K⁺/Cl⁻ cotransporter KCC2 membrane trafficking and functionality is regulated by transforming growth factor beta 2

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ABSTRACT

Functional activation of the neuronal K⁺-Cl⁻-cotransporter KCC2 is a prerequisite for shifting GABAergic responses from depolarizing to hyperpolarizing during development. Here we introduce transforming growth factor beta 2 (TGF-β2) as a novel regulator of KCC2 membrane trafficking and functional activation. TGF-β2 controls membrane trafficking, surface expression and activity of KCC2 in developing and mature mouse primary hippocampal neurons, as determined by immunoblotting, immunofluorescence, biotinylation of surface proteins, and KCC2-mediated Cl⁻ extrusion, respectively. We also identify the signalling pathway TGF-β2/cAMP response element-binding protein (CREB)/Ras-associated binding protein 11b (Rab11b) as the underlying mechanism for TGF-β2-mediated KCC2 trafficking and functional activation. TGF-β2 increases colocalization and interaction of KCC2 with Rab11b, as determined by 3D stimulated emission depletion (STED) microscopy, and co-immunoprecipitation, respectively, induces CREB phosphorylation, and enhances Rab11b gene expression. Loss of function of either CREB or Rab11b suppressed TGF-β2-dependent KCC2 trafficking, surface expression, and functionality. Thus, TGF-β2 is a novel regulatory factor of KCC2 functional activation and membrane trafficking and putative indispensable molecular determinant for the developmental shift of GABAergic transmission.

CREB / growth factor / neuronal development / Rab11b
Introduction

Regulation of KCC2, the neuron-specific electroneutral K\(^+\)-Cl\(^-\) co-transporter, is crucial for development and maturation of GABAAergic neurotransmission. In immature CNS neurons, GABA produces depolarizing postsynaptic potentials, which are likely involved in stabilizing synapses during development (Ben-Ari, 2002; Blaesse et al., 2009). GABA-mediated depolarization is sustained through a high expression of NKCC1, a cation-chloride cotransporter that mediates intracellular Cl\(^-\) accumulation above its electrochemical equilibrium. During maturation of most central neurons, however, expression of the Cl\(^-\) extruder KCC2 is up-regulated resulting in an intracellular Cl\(^-\) concentration below its electrochemical equilibrium, thereby shifting GABA\(_A\) responses from depolarizing to hyperpolarizing (Rivera et al., 1999). The mechanisms underlying KCC2 regulation have been extensively investigated but still are not fully understood. BDNF has been shown to upregulate KCC2 expression in immature neurons, whereas in mature neurons BDNF/TrkB signaling results in an activity-dependent decrease of KCC2 expression (Rivera et al., 2002; Rivera et al. 2004). Accordingly, KCC2 expression is decreased in early postnatal TrkB deficient mice (Carmona et al., 2006). However, recent observations have challenged the dominant role of BDNF in the developmental up-regulation of KCC2 by demonstrating that BDNF, although a potent regulator, is not a necessary molecular determinant for the required KCC2 upregulation during development (Puskarjov et al., 2015). Indeed, other trophic factors, such as Neurturin, possess the ability to regulate this transporter too (Ludwig et al., 2011).

KCC2 functionality is achieved not only through transcriptional control, but also through regulation of KCC2 membrane trafficking, integration, and stabilization in the membrane. KCC2 protein (de)phosphorylation is thought to be a crucial regulatory mechanism for KCC2 surface expression, surface stability, and trafficking.
Apparently, the functional consequences of phosphorylation on KCC2 depend on the specific residue (reviewed by Kahle et al., 2013). Along this line, PKC-dependent phosphorylation of KCC2 at Ser940 increases its cell surface expression and promotes KCC2 membrane stability in cultured hippocampal neurons (Lee et al., 2007), whereas WNK kinase-dependent phosphorylation at T906 and T1007 inhibits KCC2 transport function (Rinehart et al., 2009). In contrast, Src-mediated Y903/1087 phosphorylation regulates the membrane trafficking of KCC2 and decreases the cell surface stability of KCC2 by enhancing its lysosomal degradation (Lee et al., 2010).

Besides (de)phosphorylation, other molecular pathways that affect KCC2 membrane trafficking in immature neurons include TrkB and 5-HT₂A serotonin receptors (Khirug et al., 2010; Bos et al., 2013; Khirug et al., 2005), whereas for mature neurons the molecular mechanisms underlying KCC2 membrane trafficking need to be elucidated.

TGF-βs are multifunctional, extracellular signaling molecules that exert a wide range of biological responses on different cell types, including cells of the nervous system. In the CNS, TGF-βs are required for cell fate decisions (Farkas et al., 2003; Lu et al., 2005; Roussa et al., 2006; Roussa et al., 2008) and regulate neuronal survival and apoptosis during nervous system development (Dünker and Krieglstein, 2000; Krieglstein et al., 2000). Furthermore, an impact of TGF-βs on synaptogenesis, neural network function, and neuronal plasticity has been shown (Krieglstein et al., 2011). Already early studies have documented TGF-β1-induced long-term facilitation in *Aplysia* sensory-motor synapses and increase in neuronal excitability (Zhang et al., 1997; Chin et al., 1999), effects mediated through activation of MAPK signaling (Chin et al., 2006) and modulation of synapsin distribution by phosphorylation (Chin et al., 2002). TGF-β2 has been also identified as a local modulator of the neuromuscular junction through the control of presynaptic quantal size (Fong et al., 2010). The TGF-
β isoforms, i.e. TGF-β1, TGF-β2 and TGF-β3, exhibit a distinct spatial and temporal expression pattern and, although targeted mutations of individual TGF-β genes are lethal, the phenotypes are distinct and isoform-specific. *Tgf-β2*-/− mutants die at birth due to congenital cyanosis, yet cardio-vascular and pulmonary causes of lethality have been excluded (Sanford et al., 1997). Interestingly, impaired synaptic transmission of spontaneous GABAergic/glycinergic and glutamatergic postsynaptic currents in the respiratory control area, the pre-Bötzinger complex (preBötC), has been demonstrated (Heupel et al., 2008).

In the present study we show that TGF-β2 can control KCC2 trafficking and activity in mature hippocampal neurons. We also identify the signalling pathway TGF-β2/CREB/Rab11b as the underlying mechanism for TGF-β2-mediated KCC2 trafficking and activity. Our results introduce TGF-β2 as a novel regulator of KCC2 functionality and as putative crucial determinant for the developmental shift of GABAergic transmission.
RESULTS

KCC2 membrane trafficking is controlled by TGF-β2

The mechanisms regulating KCC2 membrane expression and activity are complicated but appear to involve signalling induced by trophic factors (Rivera et al., 2002; Rivera et al., 2004; Ludwig et al., 2011). With this in mind, we first addressed the question whether TGF-β2 regulates KCC2 mRNA and protein expression during neuronal development. Hippocampal neurons were isolated at E18.5 and cultured for 12 or 18 days in vitro (DIV). As shown in Figure 1A, KCC2 transcript expression (397bp) was detectable in neurons cultured for 12 days (Rivera et al., 2002; Ludwig et al., 2003) and a TGF-β2 60 min pulse did not further increase KCC2 transcript expression (Figure 1A). However, TGF-β2 treatment of the cultures induced a ~270kDa KCC2 band (Figure 1B). In contrast, NKCC1 (Fiumelli and Woodin, 2007) transcript (235bp; Figure 1C) and protein expression (Figure 1D) remained unchanged following a TGF-β2 60 min pulse. In more mature neurons, cultured for 18 days, (Dotti et al., 1988), application of TGF-β2 for 60 min had no effect on KCC2 transcript (Figure 1E) and protein expression (Figure 1F).

