

# Brain-derived neurotrophic factor attenuates mouse cerebellar granule cell GABA<sub>A</sub> receptor-mediated responses via postsynaptic mechanisms

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In addition to exerting long-term neurotrophic influences on developmental process such as neuronal survival and neuritic outgrowth, brain-derived neurotrophic factor (BDNF) has been reported to modulate synaptic transmission in the short-term. Considerable evidence indicates that BDNF acutely modulates NMDA receptor-mediated synaptic activity. However, whether BDNF modulates inhibitory synaptic transmission remains to be firmly established. In the present study, we examined the effect of acute BDNF exposure on GABA-evoked whole-cell responses as well as GABAergic synaptic activity in cultured mouse cerebellar granule cells. GABA-evoked responses were reduced by  $39.5 \pm 4.7\%$  upon acute and focal application of BDNF ( $100 \text{ ng ml}^{-1}$ ). The reduction of the GABA response recovered only partially even minutes after removal of BDNF. TrkB-IgG and K252a, but not K252b, prevented the BDNF-induced attenuation of the GABA response. BDNF exposure shifted the cumulative peak amplitude distribution leftward for both spontaneous IPSCs (sIPSCs) and miniature IPSCs (mIPSCs) without affecting the rise time and decay time constants. Acute exposure to BDNF also resulted in internalization of GABA<sub>A</sub> receptors in cultured cerebellar granule cells, as reflected by diminished immunostaining with an antibody against the GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit. Although the BDNF-induced GABA<sub>A</sub> receptor internalization was sensitive to K252a, it did not become manifest until 5 min after exposure to BDNF. Therefore, receptor internalization alone cannot account for the prompt BDNF-induced attenuation of GABA-mediated activity. We conclude that BDNF modulates GABA<sub>A</sub> receptor-mediated activity through TrkB receptor signalling that triggers a kinase-dependent short latency effect and a delayed longer latency effect hallmarked by receptor internalization.

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Brain-derived neurotrophic factor (BDNF) plays a pivotal role in regulating synaptic plasticity in the central nervous system (see McAllister *et al.* 1999 for review). It is present in both the developing and mature central nervous system, and exerts its effects through the activation of the tyrosine receptor kinase B (TrkB) receptor, which, in turn, triggers a multitude of downstream signalling events (Huang & Reichardt, 2001). The expression and release of BDNF are regulated by synaptic activity. For example, activation of glutamate receptors or depolarization by high  $[K^+]$  increases the expression of BDNF mRNA in cultured rat hippocampal neurons and cortical neurons (Lu *et al.* 1991; Zafra *et al.* 1992; Lindholm *et al.* 1994; Ghosh *et al.* 1994). Repetitive seizure activity augments BDNF mRNA levels in the hippocampus (Zafra *et al.* 1990). In hippocampal slices, patterned electrical stimulation or  $K^+$ -induced depolarization results in increased secretion of BDNF (Blochl & Thoenen, 1995; Goodman *et al.* 1996).

Acute exposure to BDNF enhances the frequency and amplitude of glutamate-mediated excitatory postsynaptic currents (EPSCs) in the hippocampus (Lessmann *et al.* 1994; Kang & Schuman, 1995; Levine *et al.* 1996, 1998). In addition, disruption of BDNF or TrkB receptor expression results in deficits in long-term potentiation in hippocampal slices (Korte *et al.* 1995; Patterson *et al.* 1996; Kang *et al.* 1997). Thus, beyond its role as a permissive survival factor, the neurotrophin has been implicated in modulating synaptic transmission and plasticity. One proposed mechanism is that BDNF increases  $Ca^{2+}$  in presynaptic glutamatergic terminals to facilitate transmitter release (Kang & Schuman, 1995; Li *et al.* 1998). However, other studies propose mechanisms favouring a postsynaptic enhancement of NMDA receptor-mediated synaptic transmission (Levine *et al.* 1998). With regard to GABA-mediated inhibitory transmission, BDNF either attenuates or potentiates the amplitude of IPSCs, and both pre- and

postsynaptic mechanisms have been independently proposed (Takana *et al.* 1997; Frerking *et al.* 1998; Boxall, 2000; Brunig *et al.* 2001). Overall, elucidating the nature of a BDNF-induced modulation of synaptic transmission will contribute toward identifying mechanisms underlying the cross-talk between BDNF, TrkB receptor signalling and neurotransmitter receptor function.

In this study, we investigated whether acute exposure to BDNF modulates responses of cerebellar granule cells to GABA. We report that BDNF suppresses GABA-mediated responses, hallmarked by a reduction in GABA-activated whole-cell current response as well as suppression of inhibitory synaptic events. Mechanistically, our results suggest that a sequel of at least two phases account for the BDNF-induced attenuation of GABAergic synaptic transmission. Specifically, by triggering the TrkB receptor-coupled intracellular cascade, BDNF suppresses postsynaptic GABA-mediated activity in an early phase and internalization of GABA<sub>A</sub> receptors in a later phase.

## METHODS

### Cerebellar granule cell culture

All procedures involving animals were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. C57BL/6J mouse pups (5–6 days postnatal) were decapitated and cerebella were isolated and immersed in ice-cold PBS. The cerebellar tissue was minced and enzymatically digested with trypsin (0.025%; Worthington, Lakewood, NJ, USA) for 12 min at 37°C. Cells were dissociated by triturating in 0.1% DNase (Sigma, St Louis, MO, USA), plated onto laminin-coated 12 mm round glass coverslips at  $5 \times 10^4$  cells coverslip<sup>-1</sup>, and maintained at 37°C in a humidified 95% O<sub>2</sub>–5% CO<sub>2</sub> environment. The culture medium was Neurobasal medium (Gibco) to which was added 2 mM L-glutamine, B-27 supplement and penicillin–streptomycin–fungizone (Gibco). The concentrations of glucose and KCl in the medium were adjusted to 5 mg ml<sup>-1</sup> and 25 mM, respectively. Forty-eight hours after plating, the cultures were treated with 6 μM fluorodeoxyuridine for 24–48 h to suppress the growth of glia (Rieff *et al.* 1999). Thereafter, the medium was changed every 2 days until used for experiments. After 7–10 days *in vitro* (DIV) cerebellar granule cells were used for the experiments reported in the present study.

### Whole-cell patch-clamp recording, data acquisition and analysis

Patch electrodes were pulled from borosilicate capillary glass (1.5 mm o.d.; Sutter Instrument Co., Novato, CA, USA) and fire-polished to a bubble number of 4.2. The electrodes had resistances ranging between 8 and 10 MΩ when filled with internal solution. The internal (recording) solution was composed of (mM): 140 KCl, 4 NaCl, 2 MgCl<sub>2</sub>, 10 potassium EGTA, 10 Hepes, 2 magnesium ATP and 3 magnesium GTP (pH 7.3 with KOH). The external (bath) solution contained (mM): 140 NaCl, 2.8 KCl, 1 MgCl<sub>2</sub>, 3 CaCl<sub>2</sub>, 10 Hepes, 10 glucose (pH 7.4 with NaOH). Recordings were made using an AxoPatch 200A amplifier (Axon Instruments, Foster City, CA, USA). Membrane currents were filtered at 5 kHz, digitized using Clampex v8.0 and analysed with Clampfit v8.0 (Axon Instruments) and Mini Analysis software (Version 5.1, Synaptosoft, Decatur, GA, USA) software. Analog signals were

monitored on-line using a chart recorder (Gould, Valley View, OH, USA). Statistical analysis was performed using Sigmaplot 5.1 (SPSS Inc., Chicago, IL, USA). Mean peak current and frequency were analysed using Student's paired *t* test. The peak amplitude of GABA-mediated synaptic events was analysed using the Kolmogorov-Smirnov two-sample distribution test. Data are reported as mean ± S.E.M.

