A kainate receptor subunit promotes the recycling of the neuron-specific K\(^+\)-Cl\(^-\) co-transporter KCC2 in hippocampal neurons

Received for publication, November 10, 2016, and in revised form, February 24, 2017  Published, JBC Papers in Press, February 24, 2017, DOI 10.1074/jbc.M116.767236

Jessica C. Pressey‡1,2, Vivek Mahadevan1,3, C. Sahara Khademullah‡, Zahra Dargaei‡, Jonah Chevrier‡, Wengye Ye‡, Michelle Huang‡, Alamjeet K. Chauhan‡, Steven J. Meas‡, Pavel Uvarov‡, Matti S. Airaksinen§ and Melanie A. Woodin‡4

From the ‡Department of Cell and Systems Biology, University of Toronto, Toronto, Ontario M5S 3G5, Canada and the §Department of Anatomy, University of Helsinki, 00014 Helsinki, Finland

Edited by Roger J. Colbran

Synaptic inhibition depends on a transmembrane gradient of chloride, which is set by the neuron-specific K\(^+\)-Cl\(^-\) co-transporter KCC2. Reduced KCC2 levels in the neuronal membrane contribute to the generation of epilepsy, neuropathic pain, and autism spectrum disorders; thus, it is important to characterize the mechanisms regulating KCC2 expression. In the present study, we determined the role of KCC2-protein interactions in regulating total and surface membrane KCC2 expression. Using quantitative immunofluorescence in cultured mouse hippocampal neurons, we discovered that the kainate receptor subunit GluK2 and the auxiliary subunit Neto2 significantly increase the total KCC2 abundance in neurons but that GluK2 exclusively increases the abundance of KCC2 in the surface membrane. Using a live cell imaging assay, we further determined that KCC2 recycling primarily occurs within 1–2 h and that GluK2 produces an ∼40% increase in the amount of KCC2 recycled to the membrane during this time period. This GluK2-mediated increase in surface recycling translated to a significant increase in KCC2 expression in the surface membrane. Moreover, we found that KCC2 recycling is enhanced by protein kinase C-mediated phosphorylation of the GluK2 C-terminal residues Ser-846 and Ser-868. Lastly, using gramicidin-perforated patch clamp recordings, we found that the GluK2-mediated increase in KCC2 recycling to the surface membrane translates to a hyperpolarization of the reversal potential for GABA (E\(_{\text{GABA}}\)). In conclusion, our results have revealed a mechanism by which kainate receptors regulate KCC2 expression in the hippocampus.

The classic fast hyperpolarizing inhibition of the mature brain results primarily from the activation of GABA\(_A\) receptors. These receptors are Cl\(^-\)-permeable ion channels, and inhibition results from the influx of Cl\(^-\) into the neuron (1). This inward gradient for Cl\(^-\) is set by the neuron-specific K\(^+\)/Cl\(^-\) co-transporter KCC2 (2, 3). Despite the requirement of KCC2 for hyperpolarizing inhibition, the mechanisms that regulate KCC2 expression and function are still under intense investigation.

In addition to the critical role KCC2 plays in synaptic inhibition, KCC2 is highly localized to excitatory synapses (4, 5), where it plays important roles in the development (6) and the function of glutamatergic synapses (7, 8). In fact, single-particle tracking revealed that KCC2 is tightly confined to excitatory synapses (5), which may result from local protein interactions. Thus, understanding how proteins associated with excitatory synapses regulate KCC2 function may provide critical insight to the function of KCC2.

KCC2 exists in a multiprotein complex and is regulated by components of excitatory synaptic transmission (9–11). Specifically, KCC2 interacts with both the kainate-type ionotropic glutamate receptor subunit GluK2 (9) and its auxiliary subunit Neto2 (10). Neto2 regulates KCC2-mediated Cl\(^-\) extrusion by binding to the active oligomeric form of KCC2 (10), whereas the GluK2-KCC2 interaction is required for KCC2 oligomerization (9), and the loss of either protein reduces KCC2-mediated Cl\(^-\) extrusion (9, 10). Although GluK2 and Neto2 can regulate KCC2 independently (9), their key individual roles in regulating total and surface abundance have not been systematically determined. In the current study, we determined the roles of Neto2 and GluK2 in regulating both total and surface KCC2 abundance using a combination of biochemistry, live cell imaging, and electrophysiology. We determined that although both proteins can regulate total KCC2 abundance, GluK2 exclusively regulates surface abundance. Moreover, we determined that GluK2 increases surface abundance by increasing KCC2 recycling to the membrane and that this recycling can be enhanced by GluK2 phosphorylation.