We next investigated the cellular localization of KCC2 in response to the TGF-β2 treatment, using DIV12 and DIV18 cultures. As shown in Figure 2A, KCC2 immunoreactivity under control conditions at DIV12 was predominantly associated with small intracellular vesicles which partly co-localized with the Golgi marker Golgi58k (asterisk). A 60 min TGF-β2 pulse cleared the majority of these KCC2 immunoreactive vesicles from intracellular stores and shifted immunoreactivity to the cell membrane (arrows). Quantification shown in Figure 2B revealed that TGF-β2 treatment significantly reduced KCC2/Golgi58K co-localization (**P=0.0015, n=4), Moreover, immunoblotting following biotinylation of cell surface proteins showed a
significant increase for both the KCC2 ~135kDa and ~270kDa band (*P=0.014, n=3, Figure 4C), but not for NKCC1 (Figure 2D) upon TGF-β2 treatment at DIV12.

In 18 day old cultures (Figs. 3A and 3B), following TGF-β2 treatment for 60 min KCC2/Golgi58K co-localization was significantly reduced (**p=0.0084, n=4) Immunoblotting following biotinylation of cell surface proteins (Fig. 3C) showed no significant differences for both the KCC2 ~135kDa and ~270kDa band upon TGF-β2 treatment at DIV18. Since TGF-β is endogenously expressed in hippocampal neurons we have treated the cultures either with TGF-β2 or with anti-TGF-β, a TGF-β function blocking antibody, at DIV12 and DIV15, and assessed KCC2 immunoreactivity at DIV18 (Fig. 3D). Representative line scans from cells under each experimental condition illustrate distribution profile for KCC2 (red) and Golgi58k (green) immunoreactivity. Peaks of KCC2 labeling were detected in the periphery of cell bodies of control and TGF-β2-treated cells, suggesting membrane labelling, whereas after neutralizing endogenous TGF-β, KCC2 was exclusively intracellularly distributed.

Taken together, these results provide first evidence for a TGF-β2-dependent trafficking of KCC2 from the Golgi to the cell membrane in developing and mature neurons. These changes may induce a functional activation of KCC2.

**Functional expression of KCC2 is controlled by TGF-β2**

TGF-β2-dependent translocation of KCC2 to the cell membrane in developing neurons does not necessarily imply functional integration of KCC2. We therefore investigated whether TGF-β2 may activate KCC2, by means of altering efficacy of KCC2-mediated Cl⁻ extrusion in hippocampal neurons. Therefore, whole cell patch-
clamp recordings were performed from primary hippocampal neurons at DIV10. Under a constant Cl⁻ load via a somatic patch pipette, transport-active KCC2 generates a negative somatodendritic electrochemical Cl⁻ gradient (Khirug et al., 2005) (Fig. 4A). We determined E_{Cl⁻} at the soma and at proximal dendrites and we observed declining somatodendritic Cl⁻ gradients after treatment with exogenous TGF-β2. Fig. 3B and Supplementary material Fig. S1 illustrate examples of neurons with distinct KCC2 activity. In the neuron illustrated in Figure 4A, soma E_{Cl⁻} was -43mV close to the calculated value (-50mV) expected following a constant load with 19mM [Cl⁻] via the patch pipette. E_{Cl⁻} in the dendrite was similar to that of the soma, demonstrating that in this particular neuron KCC2 is not yet active. Treatment with TGF-β2 (arrow) caused a negative shift in somatodendritic Cl⁻ gradients, demonstrating effective KCC2-mediated Cl⁻ extrusion activity. In more mature neurons (see Supplementary material Fig. S1) soma E_{Cl⁻}, was also close to -50mV, as expected from the chloride load but dendrite E_{Cl⁻} was more negative (-55mV), due to activated KCC2. Exposure to TGF-β2 (arrow) caused a small decline in somatodendritic Cl⁻ gradient. Somatic and dendritic E_{Cl⁻} in less mature neurons prior to TGF-β2 treatment were -47.32 ± 2.01 and -46.94 ± 1.71 mV, respectively, and the somatodendritic Cl⁻ gradient was 0.38 ± 0.41 mV (Figure 4C). TGF-β2 treatment of these neurons induced a negative shift in dendritic E_{Cl⁻} of -4.58 ± 0.62 mV (n=4; dendritic E_{Cl⁻} being -51.51 ± 1.23 mV, p<0.01 when paired t-test was applied for dendritic E_{Cl⁻} prior to and during exposure to TGF-β2; Figure 4C) when measured as an average over the time window of 20-40 min after onset of TGF-β2 application. The above data demonstrate that TGF-β2 can activate KCC2, indicated by increased cellular Cl⁻ extrusion.
TGF-β2-induced CREB phosphorylation affects KCC2 trafficking

We next addressed putative mechanisms underlying TGF-β2-mediated trafficking of KCC2 to the membrane. In rat hippocampal neurons TGF-β2 has been shown to induce CREB phosphorylation (Fukushima et al., 2007). We therefore investigated protein abundance and cellular distribution of total CREB and of phosphorylated CREB in primary cultured hippocampal neurons (DIV12) and in neurons exposed to recombinant TGF-β2 for 5, 10 and 15 min. Immunoblot analysis (Figure 5A) revealed that treatment with TGF-β2 for 5 min significantly upregulated CREB phosphorylation, compared to the untreated controls (*P<0.05, n=7; unpaired Student’s t-test). Immunofluorescence (Figure 5B) confirmed these data on the cellular level (**P<0.01). We next investigated whether TGF-β2-induced CREB phosphorylation promotes KCC2 trafficking to the cell membrane. Hippocampal neurons were transfected either with negative siRNA or with Alexa 488-labeled specific siRNA against CREB, as previously described (Oehlke et al., 2012). Cells that had been exposed to the transfection reagent only served as controls (no siRNA). After determination of efficiency of CREB transcript knock-down following transfection (Supplementary material Fig. S2), cells were treated with TGF-β2 for 60 min and cellular localization of KCC2 (red) was assessed. Figure 6 illustrates KCC2 distribution pattern together with the corresponding line scans for randomly depicted neurons for the experimental conditions used. In controls, localization of KCC2 was comparable in non transfected cells (Figure 6A), in cells transfected with negative siRNA (Figure 6B), and in cells transfected with specific CREB siRNA (Figure 6C). In these experiments KCC2 consistently revealed intracellular localization (asterisks and line scan). After treatment with TGF-β2 (Figures 6E-6G), KCC2 localization was shifted to the plasma membrane in both non transfected cells (Figure 6E) and in cells...
transfected with negative siRNA (Figure 6F) (arrows). As shown in representative line scans from cells for each experimental condition, peaks for KCC2 immunolabeling (arrows) are present at the periphery of neuronal cell bodies, suggesting labeling of the plasma membrane. In contrast, KCC2 remained localized within the cytosol in cells transfected with specific CREB siRNA (Figure 6G; asterisk and respective line scan). Thus, interfering with CREB prevented TGF-β2-mediated KCC2 translocation from intracellular pools to the plasma membrane.