### Focal applications of drugs

All drugs, including GABA (20 μM) and BDNF (20 or 100 ng ml<sup>-1</sup>), were dissolved in external solution, stored as frozen stock solutions and diluted to working concentrations with external solution immediately prior to each recording session. Drug solutions were loaded into separate barrels of an 8-barrel glass pipette assembly. The multibarrel assembly was constructed using fibre-filled borosilicate glass capillary tubes (Sutter Instrument Co.), pulled to a fine point, filled with the desired drug solutions and the tip was broken under microscopic control such that the outer diameter of each barrel was approximately 1.5 μm. The multibarrel assembly was mounted onto a hydraulically driven micromanipulator and navigated to within 10 μm of the cell under study for focal delivery of drugs. One of the barrels of the multibarrel assembly was routinely filled with external solution, which was applied between drug applications to clear drugs from the vicinity of the cell, and to control for mechanical artifacts due to bulk flow. In addition, since all drugs were dissolved in external solution, this also served as vehicle control to confirm drug effects. The application of drugs was always directed at the soma of cerebellar granule cells under study. Drug solutions were ejected by regulated pulses of pressure (< 3 p.s.i.; Picospritzer, General Valve Company, Fairfield, NJ, USA), with timing and duration controlled by a digital timing unit (Pulsemaster A300, World Precision Instruments, New Haven, CT, USA). Recombinant human BDNF was purchased from Peptide Inc. (Rocky Hill, NJ, USA); TrkB-Fc protein was a gift from Regeneron (Tarrytown, NY, USA); K252a and K252b were purchased from Alomone Labs (Jerusalem, Israel); all other chemicals were purchased from Sigma.

### Immunohistochemistry and analysis of immunofluorescence

Cerebellar granule cell cultures were fixed in 4% paraformaldehyde in PBS (PFA-PBS) for 20 min and then incubated in 10% normal goat serum for 30 min under either non-permeabilized or permeabilized conditions (0.2% Triton X-100 in PBS). All subsequent reactions were carried out in the presence of 2% normal goat serum. Double immunohistochemical labelling of TrkB receptor and GABA<sub>A</sub> receptor β<sub>2/3</sub> subunit proteins was accomplished by overnight incubation with both mouse anti-β<sub>2/3</sub> antibody (1:100) (Upstate Technology, Lake Placid, NY, USA) and rabbit anti-TrkB receptor antibody (1:1000) (Chemicon, Temecula, CA, USA) at 4°C followed by exposure for 1 h at room temperature to anti-mouse IgG-Alexa 488 and anti-rabbit IgG-Alexa 594 secondary antibodies (Molecular Probes, both at 1:1000). Sister cultures processed in parallel without the addition of primary antibodies served as negative controls.

Immunofluorescence images were viewed and captured using the Olympus confocal microscope system FV300 (Olympus, Melville, NY, USA), under × 60 or × 100 oil objectives (aperture: 1.4), and edited using Photoshop 5.5 (Adobe System Incorporated, San Jose, CA, USA). The images were then digitized to a 256 grey-scale level, and the relative intensity of immunofluorescence was analysed based on a threshold segmentation algorithm using the

MCID elite software (Image Research Inc., Ontario, Canada). Density thresholds were set such that immunoreactivity was distinguished from background, and subtracted from each measure. The median density levels were normalized to control. For each treatment, randomly selected neurites from 30–40 neurons were measured.

## RESULTS

### BDNF attenuates GABA-evoked whole-cell current in cultured cerebellar granule cells

We first assessed whether BDNF alters GABA<sub>A</sub> receptor-mediated current response. Whole-cell current responses of cerebellar granule cells (7–10 DIV) to 1 s pressure pulses of GABA (20  $\mu$ M) applied at 20 s intervals for periods of up to 10 min were monitored continuously before, during and after exposure to BDNF (100 ng ml<sup>-1</sup>). Figure 1A illustrates a penwriter record taken from a cerebellar granule cell. Acute and focal application of BDNF resulted in a prompt attenuation in the amplitude of GABA-evoked current response that stabilized within 1 min of BDNF exposure. Overall, BDNF exposure attenuated GABA responses in 28 of 35 cells tested, with the magnitude of attenuation ranging from 18 to 56% (39.5  $\pm$  4.7%; mean  $\pm$  S.E.M.;  $P < 0.01$ , Student's *t* test) (Fig. 1C). A consistent observation was that recovery of responses to GABA from BDNF exposure was only partial (approximately 70%), even after 5 min of removal of BDNF. The degree of recovery appeared to be dependent on the duration of exposure to BDNF insofar as shorter exposure times resulted in more complete recovery of GABA response to control levels. Two lines of evidence indicate that the observed attenuation of GABA response and partial recovery are not due to run-down or desensitization under our experimental protocol and recording conditions. Firstly, as illustrated in Fig. 1A, whole-cell current responses to GABA were reproducible in amplitude prior to exposure to BDNF. Secondly, in control experiments in which GABA responses were monitored continuously without exposure to BDNF over equivalent lengths of time, the GABA responses remained stable throughout the recording, displaying only 1–5% fluctuation in amplitude (Fig. 2A).

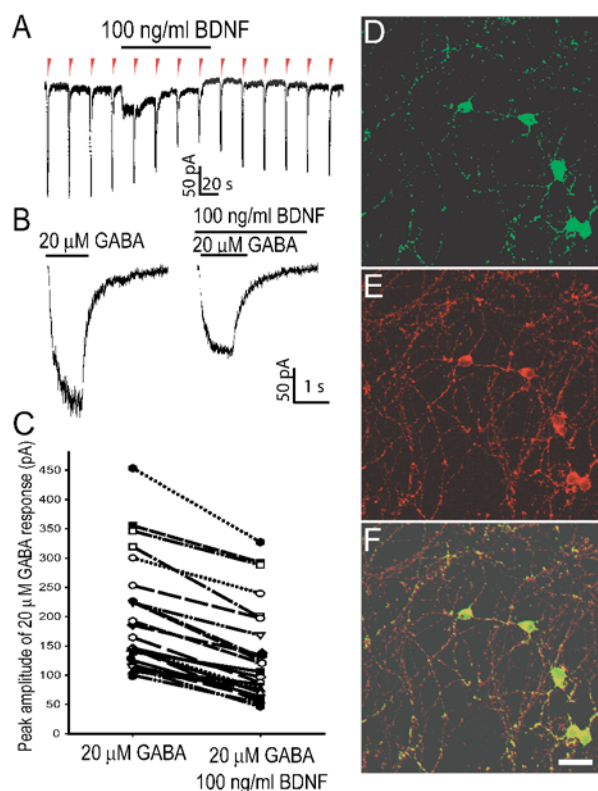
Acute BDNF exposure also induced a depolarization current in 20 of 35 granule cells tested. Although not examined in detail in the present study, this is likely to be due to the opening of Na<sup>+</sup> channels, as been reported previously (Kafitz *et al.* 1999; Urbano & Buno, 2000). Since an attenuation of GABA response has been observed in cells that do not display the depolarizing current response (data not shown), these two BDNF-induced effects appear to be independent of each other.