Results

Neto2 and GluK2 independently increase recombinant KCC2 abundance

To determine whether Neto2 and/or GluK2 can regulate total and surface KCC2 abundance, we used heterologous cells
(COS-7), which do not endogenously express our proteins of interest, allowing us to control protein expression via transfection.

We first asked whether the Neto2-KCC2 interaction can increase total KCC2 abundance by transfecting cells with increasing amounts of full-length Neto2 cDNA (containing an HA tag, Neto2-FL-HA) while keeping the amount of transfected KCC2 cDNA constant. We observed that total KCC2 abundance increased in relation to increasing Neto2, despite transfecting with the same amount of KCC2 cDNA (Fig. 1Ai; p = 0.029).

Neto2 is a single-pass transmembrane protein containing two extracellular CUB domains, which are critical for maintaining the Neto2-KCC2 interaction (10). Based on this requirement for the CUB domains, we hypothesized that co-expressing Neto2-HA lacking these domains (Neto2-ΔCUB1&2-HA) would prevent the increase in KCC2 abundance observed in Fig. 1Ai. Surprisingly, we observed that in the absence of the Neto2-KCC2 interaction, increasing Neto2-ΔCUB1&2-HA led to a decrease in total KCC2 abundance (Fig. 1Aii; p = 0.049).

Despite the fact that the Neto2-KCC2 interaction is mediated by the extracellular CUB domains, the Neto2 tail alone can rescue the deficiency of KCC2 transport in Neto2−/− neurons (10). This suggests an important Neto2 tail-dependent regulation of KCC2 function that does not require physical interaction. To determine whether the Neto2 tail-dependent regulation of KCC2 includes regulation of total KCC2 abundance, we co-expressed Neto2-HA lacking the cytoplasmic tail (Neto2-Acyto-HA). We observed that total KCC2 abundance did not increase in response to increasing Neto2-Δcyto-HA cDNA, and again we found a decrease in total KCC2 abundance (Fig. 1Aiii; p = 0.004). Expression of Neto2 truncation mutants resulted in putative degradation products as indicated by the appearance of faster migration bands in Fig. 1 (Ai and Aiii), which is consistent with previous studies (10, 18). Together, these results suggest that when Neto2 associates with KCC2, there is an increase in total KCC2 abundance; however, when this association is disrupted, either physically or through its cytoplasmic tail-dependent regulation, KCC2 is not stably expressed.

Using the same assay system, we asked whether increasing GluK2 could also increase total KCC2 abundance. We found that increasing amounts of GluK2 transfection (GluK2-MYC) also increased total KCC2 abundance, despite maintaining the same amount of KCC2 cDNA during transfection (Fig. 1Bii; p = 0.015). To determine whether the effect on KCC2 abundance was due to GluK2 specifically, we repeated these experiments with another kainate receptor subunits GluK1 (GluK1-MYC). We found that increasing GluK1-MYC did not increase KCC2 abundance (Fig. 1Bii; p > 0.05). Taken together, the above results demonstrate that both Neto2 and GluK2 can independently increase total KCC2 abundance in vitro.

We next asked whether the regulation of KCC2 abundance is unique to Neto2 and GluK2 or whether other ionotropic glutamate receptors can also regulate KCC2 abundance. To answer this question, we turned to NMDA receptors (NMDARs), which we selected because their activation is known to modulate KCC2 function (12). Because NMDA receptors are heterotetramers, we chose to perform this experiment using the obligatory GluN1 subunit. We found that increasing concentrations of transfected GluN1 did not significantly increase KCC2 abundance (Fig. 1C; p = 0.852), which suggests that the effect of GluK2 on KCC2 may be specific to kainate receptors, at least in the hippocampus.

