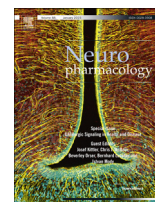Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

BDNF is required for seizure-induced but not developmental up-regulation of KCC2 in the neonatal hippocampus

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ARTICLE INFO

ABSTRACT

A robust increase in the functional expression of the neuronal K−Cl cotransporter KCC2 during CNS development is necessary for the emergence of hyperpolarizing ionotropic GABAergic transmission. BDNF-TrkB signaling has been implicated in the developmental up-regulation of KCC2 and, in mature animals, in fast activity-dependent down-regulation of KCC2 function following seizures and trauma. In contrast to the decrease in KCC2 expression observed in the adult hippocampus following trauma, seizures in the neonate trigger a TrkB-dependent up-regulation of neuronal Cl⁻ extrusion capacity associated with enhanced surface expression of KCC2. Here, we show that this effect is transient, and impaired in the hippocampus of $Bdnf^{-/-}$ mice. Notably, however, a complete absence of BDNF does not compromise the increase in KCC2 protein or K-Cl transport functionality during neuronal development. Furthermore, we present data indicating that the functional up-regulation of KCC2 by neonatal seizures is temporally limited by calpain activity.
This article is part of the Special Issue entitled 'GABAergic Signaling in Health and Disease'.

1. Introduction

Progressive up-regulation of the neuron-specific multifunctional K−Cl cotransporter KCC2 during rodent postnatal development is required for the emergence of hyperpolarizing GABA_A receptor (GABA_AR)-mediated responses in hippocampal and neocortical principal neurons (Blaesse et al., 2009; Blaesse and Schmidt, 2014; Kaila et al., 2014a; Rivera et al., 1999). This straightforward dependence of the ontogeny of hyperpolarizing inhibition on the developmental expression of KCC2 belies a much more intricate mechanistic scenario where the expression of KCC2 protein is a necessary but not sufficient condition for the above functional outcome (Blaesse et al., 2009; Kahle et al., 2013). For instance, work on the rodent auditory brainstem has demonstrated that, despite similarly high expression levels of KCC2 protein in the early postnatal period and during the first month of life, the capacity to extrude Cl⁻ in neurons of the lateral superior olive emerges gradually during the first two weeks of life (Balakrishnan et al., 2003; Blaesse et al., 2006). Indeed, mounting evidence suggests that KCC2 protein is functionally activated by phosphorylation mechanisms targeting its C-terminal domain (Chamma et al., 2012; Kahle et al., 2013; Kaila et al., 2014a; Medina et al., 2014). Observations of this kind, underscored by the fast membrane trafficking of KCC2 (Khirug et al., 2010; Lee et al., 2007), point to powerful post-translational control of KCC2 functionality (Blaesse et al., 2009; Kahle et al., 2013; Kaila et al., 2014a).

The development of postsynaptic GABA_AR-mediated responses is also strongly influenced by the Na−K−2Cl cotransporter NKCC1. While NKCC1 has gained considerable attention in the context of neonatal seizures (for review, see Briggs and Galanopoulou, 2011; Löscher et al., 2013; Nardou et al., 2013; Puskarjov et al., 2014a), only a few studies have addressed the involvement of KCC2 (Galanopoulou, 2007, 2008; Khirug et al., 2010; Nardou et al., 2011). We have recently shown that a single episode of neonatal seizures in rats at postnatal day (P) 5−7 in vivo and epileptiform activity in vitro both result in enhanced membrane insertion and fast functional activation of KCC2 without altering KCC2 synthesis (Khirug et al., 2010). The activity-dependent functional KCC2 up-regulation was fully blocked by tetrodotoxin (TTX) and K252a (Khirug et al., 2010), an efficient inhibitor of the BDNF receptor TrkB. Here we show that the rapid but transient seizure-induced functional up-regulation of KCC2 is absent in neonatal $Bdnf^{-/-}$

mice that show dramatically diminished TrkB signaling in early postnatal life (Di Lieto et al., 2012). Unexpectedly (cf. Aguado et al., 2003; Ludwig et al., 2011b), no difference was detected in the developmental up-regulation of either KCC2 total protein or functionality between $Bdnf^{-/-}$ and $Bdnf^{+/+}$ genotypes. Thus, the present findings indicate that BDNF-TrkB signaling is required for short-term, seizure-induced post-translational functional activation of KCC2. BDNF itself does not appear to be a necessary factor for the developmental up-regulation of KCC2 protein and neuronal Cl$^-$ extrusion, but, as previously demonstrated it may act as a powerful modulator of KCC2 mRNA and protein expression in developing neurons (cf. Aguado et al., 2003; Ludwig et al., 2011b).