Rab11b mediates TGF-β2-dependent KCC2 trafficking

Rab GTPases are established players of vesicular trafficking (Hutalung and Novick, 2011). Rab11b has been shown to mediate vesicular transport of transmembrane proteins (Silvis et al., 2009; Oehlke et al., 2011; Butterworth et al., 2012). In addition, Rab11b has been found in other cellular systems to be under transcriptional control of CREB (Zhang et al., 2005). We therefore explored whether Rab11b was a target of TGF-β2 in the transport of KCC2-carrying vesicles to the neuronal plasma membrane. Using 3D stimulated emission depletion (STED) microscopy we first investigated co-localization of KCC2 (red) with Rab11b (green) in control and TGF-β2 treated DIV12 hippocampal neurons. Representative pictures at low and high magnification are shown in Figure 7. KCC2 co-localization with Rab11b in dendrites of control neurons (Fig. 7A) was very low, yellow KCC2/Rab11b clusters are hardly detectable. In contrast, after TGF-β2 treatment (Fig. 7B), co-localization of KCC2 with Rab11b in dendrites was considerably increased, represented by increased appearance of KCC2/Rab11b yellow clusters (arrows). Quantification of co-localization revealed that Pearson’s correlation coefficient was significantly increased in cells treated with TGF-β2 for 15 min (r=0.148 ± 0.01 and r=0.257 ± 0.07, for
controls and TGF-β2-treated neurons, respectively; ***P=0.0005, Figure 7C).
Manders’ coefficients M1 and M2, representing colocalization of KCC2 with Rab11b and Rab11b with KCC2, respectively, were significantly increased in TGF-β2 treated cells as well (M1: 0.783 ± 0.05 (controls) and 0.921 ± 0.02 (treated), *P=0.015; M2: 0.614 ± 0.07 (controls) and 0.814 ± 0.05 (treated), *P=0.025, Student’s t-test). In addition, the size of the KCC2/Rab11b clusters was apparently increased in TGF-β2-treated cells. We next examined regulation of Rab11b expression in DIV12 cultured primary hippocampal neurons and in neurons exposed to TGF-β2 for 5 up to 30min. Quantitative real-time PCR analysis (Figure 7D) revealed that treatment with TGF-β2 significantly up-regulated Rab11b expression with a peak at 10min.

Confirmation that KCC2 and Rab11b co-localization is significantly increased in TGF-β2-treated hippocampal neurons does not necessarily implicate that these proteins interact with each other. To ensure a physical association of Rab11b and KCC2, co-immunoprecipitation experiments have been performed. As illustrated in Fig. 7E, Rab11b co-immunoprecipitated KCC2 in controls, and TGF-β2 treatment significantly increased the amount of immunoprecipitated KCC2 (1.00 ± 0.06 and 1.48 ± 0.16 for controls and TGF-β2-treated cells, respectively; *p<0.05, n=3, Student’s t-test). Vice versa, immunoprecipitation of KCC2 co-immunoprecipitated Rab11b as well, however, without differences between controls (1.00 ± 0.10 fold) and TGF-β2-treated cultures (0.96 ± 0.04 fold; n=3). The efficiency of association between Rab11b and KCC2 has been further determined by detection of the respective input in the co-immunoprecipitation.

These data demonstrate that Rab11b and KCC2 can indeed be associated with each other in mouse hippocampal neurons and that TGF-β2 may increase their interaction.
To investigate whether TGF-β2–dependent KCC2 translocation to the plasma membrane is mediated by Rab11b we knocked down Rab11b expression, as described earlier (Oehlke et al., 2011), by transfecting the neurons with Alexa 488-labeled specific siRNA against Rab11b (See Supplementary Material Fig. S2). Cells that had been exposed to the transfection reagent only served as controls. Similar to the results obtained after knock-down of CREB, cells transfected with siRNA specific for Rab11b and treated with TGF-β2, revealed intracellular KCC2 localization (Figure 6H, asterisk and corresponding line scan).

Thus, interfering with Rab11b precluded TGF-β2-mediated KCC2 translocation from intracellular pools to the plasma membrane.

To analyze whether Rab11b acts downstream of CREB, we determined Rab11b transcript and protein expression following knocking-down of CREB. As shown in Supplementary Material Fig. S2, transfection of cells with specific siRNA against CREB downregulated Rab11b mRNA and protein. These data support the notion that Rab11b acts downstream of CREB and mediates TGF-β2-dependent KCC2 trafficking.

**Rab11b mediates TGF-β2-dependent KCC2 functional expression**

To investigate whether the identified molecular pathway TGF-β2/CREB/Rab11b is the mechanism underlying TGF-β2–dependent KCC2 activation, we have measured somatodendritic Cl− gradients in cultured neurons transfected with ShRab11b and with negative shRNA (ShRab11b negative) and treated with TGF-β2 (Fig. 8). In cells transfected with Sh-neg, treatment with TGF-β2 (arrow) caused a negative shift in somatodendritic Cl− gradients, demonstrating effective KCC2-mediated Cl− extrusion activity (Fig. 8B). In contrast, in cells transfected with Sh-Rab11b (Fig. 8A) application
of TGF-β2 had no effect on the somatodendritic Cl⁻ gradient. The TGF-β2-induced shift in dendritic $E_{\text{CL}}$ (control) was $-5.49\pm0.8\text{mV}$ (n=9) and the somatodendritic gradient in the presence of TGF-β2 was measured $-7.26\pm2.21\text{mV}$ (n=5) for the neurons transfected with Sh negative and $-0.03\pm1.04\text{mV}$ (n=6) for neurons transfected with Sh Rab11b. These data clearly demonstrate that Rab11b mediates TGF-β2-dependent functional expression of KCC2.

Taken together, these results provide evidence that TGF-β2-dependent KCC2 trafficking and activity to the plasma membrane is mediated by a signaling cascade that implies CREB and Rab11b.
DISCUSSION

In the present study we show for the first time that trafficking, membrane expression, and activity of the neuron-specific K⁺-Cl⁻ cotransporter KCC2, a key element for the “developmental shift” from depolarizing to hyperpolarizing GABAergic responses (Rivera et al., 1999), can be regulated by TGF-β2.

KCC2 functionality has previously been shown to be regulated by transcriptional control, alternative splicing, trafficking, and posttranslational modifications (Blaesse et al., 2009). Meanwhile KCC2 has been identified as a crucial molecular player not only during development but also acting in a transport-independent mode to promote dendritic spine formation (Li et al., 2007; Gauvain et al., 2011; Fiumelli et al., 2013; Puskarjov et al., 2014; Llano et al., 2015). Moreover, impaired synaptic inhibition due to decreased KCC2 function has been demonstrated in many pathophysiological conditions, including epilepsy, spasticity, and chronic pain (Boulenguez et al., 2010; Puskarjov et al., 2012; Gagnon et al., 2013). According to the current view, developmental upregulation of KCC2 reduces the depolarizing action of GABA, however this may be resumed post-traumatically, where neurons likely acquire an immature state (reviewed in Kaila et al., 2014). Interestingly, during the last years several studies have highlighted that the fast functional modulation of KCC2 during physiological and pathophysiological conditions is achieved through post-translational events rather than regulation of the transporter at the transcriptional level (Puskarjov et al., 2012; Zhou et al., 2012).

Although some work has shown that the developmental regulation of KCC2 can be affected by growth factors the mechanisms underlying regulation of KCC2 in vivo are far from being understood. Previous experimental evidence has suggested that KCC2 is transcriptionally regulated by BDNF signalling through a complex signaling
cascade (Rivera et al., 2002; 2004) but recent observations have demonstrated that in Bdnf-/− mice developmental upregulation of KCC2 protein and functionality was comparable to the wild type (Puskarjov et al., 2015). In contrast, BDNF-TrkB signalling was necessary for seizure induced post-translational functional activation of KCC2. Indeed, during development, KCC2 can be transcriptionally regulated by other trophic factors such as Neurturin (Ludwig et al., 2011) and insulin like growth factor (Kelsch et al., 2001) as well. In our previous work we have shown impaired GABAergic postsynaptic currents in neurons of the preBötC of Tgf-β2-/− mice (Heupel et al., 2008). We have hypothesized that TGF-β2 is required for the establishment of a neuronal network by regulating functional expression of KCC2.

