BDNF-induced TrkB activation down-regulates the K\(^{+}\)–Cl\(^{-}\) cotransporter KCC2 and impairs neuronal Cl\(^{-}\) extrusion

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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 quantal 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 intensity of KCC2 protein expression. 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 by specifically scavenging these growth factors with 200 ng/ml of the TrkB-soluble receptor body (TrkB-Fc; Fig. 1 C). An intriguing finding was that application of TrkB-Fc or K252a produced a significant increase in KCC2 mRNA as well as protein levels (Fig. 1, A and C), indicating that KCC2 levels were regulated by an endogenous BDNF/TrkB-mediated action. TTX (1 μM) or the glutamate antagonists AP5 (20 μM) and CNQX (50 μM) did not inhibit the BDNF-induced decrease in KCC2 protein (Fig. 1 D), which shows that the TrkB-mediated effects on KCC2 were not caused by a general increase in neuronal excitability and network activity (Scharfman, 1997; Kafitz et al., 1999; Scharfman et al., 1999). Application of NGF, which acts via TrkA, or heat-inactivated BDNF and NT-4 had no effect on KCC2 expression levels (unpublished data).

To examine the consequences of a BDNF-induced reduction in KCC2 expression on neuronal Cl− extrusion, we exposed acute hippocampal slices to 100–200 ng/ml BDNF for different periods of time. Free-floating in situ hybridization showed that KCC2 mRNA levels were down-regulated in all hippocampal regions (Fig. 2 A), and a fast reduction of KCC2 protein was seen in Western blots (Fig. 2 B). The decrease in KCC2 protein expression was already observed after 2 h of incubation, and became even more pronounced after 4 h (Fig. 2 B). Here, one should note that this surprisingly brief time delay reflects a number of steps, including the diffusion of BDNF into the slice and the activation of TrkB-mediated signaling cascades, as well as the consequent changes in
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 Cl\(^-\) imposed by leakage from sharp microelectrodes filled with a solution containing 0.5 M Cl\(^-\), a fall in the level of functional KCC2 should lead to a higher steady-state level of [Cl\(^-\)], and to a consequent positive shift in the reversal potential of pharmacologically isolated IPSPs (E\(_{\text{IPSP}}\)). Therefore, we injected constant current pulses into CA1 pyramidal neurons in order to bring the membrane potential (V\(_m\)) to different steady levels. Subsequently, IPSPs were evoked by stimulating the GABAergic inputs to the pyramidal neurons (Fig. 3 A, arrow). With the exogenous Cl\(^-\) load, the control cells were able to maintain hyperpolarizing IPSPs at the resting membrane potential (V\(_r\)) level, whereas those in slices exposed to BDNF were depolarizing (Fig. 3 A), indicating, indeed, a significant reduction in functional Cl\(^-\) extrusion capacity. The synaptic responses were fully blocked by 100 \(\mu\)M picrotoxin or 50 \(\mu\)M bicuculline (unpublished data). We plotted the amplitudes of IPSPs (IPSP \(- V_m\)) against the V\(_m\) to obtain reversal potentials (Fig. 3 B). IPSPs such as those evoked presently are caused by mass stimulation of both somatic and dendritic inputs (Buhl et al., 1995), which explains the observation that they had distinct reversal potentials at various points of time (Fig. 3, A and B). The fastest post synaptic response, which had the most positive reversal potential with respect to V\(_{\text{rest}}\) in both control cells and those preexposed to BDNF (Fig. 3 B), peaked between 7–9 ms, most likely reflecting the input from basket cells (Buhl et al., 1995) that target the somata of pyramidal neurons. Using three time windows when measuring the values of E\(_{\text{IPSP}}\) under steady-state conditions (Fig. 3, B and C), the IPSP driving force (defined here as E\(_{\text{IPSP}}\)–V\(_{\text{rest}}\)) at 7–9, 20–25, and 35–40 ms after the stimulus pulse was -3.52 ±