2. Materials and methods

2.1. Induction and scoring of neonatal seizures in $Bdnf^{-/-}$ and $Bdnf^{+/+}$ mice

$Bdnf^{+/-}$ mice (Ernfors et al., 1994) used for generation of homozygous $Bdnf^{-/-}$ and $Bdnf^{+/+}$ pups were kindly provided by Dr. Eero Castrén, Neuroscience Center, University of Helsinki. The genetic background of the mice was C57BL/6. Experiments were carried out with approval by the National Animal Ethics Committee of Finland and the local Animal Ethics Committee of the University of Helsinki. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to consider alternatives to *in vivo* techniques.

Seizures were induced in P6 $Bdnf^{-/-}$ and $Bdnf^{+/+}$ pups with a single 10 μl intraperitoneal injection of kainate (2 mg/kg; Tocris) dissolved in saline (0.9% NaCl). Following injection, the pups were maintained for 90 min in a chamber preheated to a temperature of 33 ± 1 °C. Continuous behavioral monitoring was performed throughout this period via video recording and seizure severity was scored offline. Due to vestibular deficits, naïve $Bdnf^{-/-}$ mice are characterized by difficulty remaining upright and by ataxia (Ernfors et al., 1994; Jones et al., 1994). Obviously, this precludes the use of loss of righting reflex or clonic movements for scoring of behavioral seizures. Tonic hindlimb extension, a sign of severe behavioral seizures in neonatal rodents (Velisek et al., 1995), was not observed in naïve $Bdnf^{-/-}$ mice, and was used to assess the severity of seizures in $Bdnf^{+/+}$ and $Bdnf^{-/-}$ pups. To this end, individual video recordings were reviewed in an experimenter-blind manner at intervals of one minute. At every 60 s, the video was viewed for ten seconds and, provided during this period hindlimb tonus was observed, a score of 1 was given; otherwise the animal scored 0 for the period. A cumulative score was deduced for each animal by the sum of scores given over 90 min after injection. The latency to onset of tonic seizures was taken as the time to score two consecutive hindlimb extensions.

2.2. Preparation of acute brain slices

Coronal 400 μm thick brain slices containing the dorsal hippocampus were prepared 90 min after the injection of kainate or saline (Fig. 1A) as described previously (Khirug et al., 2010). Following decapitation, brains were quickly removed and immersed into ice-cold physiological solution containing (in mM): 124 NaCl, 3 KCl, 2 CaCl$_2$, 25 NaHCO$_3$, 1.1 NaH$_2$PO$_4$, 2 MgSO$_4$, and 10 D-glucose, equilibrated with 95% O$_2$ and 5% CO$_2$. Slices were cut using a Campden 7000smz vibrating microtome (Campden Instruments). Before experiments were started, slices were allowed to recover for 1 h at 35 ± 1 °C.

2.3. Assessment of the efficacy of neuronal Cl$^-$ extrusion

To gain a quantitative estimate of the efficacy of neuronal Cl$^-$ extrusion, we used our standard assay where a constant somatic Cl$^-$ load (19 mM) is imposed on the neuron via a whole-cell patch pipette (Khirug et al., 2005). The steepness of the ensuing bumetanide-insensitive somatodendritic E_{GABA} gradient (ΔE_{GABA}), determined as the difference between the E_{GABA} at the soma and at 50 μm away along the apical dendrite, has been demonstrated to provide a quantitative estimate of the efficacy of KCC2-mediated Cl$^-$ extrusion (Blaesse et al., 2006; Gauvain et al., 2011; Jarolimek et al., 1999; Khirug et al., 2005, 2008, 2010; Li et al., 2007; Puskarjov et al., 2012, 2014b). Whole-cell patch-clamp recordings were done in the presence of 10 μM bumetanide (Tocris) to inhibit NKCC1, 0.5 μM TTX (Abcam) to block voltage-gated Na$^+$ channels, 10 μM CNQX (Abcam) to inhibit AMPA/kainate signaling, and 1 μM CGP 55845 (Abcam) to block GABA$_B$ receptor-mediated signaling. Part of the P6 slices were exposed to the calpain inhibitor MDL-28170 (30 μM; Tocris) (Puskarjov et al., 2012) throughout the post-recovery period and used for electrophysiological recordings or biochemical analysis during the 4th hour after recovery. After establishing whole-cell configuration, each neuron was allowed to equilibrate with the pipette solution for at least 5–10 min. The pipette solution consisted of (in mM): 18 KCl, 111 K-gluconate, 0.5 CaCl$_2$, 2 NaOH, 10 glucose, 10 HEPES, 2 Mg-ATP, and 5 BAPTA, 0.1 Alexa Fluor 488; pH was adjusted to 7.3 with KOH. Data obtained from cells with stable access resistance between 10 and 20 MΩ and resting membrane potential below −55 mV were used for further analysis. Membrane potential values were corrected for calculated liquid junction potentials