**TGF-β2 regulates KCC2 membrane trafficking**

Our results show that TGF-β2 mode of action on KCC2 apparently differs from that reported for BDNF, Neurtrurin (Ludwig et al., 2011), and insulin like growth factor (Kelsch et al., 2001), all capable to upregulate KCC2 mRNA and protein expression in not yet fully developed neurons. Treatment of hippocampal differentiating (after 12 days *in vitro*) neurons with TGF-β2 for 60 min caused a shift in KCC2 immunoreactivity to the periphery of the neurons, decreased KCC2/Golgi58k co-localization, and increased the amount of surface KCC2. Collectively, this argues in favour of TGF-β2-mediated translocation of KCC2 from intracellular pools to the plasma membrane (Figure 2). To our knowledge this is the first demonstration of growth factor-dependent KCC2 membrane trafficking. Moreover, TGF-β2 treatment of hippocampal neurons significantly increased a ~270kDa band, likely representing oligomeric KCC2 protein. Previous results indicate that oligomerization is involved in the functional activation of KCC2 and are in agreement with the potentiating effect of
TGF-β2 on the efficacy of chloride extrusion, as discussed below. However, the question whether oligomerization of KCC2 is required for transport activity, is not yet definitively answered (for review see Hartmann and Nothwang, 2015). DIV18 neurons already express KCC2 in the membrane, and treatment with exogenous TGF-β2 decreased KCC2/Golgi58k co-localization (Fig. 3F), without altering the amount of surface KCC2 (Fig. 3G). The effect of exogenous TGF-β2 on KCC2 trafficking at DIV18 is not detectable due to endogenous TGF-β expression, as shown in Fig. 3J. The requirement for TGF-β for KCC2 trafficking and membrane expression was demonstrated after neutralizing endogenously expressed TGF-β from DIV12 to DIV18. In these neurons, KCC2 has not reached the membrane at DIV18, highlighting the biological significance of TGF-β.

Our data also show that the TGF-β2 effect is specific for KCC2, since gene expression, protein abundance, and trafficking of NKCC1 (Fiumelli and Woodin, 2007) are independent of TGF-β2 (Fig. 1). In contrast, BDNF downregulates KCC2 transcript and protein in an activity-dependent manner in differentiating and mature hippocampal neurons (Rivera et al., 2002; Rivera et al., 2004).

Obviously, the mere presence of plasmalemmal KCC2 protein in a neuron does not necessarily imply that it is functionally active. Proof that TGF-β2-induced KCC2 translocation to and incorporation into the plasma membrane renders KCC2 functional was provided by assessing the efficacy of neuronal Cl− extrusion following application of TGF-β2 (Figure 4). Interestingly, the potency of exogenous TGF-β2 to regulate KCC2 activity differed, depending whether neurons had already established a KCC2-dependent Cl− gradient (more mature neurons) or not (less mature neurons) (Figures 4A and Supplementary Material Fig. S1), and matching the biochemical data in Figs 1 and 2. In not yet mature neurons with inactive KCC2 (Figure 4A), TGF-β2
initiated KCC2-mediated Cl⁻ extrusion, a prerequisite for the ontogenetic change in GABA mediated responses from depolarizing to hyperpolarizing. In mature neurons with abundant functional KCC2 (Supplementary Material Fig. S1) application of TGF-β2 showed reduced potentiation of KCC2 activity probably reflecting that KCC2 in these neurons was already working at nearly maximal efficiency. Interestingly, the Cl⁻ gradient elicited by TGF-β2 in developing neurons reached a level close to the one found in mature neurons. These data highlight a distinct mode of action of TGF-β2 on KCC2 during maturation of neuronal networks by regulating its trafficking and KCC2-mediated Cl⁻ transport efficacy.

KCC2 membrane expression has been reported to be regulated by multiple post translational mechanisms including (de)phosphorylation (reviewed in Kahle et al., 2013), oligomerization (Blaesse et al., 2006), and cleavage by proteases (Puskarjov et al., 2012). Moreover, interaction of KCC2 with the kainate receptor subunit GluK2 (Mahadevan et al., 2014) and its auxiliary subunit Neto2 (Ivakine et al., 2013), as well as the adhesion molecule Neuroligin-2 (Sun et al., 2013) may also regulate KCC2 surface expression. However, Neto2 deficient mice reveal comparable reduction of total KCC2 protein and surface KCC2 abundance, implicating that Neto2 may be required for KCC2 biogenesis and total protein stability rather than membrane trafficking (Mahadevan et al., 2015). Similarly, knock down of Neuroligin 2 in mouse cortical neurons reduces total KCC2 levels, an effect accompanied by decreased KCC2 membrane expression and delayed GABA functional switch (Sun et al., 2013). On the other side, subunits of kainate-type glutamate receptors, such as GluK1/2, are required for KCC2 oligomerization and surface expression (Mahadevan et al., 2014). In spinal motoneurons, activation of 5-HT₂A serotonin receptors increased surface KCC2 after spinal cord injury (Bos et al., 2013). In the present study the KCC2 mRNA
levels are not changed after TGF-β2 treatment. However, TGF-β2 treatment significantly increased a ~270kDa band without having any significant effect on total ~135kDa KCC2 protein, suggesting a more complicated multisite TGF-β2-dependent post-transnational mechanism. Thus, the results of the present study differ from previous reports, our data introduce a molecular determinant acting predominantly on KCC2 trafficking. The growth factor TGF-β2 may regulate surface KCC2 expression and functionality with no net change in total KCC2 mRNA levels.

