immunoassay (EIA), we used modified techniques developed by Nawa et al. (1995). The cerebellar tissues were isolated from adult +/+ and *stg/stg* mice ($n = 3$), weighed, and immediately frozen on dry ice. The tissues were homogenized for 60 sec with 100 volumes of homogenization buffer containing 50 mM Tris, 0.3 M NaCl, 0.1% Triton X-100, 1% bovine serum albumin (Corn Fraction V; Sigma, St. Louis, MO), aprotinin (200 kallikrein inhibitor units/ml), 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM benzethonium chloride, and 1 mM benzamide. Homogenates were centrifuged for 20 min at $15,000 \times g$ at 4°C, and supernatants were stored at –70°C until use. Anti-BDNF antibodies were kindly provided by Josette Carnahan (Amgen, Thousand Oaks, CA). Polystyrene 96-well microtiter plates (Corning, Corning, NY) were coated with 100 ng/well low-affinity antibody in 0.1 M Tris buffer, pH 9.0, for 12–18 hr and then blocked with EIA buffer containing 50 mM Tris, 0.3 M NaCl, 0.05% Thiomosal, 0.1% Triton X-100, 1% BSA, and 1% gelatin, pH 7.5, at 4°C overnight. One hundred microliters of tissue extracts or BDNF standards (recombinant human BDNF; Peprotech, Rocky Hill, NJ) in EIA buffer were loaded to wells for 12–18 hr. After washing with EIA buffer without BSA, 100 μ l of biotinylated high-affinity anti-BDNF antibody (10 ng/ml) in EIA buffer were added and incubated for 12–18 hr at room temperature. Bound biotinylated secondary antibodies were detected by incubation with streptavidin/peroxidase (1:250) for 3 hr. After extensive wash, the enzyme activity retained in each well was measured by incubation with 200 μ l of 3,3',5,5'-tetramethylbenzidine substrates for 30 min. The blue end product was read on a SPECTRAMax 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 370 and 655 nm using the SOFTmax computer program. A standard curve in the range of 1–300 pg of recombinant human BDNF was determined for each titer plate.

TrkB receptor signal transduction assay. Standard procedures of TrkB receptor signal transduction assays were followed as described in detail previously (Knusel et al., 1994). Briefly, cerebellums from 2- and 6-month-old wild-type and *stg/stg* mice were homogenized in 1 ml of lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 500 μ M orthovanadate). Soluble proteins were separated by centrifugation, and the supernatant was precipitated with wheat germ lectin agarose to partially purify TrkB proteins. The glycoproteins were then subjected to SDS-PAGE and probed by Western blotting with a polyclonal rabbit anti-TrkB antibody directed against extracellular domain sequences (antibody was kindly provided by Dr. David Kaplan, National Institute of Cancer, Frederick Cancer Research and Development Center). Phosphorylation assays were performed on microslides (300 $\times$ 300 μ m) prepared from cerebellum of 2-month-old +/+ and *stg/stg* mice according to previously published procedures (Knusel et al., 1994). Briefly, the slices were maintained in gassed Krebs' buffer and then incubated for 4 min with recombinant human NT-4/5 (kindly provided by Genentech, San Francisco, CA) at 37°C. Immediately after incubation, the tissue was lysed and immunoprecipitated with mouse anti-phospholipase-C γ 1 (PLC γ 1; Upstate Biotechnology, Lake Placid, NY). By Western blotting, the precipitate was analyzed for the presence of phosphotyrosine on PLC γ 1 with anti-phosphotyrosine mouse monoclonal antibody (4G10; Upstate Biotechnology). The same microslice preparation was used to measure the phosphotyrosine levels of erk1 and erk2, another two signal transduction molecules. After incubation with NT-4/5, the tissue was lysed, and an aliquot of the lysate was separated by SDS-PAGE. Tyrosine-phosphorylated proteins were detected by Western blotting with anti-phosphotyrosine antibody.

Tissue processing for histology. Mice were anesthetized with Avertin (1.25% tribromoethanol–amylalcohol solution) and perfused transcardially with 4% paraformaldehyde in PBS (0.1 M sodium phosphate and 0.9% NaCl, pH 7.4). The brains were post-fixed overnight and subsequently equilibrated in 30% sucrose solution. Coronal sections were cut and stained with either 1% cresyl violet and eosin or hematoxylin and eosin.