(Barry, 1994). Somatic and dendritic E_{GABA} values were consecutively determined from the current–voltage relations, obtained by sequentially clamping the membrane potential for 200 ms at six different voltage levels with a 5 mV increment and a 10 s step interval. DPNI-caged GABA (1 mM; Tocris) (Puskarjov et al., 2012; Trigo et al., 2009, 2010) was dissolved in the physiological solution containing the above drugs and delivered in the vicinity of the patched neuron at a flow rate of 1–2 μl/min via an UltraMicroPump II (WPI) equipped with a syringe and a 100 μm inner tip diameter quartz needle (WPI). To evoke local GABA$_A$R-mediated currents at the soma or at the apical dendrite, GABA was locally uncaged using a ~10 μm diameter 10 ms long UV-flash (372 nm) 50 ms after the start of each voltage step (Khirug et al., 2005; Puskarjov et al., 2012). The 50 μm distance from the soma along the apical dendrite was traced using a Radiance 2100 confocal microscope (Bio-Rad) and Alexa Fluor 488 in the patch pipette (Khirug et al., 2008; Puskarjov et al., 2012).

An earlier study reported a gender-related difference in the reversal potentials of GABA$_A$R-mediated currents in CA1 pyramidal neurons of P4–7 rats that had undergone multiple saline injections (Galanopoulou, 2008). However, presently, no significant difference in the Cl$^-$ extrusion capacity of CA1 pyramidal neurons between P6 male and female $Bdnf^{+/+}$ mice was observed after a single injection of saline (ΔE_{GABA} in males: −3.03 ± 0.24 mV/50 μM; n = 11 neurons; ΔE_{GABA} in females: −3.54 ± 0.32 mV/50 μM; n = 13 neurons; p = 0.232; Mann–Whitney Rank Sum Test). Thus, data from male and female mice were pooled in the present study.

2.4. Analysis of KCC2 surface expression

A protease approach (Ahmad et al., 2011; Khirug et al., 2010; Puskarjov et al., 2012) was used to measure the surface expression of KCC2. In brief, 30 min after recovery, slices were treated with cod trypsin (Zymetech), a cold-adapted protease that retains its activity at low temperatures while trafficking of membrane proteins is prevented (Ahmad et al., 2011). Slices were incubated on ice for 60 min in the presence of cod trypsin (2 U/ml). The cleavage reaction was stopped by adding the trypsin inhibitor phenylmethylsulfonyl fluoride (100 μM; 5 min on ice). After homogenization the samples were analyzed using immunoblotting.

2.5. Quantitative immunoblotting

Immunoblot analysis was performed on hippocampal slices and whole hippocampi as described previously (Puskarjov et al., 2012). A polyclonal rabbit-anti-panKCC2 antibody (1:1000; Ludwig et al., 2003) was used for KCC2 and rabbit-anti tubulin (1:20,000; Nordic BioSite, catalog #PRB-435P) for reference. For some blots, affinity-purified rabbit-anti-panKCC2 antibody was used to detect the cod trypsin cleavage product of KCC2. The secondary antibody used was horseradish peroxidase-conjugated donkey-anti-rabbit IgG (1:3000; GE Healthcare). Immunoreactivity was detected using an enhanced chemiluminescence detection kit (Pierce) and a LAS-3000 documentation system (Fujifilm). Quantification was performed using AIDA software (Raytest).

3. Results

3.1. Seizure-induced activation of KCC2 is impaired in the neonatal $Bdnf^{-/-}$ hippocampus

Consistent with previous work in P5-7 rats (Khirug et al., 2010), an episode of neonatal seizures induced by kainate injection in P6 $Bdnf^{+/+}$ mice resulted in a fast increase in the Cl$^-$ extrusion capacity of CA1 pyramidal neurons (Figs. 1B–F; mean ΔE_{GABA} of neurons recorded up to 90 min after recovery from slicing: −5.58 ± 0.38 mV/50 μm; n = 18 neurons; p = 0.002, when compared to saline-injected controls: −3.44 ± 0.43 mV/50 μm; n = 10 neurons). No significant sex-related difference (see Methods) in seizure-induced up-regulation of Cl$^-$ extrusion capacity was observed (respective ΔE_{GABA} values in males −5.18 ± 0.42 mV/50 μm, n = 13, and females −6.03 ± 1.05 mV/50 μm, n = 5; p = 0.186; one-tailed Student's t-test). In contrast, the mean ΔE_{GABA} value in neurons from kainate-injected $Bdnf^{-/-}$ animals was only −3.89 ± 0.37 mV/50 μm (n = 13 neurons), which is significantly lower than the ΔE_{GABA} from kainate-injected $Bdnf^{+/+}$ pups (p = 0.007) and not different from saline-injected $Bdnf^{+/+}$ animals (p = 0.477; one-way ANOVA with Holm-Sidak *post hoc* test). This level is identical to that observed in the presence of furosemide (Khirug et al., 2010) indicating the absence of KCC2 activity in neurons from kainate-injected $Bdnf^{-/-}$ and saline-injected $Bdnf^{+/+}$ mice at P6.