**CREB/Rab11b signaling underlies TGF-β2-dependent KCC2 trafficking**

Phosphorylation has been proposed as a regulatory mechanism for KCC2 trafficking. PKC-dependent phosphorylation of KCC2 (Ser940) increases its cell surface expression, whereas Src-mediated phosphorylation (Y903/1087), by regulating KCC2 membrane trafficking, decreases its cell surface stability (Lee et al., 2010). In the present study we have identified a novel signaling pathway, TGF-β2/pCREB/Rab11b, as the underlying mechanism for TGF-β2-mediated KCC2 trafficking and functional expression. Several lines of experimental evidence support this view: (i) TGF-β2 induced phosphorylation of CREB(Ser133) (Figure 5) and increased Rab11b transcription in cultured mouse hippocampal neurons (Figure 7); (ii) TGF-β2 treatment increased KCC2/Rab11b co-localization (Fig. 7C) and their interaction (Fig. 7E); (iii) either CREB or Rab11b knock down significantly impaired TGF-β2-induced KCC2 trafficking (Figure 6); (iv) knock down of CREB down-regulated Rab11b transcript and protein (Supplementary Material Fig. S2); and (v) knock down of Rab11b abolished TGF-β2-mediated KCC2 activity by means of KCC2-mediated Cl− transport efficacy (Figure 8). TGF-β-dependent CREB phosphorylation and its physiological significance have been studied in many non-neural paradigms,
including epithelial-mesenchymal transition, tumor growth inhibition (Yang et al., 2013), and fibronectin expression in mesangial cells (Peng et al., 2008). In contrast, little is known on the impact of TGF-β-dependent CREB phosphorylation in neurons. TGF-β1 enhances CREB phosphorylation in *Aplysia* sensory neurons in a MAPK-dependent manner (Chin et al., 2006), and TGF-β2 induces CREB phosphorylation in rat hippocampal neurons (Fukushima et al., 2007). The latter observation has been postulated as the mechanism underlying TGF-β2-dependent modulation of synaptic efficacy and plasticity, however, the causative link is missing. The present study, for the first time, deciphers a putative biological role of TGF-β2-dependent CREB phosphorylation in neurons. Our data propose a scenario, in which TGF-β2 may activate CREB by inducing its phosphorylation, which in turn induces Rab11b expression. Rab11b acting downstream of CREB mediates TGF-β2-dependent KCC2 trafficking and incorporation in the neuronal plasma membrane, ultimately leading to activation of KCC2. Rab11b differentially regulates the trafficking of distinct cargo, impacts plasma membrane expression of several proteins and is an established regulator of endosomal recycling (Hutalung and Novick, 2011). It regulates degradation and decreases the amount of surface L-type Cav1.2 channels in cardiomyocytes (Best et al., 2011), while it increases surface expression of ENaC in renal cortical collecting duct cells (Butterworth et al., 2012), of CFTR in intestinal epithelial cells (Silvis et al., 2009), and of V-ATPase in salivary duct cells (Oehlke et al., 2011). In contrast, data related to actions of Rab11b in neurons are scarce. Rab11b is moderately enriched with the synaptic vesicle fraction of rat hippocampal neurons (Pavlos et al., 2010), and present in secretory vesicles of PC12 cells (Khvotchen et al., 2003). Our results show that TGF-β2 treatment has led to KCC2 enrichment after CoIP with Rab11b, indicating increased KCC2/Rab11b interaction to
facilitate KCC2 trafficking to the membrane. However, the co-immunoprecipitated KCC2 is only a fraction of total KCC2, a pool of non-interacting KCC2 with Rab11b KCC2 is still present. The latter fraction is included when immunoprecipitation for KCC2 is performed. This fact together with the result that TGF-β2 treatment increases Rab11b expression but not KCC2 expression likely results to lack of enrichment of Rab11b after CoIP with KCC2 (Fig. 7E). Taken together, our results provide the first evidence for a crucial physiological role of Rab11b in neurons, by regulating trafficking of KCC2 to the membrane.

In summary, we introduce TGF-β2 as a novel regulator of the neuronal KCC2 membrane trafficking, membrane expression and activity and provide mechanistic insight by identifying CREB/Rab11b as molecular determinants underlying TGF-β2-dependent KCC2 trafficking and activity. We propose an overall requirement for TGF-β2 for the developmental shift of GABAergic transmission and the development of a functional neuronal network.
Material and Methods

Animals

All protocols were carried out in accordance with German ethical guidelines for laboratory animals and approved by the Institutional Animal Care and Use Committee of the University of Freiburg (authorizations: X-10/27S and X-10/08S). Adult C57BL/6N mice of either sex were maintained on a 12-h dark/light cycle with food and water ad libitum. Mice were sacrificed by cervical dislocation and all efforts were made to minimize suffering.

Primary cultures of mouse E18.5 hippocampal neurons

Hippocampal neurons were isolated from C57Bl6 mice at embryonic (E) day 18.5 of gestation, as described earlier (Lacmann et al., 2007). Cultures were treated with human recombinant TGF-β2 (2ng/ml; R&D Systems) for 5, 10, 15, 30, and 60 min or with anti-TGF-β1,2,3 (10µg/ml; R&D Systems). At day in vitro (DIV) 12, or DIV18 cells were processed for RT-PCR, immunoblotting or immunocytochemistry.

Efficacy of KCC2-mediated Cl⁻ extrusion

KCC2-mediated Cl⁻ extrusion in hippocampal neurons was determined as described earlier (Khirug et al., 2005). The assay of neuronal Cl⁻ extrusion is based on imposing a somatic chloride load via a whole-cell patch-clamp electrode and measuring the somato-dendritic gradient of the reversal potential of GABA_A receptor-mediated current responses (E_GABA) induced along the dendrite by local iontophoretic application of GABA. Somatic recordings in immature cultured hippocampal neurons (DIV 10-14) were performed in standard extracellular solution at room temperature in the whole-cell voltage-clamp configuration using a patch-clamp amplifier, according to Khirug et al. (2005). For local iontophoretic application of GABA, brief (100 ms)
positive current pulses (30-100 nA) were delivered to sharp micropipette (100-200 MOhm when filled with 250 mM GABA in 250 mM HCl). Iontophoretic GABA injections were given not more often than once in 2 min. Constant negative current of -4 nA was applied to the micropipette in order to compensate for the passive leak of GABA. GABA was applied at the soma and at the dendrite (approx. 100 μm from the soma) of a given neuron. NKCC1 was blocked throughout the experiments with 10μM bumetanide, action potentials with 1μM TTX, and GABA_B receptors with 1μM CGP 55845. Under these conditions, the somatodendritic gradient of $E_{GABA}$ provides a quantitative estimate of the efficacy of KCC2-mediated Cl\(^{-}\) extrusion.

**Immunocytochemistry**

Cells were fixed with methanol for 20 min at -20°C or with 4% PFA for 30 min and washed three times with PBS for 5 min. For double immunofluorescence cells were treated for 15 min with 1% BSA, followed by incubation with primary antibodies (anti-KCC2 at 1:1,500 (C2366, Sigma Aldrich, Seelze, Germany), anti-Golgi58K at 1:75 (G2404, Clone 58K-9, Sigma Aldrich, Seelze, Germany), anti-CREB at 1:500 (48H2, New England Biolabs, Frankfurt/Main, Germany), anti-phospho-CREB 1:50 (87G3, New England Biolabs, Frankfurt/Main, Germany), anti-Rab11b 1:250 (BO2P, Abnova, Taipei city, Taiwan) overnight at 4°C. Cells were incubated with goat anti-rabbit IgG coupled to Alexa 568 (1:500) and goat anti-mouse IgG coupled to Alexa 488 (1:200) (Jackson Immuno Research, Suffolk, UK) for 1h at RT. Cells were washed with PBS, coverslips mounted with Vectashield and viewed either with a Zeiss Axioplan2 fluorescence microscope with ApoTome module or with a Leica confocal SP8 microscope (Wetzlar, Germany).
Immunoblotting

Primary hippocampal neurons were washed with ice-cold homogenizing buffer containing (in mM) 280 mannitol, 10 HEPES, 10 KCl, 1 MgCl$_2$, adjusted to pH 7.0 and a protease inhibitor “cocktail” (10µM leupeptin, 2mM benzamidine and 0.1mM Pefabloc®SC), scraped off the culture flasks with a rubber policeman, pelleted by centrifugation at 250 g for 5 min, and resuspended in homogenization buffer. Homogenization was performed by sonication. Protein concentration was determined according to Bradford (1976) and samples were processed for immunoblotting. Electrophoresis and blotting procedures were performed as previously described (Brandes et al., 2007). Blots were incubated with primary antibody overnight at dilution 1:5,000 for KCC2, 1:4,000 for $\alpha_1$ subunit of Na$^+$/K$^+$-ATPase (05-369, Upstate/Millipore, Schwalbach, Germany), 1:2,000 for NKCC1 (AB3560P, Upstate/Millipore, Schwalbach, Germany) and β-III-tubulin (Developmental Studies Hybridoma Bank, Iowa, USA), 1:1,000 for CREB and phospho-CREB, and 1:10,000 for GAPDH (ab8245, [6C5], Abcam, Cambridge, UK). After incubation with secondary antibodies (Amersham/GE Healthcare, Freiburg, Germany), blots were developed in enhanced chemiluminescence reagents and signals were visualized on X-ray film. Subsequently, films were scanned using a flat-bed scanner and the signal ratio KCC2:GAPDH, NKCC1:GAPDH, KCC2:Na$^+$/K$^+$-ATPase, pCREB:CREB was quantified densitometrically for controls and TGF-β2-treated cells. The signal ratio KCC2:GAPDH, NKCC1:GAPDH, KCC2:Na$^+$/K$^+$-ATPase, pCREB:CREB for untreated cells was set to 1. Relative protein abundance (signal ratio of treated cells / signal ratio of untreated cells, i.e. fold change) of the protein of interest was then plotted.
Surface biotinylation

Hippocampal cultures were subjected to control or experimental conditions (application of recombinant TGF-β2) and then kept on ice. Isolation of cell surface proteins was performed using the Pierce® (Thermo Fisher Scientific) cell surface protein isolation kit following the manufacturer’s instructions. Proteins were then processed for immunoblotting with antibody for KCC2, NKCC1, and Na⁺/K⁺-ATPase, as described above.