**Neto2 and GluK2 together increase KCC2 total abundance**

GluK2 normally exists in heteromeric combinations with other GluK subunits (15) and associates with Neto2 (16–18). To test whether GluK2 and Neto2 together can further regulate total KCC2 abundance *in vitro*, we co-expressed KCC2 in combinations with GluK subunits and Neto2. In this assay we used subsaturating amounts of GluK and Neto2 cDNA (125 ng) as determined above (Fig. 1), in combination with KCC2 cDNA (125 ng). As we reported above, co-expression of KCC2 with either Neto2 or GluK2 significantly increased total KCC2 abundance (Fig. 2; p = 0.023), whereas co-expression of KCC2 with GluK1 did not (Fig. 2; p > 0.05). However, when KCC2 was co-expressed with both GluK2 and Neto2, there was a significant increase in total KCC2 abundance as compared with when KCC2 was expressed with either GluK2 or Neto2 alone (Fig. 2; p = 0.002). These results demonstrate that although KCC2 abundance can be increased by either Neto2 or GluK2, both Neto2 and GluK2 produce a further significant increase in total KCC2 abundance in heterologous cells.

**KCC2 abundance and surface expression depend on both Neto2 and GluK2 in hippocampal neurons**

We have demonstrated that in combination Neto2 and GluK2 can significantly increase KCC2 abundance in a heterologous system. To determine whether this is also true in neurons and to determine the role of Neto2 and GluK2 in regulating KCC2 surface abundance, we quantified KCC2 expression in neurons using immunofluorescence. We chose to perform this experiment in GluK1/2−/− hippocampal neurons because, in addition to lacking kainate receptor subunits, these neurons also have a reduction in Neto2 expression (9, 16). To quantify KCC2 abundance, we performed immunocytochemistry in neurons transfected with a KCC2 construct containing an extracellular FLAG-tag (Fig. 3A). In contrast to recombinant systems, we found that co-expression of either Neto2 alone (Fig. 3B) or GluK2 alone (Fig. 3C) did not alter total KCC2 abundance (Fig. 3E). However, overexpressing GluK2 alone (Fig. 3C) significantly increased surface KCC2 immunofluorescence (Fig. 3F; p = 0.004), whereas overexpressing Neto2 alone did not (Fig. 3B and F). When both Neto2 and GluK2 were present (Fig. 3D), there was a significant increase in both total (Fig. 3E; p = 0.04) and surface abundance of KCC2 (Fig. 3F; p = 0.004). Taken together, this suggests that although both Neto2 and GluK2 are required to increase total KCC2 abundance in neurons, only GluK2 increases KCC2 expressed at the surface. Interestingly, however, only GluK2 regulated KCC2 expressed at the surface even in the absence of any increase in total KCC2 abundance. We therefore hypothesized that the GluK2-mediated increase in KCC2 surface expression resulted from a GluK2-mediated increase in KCC2 recycling to the surface.

**GluK2 promotes KCC2 recycling to the neuronal membrane**

To test whether GluK2 regulates KCC2 recycling to the neuronal surface, we performed a live cell recycling assay. In this
assay we labeled the pool of surface KCC2 that was subsequently endocytosed and then used live imaging to quantify the recycling of that initial membrane pool back to the surface. Similar techniques are routinely used to characterize excitatory neurotransmitter receptor endocytosis and recycling (19–21). As illustrated in the schematic (Fig. 4A), we first labeled over-