Partial suppression of electrical kindling has been demonstrated in adult $Bdnf^{+/-}$ and $Bdnf^{-/-}$ mice (Barton and Shannon, 2005; He
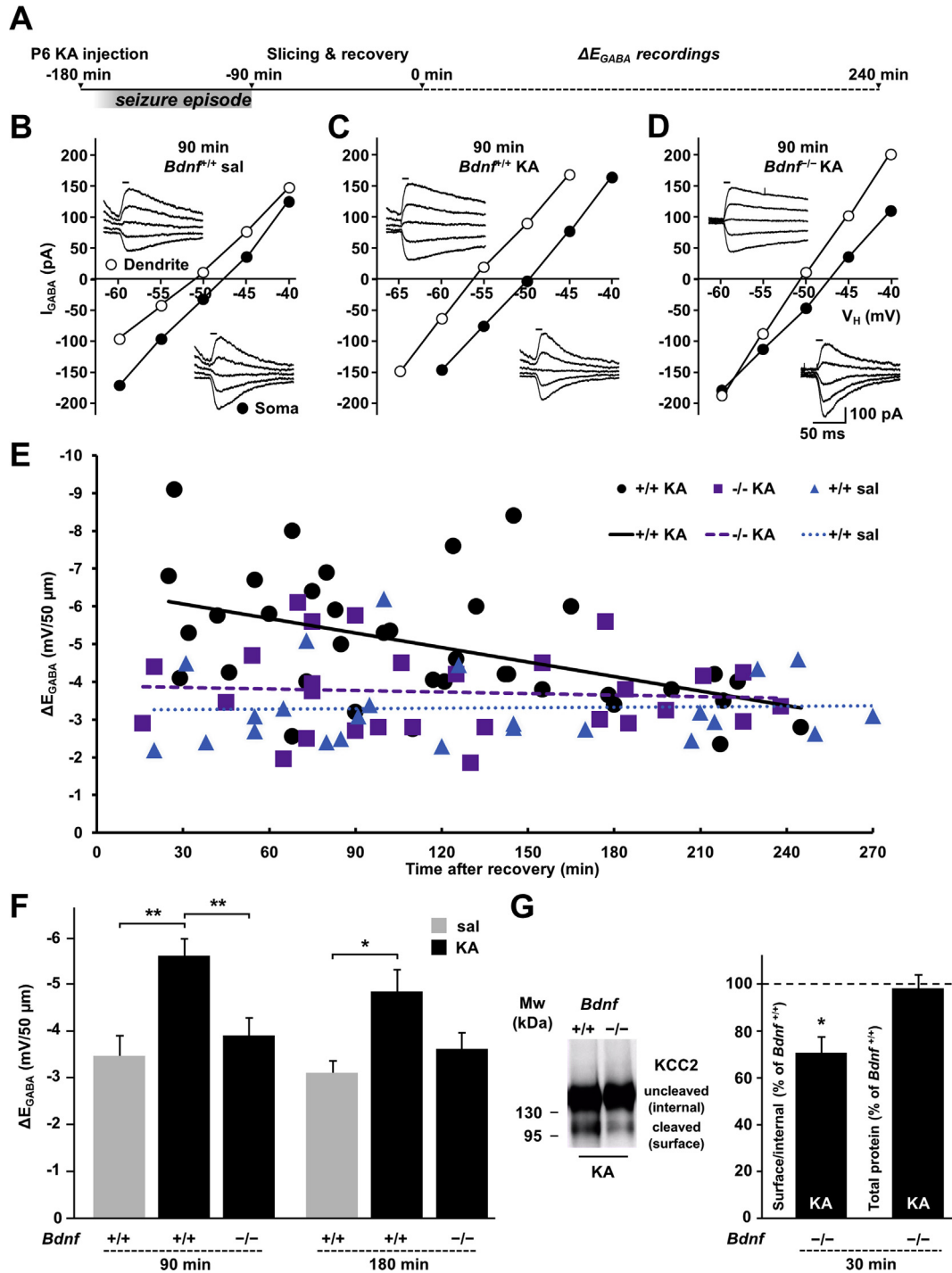
Fig. 1. Activation of KCC2 after a single episode of kainate-induced neonatal seizures in the P6 hippocampus is impaired in the absence of BDNF. **A,** Schematic timeline of the experimental protocol for P6 $Bdnf^{-/-}$ and $Bdnf^{+/+}$ mice. **B–F,** Whole-cell patch-clamp recordings of uncaging-induced $GABA_A$R-mediated currents (I_{GABA}) and E_{GABA} in CA1 pyramidal neurons under an imposed somatic Cl^- load demonstrated an increase in the somatodendritic E_{GABA} gradient (ΔE_{GABA}) after an episode of kainate-induced seizures in $Bdnf^{+/+}$ but not in $Bdnf^{-/-}$ mice (sal, saline; KA, kainate). **B–D,** Sample E_{GABA} recordings during the first 90 min after recovery from slicing. Uncaging flash indicated by horizontal bars. **E,** Scatter plot of ΔE_{GABA} over time in CA1 pyramidal neurons in slices from kainate- or saline-treated $Bdnf^{+/+}$ and kainate-treated $Bdnf^{-/-}$ animals. Linearly fitted lines illustrate the main trends in the respective data sets. **F,** The mean ΔE_{GABA} value in kainate-injected $Bdnf^{+/+}$ mice was significantly higher compared to both saline-injected $Bdnf^{+/+}$ and kainate-injected $Bdnf^{-/-}$ during the first ~90 min, with progressive down-regulation of ΔE_{GABA} ensuing thereafter. **G,** Surface expression of KCC2 was significantly lower after the seizure episode in hippocampal slices from $Bdnf^{-/-}$ mice compared to slices from wildtype littermates. The surface/internal ratio (ratio of trypsin-cleaved KCC2 [~100 kDa; surface] to uncleaved KCC2 [~140 kDa; internal]) measured in slices from $Bdnf^{-/-}$ was normalized with respect to $Bdnf^{+/+}$. No difference in total KCC2 protein was observed between kainate-injected $Bdnf^{-/-}$ and $Bdnf^{+/+}$ mice. *$p < 0.05$; **$p < 0.01$. Error bars denote SEM.