Immunoprecipitation

Immunoprecipitation was performed as described earlier (Oehlke et al., 2011). Hippocampal neurons at DIV12 were scrapped off a Petri dish into 500µl non-denaturing lysis buffer (50mM Tris, pH 7.4; 300mM NaCl; 5 mM EDTA; 1% Triton X-100, Complete™ Proteinase Cocktail (Roche Diagnostics, Mannheim, Germany) and 0.1mM Pefabloc®SC). Cells were pelleted by centrifugation at 250 x g for 5min, and resuspended in non-denaturing lysis buffer. Efficient homogenization was assured by sonication. Protein concentration was determined according to Bradford (1976). Total protein (500µg) was mixed with 75µl protein A sepharose beads (Invitrogen, Carlsbad, CA, USA; 1:1 in immunoprecipitation buffer, i.e. non-denaturing lysis buffer containing 0.1% Triton X-100) and incubated overnight at 4°C with agitation. After centrifugation for 5min at 5000rpm to remove proteins non-specifically bound to protein A sepharose, the precleared cell lysate was added to antibody-conjugated beads and incubated overnight at 4°C with permanent agitation. To prepare antibody-conjugated beads 3µg of antibody (KCC2 or Rab11b) and 20µl 1% BSA in immunoprecipitation buffer were added to 75µl of protein A sepharose beads and incubated overnight at 4°C. The beads were spun down for 5min at 5000 and the precleared cell lysate was added as described above. Subsequently, the beads were
spun down, washed three times in immunoprecipitation buffer and the supernatant was discarded. Bound proteins and their interaction partners were resuspended in 30µl 6× Laemmli buffer (62.5mM Tris, pH 6.8; 2% SDS, 10% glycine, 5% β-mercaptoethanol, 0.001% bromophenolblue), heated for 5min at 95°C and immediately cooled down on ice, and processed by SDS-PAGE.

RT-PCR
Total RNA was isolated from hippocampal cells using the Qiagen RNeasy kit (Qiagen) according to the manufacturer’s instructions, and reverse transcribed as previously described (Brandes et al., 2007). For detection of the transcripts, the following protocol was used: denaturation at 95°C for 5min followed by 35 cycles of PCR amplification performed under the following conditions: denaturation at 95°C for 30sec, annealing at the appropriate temperature according to the primer pairs for 45sec, and elongation at 72°C for 60sec. Final extension at 72°C for 10min was terminated by rapid cooling to 4°C. PCR products were analyzed by agarose gel electrophoresis. Subsequently, the signal ratios KCC2:GAPDH and NKCC1:GAPDH, were quantified densitometrically. For PCR, the following primers were used: GAPDH (Genbank accession number: NM_008084.3): Forward; 5’-CGGCGCATCTTTTGT-3’ nucleotides (nt196-213). Reverse; 5’-TGACAGAAGACGCCAATAC-3’ (nt289-272); for KCC2 (Genbank accession number: NM_020333.2): Forward; 5’-CTCAACACCTGGACGGACTG-3’ (nt 419-438). Reverse: 5’-GCAGAAGACCTCAGATGCCCTGACG-3’ (nt816-797); for NKCC1 (Genbank accession number NM009194.3): Forward; 5’-CATGCTGTCAGGATTTGAC (nt1893-1912), Reverse: 5’-CGTTCAATCCAGCAATTCAG-3’ (nt2128-2109).
Quantitative Real-Time PCR

Isolation of total RNA from mouse E18.5 primary hippocampal cultures and subsequent quantitative real time PCR was performed as described earlier (Rickmann et al., 2007). Primers and probes were validated by analysis of a standard curve in a template dissociation curve. Real-time PCR was performed according to the manufacturer’s instructions (BioRad, Munich, Germany). Cycle conditions: denaturation at 95°C for 10min, and 40 cycles of PCR amplification at 95°C for 30sec and at the appropriate temperature according to the primer pair for 30sec and elongation at 72°C for 1min. For QRT-PCR, the following primers were used: CREB (Genebank accession number NM_001037726.1) Forward: 5’-GCCTCTGGTGATGTACAAACATACC-3’ (nt794-818), Reverse: 5’-GGGAGGAGCCATAACAACATACC-3’ (nt794-818); for Rab11b (Genebank accession number NM_008997.3) Forward: 5’-GAAGCAATCGCTGACCGTG-3’ (nt758-777), Reverse: 5’-GCTTGTTGGGTCTCTGTCCA-3’ (nt858-839); for GAPDH (Genebank accession number NM_001289726.1), Forward: 5’-TGACGTGCCGCTGGAGAAA-3’ (nt820-839), Reverse: 5’-AGTGTAGCCCAAGATGCCCTTCAG-3’ (nt917-894).

All PCRs were performed in triplicate on a MyiQ Optic I Cycler (Biorad) The mean ± SD of the Ct values for Rab11b, CREB, and GAPDH were determined and analyzed for statistical significance. For documentation of the data shown in Supplementary Material Fig. S2 relative mRNA levels were calculated using the comparative C_t method ($2^{-\Delta\Delta C_t}$).

Transient transfection of mouse primary hippocampal cultures

Mouse primary hippocampal neurons grown on cover slips or on 6well plates were transiently transfected with 100ng Alexa 488-labeled siRNA specifically targeting mouse Rab11b mRNA or with Alexa 488-labeled siRNA specifically targeting CREB
mRNA (purchased from Qiagen), or with control (negative) siRNA, a sequence that reveals no homology with any known mammalian gene labeled with Alexa 488 (AllStars Negative Control siRNA, Qiagen), as previously described (Oehlke et al., 2011; 2012). Cells solely exposed to the transfection reagent were considered as controls. Cells were treated with 2ng/ml TGF-β2 for 60 min 24h after transfection, harvested and processed for either RT-PCR or immunocytochemistry. Efficacy of Rab11b and CREB transcript and protein knock-down was quantified using quantitative real-time PCR, as described above, and Rab11b and CREB immunolabeling, respectively. For functional experiments primary cultures were treated with 1µg/ml shRNA (SureSilencing shRNA Plasmid for Rat Rab11b from SABIOSCIENCES (KR43269G GFP carrying plasmid).