Cell count and electron microscopy. Mice were perfused transcardially with 0.1 M cacodylate buffer for 2 min and then with fixative of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer

with 0.02% CaCl₂ for 20 min. The medial cerebellum was sliced into 1 mm translobular (sagittal) sections. The sections were post-fixed in the fixative for 2 d and in 1% osmium tetroxide for 1 hr. Tissues were washed in the buffer, dehydrated, and infiltrated with propylene oxide. Tissues were then embedded in Epon and polymerized overnight at 67°C. Semi-thin sections (3 μ m) were cut for light microscopic evaluation and granule cell count. Sections were deplastic in NaOH ethanol solution before hematoxylin and eosin staining. External granule cells from lobules 5–8 were counted in 10 sections from each animal. Means of granule cells along the pia surface (millimeters) were calculated. Student's *t* test was used for statistical significance between the genotypes. Ultrathin sections were then cut from the blocks, counterstained with 2% uranyl acetate and lead citrate, mounted on grids, and examined with a JEOL JEM-1200EX electron microscope.

Behavioral analysis. Eyeblick conditioning procedures were followed as described in detail previously (Chen et al., 1996). Briefly, adult male *stg/stg* ($n = 8$) and wild-type littermate (+/–) ($n = 8$) mice of C3B6Fe+ strain were anesthetized with ketamine (80 mg/kg) and xylazine (20 mg/kg) and implanted with four wires (Teflon-coated stainless steel; A-M Systems, Everett, WA) subcutaneously to the left upper eyelid. The tips of the wires were exposed, and two of the wires were used to record differential electromyography (EMG) from obicularis oculi, and the remaining two wires were used to deliver periorbital shock. A four-pin strip connector to which the wires were soldered was cemented to the animal's skull with dental acrylic. After 1 d of habituation, animals underwent 6 d of paired behavioral training. The daily paired training consisted of 100 trials grouped in 10 blocks. Each block included one conditioned stimulation (CS) alone trial (80 dB, 1 kHz tone), one unconditioned stimulation (US) alone trial (100 msec shock, 100 Hz biphasic square pulses), and eight CS–US paired trials with a 252 msec interstimulus interval. The US intensity used was the minimal voltage required to elicit an eyeblink and head turn response, and the US intensity was adjusted daily for each animal. The EMG activity was digitized using a window discriminator and stored onto a computer with a sampling rate of 4 msec/bin. The “bad” trials were excluded using the following criteria: (1) during the 252 msec pre-CS period, the average unit counts per bin were >2 ; (2) the SD was greater than the average unit counts per bin during the pre-CS period; or (3) the average unit count within 28 msec after the CS onset was 1 SD larger than the average of pre-CS activity. The eyeblink conditioned response (CR) was defined as the average unit activity of any consecutive seven bins during the CR period (from 84 msec after the CS onset to the US onset for paired trials or to the termination of the CS for CS alone trials) that was higher than “average plus SD plus 1” of the pre-CS unit activity. The CR onset latency was computed as the first time during the CR period at which the average unit count of any 28 msec was higher than average plus SD plus 1 of the pre-CS unit activity. The unconditioned response (UR) amplitude was defined as the difference between averaged unit activity during the UR period [100 msec period 50 msec after the termination of the unconditioned stimulation (US)] and averaged unit of the pre-CS period.

RESULTS

Lack of BDNF protein in the *stg* mutant cerebellum

In a previous paper, we have shown a pronounced selective failure of BDNF mRNA expression in cerebellar granule cells of the *stg* mouse (Qiao et al., 1996). To test whether the lack of BDNF mRNA is reflected at the protein level, BDNF was measured with a two-site EIA for this protein. We found a 70% reduction in BDNF protein in the adult *stg* cerebellum compared with normal control mice [$+/+$, 1.20 ± 0.08 ng/gm (mean $\pm$ SE); *stg/stg*, 0.35 ± 0.03 ng/gm; $n = 3$; $p < 0.01$]. This result is consistent with the decreased BDNF mRNA level found previously by Northern blotting and *in situ* hybridization (Qiao et al., 1996).