### The effect of BDNF is mediated by TrkB receptor

In a second series of experiments, we asked whether the observed effect of BDNF was mediated by the TrkB receptor.

Both *in situ* hybridization and immunohistochemistry revealed the presence of TrkB receptor in cerebellar granule cells (Klein *et al.* 1990; Segal *et al.* 1995). We confirmed the expression of TrkB receptor in cultured cerebellar granule cells. Figure 1D, E and F shows that TrkB immunoreactivity overlaps with that of synaptophysin, a presynaptic protein used as synaptic marker, indicating that TrkB receptor is present in both somatic and synaptic membranes. Our imaging system precluded identification of presynaptic *versus* postsynaptic locus of the TrkB receptors.

We then tested whether the addition of 200 nM K252a, an inhibitor of autophosphorylation of tyrosine receptor kinases, or 200 nM K252b, its non-functional analogue (Knusel & Hefti, 1992), alters the effect of BDNF on



**Figure 1. Acute exposure to brain-derived neurotrophic factor (BDNF) attenuates GABA-evoked whole-cell current in cerebellar granule cells**

A, representative penwriter record displays current responses evoked by a 1 s application of GABA (20  $\mu$ M) delivered every 20 s as denoted by the red arrowheads. Exposure to BDNF (100 ng ml<sup>-1</sup>) (continuous line above the arrowheads) reduces the peak amplitude of GABA responses. Note the depolarizing current during acute BDNF exposure in this example. B, digitized traces recorded in the absence (left) and presence (right) of BDNF are shown in expanded time scale. C, scatter plot summarizing the change in peak amplitude of GABA responses upon exposure to BDNF monitored in 20 granule cells. Each pair of connected points represents data derived from one cell. Confocal images of cultured cerebellar granule cells stained with antibodies against TrkB receptor (D) and synaptophysin (E), using green and red fluorescence respectively. F, overlay of D and E. Scale bar = 20  $\mu$ m applies to D, E and F.

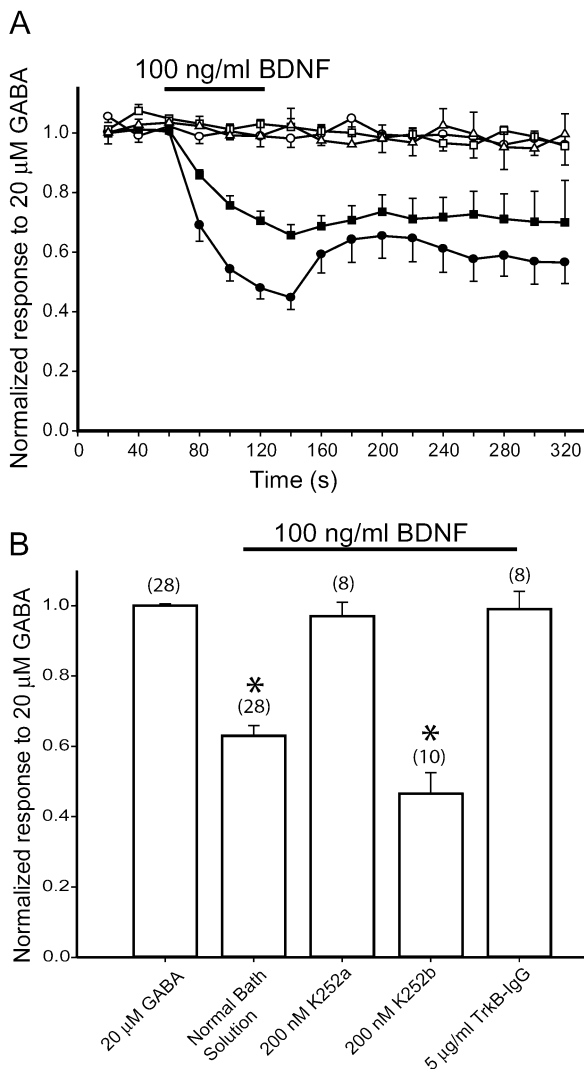


GABA-evoked current responses. Granule cell cultures were pre-incubated with either K252a or K252b for 15 min. Then experiments were conducted in the presence of either K252a or K252b. In the presence of K252a, the peak response to 20  $\mu\text{M}$  GABA fluctuated about  $4.5 \pm 0.6\%$  around the mean value but was not reduced upon addition of 100  $\text{ng ml}^{-1}$  BDNF. In contrast, the reduction of the GABA response induced by BDNF persisted in the presence of 200 nM K252b (Fig. 2A and B). In Fig. 2B, the reduction in the mean peak amplitude of GABA response appeared to be greater upon exposure to

K252b and BDNF than to BDNF alone. However, this difference was not statistically significant. Since K252a also inhibits tyrosine receptor kinases other than TrkB, we confirmed further the involvement of the TrkB receptor by using the TrkB receptor antibody (TrkB-IgG), which competes with native TrkB receptors to sequester BDNF and prevent TrkB receptor activation (Davis *et al.* 1994). TrkB-IgG (5  $\mu\text{g ml}^{-1}$ ) has been shown to be effective in blocking BDNF-dependent long-term potentiation in hippocampal slices (Kang *et al.* 1997). At this concentration of TrkB-Fc, GABA-evoked responses remained unaltered after exposure to 100  $\text{ng ml}^{-1}$  BDNF. Thus, consistent with the experiments employing K252a, TrkB-Fc blocked BDNF-induced modulation. These results indicate that the acute effect of BDNF depends on activation of the TrkB receptor.

### BDNF decreases the amplitude and frequency of spontaneous IPSCs

To determine whether the BDNF-induced attenuation of GABA-mediated responses could be resolved at the synaptic level, we recorded spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) in the absence and presence of BDNF in the same cerebellar granule cells. The antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10  $\mu\text{M}$ ) and DL-2-amino-5-phosphonovaleric acid (APV, 20  $\mu\text{M}$ ) were added to the external solution to block AMPA and NMDA receptor-mediated synaptic currents, respectively. Under each condition, sIPSC events were collected at 34  $^{\circ}\text{C}$  over a period of at least 5 min in order to obtain sufficient number of events (> 100 events) for analysis. Figure 3A shows inwardly directed events recorded in the presence of CNQX and APV from a cultured cerebellar granule cell held at  $-60$  mV. These sIPSCs were completely and reversibly blocked by bicuculline (10  $\mu\text{M}$ ), indicating mediation by synaptic GABA<sub>A</sub> receptors (data not shown). In the absence of BDNF, the frequency of spontaneous IPSCs ranged between 0.3 and 1.8 Hz, with a mean amplitude of  $36.5 \pm 3.7$  pA (mean  $\pm$  S.E.M.). Upon a 5 min focal exposure to BDNF (20  $\text{ng ml}^{-1}$ ), the amplitude of sIPSCs were reduced to  $27.3 \pm 2.3$  pA and remained at this level for at least 10 min (Fig. 3B). Cumulative distribution of amplitude (Fig. 3C) revealed a leftward shift during exposure to BDNF ( $n = 9$ ,  $P < 0.05$  by two-sample Student's *t* test and Kolmogorov-Smirnov two-sample test). The frequency of sIPSCs decreased from  $0.94 \pm 0.2$  to  $0.34 \pm 0.1$  Hz. Cumulative distribution of inter-event interval (Fig. 3D) revealed a rightward shift during exposure to BDNF ( $n = 9$ ,  $P < 0.05$  by two-sample Student's *t* test and Kolmogorov-Smirnov two-sample test).