**Image acquisition and analysis.**

Images were acquired with a Leica TCS SP8 confocal microscope using a 20x/0.75HC PL APO immersion objective lens (to acquire images to determine CREB and p-CREB immunofluorescence intensity following TGF-β2 treatment at different time points) and CS2 63x/1.40 oil objective lens (to acquire images used for calculation of KCC2 fluorescence intensity after knocking down either Rab11b or CREB). Within each experiment, confocal microscope settings (laser power, detector gain, and amplifier offset) were kept the same for all scans in which protein expression was compared. Slide labels were covered and only revealed after data collection. The z-stacks of 5-6 optical sections with a step size of 0.5μm were taken for at least 5 separate fields of view for each condition to ensure random image collection. Maximum intensity projections were created from the z-stacks. To quantify nuclear CREB and phospho-CREB protein expression ImageJ (NIH) was used to measure the average intensity within the nucleus. After quantification data were
normalized to the mean of controls (non-treated neurons) in every experiment. Representative images in each figure were processed identically.

**Image acquisition using stimulated emission depletion (STED) microscopy and analysis.**

For co-localization of KCC2 and Rab11b images were acquired with Leica TCS SP8 gated stimulated emission depletion (STED 3X) microscopy using HCX PLAPO 100x/1.40 oil objective lens. Samples were prepared according to Leica microsystems quick guide with slight modifications. Briefly, primary hippocampal neurons were plated on 0.17mm thick coverglass (Harvard apparatus, 64-0713) and maintained for 12 DIV. After treatment with 2ng/ml TGF-β2 for 1 hour neurons were fixed with 4% PFA for 15 min and washed three times with PBS for 5 min. Cells were blocked with 2% BSA/PBS for 1h and subsequently, incubated with mouse monoclonal KCC2 (1:150) and rabbit polyclonal Rab11b (1:200) overnight at 4ºC. The following day neurons were incubated with goat anti-mouse IgG coupled to Alexa532 (1:100) and goat anti-rabbit IgG coupled to Tetramethylrhodamine (TRITC) (1:100) for 1h at RT. Cells were washed with PBS, and coverslips were mounted with Prolong Gold (Molecular Probes).

Within each experiment, identical settings for laser power, STED power and gate were used to acquire images. The wavelength of the laser was 660nm and was adjusted to 50% of power. Images with the z-stacks of 5-6 optical sections with a step size of 0.21μm were de-convolved, using Huygens Softwar. Co-localization of KCC2 with Rab11b was assessed by analysis of Pearson’s correlation coefficient and Mander’s co-localization coefficient. The whole cell soma of each condition was used for quantification. Manders’ M1 and M2 coefficients are defined separately for each channel so that they measure the portion of the intensity in each channel that
coincide with some intensity in the other channel. Pearson’s r-values were calculated for all planes of the z stack of each picture, essentially calculating values for a 3D image. Pictures were visualized with Huygens Surface renderer.

Statistics

No statistical methods were used to pre-determine sample sizes. We used GraphPad Prism 5 software for statistical analysis. Statistical significance was assessed by unpaired, two-tailed Student's t test unless otherwise specified and was accepted at the $P < 0.05$ level. The data distributions were assumed to be normal, but this was not formally tested.
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Competing interests

The authors declare that they have no conflict of interest.

Author contributions:

E.R. was responsible for study conception and design, data acquisition, data analysis and interpretation. J.-M.S., S.K., I.C., and S.S. carried out experiments and analyzed data. C.R. was responsible for study design, data interpretation and critical manuscript revision. K.K. was responsible for study conception and design, data analysis and interpretation. E.R. and K.K. wrote the paper. All authors read and approved the final manuscript.

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Fig. 1 Regulation of KCC2 in neurons at different developmental stages by TGF-β2.
(A) Developing (DIV12) cultured mouse hippocampal neurons were treated with 2ng/ml TGF-β2 for 60 min. KCC2 transcript (397bp) expression was normalized to GAPDH. Data are given as fold changes compared to control. (B) DIV12 cultured neurons were treated with TGF-β2 before immunoblotting with anti-KCC2 antibody. Arrows point to the ~135kDa and ~270kDa KCC2 bands. The ratio of KCC2:GAPDH immunoreactivity was determined. *indicates statistically significant differences.
relative to control as assessed by an unpaired \( t \)-test (\( p=0.016, n=4 \)). (C–D) DIV12 cultured mouse hippocampal neurons were treated with 2ng/ml TGF-\( \beta \)2 for 60 min followed by RT-PCR analysis (C) or immunoblotting (D). The ratio NKCC1:GAPDH transcript expression (in control set to 1) and the ratio of NKCC1:GAPDH immunoreactivity were then determined. Error bars represent SEM. (E) RT-PCR analysis in more mature (DIV18) cultured mouse hippocampal neurons treated with 2ng/ml TGF-\( \beta \)2 for 60 min, (\( n=4 \)). (F) Immunoblot analysis for \~{}135kDa (arrow) and \~{}270kDa (arrow) KCC2 protein in cultures of more mature hippocampal neurons upon TGF-\( \beta \)2 treatment. Data are given as mean ± SEM from four independent cultures and experiments.
Fig. 2 KCC2 membrane trafficking in hippocampal neurons is controlled by TGF-β2. (A) Mouse hippocampal neurons cultured for 12 days were treated for 60 min with 2ng/ml TGF-β2, followed by immunolabeling for KCC2 (red) and the Golgi marker Golgi58k (green). Asterisk indicates intracellular KCC2/Golgi58k co-localization and arrows point to membrane KCC2 labeling. Scale bar: 10 µm. (B) Co-localization of KCC2 and Golgi58k was quantified by determination of the Pearson’s correlation coefficient. **indicate statistically significant differences from control condition (P=0.0015, unpaired Student’s t-test). Data are given as mean ± SEM from 4 independent experiments. (C-D) Cultured neurons were treated with TGF-β2 for 60 min followed by biotinylation of surface proteins. The ratio of ~135kDa surface KCC2:total KCC2, ~270kDa surface KCC2:total KCC2 (C), and of surface
NKCC1: total NKCC1 (D) in untreated (Ctl) and TGF-β2-treated cultures were then determined and values for controls were set to 1. * indicates statistically significant differences from control condition ($p=0.014$, unpaired Student’s $t$-test, $n=3$). Error bars represent SEM.
Fig. 3: (A) Double immunofluorescence for KCC2 (red) and the Golgi marker Golgi58K (green) in DIV18 hippocampal cultures following application of TGF-β2.
(2ng/ml) for 60 min. Arrows and asterisk indicate membrane and intracellular KCC2, respectively. Scale bar: 10µm (B) Trafficking of KCC2 was assessed by determination of co-localization between KCC2 and Golgi58k using the Pearson’s correlation coefficient. After TGF-β2 treatment Pearson’s correlation coefficient for KCC2 and Golgi58k was significantly decreased. Data are given as mean ± SEM from four independent cultures and experiments. (C) Cultured neurons at DIV18 were treated with TGF-β2 for 60 min followed by biotinylation of surface proteins. The ratio of ~135kDa surface KCC2:total KCC2, ~270kDa surface KCC2:total KCC2 in untreated (Ctl) and TGF-β2-treated cultures were then determined and values for controls were set to 1. Error bars represent SEM. (D) Double immunofluorescence for KCC2 (red) and the Golgi marker Golgi58k (green) in DIV18 hippocampal cultures following application of TGF-β2 (2ng/ml) or anti-TGF-β (10µg/ml) at DIV12 and DIV15. Line scans illustrate KCC2 (in red) and Golgi58K (in green) distribution profile and arrows indicate peaks of KCC2 immunofluorescence (representative line scans from 3 independent experiments).
Fig. 4 Treatment with TGF-β2 augments extrusion of intracellular Cl⁻ from cultured hippocampal neurons. (A) Schematic representation of experimental paradigm for
assessing changes in dendritic chloride extrusion. GABA is locally applied at the soma and at a primary dendrite (100 µm distal from the soma) of a neuron recorded in whole-cell voltage clamp mode with a pipette containing a slight load of chloride. The reversal potential in for GABA is estimated in the two positions. (B) Time course of Cl⁻ reversal potential measured in soma (a) and dendrite (b, c) of a cultured (DIV 10) hippocampal neuron. Onset of TGF-β2 application corresponds to time point zero. In this particular neuron, almost no somatodendritic Cl⁻ gradient was observed before exposure to TGF-β2 as the difference in $E_{\text{Cl}^-}$ measured in soma (a) and in dendrite (b) was almost zero. After application of TGF-β2, dendritic $E_{\text{Cl}^-}$ and therefore somatodendritic Cl⁻ gradient shifted negative (c). The insets are example traces of voltage ramps before (light grey) and during local application of GABA (dark grey) at the soma (a) and the dendrites (b-c). The intercept between these traces give an estimation of the GABAₐ reversal potential at the specific location. (b) and (a) are example traces before and after application of TGF-β. (C) Quantification of dendritic $E_{\text{Cl}^-}$ in control and TGF-β2-treated neurons. * indicates statistically significant difference from control condition ($p<0.05$, unpaired Student’s $t$-test, $n=4$). Error bars represent SEM from four independent experiments. Data picked up for this panel include only those experiments where no statistically significant difference between somatic and dendritic $E_{\text{Cl}^-}$ measured in the same cell was observed prior to TGF-β2 application (cf. Supplementary Material Figure S1).
**Fig. 5.** TGF-β2 activates CREB by increasing its phosphorylation. (A) Western blot analysis of total and of phosphorylated CREB in cultured hippocampal neurons (DIV12) treated with 2ng/ml TGF-β2 for either 5, 10, or 15 min (dotted line represents values for control; n=7). (B) Immunofluorescence for CREB and pCREB of mouse hippocampal cultures (DIV12) under control conditions and following treatment with TGF-β2 for 10 min. Scale bar: 10µm. Quantification of relative CREB and phospho-CREB fluorescence intensity following application of TGF-β2 for 5 and 10 min (representative out of 4 experiments). Data are shown as mean ± SEM. *p<0.05, ** p<0.01.
**Fig. 6** Loss-of-function of either *CREB* or *Rab11b* impairs TGF-β2-mediated KCC2 trafficking to the plasma membrane. Immunofluorescence for KCC2 in primary mouse hippocampal neurons transiently transfected either with negative siRNA (green in B and F), with specific siRNA against *CREB* (green in C and G), or with specific siRNA against *Rab11b* (green in D and H). Cells shown in (A) and (E) represent non-transfected neurons. 24h following transfection cells were treated with 2ng/ml TGF-β2 for 60 min. Nuclei were labelled with DAPI (blue). Scale bar: 10μm. Asterisks
indicate cytosolic KCC2 localization, white arrows point to plasma membrane labeling. KCC2 distribution profile was visualized by line scans for KCC2 immunofluorescence. Black arrows indicate peaks of KCC2 immunoreactivity. Representative images and line scans from 4 independent experiments
Fig. 7 Rab11b co-localizes and interacts with KCC2 and its expression depends on TGF-β2. (A-B) 3D STED images illustrating part of dendrite of an untreated control neuron (inset in A) and a TGF-β2-treated neuron (inset in B) were acquired. High magnification images correspond to the white-boxed area of the inset. Scale bar: 500nm. C. Pearson’s correlation coefficient and Manders’ coefficients for KCC2 and Rab11b were calculated and were significantly increased in neurons treated with TGF-β2 for 15 min (**p=0.0005, *p=0.015 and *p=0.025 for M1 and M2, respectively). (D) DIV12 cultured mouse hippocampal neurons were treated for 5-30
min with 2ng/ml TGF-β2, followed by quantitative RT-PCR analysis for Rab11b expression. * indicates significant increase of relative Rab11b expression after TGF-β2 application, (*p=0.033, unpaired Student’s t-test, n=4 independent experiments).