Deficit on TrkB signal transduction in the *stg* cerebellum

Our previous study has shown a normal pattern and level of trkB mRNA expression in the cerebellum of *stg* mouse, despite the striking reduction of its ligand BDNF. To measure the levels of TrkB protein, we performed Western blotting using a polyclonal

Figure 1. Western blot analysis of levels of TrkB receptor protein (*A*) and Trk signaling in cerebellum (*B*, *C*) of wild-type and *stg/stg* mice. *A*, Glycoproteins were precipitated by incubation with wheat germ lectin and were then probed with a polyclonal rabbit anti-TrkB receptor antibody directed against extracellular domain sequences of TrkB. The antibody identified two major bands at 145 kDa and 95 kDa corresponding to the full-length and truncated forms of TrkB receptor. No significant difference was found between the wild-type and *stg* mutant cerebellum for either form of TrkB receptor protein at 2 and 6 months of age. The same antibody detected a third band with molecular weight of ~75 kDa, indicated in the figure with an arrow. This protein was clearly reduced in *stg/stg* compared with wild type. *B*, PLC γ 1 was immunoprecipitated from untreated and NT-4/5-treated cerebellar slices with anti-PLC γ 1 and analyzed by Western blotting with anti-phosphotyrosine. Both constitutive and NT-4/5-induced tyrosine phosphorylation of PLC γ 1 was significantly reduced in *stg* mouse cerebellum when compared with wild-type cerebellum. *C*, Tyrosine phosphorylated proteins were detected by Western blotting with anti-phosphotyrosine antibody in lysates from untreated and NT-4/5-treated cerebellar slices. In contrast to the equivalent levels of a third band on the same blot, both constitutive and NT-4/5-induced phosphotyrosine signals of two proteins with molecular weights identical to erk1 and erk2 were reduced in *stg* cerebellum compared with wild-type cerebellum.

rabbit anti-TrkB antibody directed against extracellular domain sequences. As shown in Figure 1*A*, the antibody identified two major bands corresponding in molecular size to full-length TrkB at 145 kDa with the intracellular kinase domain and to truncated TrkB at 95 kDa lacking the kinase domain. In agreement with the mRNA levels, no significant difference was found in either form of TrkB receptor protein between the wild-type and *stg* mutant cerebellum of the 2- and 6-month-old mice tested. The antibody recognized a third band that was less intense with molecular weight at ~75 kDa in our blots of wheat germ lectin-precipitated proteins (Fig. 1*A*). Although the molecular identity of this band is unknown at present, the reduced prevalence of this protein in *stg* indicates reduced levels in the mutant cerebellum of a protein with a very similar epitope as TrkB or of a protein that coprecipitates with one or both of the TrkB forms.

To further assess the functional response of TrkB receptors in *stg*, we performed tyrosine phosphorylation assays in freshly prepared microslices from *stg* cerebellum for several proteins involved in TrkB signal transduction, including PLC γ 1, erk1, and erk2 (Knusel et al., 1994). Our results indicate strongly reduced

constitutive and NT-4/5-induced tyrosine phosphorylation of PLC γ 1 in *stg* cerebellum (Fig. 1*B*). To confirm that the difference of PLC γ 1 signal was not attributable to decreased PLC γ 1 level, the PLC γ 1 protein levels were found in *stg* and wild-type cerebellum (data not shown). Phosphotyrosine signals of untreated and NT-4/5-treated microslices for both erk1 and erk2 were also reduced in *stg* cerebellum compared with controls (Fig. 1*C*). Together, our results reveal a consistent deficit in constitutive and NT-induced activation of cellular messengers involved in TrkB signal transduction.