---

**Figure 1. Neto2 and GluK2 independently increase recombinant KCC2 abundance.** Ai, representative immunoblot from COS-7 cells transfected with equal amount of KCC2 cDNA (125 ng) in combination with increasing amounts of full-length Neto2 cDNA (Neto2-FL-HA). The blot was probed with the antibodies indicated on the left. Summary figure below shows total KCC2 abundance, normalized to cells transfected with KCC2 alone. Aii and Aiii, similar to Ai, but cells were transfected with cDNA for Neto2 lacking the CUB domains (Neto2-ΔCUB1&2-HA) and cDNA for Neto2 lacking the cytoplasmic tail (Neto2-Δcyto-HA). B, similar to A, but cells were transfected with cDNA for GluK2 (GluK2-MYC) (Bii) or GluK1 (GluK1-MYC) (Biii). C, similar to A and B, but cells were transfected with GluN1 (GluN1-eGFP). All immunoblots are representative of n = 3 independent biological replicates. The bars represent means ± S.E. Statistical significance was determined using one-way ANOVAs followed by Dunnett’s post hoc test. Asterisks denote significance from control (KCC2 alone, black bars), *p < 0.05, **p < 0.01.
expressed KCC2 containing an extracellular FLAG tag using anti-FLAG antibody (step 1). Following labeling and subsequent endocytosis, we removed any remaining anti-FLAG antibody at the surface using an ice-cold acetic acid buffer wash (step 2). Following a time interval to allow for vesicle recycling (step 3), we visualized KCC2-FLAG recycling back to the membrane by staining for surface FLAG with Alexa Fluor 488 as a secondary antibody (step 4). Lastly, we permeabilized neurons and stained them for intracellular pool of non-recycled KCC2 using Alexa Fluor 555 as secondary antibody (step 5). We first verified that the acetic acid wash removed all surface anti-FLAG antibodies while leaving the cytosolic pool of FLAG-KCC2 intact (Fig. 4Aii). We found that when we overexpressed KCC2-FLAG, we could clearly distinguish recycled from intracellular KCC2 (Fig. 4B). To test our hypothesis that GluK2 regulates KCC2 recycling to the neuronal surface, we compared KCC2 recycling in the somatodendritic compartment (which included the soma and first 50 μm of the proximal dendrites) in GluK1/2/Neto2 neurons overexpressing KCC2-FLAG versus KCC2-FLAG + GluK2. Although we found that KCC2 underwent recycling in the absence of GluK2, KCC2 recycling was significantly increased in the presence of GluK2 (Fig. 4C; 2 h, \( p < 0.01 \); 4 h, \( p < 0.04 \)). KCC2 recycling was determined by normalizing surface KCC2-FLAG to total KCC2-FLAG (surface + internal) to account for potential GluK2-mediated increases in total KCC2 expression. The GluK2-mediated increase in KCC2 surface recycling translated into an increase in surface KCC2-HA puncta as measured using standard immunofluorescence (Fig. 4D; \( p = 0.02 \)). To confirm that GluK2 increases KCC2 expression in the membrane, we performed total internal reflection microscopy (TIRF)\(^5\) in COS-7 cells overexpressing KCC2-HA, which allows the imaging of proteins exclusive to the surface membrane. We observed a signif-

\(^5\) The abbreviations used are: TIRF, total internal reflection microscopy; PMA, phorbol 12-myristate 13-acetate; ANOVA, analysis of variance.
GluK2 regulates KCC2 surface recycling.

Figure 4. GluK2 promotes KCC2 recycling. Ai, schematic diagram of the live cell imaging recycling assay. Aii, representative confocal image of live cell imaging recycling assay in GluK1/2−/− neurons showing the surface KCC2-FLAG before and after stripping (compare top to bottom). B, recycled KCC2-FLAG was labeled using Alexa Fluor 488 (green), and non-recycled/internal KCC2-FLAG was labeled with Alexa Fluor 555 (red). Neurons were co-stained for MAP2 (purple). A dotted box depicts enlarged region of proximal dendrite showing recycled (green) and non-recycled (red) KCC2-FLAG. C, surface KCC2-FLAG fluorescence (fluorescence/volume) recorded over time in GluK1/2−/− neurons transfected with either KCC2-FLAG (black, n = 15) or KCC2-FLAG+GluK2 (gray, n = 15). Surface KCC2-FLAG fluorescence is normalized to total KCC2-FLAG fluorescence (surface + internal). Di, representative immunofluorescent confocal images of surface KCC2-HA puncta in a GluK1/2−/− neuron transfected with KCC2 (left) or KCC2 + GluK2 (right). Scale bars, 5 μm. Dii, summary of quantified puncta density (#/μm²) for all images similar to Di (KCC2, n = 13; KCC2+GluK2, n = 12). Ei, representative images of COS-7 cells transfected with KCC2-HA (left) or KCC2-HA + GluK2 (right) and imaged using TIRF microscopy. Scale bars, 5 μm. Eii, summary of quantified puncta density (#/μm²) for all images similar to Ei (KCC2, n = 18; KCC2+GluK2, n = 15). The bars represent means ± S.E. Statistical significance was determined using: C, one-way ANOVA followed by Dunnett’s post hoc test. Di and Ei, Student’s t test. Asterisks denote significance from control (KCC2 alone, black line in C, bars in D and E), unless otherwise indicated: *, p < 0.05; and **, p < 0.01. The n values represent individual cells on coverslips and were obtained from at a minimum of three independent sets of cultures.
PKC-mediated phosphorylation of GluK2 is required for GluK2 to promote KCC2 recycling and GABAergic inhibition