et al., 2004; Kokaia et al., 1995), and higher doses of kainate but lower doses of pilocarpine are needed to produce limbic seizures in adult $Bdnf^{+/-}$ mice (Barton and Shannon, 2005). Therefore we investigated whether the absence of KCC2 activation in $Bdnf^{-/-}$

animals may be due to potentially suppressed seizure generation. No significant difference in severity of kainate-induced seizures (see Methods) was observed between P6 $Bdnf^{-/-}$ and $Bdnf^{+/+}$ animals (seizure severity score: 39.6 ± 5.2; $n = 10$ $Bdnf^{-/-}$ pups vs.

29.9 ± 5.7; $n = 14$ $Bdnf^{+/+}$ pups; $p = 0.169$; Mann–Whitney Rank Sum Test). The difference in the latency to onset of tonic seizures was also not statistically significant ($Bdnf^{-/-}$: 18.2 ± 4.1 min; $n = 10$ pups $vs.$ $Bdnf^{+/+}$: 30.6 ± 6.6 min; $n = 14$ pups; $p = 0.127$; Mann–Whitney Rank Sum Test), supporting the earlier observation by Barton and Shannon (2005) of no difference between BDNF-deficient and wildtype mice in seizure thresholds for tonic-clonic seizures induced by electrical stimulation. The above results suggests that under the present conditions, the difference in KCC2 activation between the two genotypes is unlikely to stem from differences in the severity of the seizures experienced by the $Bdnf^{-/-}$ and $Bdnf^{+/+}$ animals.

The seizure-induced enhancement of neuronal Cl^- extrusion capacity in neonatal rats is not associated with increased KCC2 total protein, but it is paralleled by a robust increase in the surface expression of KCC2 that is sensitive to TTX and the kinase inhibitor K252a (Khirug et al., 2010). Accordingly, KCC2 surface expression (surface/internal ratio) was significantly lower in slices from kainate-injected $Bdnf^{-/-}$ pups than in kainate-injected wildtype littermates, quantified immediately after slicing recovery (Fig. 1G; 68.8 ± 8% of the level in kainate-injected $Bdnf^{+/+}$ littermates; with $n = 6$ slices from both genotypes; $p = 0.031$; Wilcoxon matched pairs test). As expected, no difference was observed in the total protein level between kainate-injected $Bdnf^{-/-}$ and $Bdnf^{+/+}$ littermates (Fig. 1G; 98.3 ± 6.0% of $Bdnf^{+/+}$; $n = 4$ slices from both genotypes; $p = 0.4$; one-tailed Student's paired t-test).