Normal cerebellar-vestibular morphology in *stg* homozygotes

We have reported normal foliation and laminar structure in *stg* cerebellar cortex (Qiao et al., 1996). In the present study, we further investigated the cerebellar-vestibular system, which might contribute to the behavioral deficits in motor coordination and in which neurotrophins are known to be involved in development. Coronal brain sections through medial cerebellum revealed a

Figure 2. Coronal brain sections through the cerebellar-vestibular system of adult wild-type (*A*) and *stg/stg* (*B–E*) mice. *A, B*, Comparable gross histological appearance in wild-type (*A*) and *stg* (*B*) mice is evident in cerebellar cortex, cerebellar deep nuclei (*DN*), and vestibular nuclei (*VN*). At high magnification, no qualitative changes were observed in the interpositus nuclei (*C*), the vestibular nuclei (*D*), as well as the vestibular ganglion (*E*) in *stg* mutants. *C*, *Top* is medial and *bottom* is lateral. Scale bars: *B*, 1 mm; *C–E*, 100 μ m.

normal layout of cerebellar cortex, cerebellar deep nuclei, and vestibular nuclei in *stg* mouse when compared with the wild type (Fig. 2*A,B*). At high magnification, no qualitative changes were observed in the interpositus nucleus (Fig. 2*C*), the vestibular nuclei (Fig. 2*D*), as well as the vestibular ganglion (Fig. 2*E*), in the appearance, the density, and the size of the neurons in the adult *stg* mutants.

Increased external granule cells during *stg* development

To test the possible influence of BDNF on cerebellar granule cells during development, we examined the cerebellar structure in *stg/stg* and $\pm$ littermates at P15, P20, and P30. At P15, few scattered external granule cells (EGCs) were seen near the surface of the cerebellar cortex in wild-type animals (Fig. 3*A*). In contrast, multiple layers of EGCs were found in the *stg* cerebellar cortex at the same developmental stage (Fig. 3*B*). At P20, the EGC layer had virtually disappeared in both *stg* and wild-type mice (Fig. 3*C,D*), and no difference was found at P30 (data not shown). Quantitative cell counts of EGCs ($n = 3$) confirmed significantly more external granule cells in the mutant cerebellum than in the wild type at P15 (226% of control; $p < 0.01$), whereas no difference at P20 ($p > 0.05$) was found between the two genotypes (Fig. 3*E*). This finding is consistent with the observation of delayed disappearance of the EGC layer in the cerebellum

of BDNF knock-out mice (Jones et al., 1994), suggesting a regulatory role of BDNF in the cerebellar granule cell migration.

Abnormal granule cell morphology in adult *stg* cerebellum

BDNF has been proposed to play a direct role in synaptogenesis and adaptive changes in mature brain (Gall, 1992). Excess BDNF has been shown to inhibit ocular dominance column formation in the visual cortex and, therefore, may influence the activity-dependent control of axonal branching during development (Cabello et al., 1995). To determine whether BDNF is involved in synaptic formation during development, we examined the cerebellar ultrastructure in adult mice from both genotypes by electron microscopy. Qualitative examination of the input and output synapses of granule cells revealed normal synaptic structures in *stg* cerebellum. Figure 4*C* shows the structure of a typical glomerulus in the granule cell layer of adult *stg* cerebellum. Large mossy fiber rosettes filled with clear synaptic vesicles and dense core vesicles form asymmetrical synapses with granule cell dendrites. Translobular (sagittal) section through the outer molecular layer in adult *stg* cerebellum is shown in Figure 4*D*. Numerous transverse cut parallel fibers are packed in this region. Several parallel fiber varicosities containing a large concentration of tightly packed round synaptic vesicles have asymmetrical contact with presumed Purkinje cell spines featuring a densely staining

Figure 3. Postnatal cerebellar development in wild-type and *stg/stg* mice. *A, B*, Horizontal cerebellar sections at P15 reveal few scattered EGCs (arrows) in wild-type mice (*A*) but multiple layers of EGCs in *stg* cerebellum (*B*). *C, D*, At P20, the EGC layer (arrows) is hardly visible in both the wild-type and *stg* mouse. *E*, Quantitative cell counts of EGCs from 10 semithin sagittal sections of medial cerebellum ($n = 3$ for all the groups) have shown significantly more EGCs (226% of control; $p < 0.01$) in the mutant cerebellum than the corresponding wild type at P15, whereas there was no difference at P20 ($p > 0.05$) between the two genotypes. Scale bar, 0.5 mm.

