BDNF-induced TrkB activation down-regulates the K⁺–Cl⁻ cotransporter KCC2 and impairs neuronal Cl⁻ extrusion

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Pathophysiological activity and various kinds of traumatic insults are known to have deleterious long-term effects on neuronal Cl⁻ regulation, which can lead to a suppression of fast postsynaptic GABAergic responses. Brain-derived neurotrophic factor (BDNF) increases neuronal excitability through a conjunction of mechanisms that include regulation of the efficacy of GABAergic transmission. Here, we show that exposure of rat hippocampal slice cultures and acute slices to exogenous BDNF or neurotrophin-4 produces a TrkB-mediated fall in the neuron-specific K⁺–Cl⁻ cotransporter KCC2 mRNA and protein, as well as a consequent impairment in neuronal Cl⁻ extrusion capacity. After kindling-induced seizures in vivo, the expression of KCC2 is down-regulated in the mouse hippocampus with a spatiotemporal profile complementary to the up-regulation of TrkB and BDNF. The present data demonstrate a novel mechanism whereby BDNF/TrkB signaling suppresses chloride-dependent fast GABAergic inhibition, which most likely contributes to the well-known role of TrkB-activated signaling cascades in the induction and establishment of epileptic activity.

Introduction

Brain-derived neurotrophic factor (BDNF)* has well-documented long-term effects on neuronal survival and differentiation, as well as on synapse formation and functional maturation (Marty et al., 1997; Huang and Reichardt, 2001). In the adult brain, an enhancement in the expression of BDNF and its receptor TrkB is thought to predispose cortical areas to seizure (Binder et al., 2001). BDNF-induced hyperexcitability has been proposed to be partly due to effects on fast GABAergic inhibition. In rat hippocampal slices, BDNF acting via TrkB receptors inhibits GABA_A synaptic responses of CA1 pyramidal neurons (Kim et al., 1994; Tanaka et al., 1997; Frerking et al., 1998; Brunig et al., 2001).

GABA_A receptor–mediated inhibition can be modulated by a variety of mechanisms, including changes in the firing rate of GABAergic interneurons, the kinetics of quantum release, or by postsynaptic changes at the GABA_A receptor level (Ben Ari and Cossart, 2000; Dalby and Mody, 2001). In addition, GABA_A-mediated responses are sensitive to changes in the electrochemical gradients for the permeant anions (Kaila, 1994), a factor that is often ignored. In most central neurons, fast inhibition is based on a postsynaptic intracellular [Cl⁻] that is lower than expected from passive distribution (Thompson et al., 1988; Thompson and Gähwiler, 1989). The generation and maintenance of the chloride gradient required for hyperpolarizing ionotropic responses is attributable to the neuron-specific K⁺–Cl⁻ cotransporter, KCC2 (Rivera et al., 1999; DeFazio et al., 2000; Kakazu et al., 2000; Hubner et al., 2001). Here, we present several lines of evidence showing that BDNF, acting via TrkB, down-regulates KCC2, leading to impairment of Cl⁻ extrusion from mature hippocampal neurons.

Results and discussion

We examined the effects of the TrkB receptor ligands BDNF and neurotrophin-4 (NT-4) on the expression of KCC2 in rat organotypic hippocampal cultures. RT-PCR
analysis (Rivera et al., 1999) showed a clear down-regulation of KCC2 mRNA expression by BDNF or NT-4, indicating that this effect is mediated by TrkB (Fig. 1 A) and acts at the transcriptional level. Treatment of the organotypic cultures with increasing concentrations of BDNF in the culture medium (1–100 ng/ml) for 17–19 h (Fig. 1 B) resulted in a dose-dependent decrease in the expression of KCC2 protein. At 10 ng/ml of BDNF, there was a 61% decrease, and at 100 ng/ml, an 82% decrease in the expression of KCC2 as compared with control levels. A similar down-regulation of KCC2 protein was observed when NT-4 was applied (Fig. 1 C). The effects of both BDNF and NT-4 on KCC2 expression were blocked by either inhibiting tyrosine kinase activity with 1 μM K252a or TrkB-Fc receptor body inhibited this effect. Note the significant increase in KCC2 protein level after treatment with TrkB-Fc or K252a only. The bottom panel shows the average normalized optical densities displayed as percentage of control (mean ± SD, n = 24). (D) Inhibition of network activity with TTX or with glutamate blockers (CNQX and AP5) does not inhibit the down-regulation of KCC2 by BDNF in organotypic slices. The top panel shows a representative Western blot, and the bottom one the normalized optical densities (mean ± SD, n = 7; ***, P < 0.001; **, P < 0.05 as compared with control using the t test).