(E) Interaction of KCC2 with Rab11b in controls and TGF-β2-treated DIV12 hippocampal neurons. Antibodies against Rab11b were able to immunoprecipitate (IP) KCC2 expressed in control hippocampal neurons, as detected by immunoblotting with KCC2 antibody. Antibodies against KCC2 were also able to immunoprecipitate Rab11b expressed in control hippocampal neurons, as detected by immunoblotting with Rab11b antibody. The ratio of ~135kDa KCC2:input Rab11b, ~25kDa Rab11b:input KCC2 in untreated (Ctl) and TGF-β2-treated cultures were then determined and values for controls were set to 1. Error bars represent SEM. Amount of co-immunoprecipitated KCC2 was significantly increased in TGF-β2-treated neurons, compared to the untreated controls (*p=0.04, unpaired Student’s t-test, n=3 independent experiments).
Fig. 8 Rab11b mediated TGF-β2-dependent KCC2 functional expression. (A) Somatodendritic Cl⁻ gradients measured in cultured neurons (DIV10-15) transfected with ShRab11b (n=6 independent experiments) and treated with TGF-β2. (B) Somatodendritic Cl⁻ gradients measured in cultured neurons (DIV10-15) transfected with ShRab11b negative (n=5 independent experiments). Control cells (n=9) include those characterized in Fig.4. (C) Quantification of $E_{\text{GABA}}$ gradient after TGF-β2 application. Statistics for the cells transfected with ShRab11b (*p<0.01, n=6) includes only those experiments where “external control” was positive, i.e. non-transfected cells from the same coverslip showed clear difference in somatic and dendritic $E_{\text{Cl}^-}$. Data are shown as mean ± SEM.
Supplementary Material Figure S1

Treatment with TGF-β2 provokes extrusion of intracellular Cl⁻ from cultured mature hippocampal neurons. Same experiment as in Fig.4B but in this particular neuron some somatodendritic Cl⁻ gradient was already formed before exposure to TGF-β2 (arrow). Time course of Cl⁻ reversal potential measured in soma (a) and dendrite (b, c) of a cultured mature hippocampal neuron. Treatment with TGF-β2 evoked further negative shift in dendritic $E_{Cl^-}$, although the effect was smaller, compared to that shown in Fig. 4A. The insets are example traces of voltage ramps before (light grey) and during local application of GABA (dark grey) at the soma (a) and the dendrites (b-c). The intercept between these traces give an estimation of the GABAₐ reversal potential at the specific location. (b) and (a) are example traces before and after application of TGF-β2.
**Supplementary Material Figure S2**

Validation of CREB and Rab11b knock down following transfection with specific siRNAs. (A-B) Quantitative real-time PCR analysis for CREB (A) or Rab11b (B) in primary mouse hippocampal neurons transiently transfected either with negative siRNA, or with specific siRNA against CREB or Rab11b (n=4). Relative mRNA levels calculated using the comparative Ct method (2^{-\Delta\Delta Ct}). *p<0.05 and **p<0.01 indicate significantly decreased relative CREB and Rab11b expression. (C) Immunofluorescence for Rab11b shows knock-down of Rab11b protein in hippocampal neurons following transfection with specific siRNA against Rab11b. Scale bar: 10µm. (D) Primary hippocampal neurons were transiently transfected with either negative siRNA or with 100ng specific siRNA against CREB, and relative Rab11b expression was determined by quantitative real-time PCR. * indicates significant decrease of relative Rab11b expression after transfection with siCREB (p<0.05 by unpaired Student’s t-test, n=3). Downregulation of Rab11b protein in cells transfected with siCREB validated by immunofluorescence. Scale bar: 20µm.