To investigate whether the seizure-induced functional up-regulation of KCC2 is delayed in the absence of BDNF expression, we extended ΔE_{GABA} measurements to time points later than 90 min after recovery (Figs. 1A and E). No change in ΔE_{GABA} was observed during the entire experimental time window of 240 min in the kainate-injected $Bdnf^{-/-}$ group (mean ΔE_{GABA} at 90–180 min: −3.6 ± 0.35 mV/50 μm; $n = 10$ neurons; at 180–240 min: −3.59 ± 0.26 mV/50 μm; $n = 5$ neurons; $p = 0.803$, when comparing the mean ΔE_{GABA} values recorded during 0–90, 90–180, and 180–240 min after recovery; one-way ANOVA). In contrast, a progressive down-regulation of the initial negative shift in ΔE_{GABA} was observed in neurons from kainate-injected $Bdnf^{+/+}$ mice, reaching statistical significance during the 4th hour (180–240 min) after recovery (Fig. 1E, F and 2A; mean ΔE_{GABA} at 90–180 min: −4.82 ± 0.47 mV/50 μm; $n = 13$ neurons; mean ΔE_{GABA} at 180–240 min: −3.44 ± 0.30 mV/50 μm; $n = 6$

neurons; $p = 0.018$ for the mean ΔE_{GABA} at 180–240 min $vs.$ the mean ΔE_{GABA} values recorded during 0–90 min; and $p = 0.891$ $vs.$ mean saline ΔE_{GABA} values at 180–240 min: −3.33 ± 0.31 mV/50 μm; $n = 7$ neurons; one-way ANOVA with Holm-Sidak $post$ hoc test). Interestingly, during the 4th hour after recovery, we observed also a significant decrease in the total KCC2 protein levels in slices from kainate-injected $Bdnf^{+/+}$ pups (Fig. 2B; 80.1 ± 5% at 240 min of the level measured immediately after recovery [0 min in Fig. 1A] in slices from the same animal; $n = 10$ slices for both groups; $p = 0.011$; one-way repeated measures ANOVA with Tukey's $post$ hoc test). This effect is unlikely to be attributable to putative progressive deterioration of brain tissue due to damage caused by slicing as evidenced by the fact that, under control conditions in hippocampal slices, the total and functional KCC2 protein pools are stable for several hours (Puskarjov et al., 2012).

Recent work (Chamma et al., 2013; Puskarjov et al., 2012; Zhou et al., 2012) has shown that KCC2 is down-regulated by the Ca^{2+}-activated protease calpain as a consequence of strongly enhanced glutamatergic signaling. Kainate-induced seizure activity has been reported to result in calpain activation in the CA1 stratum radiatum of P7 rats 4 h after seizure onset (Bi et al., 1997). Thus, we tested whether the delayed down-regulation of KCC2 following neonatal seizures is sensitive to the calpain inhibitor MDL-28170 applied immediately after recovery. In the absence of calpain activity, significantly higher ΔE_{GABA} values were observed in slices from kainate-injected $Bdnf^{+/+}$ animals during the 4th hour after recovery (Fig. 2A; −5.18 ± 0.31 mV/50 μm; $n = 6$ neurons; $p = 0.003$, $vs.$ respective 180–240 min values without MDL-28170; one-way ANOVA with Holm-Sidak $post$ hoc test). Likewise, down-regulation of KCC2 protein was fully blocked by MDL-28170 (Fig. 2B; 109 ± 6.4% of the level measured at 0 min; $n = 10$ slices; $p = 0.965$ and 0.006, respectively, $vs.$ levels in slices from the same animal at 0 and 240 min after recovery; one-way repeated measures ANOVA with Tukey's $post$ hoc test). It has been previously demonstrated that, under control conditions, blocking protein degradation, including constitutive calpain activity, does not result in significant up-regulation of KCC2 protein in the present timescale of 4 h (Puskarjov et al., 2012), suggesting that the effect obtained with MDL-28170 is unlikely to be accounted by unmasking of KCC2 de $novo$ synthesis.
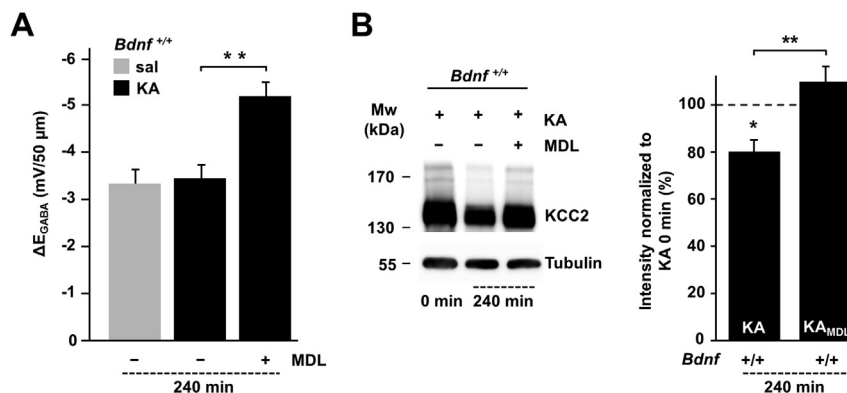
Fig. 2. Neonatal seizure-induced activation of KCC2 is temporally limited by calpain activity. **A,** During the 4th hour after recovery (240 min) ΔE_{GABA} values of neurons in slices from kainate-injected $Bdnf^{+/+}$ animals were no longer different from the respective level measured from saline-injected controls. ΔE_{GABA} values measured in MDL-28170 (MDL; 30 μM)-incubated slices from kainate-injected $Bdnf^{+/+}$ animals were significantly higher than those measured during the 4th hour without MDL and remained at the level of the 0–90 min group (see Fig. 1F). **B,** Down-regulation of KCC2 total protein observed at the 4th hour after recovery in slices from kainate-injected $Bdnf^{+/+}$ animals is prevented by MDL-28170. Representative Western blot analysis and quantification of KCC2 total protein after the seizure episode in $Bdnf^{+/+}$ slices incubated for 4 h with MDL or DMSO. Signal intensity at 240minutes ± MDL was normalized with respect to $Bdnf^{+/+}$ to the 0 min level in slices from the same animal. $^*p < 0.05$; $^{**}p < 0.01$. Error bars denote SEM.