Figure 1. Exogenous BDNF and NT-4 down-regulate KCC2 mRNA and protein expression in organotypic hippocampal slices. (A) A representative RT-PCR experiment (out of four similar ones) showing the down-regulatory effect of BDNF and NT-4 on KCC2 mRNA expression. The effects of BDNF and NT-4 were inhibited by 200 ng/ml TrkB-Fc. The amplified light neurofilament (NF-L) fragment indicates an equal amount of mRNA in the reactions. Note the increase in KCC2 mRNA brought about by exposure to TrkB-Fc only. (B) Western blot analysis showing the dose-dependent effect of BDNF. A representative blot (left) and the average normalized optical densities displayed as a percentage of control (right, mean ± SD, n = 5). (C) Representative Western blot (top) showing that KCC2 protein levels are down-regulated by BDNF or NT-4. The Trk inhibitor K252a and TrkB-Fc receptor body inhibited this effect. Note the significant increase in KCC2 protein level after treatment with TrkB-Fc or K252a only. The bottom panel shows the average normalized optical densities displayed as percentage of control (mean ± SD, n = 24). (D) Inhibition of network activity with TTX or with glutamate blockers (CNQX and AP5) does not inhibit the down-regulation of KCC2 by BDNF in organotypic slices. The top panel shows a representative Western blot, and the bottom one the normalized optical densities (mean ± SD, n = 7; ***, P < 0.001; **, P < 0.05 as compared with control using the t test).
KCC2 expression. Again, the effect of exogenous BDNF was not attributable to a network-mediated action because a prompt BDNF-dependent down-regulation of KCC2 mRNA was also seen in the continuous presence of the glutamate antagonists CNQX and AP5 (Fig. 2 B).

Next, we asked whether the down-regulation of KCC2 seen in acute slices is paralleled by a decrease in the capacity of neuronal Cl⁻ extrusion in CA1 pyramidal neurons. The synaptic and ionic mechanisms underlying hyperpolarizing inhibitory post synaptic potentials (IPSPs) in these neurons have been extensively examined (Thompson et al., 1988; Thompson and Gähwiler, 1989; Kaila, 1994), and a wealth of data points to the K⁺–Cl⁻ cotransporter KCC2 as the main Cl⁻ extrusion mechanism (Rivera et al., 1999; DeFazio et al., 2000; Kakazu et al., 2000). Furthermore, exogenous BDNF is known to activate TrkB in these neurons (Huang et al., 2000; Kakazu et al., 2000). BDNF is known to activate TrkB in these neurons (Huang et al., 2000; Kakazu et al., 2000). Furthermore, exogenous BDNF is known to activate TrkB in these neurons (Huang et al., 2000; Kakazu et al., 2000). Furthermore, exogenous BDNF is known to activate TrkB in these neurons (Huang et al., 2000; Kakazu et al., 2000). 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hippocampal epileptiform activity after each stimulus was 4.6 ± 0.5 generalized (grade 4–5) seizures during the series of 40 hippocampal kindling stimulations where the mean duration of focal hippocampal epileptiform activity after each stimulus was 46 ± 2 s (n = 16). Control animals (0 h) were operated, but did not receive stimulation. Note the conspicuous down-regulation of KCC2 mRNA in the dentate gyrus (DG) 2 and 6 h after stimulation (b vs. h). KCC2 levels were low in the CA1–CA3 region even after 24 h (j and l). The contralateral side showed identical changes and is therefore not shown. Bars: left panel, 1 mm; right panel, 100 μm. (B) Representative pictures of transverse brain sections from kindled mice showing changes in the distribution of KCC2 immunostaining in the hippocampus (dark areas indicate high expression, n = 3). (a) Control section shows intense staining in CA1–CA3 stratum oriens (so) proximal to the pyramidal layer (p) and moderate staining in str. lacunosum-moleculare (slm). A lower level of KCC2 immunoreactivity is observed in str. radiatum (sr) and str. lucidum (sl). (b) Higher magnification of the DG. Here, the molecular layer (ML) shows strong KCC2 immunoreactivity, whereas the granular layer (GL) and the hilus (Hil) show weak staining. (c) Note a significant reduction of KCC2 immunoreactivity in all hippocampal regions 6 h after the last seizure. The CA1 region, especially the proximal dendrites in the str. oriens, although displaying lower levels than control, shows higher KCC2 immunoreactivity than the CA3 region and DG. (d) The ML in the DG is the region with the most conspicuous fall in KCC2 expression at 6 h. (e and f) A partial recovery of the KCC2 immunostaining intensity is observed 24 h after the last stimulus-evoked seizure. The DG still shows lower levels of KCC2 expression. Bars: left panel, 350 μm; right panel, 100 μm.