**Figure 2. TrkB-IgG and K252a, but not K252b, prevent BDNF-induced reduction of GABA response**

A, peak amplitude of responses to GABA (20  $\mu\text{M}$ ) plotted as a function of time before, during and after exposure to 100  $\text{ng ml}^{-1}$  BDNF. The continuous line denotes time and duration of BDNF application. GABA responses were monitored in normal bath solution without exposure to BDNF during recording (□), in normal bath solution with a 1 min exposure to BDNF (■), and with added K252a (200 nM; ○), K252b (200 nM; ●) and TrkB-IgG (5  $\mu\text{g ml}^{-1}$ ; △). Each point represents data averaged from 8–10 cells. B, bar graph summarizing mean peak amplitude of GABA responses as a function of the conditions in A.

While the attenuation of sIPSC amplitude is consistent with data on whole-cell GABA response, the decrease in frequency of sIPSCs, which is typically attributed to reduction of presynaptic transmitter release, could also be due to a suppression of postsynaptic GABA<sub>A</sub> receptor. To

address this issue, we included K252a (200 nM) in the internal solution and recorded sIPSCs before, during and after exposure to BDNF. With K252a present intracellularly, the BDNF-induced attenuation of GABA response amplitude was no longer observed (data not shown). These results suggest that postsynaptic mechanisms contribute to the observed BDNF-induced effect.

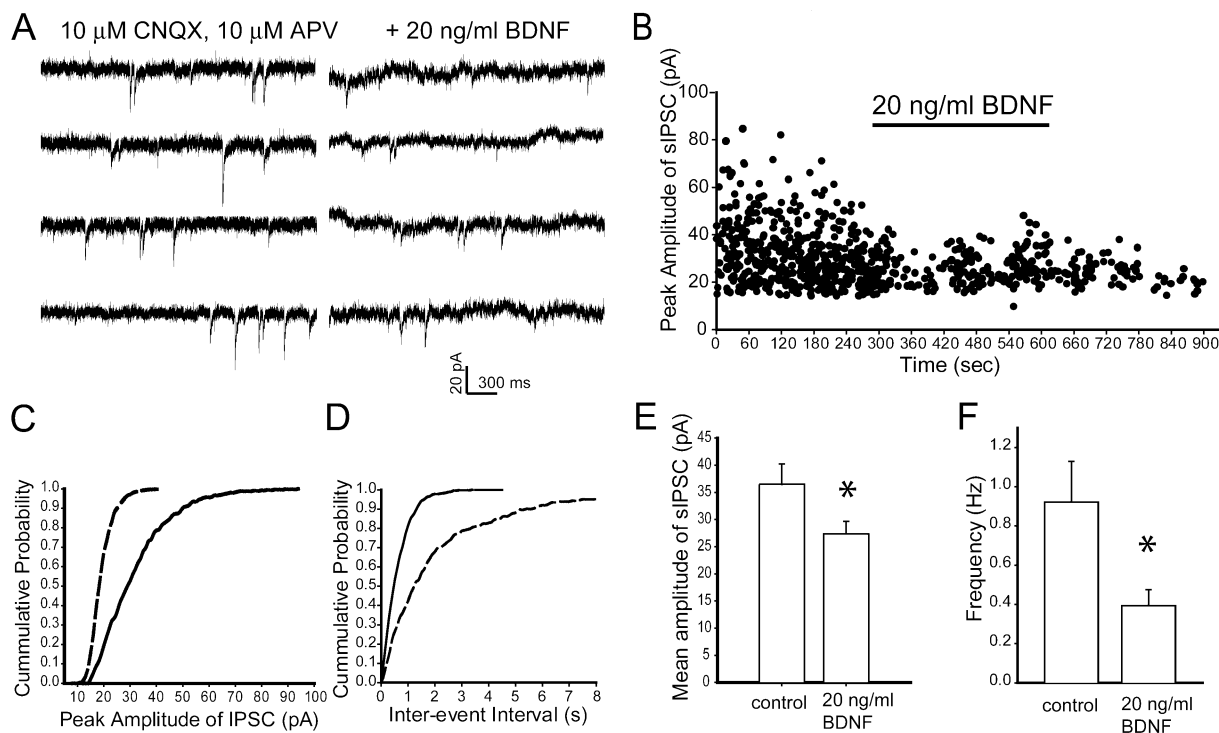
### BDNF decreases amplitude and frequency of miniature IPSCs

To assess further the BDNF-induced postsynaptic modification of GABA-mediated synaptic activity, we examined the properties of miniature IPSCs (mIPSCs) that are independent of action potential-evoked presynaptic release of neurotransmitter (Otis & Mody, 1992). In the absence of BDNF, the frequency of mIPSCs ranged from 0.2 to 1.5 Hz, with a mean amplitude of  $25.8 \pm 2.1$  pA. Multiexponential fits to averaged events revealed a fast rise time ( $t_{\text{rise}} = 2.9 \pm 0.2$  ms) and a biexponential decay time course ( $\tau_{\text{dec1}} = 7.0 \pm 0.3$  ms and  $\tau_{\text{dec2}} = 38.2 \pm 1.9$  ms) ( $n = 8$ ). In the presence of BDNF (20 ng ml<sup>-1</sup>), the amplitude of mIPSCs recorded differed significantly from

those obtained under the control condition ( $20.7 \pm 1.8$  pA) (paired *t* test,  $P < 0.05$ ). The frequency of mIPSCs decreased to  $0.3 \pm 0.1$  Hz (Fig. 4). However, the kinetic profiles of these events were indistinguishable from control ( $t_{\text{rise}} = 3.2 \pm 0.3$  ms,  $\tau_{\text{dec1}} = 6.5 \pm 0.3$  ms and  $\tau_{\text{dec2}} = 33.8 \pm 1.4$  ms;  $n = 8$ ;  $P < 0.5$  by two-sample Student's *t* test) (Table 1). These results indicate that BDNF attenuates the peak amplitude and decreases the frequency of mIPSCs through a postsynaptic alteration of GABA<sub>A</sub> receptor properties. Nonetheless, it should be noted that our results do not exclude the possibility that presynaptic mechanisms regulating probability of transmitter release may also contribute to the synaptic effects of BDNF.