Figure 4. Electron micrographs of cerebellar cortex. *A, B*, Granule cells from adult $+/+$ (*A*) and *stg/stg* (*B*) mice. The darkly staining nucleus of granule cells (*gc*) in the wild-type cerebellum has only few scattered clumps of chromatin around the nucleus membrane and is surrounded by a thin layer of cytoplasm. The cell bodies are packed densely within the granule cell layer. In contrast, some of the granule cells in the mutant cerebellum displayed elongated oval-shaped nuclei studded with clumps of coarse chromatin. Such granule cells resemble immature-looking cells and represent $<20\%$ of the granule cell population in the adult *stg* cerebellum. *C*, Typical glomerulus in the granule cell layer of *stg* cerebellum shows the normal structure of mossy fiber–granule cell synapses, large mossy fiber rosettes (*mfr*) filled with clear and dense core synaptic vesicles, as well as mitochondria forming asymmetrical synapses with granule cell dendrites (*gcd*). *D*, Translobular (sagittal) section through the outer molecular layer in *stg* cerebellum displays numerous transverse cut parallel fibers (*pf*). Several parallel fiber varicosities containing a large concentration of tightly packed round synaptic vesicles have asymmetrical contact with presumably Purkinje cell spines (*pcs*) featuring a densely staining postsynaptic web. No noticeable abnormality was found at the parallel fiber–Purkinje cell synapses. Magnification: *A, B*, 10,000 $\times$; *C, D*, 20,000 $\times$.

postsynaptic web. No noticeable abnormality was found in the synapses at either mossy fiber–granule cell or parallel fiber–Purkinje cell sites. Although not excluding the potential contribution of minimal level of BDNF released from neighboring cell types as well as possible subtle quantitative differences in the number of synapses, our findings suggest that a near total lack of BDNF in granule cells has little effect on the synaptic structure of their input and output sites in the cerebellum.

In contrast to the normal appearance of cerebellar synapses, a noticeable difference was observed in the appearance of granule cell bodies (Fig. 4*A,B*). In the wild-type cerebellum, the darkly staining nucleus of granule cells has only a few scattered clumps of chromatin around the nucleus membrane and is surrounded by a thin layer of cytoplasm. The cell bodies are packed densely within the granule cell layer (Fig. 4*A*). In contrast, some of the granule cells in the mutant cerebellum displayed elongated oval-shaped nuclei studded with clumps of coarse chromatin (Fig. 4*B*). The morphology of these granule cells resembles migrating immature granule cells at early postnatal days, as described by Altman (1972). Such abnormal granule cells represented <20% of the granule cell population in the adult *stg* cerebellum but were never seen in the wild-type cerebellar cortex.

Impaired classical eyeblink conditioning in *stg* mice

To determine whether there is any change in cerebellar function that may be correlated with the biochemical and anatomical abnormalities, we performed classical eyeblink conditioning tests in adult *stg* mutant. We found that *stg* mice, when compared with their normal littermates, were severely impaired in acquisition in this paradigm. Figure 5 shows the percentage (*A*) and the amplitude (*B*) of the eyeblink CR (+/- littermates, $n = 8$; *stg/stg*, $n = 8$). During 6 d of paired training, wild-type mice exhibited a significant level of learning (linear trend analysis, $p = 0.00009$), whereas *stg* mutants performed poorly and showed a profound impairment with little sign of learning ($p = 0.0558$). A two-way ANOVA analysis clearly demonstrated that wild-type mice learn to a significantly higher asymptotic level at a faster learning rate than *stg* mutants in both the percentage and the amplitude of the eyeblink CRs (CR percentage, group difference, $p = 0.0007$; learning rate, $p = 0.00003$; CR amplitude, group difference, $p = 0.00024$; learning rate, $p = 0.00024$). Analysis of CR performance in unpaired controls and *stg* mice fails to reveal any statistical significance (group difference, $p = 0.73$; learning rate, $p = 0.49$). The results of CR onset latency analysis are shown in Figure 6*A*. The onset latency of the wild-type littermates exhibited a normal pattern similar to those observed in other mouse strains, such as C57BL/6J and BALBC mice (data not shown). A large number of CR onset latencies were within the 88–144 msec range. However, the *stg* mice exhibited many fewer CRs with early onset latencies. In addition, essentially no peak in CR onset latency was found in *stg* mutants, with only a slightly increased number of trials of ~172 msec. The trial distribution indicates significantly delayed and virtually flat CR onset latencies in *stg* mutants compared with that in wild types. Sensitivity to the US shocks and URs to the US were also tested. Neither US intensities (Fig. 6*B*) nor UR amplitudes (Fig. 6*C*) were significantly different between the two groups.