Figure 4. Down-regulation of KCC2 in vivo after hippocampal kindling. (A) The photomicrographs show changes in the distribution of KCC2 mRNA in transverse sections from mice 2, 6, and 24 h after the last stimulus-evoked seizure (a, d, g, and j). The mice experienced 4.6 ± 0.5 generalized (grade 4–5) seizures during the series of 40 hippocampal kindling stimulations where the mean duration of focal hippocampal epileptiform activity after each stimulus was 46 ± 2 s (n = 16). Control animals (0 h) were operated, but did not receive stimulation. Note the conspicuous down-regulation of KCC2 mRNA in the dentate gyrus (DG) 2 and 6 h after stimulation (b vs. h). KCC2 levels were low in the CA1–CA3 region even after 24 h (j and l). The contralateral side showed identical changes and is therefore not shown. Bars: left panel, 1 mm; right panel, 100 μm. (B) Representative pictures of transverse brain sections from kindled mice showing changes in the distribution of KCC2 immunostaining in the hippocampus (dark areas indicate high expression, n = 3). (a) Control section shows intense staining in CA1–CA3 stratum oriens (so) proximal to the pyramidal layer (p) and moderate staining in str. lacunosum-moleculare (slm). A lower level of KCC2 immunoreactivity is observed in str. radiatum (sr) and str. lucidum (sl). (b) Higher magnification of the DG. Here, the molecular layer (ML) shows strong KCC2 immunoreactivity, whereas the granular layer (GL) and the hilus (Hil) show weak staining. (c) Note a significant reduction of KCC2 immunoreactivity in all hippocampal regions 6 h after the last seizure. The CA1 region, especially the proximal dendrites in the str. oriens, although displaying lower levels than control, shows higher KCC2 immunoreactivity than the CA3 region and DG. (d) The ML in the DG is the region with the most conspicuous fall in KCC2 expression at 6 h. (e and f) A partial recovery of the KCC2 immunostaining intensity is observed 24 h after the last stimulus-evoked seizure. The DG still shows lower levels of KCC2 expression. Bars: left panel, 350 μm; right panel, 100 μm.
KCC2 is also observed in vitro in the classical 0-Mg slice model. Here, the effects of continuous neuronal activity on KCC2 can be blocked by K252a or by scavenging endogenous BDNF with TrkB receptor bodies (unpublished data).

In conclusion, our results disclose a novel BDNF/TrkB-mediated signaling mechanism that is likely to have a profound action on neuronal Cl⁻ homeostasis. Here, it is worth pointing out that changes in intraneuronal [Cl⁻] do not only affect the amplitude and polarity of GABAergic responses (Kaila, 1994). They are also intimately involved in the control of both neuronal and interstitial volume, which play a critical role in the modulation of neuronal excitability, especially under conditions that promote epileptiform activity (Jefferys, 1995, 1998; Azouz et al., 1997).

Materials and methods
All experimental procedures were performed according to ethical guidelines approved by local authorities.

Kindling
Male adult C57BL/6 mice (n = 16) were housed under 12 h of light/12 h of dark with food and water ad libitum. Stimulating/recording electrodes were implanted in the left ventral hippocampus and 40 threshold stimulations with 5-min intervals (1-ms pulses, 10-Hz frequency, and 10-s duration) were delivered 7–10 d after electrode implantation as described previously (Kokaia et al., 1999). Animals were killed at 2, 6, and 24 h after the last stimulus-evoked seizure (four animals in each group). Four electrode-implanted nonstimulated mice were used as controls. Brains were immediately frozen on powdered dry ice. For in situ hybridization, brains were sectioned on a cryostat at 14 μm in the frontal plane at the level of the dorsal hippocampus, and thaw-mounted onto ProbeOn slides (Fisher Scientific) and stored at ~70°C. For free-floating immunohistochemistry, 40-μm sections were prepared and stored at ~20°C in a cryoprotecting solution.