3.2. Developmental up-regulation of KCC2 takes place in the $Bdnf^{-/-}$ hippocampus

Overexpression or exogenous application of BDNF has been reported to result in increased KCC2 mRNA and protein levels, suggesting that BDNF may play a role in the developmental expression of KCC2 protein (Aguado et al., 2003; Ludwig et al., 2011b). To investigate the possibility that the lack of seizure-induced functional activation of KCC2 may be a consequence of compromised developmental up-regulation of KCC2 in $Bdnf^{-/-}$ mice, we analyzed the total hippocampal KCC2 protein levels between naïve (non-injected) $Bdnf^{-/-}$ mice and their wildtype littermates at P5-6 and P13-14. Differences in the total protein levels of KCC2 between the two genotypes were observed neither at P5-6 nor at P13-14 (Figs. 3A and B; P5-6: $89 \pm 7.2\%$ of $Bdnf^{+/+}$; $n = 12$ hippocampi from both genotypes; $p = 0.129$; Wilcoxon matched pairs test; P13-14: $89.5 \pm 12.7\%$ of $Bdnf^{+/+}$; $n = 6$ $Bdnf^{-/-}$ and 8 $Bdnf^{+/+}$ hippocampi; $p = 0.312$; one-tailed Student's t-test). Moreover, at P14, when functional up-regulation of KCC2 reaches its adult levels in wildtype mouse CA1 neurons (Khirug et al., 2005), no difference was observed in ΔE_{GABA} between the two genotypes (Fig. 3C; $Bdnf^{-/-}$: -9.91 ± 0.78 mV/ 50 μm; $n = 11$ neurons; $Bdnf^{+/+}$: -9.71 ± 1.04 mV/50 μm; $n = 6$ neurons; $p = 0.44$; one-tailed Student's t-test).

4. Discussion

We show here that the fast functional up-regulation of KCC2 in the mouse hippocampus following neonatal status epilepticus is dependent on BDNF. In our previous experiments, both a kainate-induced seizure episode in P5-7 rats and seizure-like activity induced via kainate application to brain slices led to a rapid enhancement of the efficacy of neuronal Cl⁻ extrusion capacity and to a hyperpolarizing E_{GABA} shift in CA1 pyramidal neurons. This was accompanied by a large increase in the surface expression of KCC2 (Khirug et al., 2010). Interestingly, a similar effect has been recently described in P5-7 rat motoneurons in the context of spinal cord injury (Bos et al., 2013), suggesting that in the neonate this may be a general first-in-line neuronal response to seizures and trauma (cf. Kaila et al., 2014b).

After the initial functional up-regulation of KCC2 observed in the present study, a progressive calpain-dependent down-regulation of KCC2 protein and neuronal Cl⁻ extrusion capacity ensued. This is in accord with the reported down-regulation of KCC2 in the intact neonatal hippocampal preparation following sustained kainate-triggered ictal events (Nardou et al., 2011) and the calpain-dependency of KCC2 down-regulation in CA1 pyramidal neurons of juvenile rats (Puskarjov et al., 2012). Indeed, a recent

study demonstrated that hippocampal KCC2 is targeted by calpain-mediated cleavage in a rat model of late gestation hypoxia-ischemia (Jantzie et al., 2014), and this may well represent a mechanism that contributes to the brain damage-related down-regulation of KCC2 in human babies (Robinson et al., 2010).