### BDNF reduces immunoreactivity of cell surface GABA<sub>A</sub> receptor $\beta_{2/3}$ subunits

A recent study (Brunig *et al.* 2001) reported that a 30 min exposure to BDNF reduced the surface immunoreactivity of GABA<sub>A</sub> receptor  $\alpha_2$  subunits in cultured hippocampal neurons. We asked whether receptor internalization could account mechanistically for the observed postsynaptic attenuation of GABA responses by BDNF. Cerebellar



**Figure 3. BDNF decreases the amplitude and frequency of spontaneous IPSCs (sIPSCs) in cerebellar granule cells**

A, representative traces recorded in the presence of 10  $\mu\text{M}$  CNQX and 10  $\mu\text{M}$  APV, without (left panel) or with (right panel) BDNF (20 ng ml<sup>-1</sup>); holding potential  $-60$  mV. B, peak amplitude of sIPSCs plotted as a function of time. Upon a 5 min exposure to BDNF, both amplitude and frequency of sIPSCs declined. C, cumulative distribution of peak amplitude of sIPSCs in the presence of BDNF (dashed line) shifted leftward compared to that obtained under control conditions (continuous line). D, exposure to BDNF (dashed line) shifted rightward the cumulative distribution of inter-event interval of sIPSCs compared to that obtained under control conditions (continuous line). E and F, histograms summarizing mean amplitude (E) and frequency (F) of sIPSCs derived from 8 cells; the mean amplitude decreased from  $37 \pm 3.7$  to  $26 \pm 2.3$  pA; frequency decreased from  $0.94 \pm 0.2$  to  $0.34 \pm 0.1$  Hz. \* Statistically significant (Student's paired *t* test,  $P < 0.05$ ).

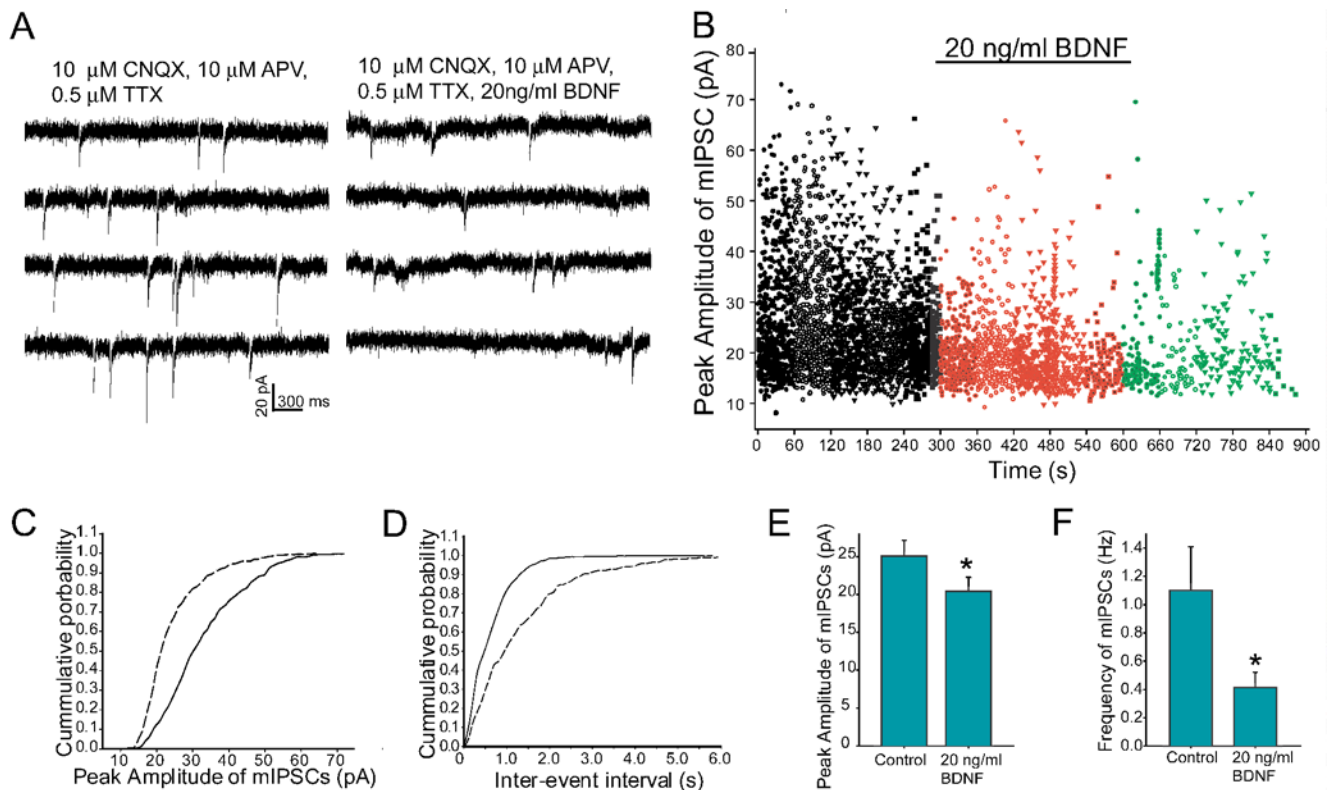
**Table 1. Properties of IPSCs in the absence and presence of 20 ng ml<sup>-1</sup> BDNF**

Mean	sIPSC	sIPSC + BDNF	mIPSC	mIPSC + BDNF
Amplitude (pA)	36.5 ± 3.7	27.3 ± 2.3 *	25.8 ± 2.1	20.4 ± 1.8 *
Frequency (Hz)	0.94 ± 0.2	0.34 ± 0.1 *	1.1 ± 0.3	0.41 ± 0.3 *
$t_{\text{rise}}$ (ms)	3.2 ± 0.4	3.3 ± 0.4	3.1 ± 0.2	2.9 ± 0.3
$\tau_{\text{dec1}}$ (ms)	3.7 ± 0.6	3.3 ± 0.7	2.9 ± 0.5	2.7 ± 0.4
$\tau_{\text{dec2}}$ (ms)	18.2 ± 2.8	16.9 ± 3.1	14.7 ± 2.3	12.9 ± 1.8
No. of cells	9	9	8	8

BDNF, brain-derived neurotrophic factor; sIPSC, spontaneous IPSC; mIPSC miniature IPSC;  $t_{\text{rise}}$ , the 10–90% rise time;  $\tau_{\text{dec1}}$  and  $\tau_{\text{dec2}}$  are time constants of the fast and slow phases of the biexponential decay, respectively. Data are presented as means ± s.e.m. \*Significant difference between the absence and presence of BDNF (20 ng ml<sup>-1</sup>) by paired Student's *t* test at  $P < 0.05$ .

granule cells were exposed to BDNF for 1, 5, 15, 30 and 60 min, fixed with 4% PFA-PBS and subsequently processed for double-label immunohistochemistry using the GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit and TrkB antibodies. As illustrated in Fig. 5A1, GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit

immunoreactivity appeared punctate, outlining the cell body and neurites of granule cells. TrkB immunostaining revealed somatic and neuritic distribution, and overlay of the two staining patterns indicated co-localization of GABA<sub>A</sub> receptors and TrkB receptors. No immuno-