In situ hybridization
The following cDNA constructs were used as templates for the synthesis of labeled cRNA probes: a 1,039-bp mouse KCC2 EST clone (EMBL/GenBank/DDBJ AA982489), corresponding to nucleotides 4,605–5,566 of the full-length rat KCC2 cDNA (Payne et al., 1996), and a 366-bp insert of rat BDNF cDNA construct (Hiltunen et al., 1996). This fragment (nucleotides 517–882) of the rat BDNF cDNA sequence (EMBL/GenBank/DDBJ M61178) is 99% identical to the corresponding mouse BDNF sequence (EMBL/GenBank/DDBJ AA982489). A 483-bp insert of mouse TrkB cDNA extracellular domain (Klein et al., 1989; Hiltunen et al., 1996). Radioactive in situ hybridization was performed on frozen sections as described previously (Kokaia et al., 1999). Free-floating in situ hybridization was performed on 100-μm sections obtained from thick acute hippocampal slices (350 μm) using digoxigenin-labeled riboprobes as described previously (Nieto et al., 1996).

Quantification of mRNA levels
Quantification of hybridization signals on the X-ray films was performed using ImageJ (National Institutes of Health, Bethesda, MD). Grey levels were assayed with increasing protein concentrations, and a protein concentration of 15–20 μg/ml was calculated to be optimal.

Immunohistochemistry
For free-floating immunostaining, 50-μm brain sections and organotypic hippocampal slices were washed several times with PBS, dehydrated through methanol series and incubated in Dent’s fixative (20% DMSO in methanol) for 12–15 h. Sections were washed with TBST (0.1% Triton X-100 in TBS), incubated overnight with rabbit anti-KCC2 antibody (1:600), and diluted in TBST (5% BSA and 0.4% sheep serum). After washing, the sections were incubated with corresponding Cy3-conjugated secondary antibody (1:200; Jackson Immunoresearch Laboratories) overnight and mounted on Superfrost plus (Menzel-Glaser) gelatin (Biomedica Corp.).

RT-PCR
Total RNA from rat hippocampal slices was reverse-transcribed and PCR-amplified as described previously (Rivera et al., 1999). Primers were synthesized over specific regions for full-length TrkB, forward, 5’-TCAAGTTGGCGGAGCATT-3’; reverse, 5’-ATGACTCTAAAGACCATGATGACG-3’. Targeting nucleotides 1,869–2,367; for KCC2, forward, 5’-CTCAGAACCC-TGACGGACTG-3’; reverse, 5’-GCAGAAGGACCTCATGATGCTGCGG-3’, targeting nucleotides 119–518; for neuron-specific neurofilament light chain, forward, 5’-GACATCTCCACCCGGTTCAGC-3’; reverse, 5’-GGATCTGGACCCCTGACTGTCG-3’.

Electrophysiology
350-μm transverse hippocampal slices from 100–150-g Wistar rats were cut using a Vibratome (Technical Products International, Inc.), and the slices were allowed to recover in standard physiological solution containing 124 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 25 mM NaHCO₃, 1.1 mM NaH₂PO₄, 2 mM MgSO₄, and 10 mM d-glucose, and were equilibrated with 95% O₂ and 5% CO₂ at 32°C. Picrotoxin and bicuculline were added to the recording solution targeting nucleotides 1,869–2,367; for KCC2, forward, 5’-CTCAGAACCC-TGACGGACTG-3’; reverse, 5’-GCAGAAGGACCTCATGATGCTGCGG-3’, targeting nucleotides 119–518; for neuron-specific neurofilament light chain, forward, 5’-GACATCTCCACCCGGTTCAGC-3’; reverse, 5’-GGATCTGGACCCCTGACTGTCG-3’.

Intracellular recordings were obtained blind in CA1 stratum pyramidale using microelectrodes filled with 0.5 M potassium acetate plus 0.5 M KCl (pH 6.6–6.8; resistance 135–220 MΩ), and 50 mM QX-314 (Tocris Cookson Ltd.) by block spiking. Data accepted for analysis were taken from cells that had a stable Vm of at least ~50 mV (control ~64.74 ± 1.91 mV; BDNF ~67.88 ± 0.90 mV) and an input resistance of 68–163 MΩ (control ~93 ± 8 MΩ; BDNF ~129 ± 12 MΩ). Pharmacologically isolated IPSPs were evoked in the presence of the ionotropic glutamate antagonists NBQX and AP5 (10 and 40 μM, respectively; Tocris Cookson Ltd.) by stimuli (5–25 V, 60–100 μsec, and frequency 1/10 or 1/15 Hz) delivered via a bipolar tungsten electrode positioned close (~500 μm) to the recording electrode (Davies et al., 1990). Picrotoxin and bicuculline were added from Sigma-Aldrich. Measurements were performed with an Axoclamp 2B amplifier (Axon Instruments, Inc.) in bridge mode. Data were digitized and analyzed offline using WinWCP v3.2 (Strathclyde University, Glasgow, UK).

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