To ascertain that the impairment in *stg* mutants was not attributable to impaired sensitivities to the US or the CS, pain and tone perceptions were tested in *stg* mice. Tail flick analgesia measurements showed no significant difference between wild-type and *stg* mice at either 55 or 60°C [55°C, +/-, 20.67 ± 1.45 sec (mean $\pm$

A

B

Figure 5. Performance of classical eyeblink conditioning during acquisition training in wild-type (open square), *stg* (filled square), and *tg* (filled circle) mice. During 6 d of paired training, wild-type and *tg* mice exhibited a significant level of learning, whereas *stg* mutants performed poorly. Both percentage (*A*) and amplitude (*B*) of eyeblink CR revealed severe impairment in the acquisition of eyeblink conditioning in the *stg* mutant.

SE); *stg/stg*, 23.08 ± 1.22 ; $p > 0.05$; 60°C, +/-, 10.61 ± 0.98 ; *stg/stg*, 11.08 ± 0.89 ; $p > 0.05$). Multiple-unit recordings from auditory cortex in *stg* mouse revealed normal evoked spikes during the presentation of a 350 msec, 80 dB, 1 kHz tone, which is comparable to that of the wild type (data not shown). These results demonstrate normal perceptions in the *stg* mouse, which in turn strongly suggest that the eyeblink conditioning impairment reflects a functional defect of the cerebellum in *stg* mutant.

To determine whether the impaired eyeblink conditioning in *stg* mice was linked specifically to the BDNF deficit or to the nonspecific feature of seizure activity, we tested eyeblink conditioning in a second ataxic mutant with the same type of spike wave seizures, the *tottering* (*tg*; chromosome 8) mouse. Adult *tg/tg* mutants display a similar ataxic gait with mild cerebellar hypoplasia (Sidman et al., 1965; Noebels and Sidman, 1979; Qiao and Noebels, 1991) but with normal cerebellar BDNF expression (Qiao et al., 1996). Unlike the *stg* mutant, the *tg* mice exhibited normal acquisition of conditioned eyelid responses in comparison to the wild-type mice (CR percentage, group difference, $p = 0.13$; learning rate, $p = 0.019$; $n = 4$) (Fig. 5*A*). The result suggests that the impairment in eyeblink conditioning is not related to the secondary effects of either spike wave seizures, which do not involve the cerebellar cortex, or cerebellar hypoplasia. Impaired cerebellar function in *stg* is more likely linked to the BDNF defect in this mutant.

Figure 6. *A*, Histogram of the CR onset latencies during training. The normal pattern of onset latency in the wild-type (filled bar) littermates is contrasted with the delayed and flat distribution of CR onset latencies in *stg* mutants (open bar). *B*, *C*, Sensitivity to the US and UR to the US in the wild-type (open square) and *stg* (filled square) mice. Neither US intensities (*B*) nor UR amplitudes (*C*) were significantly different between the two groups.

DISCUSSION

This report extends our previous finding of a specific and pronounced deficit in BDNF expression in *stg* mouse to the protein level. BDNF protein in this mutant cerebellum was reduced by 70% compared with controls. Protein levels of the BDNF receptor TrkB were normal, but ligand-induced tyrosine phosphorylation of several TrkB-activated signal transduction molecules was reduced. At the morphological level, an increased number of external granule cells was found in the *stg* cerebellum at P15. This difference to controls had disappeared at P20. Ultrastructural examination of adult *stg* revealed some immature-looking granule cells in the cerebellum but normal development of the mossy fiber–granule cell and parallel fiber–Purkinje cell synapses. In contrast to the minor effects of the *stg* mutation on morphological parameters in the cerebellum, our behavioral results show a severe deficit in cerebellar learning in this mouse. Together, our findings support a role of BDNF in functional plasticity in the CNS that is not reflected in gross morphological changes at cellular or synaptic levels.