**Figure 4. Exposure to BDNF decreases the amplitude and frequency of miniature IPSCs (mIPSCs)**

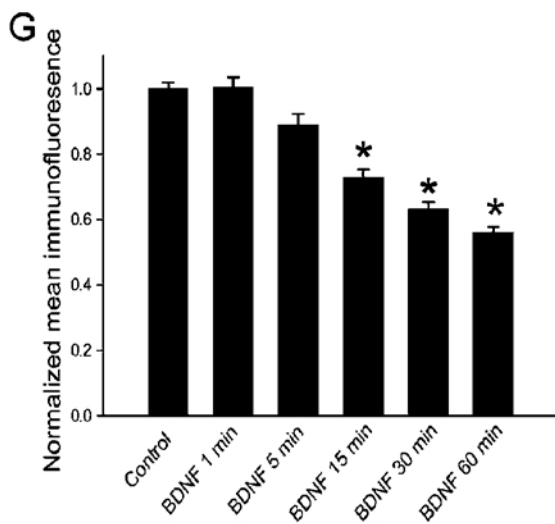
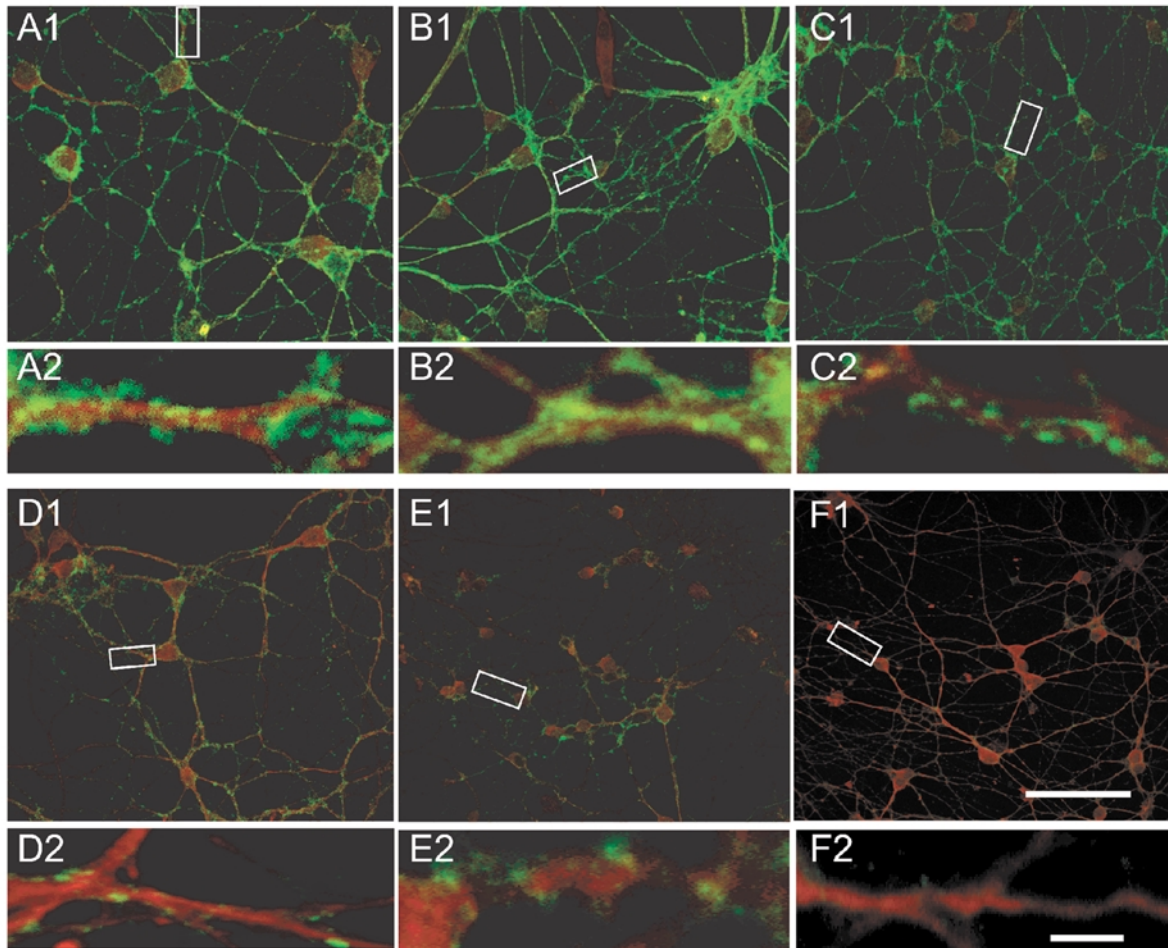
A, representative traces recorded in the presence of 10  $\mu\text{M}$  CNQX, 10  $\mu\text{M}$  APV and 0.5  $\mu\text{M}$  TTX, without (lefthand traces) or with (righthand traces) BDNF (20 ng ml<sup>-1</sup>); holding potential  $-60$  mV. B, peak amplitude of mIPSCs plotted as a function of time before, during and after exposure to BDNF. There are fewer large-size amplitude events during BDNF exposure (red dots) than during the control period (black dots). C, cumulative distribution of peak amplitude of mIPSCs in the presence of BDNF (dashed line) shifted leftward compared to that obtained under control conditions (continuous line). D, exposure to BDNF (dashed line) shifted rightward the cumulative distribution of inter-event interval of mIPSCs compared to that obtained under control conditions (continuous line). E and F, histograms summarizing data derived from 8 cells; exposure to BDNF decreased the mean amplitude (E) and frequency (F) of mIPSCs from  $25.8 \pm 2.1$  to  $20.4 \pm 1.8$  pA and  $1.1 \pm 0.3$  to  $0.41 \pm 0.3$  Hz, respectively. \*Statistically significant (Student's paired *t* test,  $P < 0.05$ ).



staining was observed in negative control cultures with the primary antibodies omitted.

After a 1 min incubation with 100 ng ml<sup>-1</sup> BDNF, there was no apparent change in GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity (Fig. 5B1). However, after a 5 min exposure to BDNF, the reduction in GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity became evident in so far as the

number and size of the puncta decreased. This can be discerned to better advantage at a higher magnification (Fig. 5C2). With longer periods of treatment with BDNF, the reduction of GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity became increasingly striking. In contrast, the TrkB receptor staining remained unchanged at all time points tested. Performing the experiments under either non-permeabilized or permeabilized conditions yielded



**Figure 5. Exposure to BDNF reduces GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity in a time-dependent manner**

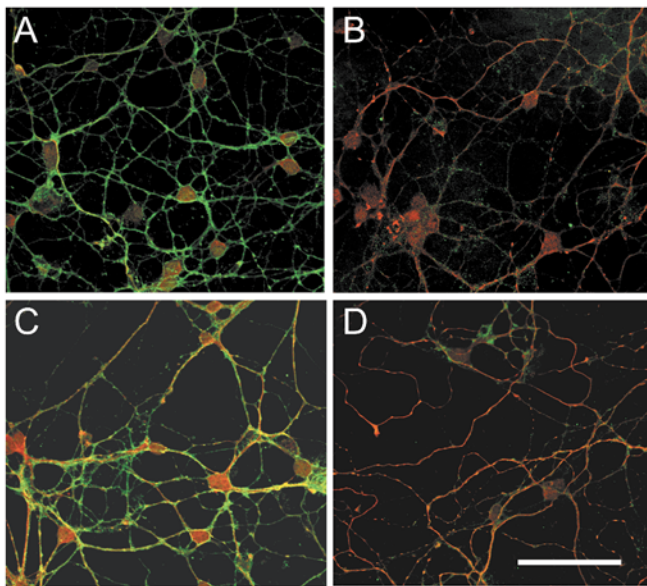
Confocal images of cultured cerebellar granule cells before (A1) and following a 1 (B1), 5 (C1), 15 (D1), 30 (E1) and 60 (F1) min exposure to BDNF (100 ng ml<sup>-1</sup>). The cultures were fixed and double-labelled with antibodies against GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunits (green fluorescence) and TrkB receptors (red fluorescence). A2, B2, C2, D2, E2 and F2, GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactive profiles corresponding to the boxed areas in A1–F1 are resolved at higher resolution ( $\times 60$  oil) to illustrate decreased immunoreactivity along neurites following a > 5 min exposure to BDNF. Scale bars, 50  $\mu$ m in A1–F1 and 5  $\mu$ m in A2–F2. The images illustrated above were digitized; relative median density level was determined for each treatment and normalized to control. G, data are presented as means  $\pm$  S.E.M. \* Significant difference between control and the corresponding times of exposure to BDNF (100 ng ml<sup>-1</sup>) (Student's *t* test at *P* < 0.01).

similar results. Figure 5G summarizes the change in mean immunofluorescence as a function of duration of exposure to BDNF (1–60 min). A statistically significant decrease in immunofluorescence was first detected after 15 min of exposure to BDNF.