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**Figure 2.** Rapid BDNF-induced down-regulation of KCC2 in acute hippocampal slices. (A) Free-floating in situ hybridization of acute hippocampal slices exposed to 100 ng/ml BDNF showing decreased KCC2 mRNA expression in all hippocampal regions as compared with control. (B) The top panel shows a representative Western blot of KCC2 expression at different time points after adding 100 ng/ml BDNF to the extracellular solution in the absence or presence of glutamate antagonists (CNQX and AP5). Normalized optical densities are shown in the bottom panel (n = 5; ***, P < 0.001 as compared with control using the t test). Note that the KCC2 protein is already down-regulated after 2 h. Bar, 1 mm.

**Figure 3.** BDNF reduces the capacity of neuronal Cl\(^-\) extrusion in rat hippocampal slices. (A) The reversal potential of IPSPs (E\(_{\text{IPSP}}\)) was measured using 0.5 M Cl\(^-\)-containing microelectrodes from acute slices incubated with 100–200 ng/ml BDNF for 2–4 h (bottom) and from control slices (top). The traces are superimposed responses to constant current pulses injected to bring the membrane potential (V\(_m\)) to different levels before evoking an IPSP by stimulation (arrow). The amplitudes of the IPSPs (i.e., IPSP \(- V_m\)) from the recordings in A have been plotted against the V\(_m\) measured 5–15 ms before (square) the time of stimulation in B. (C) Summary of the mean (± SEM) driving forces of IPSPs (E\(_{\text{IPSP}}\)–V\(_m\)) obtained from control cells (n = 9), and from cells exposed to BDNF (n = 11), all measured under steady-state conditions in the presence of the electrode-induced Cl\(^-\) load. Note that the time-dependent negative shift in reversal cannot be accounted for by inadequate space clamp because a time-dependent change in IPSP polarity was often seen in BDNF-incubated slices in the absence of injected current (A, bottom, and C).
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 (Hi) 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.

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 1.36-kb 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 X55573; 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 with the TINA program (Tamro). The linearity of the system was assayed with increasing protein concentrations, and a protein concentration of 15–20 μg/ml was calculated to be optimal.

Immunohistochemistry
Membrane fractions prepared from hippocampal slices were analyzed by Western blotting as described previously (Payne et al., 1996; Rivera et al., 1999), using the following source of pAbs: affinity-purified rabbit anti-rat KCC2 (a gift from Dr. John Payne, University of California, Davis, CA) and monoclonal anti-α-tubulin clone DM1A (Sigma-Aldrich). The corresponding bands were developed using ECL-plus (Amersham Biosciences) on X-ray films as well as on a phosphorimager (model BAS-1500; Fuji) and were analyzed with the TINA program (Tann). The linearity of the system was assayed with increasing protein concentrations, and a protein concentration of 15–20 μg/ml was calculated to be optimal.

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. Slices were placed into Falcon® tissue culture filters (treated with 2% BSA overnight) in a 6-well culture tray containing 8–10 ml of physiological solution and continuously gassed with humidified 95% O₂ and 5% CO₂ at 32°C. Slices were exposed to 100–200 ng/ml BDNF for 2–4 h before transferring to either a submerged-type (double-sided perfusion) recording chamber (32–34°C) or processing for free-floating in situ hybridization. The procedure with control slices was identical except that the BDNF application was omitted.

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.) to block spiking. Data accepted for analysis were taken from cells that had a stable V_m of at least −50 mV (control = −64.74 ± 1.91 mV; BDNF = −67.60 ± 1.09 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 DL-AP5 (10 and 40 μM, respectively; Tocris Cookson Ltd.) by stimuli (5–25 V, 60–100 μs, 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 acquired 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 v3.2.0 software (Strathclyde University, Glasgow, UK).

We would like to thank Eila Kujamäki, Miika Palvainen, and Marjo
References