BDNF effect on TrkB receptor

Neurotrophins have been found to regulate Trk receptor levels. Chronic exposure of neurons to NGF was shown to result in increased levels of the NGF receptor TrkA (Holtzman et al., 1992; Kojima et al., 1994; Venero et al., 1994; Li et al., 1995). Treatment of rat embryonic neurons with BDNF, NT-3, and NT-4/5 leads to reduction of the protein and mRNA levels for the TrkB receptor, as well as a downregulation of Trk tyrosine phosphorylation (Frank et al., 1996; Knusel et al., 1997). However, little is known about the effect of NT-deprivation on Trk receptor levels. We have shown previously that the BDNF expression defect in the granule cells was not associated with any changes in trkB receptor mRNA levels in *stg* mutant (Qiao et al., 1996). We now showed that the pronounced reduction of the BDNF protein level in *stg* cerebellum does not result in a change in TrkB protein levels. These results suggest that the expression of the BDNF receptor TrkB *in vivo* does not depend on the tissue level of its ligand, as suggested by the earlier *in vitro* findings (Frank et al., 1996; Knusel et al., 1997).

Binding of the NTs to Trks stimulates tyrosine phosphorylation of the receptor and of intracellular substrates. The process has been used as an indicator of Trk receptor activation (Kaplan and Stephens, 1994; Knusel et al., 1994). Despite unchanged levels of TrkB protein, our results demonstrate a significant deficit in tyrosine phosphorylation of several signal transduction molecules in *stg* cerebellum. Our data indicate a regulatory effect of BDNF on the efficiency of TrkB signaling without an effect on TrkB levels. These results agree with a recent report showing that Trk activation is dramatically decreased in granule and Purkinje cells of BDNF knock-out mice when assayed by specific antibodies raised against tyrosine phosphorylated Trk receptors (Schwartz et al., 1997). In a previous study, we observed a decrease in BDNF-, NT-3-, and NT-4/5-induced Trk and Trk substrate tyrosine phosphorylation at P7–P14 (Knusel et al., 1994). This finding indicates the presence of efficient posttranslational regulatory mechanisms to control the responsiveness of NT receptors.

BDNF and cerebellar morphology

Studies of BDNF gene deletion mice have found severe deficiencies in motor coordination and balance, resulting in head bobbing and tilting, circling behavior, and periods of immobility (Ernfors et al., 1994). The behavioral phenotype reflects defects in both cerebellar and vestibular systems. Indeed, neuronal degeneration in vestibular and other sensory ganglia, as well as abnormal cerebellar development, was found in these mutants (Ernfors et al., 1994, 1995; Jones et al., 1994; Conover et al., 1995; Liu et al., 1995; Schwartz et al., 1997). The *stargazer* mutants share major elements of the BDNF^{−/−} behavioral abnormalities, including ataxic gait, episodic upward head tilting, and impaired motor coordination (Noebels et al., 1990; Qiao et al., 1996). In the present study, we failed to detect any abnormalities in the vestibular ganglia and vestibular nuclei of *stg* mice. The normal morphology is correlated with the absence of circling behavior seen in BDNF^{−/−} mice. In contrast, similar presumably delayed granule cell migration and maturation were evident in *stg* cerebellum. Our results are in agreement with the previous finding that BDNF can influence granule cell migration (Gao et al., 1995). These findings also indicate that the *stg* behavioral phenotype is primarily, if not solely, the result of a cerebellar defect without direct involvement of the vestibular system.