Pretreatment of cerebellar granule cell cultures with K252a (200 nM), but not with K252b (200 nM), prevented the reduction of GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity (Fig. 6). Thus, as is the case with attenuation of GABA responses, BDNF reduces GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity through a tyrosine receptor kinase-dependent pathway.

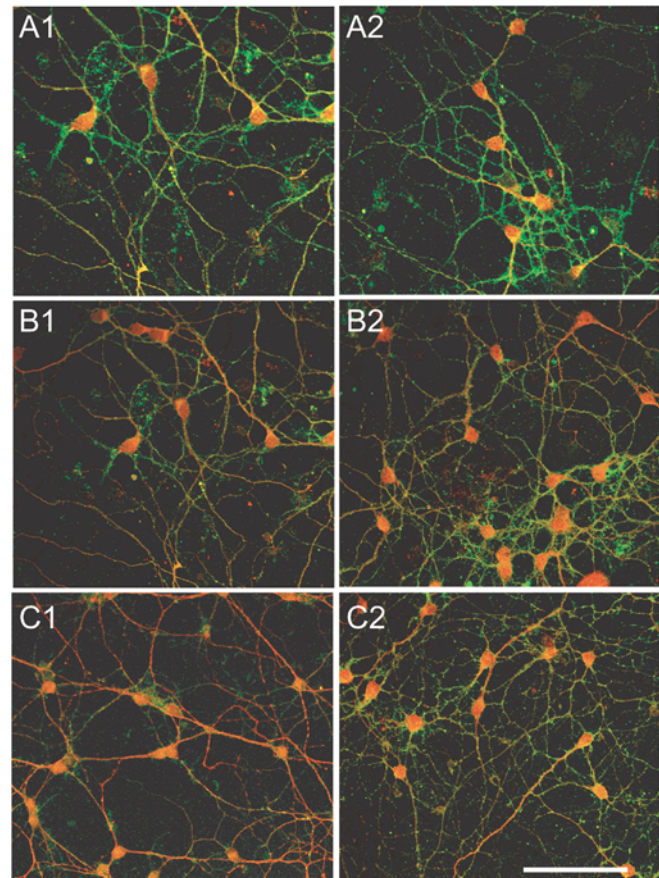
Our electrophysiological experiments demonstrating partial recovery from a BDNF-induced attenuation of the GABA response (Fig. 2) suggest that recovery is likely to be a slow process. A possible mechanism to account for such slow recovery is the time course of reversal of BDNF-induced internalization of GABA<sub>A</sub> receptors. To test this idea, cerebellar granule cell cultures were first incubated in medium containing BDNF (100 ng ml<sup>-1</sup>) for 5 and 60 min, simulating an acute and longer term exposure, respectively. The medium containing BDNF was subsequently replaced with normal medium and

individual cultures were fixed for immunohistochemical processing at selected time points between 15 min and 18 h after removal of BDNF. Figure 7 illustrates that, with both 5 min (Fig. 7B1 and B2) and 60 min (Fig. 7C1 and C2) exposure, the diminution of GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity was gradually reversible over 30 min after removal of BDNF. We have not previously recorded from cultured cerebellar granule cells for this length of time due to technical difficulties in maintaining a stable recording and reproducible responses to repeated applications of GABA. However, the relatively slow time course of recovery from receptor internalization could at least in part account for the observed incomplete recovery of GABA responses within the first 5 min following exposure to BDNF.



**Figure 6. K252a blocks BDNF-induced reduction of cell surface GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity**

Confocal images of cultured cerebellar granule cells before (A) and following a 60 min exposure to BDNF (100 ng ml<sup>-1</sup>) (B, C and D). The cultures were fixed and co-stained with antibodies against GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunits (green fluorescence) and TrkB receptors (red fluorescence). GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity, illustrated in A, is markedly reduced following exposure to BDNF (B). C and D, addition of K252a (200 nM) prevented the BDNF-induced reduction of GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity (C), which persisted in the presence of K252b (200 nM; D). Scale bar (applies to all panels), 50  $\mu$ m.



**Figure 7. Recovery from BDNF-induced reduction of GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity**

Before (A1 and A2) and following bath application of BDNF (100 ng ml<sup>-1</sup>) for 5 min (B1 and B2) and 60 min (C1 and C2), a set of cerebellar granule cell cultures were fixed and processed for GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit (green fluorescence) and TrkB receptor (red fluorescence) immunohistochemistry (A1, B1 and C1). Another set of sister cultures was returned to normal medium after the 5 and 60 min exposures to BDNF, and incubated for an additional 30 min before being processed immunohistochemically (A2, B2 and C2). In cultures exposed to BDNF for either 5 or 60 min, recovery of GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit immunoreactivity occurred by 30 min after washout of the neurotrophin. Scale bar (applies to all panels), 50  $\mu$ m.



## DISCUSSION

This study examined the interaction between acute exposure to BDNF and GABA-activated current responses in cultured cerebellar granule cells. In our culture system, as *in vivo* (Klein *et al.* 1990; Segal *et al.* 1995), cerebellar granule cells express an abundance of TrkB receptors. The major findings of our study are threefold. Firstly, focally applied BDNF acutely attenuates cerebellar granule cell responses to GABA delivered exogenously or released endogenously at synaptic sites. Secondly, the BDNF-induced attenuating effect is mediated by the TrkB receptor. Thirdly, exposure to BDNF results in internalization of GABA<sub>A</sub> receptors, as reflected by diminished cell surface immunostaining of the GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunit. Both the BDNF-induced attenuation and internalization of GABA<sub>A</sub> receptors are dependent on kinase activity downstream of receptor activation. Taken together, our results demonstrate the presence of postsynaptic mechanisms by which BDNF attenuates GABA-mediated responses, including an early phase characterized by a tyrosine receptor kinase-dependent suppression of GABA<sub>A</sub> receptor-mediated activity and a later phase hallmarked by internalization of GABA<sub>A</sub> receptors.

### BDNF attenuation of GABAergic synaptic activity

In the present study, BDNF reduced the frequency and amplitude of both the spontaneous and miniature IPSCs. Typically, a decrease in the amplitude of IPSCs implies a change in the properties and/or number of postsynaptic GABA<sub>A</sub> receptors. On the other hand, a decrease in the frequency of IPSCs implicates reduced presynaptic neurotransmitter release. We defined sIPSCs as inhibitory postsynaptic currents inherent in our culture system that were recorded without intentional manipulation or stimulation to induce transmitter release, and mIPSCs as those that were monitored in the presence of TTX and were thus independent of action potential-induced transmitter release. In fact, the amplitude and frequency of GABA-mediated synaptic currents were similar in the absence or presence of TTX, suggesting that action potential-dependent transmitter release is but a minor component of the overall spontaneous synaptic activity.