Our previous work demonstrated that both the increase in surface expression of KCC2 and the enhanced Cl⁻ extrusion capacity were abolished by the kinase inhibitor K252a. By contrast, in mature hippocampal neurons this drug has been shown to prevent TrkB-mediated *down-regulation* of KCC2 induced by exogenous BDNF or NT-4 (Rivera et al., 2002) or endogenous BDNF following NMDA receptor activation (Rivera et al., 2004). In these cells, BDNF-induced down-regulation of KCC2 appears to require activation of the TrkB-phospholipase-Cγ1 (PLCγ1) signaling pathway that is heavily implicated in the process of epileptogenesis (Kaila et al., 2014b; Rivera et al., 2004). BDNF has been shown to exert a developmental stage-dependent effect also on the function of GABA$_A$Rs. While at P6 BDNF rapidly potentiates GABA$_A$R-mediated currents of CA1 pyramidal neurons, by P10 this effect is lost, and from ~P14 onwards BDNF suppresses GABA$_A$R function (Mizoguchi et al., 2003). This kind of striking age-dependent co-regulation of KCC2 and GABA$_A$Rs is in line with the qualitative change in BDNF-TrkB signaling that takes place around P12 in the rodent hippocampus and cortex (Di Lieto et al., 2012; Knüsel et al., 1994), and may in part be explained by the apparently age-dependent response of TrkB phosphorylation at its PLCγ1 binding site (Di Lieto et al., 2012).

Findings of the above kind may also shed light on the mechanisms which underlie the well-known differences between seizures in neonates and adults that have been observed in neurological and pharmacological work (cf. Haut et al., 2004; Löscher et al., 2013; Volpe, 2008). Thus, the present data and those by Mizoguchi et al. (2003) suggest that, in a manner opposite to what is observed in adults, seizure-induced release of BDNF in the early postnatal period enhances the efficacy of inhibition by increasing the amplitude of the GABA$_A$R-mediated current and by inducing a negative shift in its reversal potential.

Another key finding in the present work is that the developmental up-regulation of hippocampal KCC2 protein and transport functionality takes place in the complete absence of BDNF expression *in vivo*. This indicates that although BDNF has been shown to exert an accelerating effect on neuronal development including the up-regulation of KCC2 (Aguado et al., 2003; Kaila et al., 2014a; Ludwig et al., 2011b), it is not a necessary component for KCC2 up-regulation to take place during ontogenesis. Indeed, other trophic factors act in a parallel and apparently redundant manner with KCC2-regulating transcriptional pathways
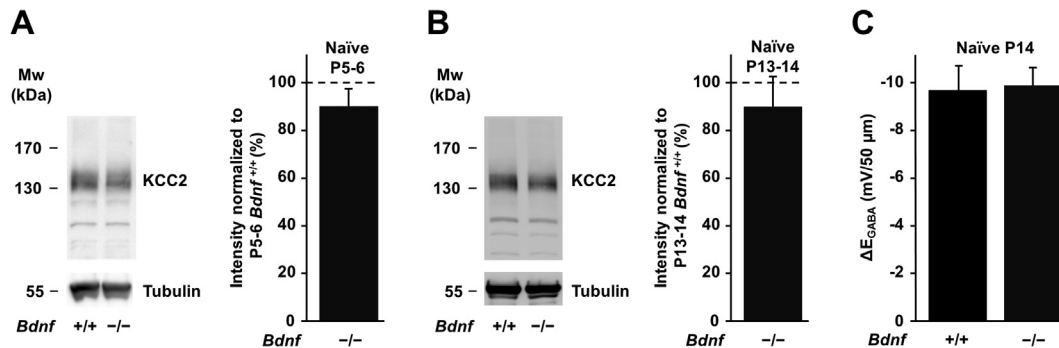
Fig. 3. Developmental up-regulation of KCC2 protein and function in the mouse hippocampus takes place in the absence of BDNF. Representative Western blot analysis and quantification of KCC2 signal (~140 kDa) from P5-6 (**A**) and P13-14 (**B**) whole hippocampi of non-injected (naïve) $Bdnf^{-/-}$ and $Bdnf^{+/+}$ littermates. Tubulin was used as a loading control. No significant differences in KCC2 protein expression was observed between the genotypes at either age. **C**, No significant difference in ΔE_{GABA} of CA1 pyramidal neurons was observed between P14 $Bdnf^{-/-}$ and $Bdnf^{+/+}$ mice. Error bars denote SEM.

downstream of BDNF (Ludwig et al., 2011a). Reduction in BDNF expression has been previously shown to result in a decrease of both frequency and amplitude of GABAergic inhibitory post-synaptic currents in various central neurons (e.g. Abidin et al., 2008; Laudes et al., 2012). The present observations suggest that such effects are unlikely to be a consequence of reduced neuronal Cl$^-$ extrusion.

Acknowledgments

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