Jones et al. (1994) reported a slight delay in granule cell migration in BDNF^{-/-} mutants. The same phenomenon was observed during *stg* cerebellar development, supporting a role for BDNF in cerebellar granule cell migration. In a more recent study, additional cerebellar abnormalities were found in BDNF transgenics, including increased death of granule cells, stunted growth of Purkinje cell dendrites, impaired formation of horizontal layers, and defects in the foliation pattern (Schwartz et al., 1997). However, our present observations in *stg* cerebellum only revealed mild morphological abnormalities, i.e., <20% abnormal granule cells in the adult. The following two facts may contribute to the differences. First, ~30% of normal BDNF mRNA and protein levels were still detected in adult *stg* cerebellum by Northern analysis and BDNF EIA, respectively. Although most of this BDNF might be produced by cell types other than granule cells, it may still influence granule cell development. BDNF mRNA has been localized in the deep cerebellar nuclei in rat (Rocamora et al., 1993). Second, although we did observe a delayed disappearance of the EGC layer during development, the normal life span of *stg* mice allowed us to perform our experiments in adult mice. In contrast, because of high postnatal mortality, most studies using BDNF^{-/-} mice are limited to the first 2–3 weeks when the cerebellum is not fully developed. Because cerebellar granule cell proliferation normally continues until P20 (Fujita et al., 1966) and migration, neurite outgrowth, and the synaptic formation proceed until >P30 (Altman, 1972), some of the findings with BDNF transgenics might reflect a possible delay in development rather than absolute loss of function.

Potential role of BDNF in cerebellar-dependent learning

NGF administrations have been reported to improve spatial memory in aged rats (Markowska et al., 1994). Anti-NGF antibody application leads to impairment in passive-avoidance learning in neonatal mice (Ricceri et al., 1994). In the present study, we provide evidence that a specific and pronounced deficit of BDNF expression in cerebellar granule cells is associated with a loss of functional plasticity, detected as severely impaired eyeblink conditioning in *stg* mutant. Unlike in BDNF knock-out mice (Ernfors et al., 1994; Jones et al., 1994), no sensory or vestibular deficiencies were found in *stargazer*. In addition, the normal eyeblink conditioning performance in the related *tottering* mutant is significant for two reasons. First, it demonstrates that the BDNF defect in *stg* cerebellum is specifically linked to the impairment in learning, because *tg* mice express normal levels of BDNF mRNA throughout the forebrain and cerebellum (Qiao et al., 1996). Second, it indicates that the neocortical seizures seen in both mutants, which do not spread to the cerebellum (Noebels and Sidman, 1979), do not account for the learning impairment in *stg* mouse. BDNF defect appears to be critical for the observed deficit in cerebellar learning in *stg* mutant and suggests that BDNF is essential for normal neuronal plasticity in the learning and memory process of the eyeblink conditioning paradigm.

As previously found in rabbits, rats, and humans (Thompson, 1986; Skelton, 1988; Daum et al., 1993; Lavond et al., 1993), recent studies have shown impaired eyeblink conditioning in cerebellum-lesioned and Purkinje cell degeneration (*pcd*) mutant mice (Chen et al., 1996). In comparison to *pcd* mice (i.e., complete functional cerebellar decortication in adult), only limited morphological changes were present in *stg* cerebellum. However, more severe deficits in eyeblink conditioning are found in *stg* mice than in *pcd* mutants. Our results suggest that the functional

integrity of cerebellum is severely compromised by the BDNF defect in cerebellar granule cells without parallel gross morphological changes to the cerebellar structure.

In addition to its traditional role as a neuronal survival factor, much evidence has shown that BDNF is involved in the modulation of synaptic transmission. Application of BDNF can potentiate the efficacy of developing neuromuscular synapses in culture (Lohof et al., 1993), enhance synaptic strength in the hippocampus (Lessmann et al., 1994; Kang and Schuman, 1995), facilitate long-term potentiation (LTP) induction (Figurov et al., 1996), and inhibit GABA_A synaptic responses in rat hippocampus (Tanaka et al., 1997). Furthermore, BDNF gene deletion impairs both synaptic transmission and LTP in the hippocampus (Korte et al., 1995; Patterson et al., 1996). Recent evidence indicates that BDNF modulates synaptic transmission by postsynaptic activation of Trk tyrosine kinase receptors (Levine et al., 1995; Tanaka et al., 1997) and subsequent phosphorylation of the NMDA receptors (Suen et al., 1997). The presence of inactive TrkB receptor signal transduction in *stg* cerebellum in conjunction with decreased Trk activation seen in BDNF knock-out transgenics (Schwartz et al., 1997) supports the hypothesis that defects in BDNF–TrkB signal transduction pathway may underlie the behavioral deficiencies in coordination and learning seen in these mutants.

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