At first glance, our data on sIPSCs suggest involvement of both pre- and postsynaptic mechanisms. However, further analysis revealed that the decrease in frequency persisted even when mIPSCs were monitored. Since mIPSCs are not influenced by action potential-dependent presynaptic release processes, the apparent reduction in frequency is likely to be a consequence of reduced IPSC amplitude. In addition, direct intracellular delivery of K252a prevented the BDNF-induced attenuation of spontaneous IPSCs. These data lead us to conclude that the neurotrophin modifies properties of postsynaptic GABA<sub>A</sub> receptors, thereby reducing GABA<sub>A</sub> receptor-mediated synaptic

events. Indeed, at the ultrastructural level, TrkB receptor immunoreactivity has been localized to the postsynaptic membrane (Aoki *et al.* 2001). In addition, BDNF has been reported to enhance glutamatergic transmission through postsynaptic modulation of NMDA receptor activity (Levine *et al.* 1996, 1998). These studies provide evidence that BDNF can modulate synaptic transmission through postsynaptic mechanisms. However, it should be noted that K252a itself reduced the frequency of postsynaptic currents, and that this confounded our ability to definitively differentiate between the contribution of presynaptic and postsynaptic components in the BDNF-induced process. Thus, in a strict sense, the presence of a presynaptic effect of BDNF in modulating GABA-mediated synaptic transmission cannot be excluded based on available data.

### BDNF–GABA<sub>A</sub> receptor cross-talk: possible mechanisms

How does acute exposure to BDNF attenuate GABA-mediated activity? We considered two possibilities, namely, modulation of GABA<sub>A</sub> receptor properties by TrkB receptor-mediated signalling and BDNF-induced internalization of GABA<sub>A</sub> receptors.

**Mediation by TrkB receptor signalling.** Pretreatment of cultured granule cells with either K252a or TrkB-Fc prevented the BDNF-induced diminution of the GABA response. Since granule cells do not express immunohistochemically detectable levels of the p75 receptor (Anderson *et al.* 1995), the BDNF-induced effect would appear to be mediated primarily through the TrkB receptor. In light of this, activation of the TrkB receptor and its downstream signalling pathways may trigger phosphorylation cascades, leading to altered affinity or channel conductance of GABA<sub>A</sub> receptors. The outcome of such a BDNF–GABA<sub>A</sub> receptor interaction would be expected to depend on several factors, including cell type-dependent expression of GABA<sub>A</sub> receptors harbouring different subunit combinations or distinct BDNF signalling pathways, or both. Indeed, BDNF exerts an attenuating effect on GABA-mediated IPSCs in rat hippocampal neurons (Tanaka *et al.* 1997) but enhances them in cerebellar Purkinje cells (Boxall, 2000). Other studies report that protein tyrosine kinase receptors modulate response to GABA in opposite ways. In cultured superior cervical ganglion neurons, intracellular application of vanadate, a tyrosine phosphatase inhibitor, increases the amplitude of GABA-activated current (Moss *et al.* 1995). In contrast, platelet-derived growth factor binds to its receptor tyrosine kinase and activates phospholipase C- $\gamma$  in rat hippocampal neurons, resulting in Ca<sup>2+</sup>-dependent decreases in both synaptic and somatic responses to GABA (Valenzuela *et al.* 1995). In recombinant GABA<sub>A</sub> receptors expressed in HEK 293 cells, the presence of the  $\beta_1$ ,  $\beta_2$  or  $\beta_3$  subunit dictates the direction of modulation by cyclic AMP (McDonald *et al.* 1998), underscoring the

dependence of phosphorylation-mediated modulation of GABA<sub>A</sub> receptors on subunit expression.

In our experiments, recovery from a BDNF-induced attenuation of the GABA-mediated response was only partial during our electrophysiological recordings. Since we have experimentally ruled out GABA<sub>A</sub> receptor run-down and desensitization as possible confounding factors, phosphorylation of TrkB receptors is likely to account for the observed partial recovery. Indeed, once phosphorylated, the TrkB receptor and its associated downstream signalling proteins can remain in this state for hours beyond removal of BDNF (Choi *et al.* 2001).

Finally, the neurotrophin 4/5 (NT4/5), via activation of the TrkB receptors, exerts effects that mimic those of BDNF (Rabacchi *et al.* 1999; Fan *et al.* 2000; Brunig *et al.* 2001). It would be reasonable to postulate that NT4/5 also attenuates GABA responses in cerebellar granule cells through TrkB receptor-mediated signalling. These and other related issues await experimentation in future studies.

**Internalization of GABA<sub>A</sub> receptors.** Another possible mechanism to account for our findings is receptor internalization. Our electrophysiological data indicate that exposure to BDNF decreases IPSC amplitude but leaves kinetic properties of GABA<sub>A</sub> receptors unchanged, suggesting a reduction in GABA<sub>A</sub> receptor numbers. Indeed, our immunohistochemical results indicate quantitatively that exposure to BDNF diminishes cell surface immunofluorescence of GABA<sub>A</sub> receptor  $\beta_{2/3}$  subunits, consistent with receptor internalization. Trafficking of receptors as a means of regulating the efficacy of synaptic transmission is quite prevalent. Activation of the insulin receptor, a member of the family of tyrosine kinase receptors, recruits GABA<sub>A</sub> receptors to the cell surface (Wan *et al.* 1997). Activation of protein kinase C reduces GABA<sub>A</sub> receptor  $\alpha_1$  subunit immunoreactivity in the short term (Chapell *et al.* 1998). In HEK 293 cells, recombinant GABA<sub>A</sub> receptors undergo clathrin-independent endocytosis (Cinar & Barnes, 2001). Activation of mGluR1 induces a loss of AMPA and NMDA receptors, resulting in decreased frequency and amplitude of mEPSCs (Snyder *et al.* 2001). In addition, BDNF exposure decreases the number of cell surface GABA<sub>A</sub> receptors in cultured hippocampal neurons (Brunig *et al.* 2001). However, in all these cases, the onset of receptor internalization is 5–60 min following BDNF exposure, which is too slow to account for the rapid suppression of the GABA response of cerebellar granule cells observed in our study. We nonetheless consider that a BDNF-induced internalization of GABA<sub>A</sub> receptors with a relatively long time course (> 30 min) of recovery could explain the partial recovery of GABA responses consistently observed in our electrophysiological studies following removal of BDNF.

Finally, our data suggest that activation of the TrkB receptor and its downstream signalling cascade may be mechanistically a point of convergence for the early and later phases of the acute action of BDNF demonstrated in this study, characterized by attenuation of GABA responses and internalization of GABA<sub>A</sub> receptors, respectively. Overall, we propose that the effects of acute exposure to BDNF of prompt attenuation of GABA<sub>A</sub> receptor-mediated activity and a longer-latency internalization of GABA<sub>A</sub> receptors are not mutually exclusive and, in fact, are complementary and occur *in tandem* to account for the sequence of events.

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