It was previously demonstrated that PKC-mediated phosphorylation of GluK2 C-terminal residues Ser-846 and Ser-868 regulates the recycling of GluK2 to the plasma membrane (19, 22–24). This led us to predict that if we promoted PKC-mediated GluK2 phosphorylation, we would also regulate KCC2 recycling. To test our prediction, we returned to our live cell imaging assay and took advantage of the previously characterized GluK2-S846A/S868A phospho-deficient mutant (23). To express this phospho-deficient mutant in the absence of endogenous GluK2 and KCC2, we performed these experiments in COS-7 cells. This system allowed us to test the sufficiency of GluK2 phosphorylation, we would also regulate KCC2 recycling. To test this prediction, we promoted PKC phosphorylation using PMA. If PKC activation increases KCC2 recycling and surface abundance via phosphorylation of GluK2, then we should see a significantly higher KCC2 transport activity compared with GluK1/2−/− neurons. Using gramicidin perforated patch clamp recordings to measure E_{GABA}, we determined that promoting PKC phosphorylation with PMA in wild type neurons significantly hyperpolarized E_{GABA} from −69.6 ± 2.6 to −89.1 ± 5.5 mV (Fig. 6, A and D; p = 0.007). In GluK1/2−/− neurons, PMA treatment hyperpolarized E_{GABA} from −67.2 ± 3.0 to −74.4 ± 3.7 mV (Fig. 6B), which also resulted in a significant hyperpolarization of E_{GABA} (Fig. 6D; p = 0.046). Based on the results from our recycling assay, which indicated that the phosphorylation of GluK2-Ser-846/868 promotes KCC2 recycling, we predicted that in the absence of GluK2-Ser-846/868 phosphorylation, PMA would not hyperpolarize E_{GABA}. To test this prediction, we expressed the phospho-deficient GluK2-S846A/S868A in GluK1/2−/− neurons and repeated the above electrophysiology experiments. We found that PMA treatment did not significantly change E_{GABA} (Fig. 6, C and D; no PMA, −55.6 ± 1.6 mV; PMA, −49.28 ± 2.6; p = 0.09). Lastly, we determined that the re-expression of GluK2 in GluK1/2−/− neurons was sufficient to rescue the significant increase in KCC2 puncta density in cells expressing KCC2 with GluK2, compared with cells expressing KCC2 alone (Fig. 4E; p = 0.02).

PKC-mediated phosphorylation of GluK2 is required for GluK2 to promote KCC2 recycling and GABAergic inhibition

Figure 5. PKC-mediated phosphorylation of GluK2 is required for GluK2 to promote KCC2 recycling and GABAergic inhibition. A, representative confocal images of recycled KCC2-FLAG (green) in COS-7 cells transfected with KCC2-FLAG alone or in combination with GluK2 or phospho-deficient GluK2. Scale bars, 5 μm. B, summary of quantified total recycled fluorescence presented in A (KCC2-FLAG, n = 17; KCC2-FLAG + GluK2, n = 11; KCC2-FLAG + phospho-deficient GluK2, n = 11). C, representative confocal images of recycled KCC2-FLAG in GluK2−/− neurons transfected with KCC2-FLAG alone or in combination with phospho-deficient GluK2. Scale bars, 5 μm. D, summary of surface KCC2-FLAG fluorescence normalized to total KCC2-FLAG fluorescence (surface + internal) (KCC2-FLAG + GluK2, n = 11; KCC2-FLAG + phospho-deficient GluK2, n = 10). Statistical significance was determined using a one-way ANOVA (B), and a Student’s t test (D). *, p < 0.05. The n values represent individual cells on coverslips and were obtained from at a minimum of three independent sets of cultures.

GluK2 regulates KCC2 surface recycling

If PKC-mediated phosphorylation of GluK2 promotes KCC2 recycling and surface expression, then we would predict that phosphorylated GluK2 should result in increased KCC2-mediated Cl− extrusion (because of the increase in surface abundance). To test this prediction, we promoted PKC phosphorylation using PMA. If PKC activation increases KCC2 recycling and surface abundance via phosphorylation of GluK2, then we need to see a significantly higher KCC2 transport activity (determined as a hyperpolarization of E_{GABA}) in wild type neurons compared with GluK1/2−/− neurons. Using gramicidin perforated patch clamp recordings to measure E_{GABA}, we determined that promoting PKC phosphorylation with PMA in wild type neurons significantly hyperpolarized E_{GABA} from −69.6 ± 2.6 to −89.1 ± 5.5 mV (Fig. 6A and D; p = 0.007). In GluK1/2−/− neurons, PMA treatment hyperpolarized E_{GABA} from −67.2 ± 3.0 to −74.4 ± 3.7 mV (Fig. 6B), which also resulted in a significant hyperpolarization of E_{GABA} (Fig. 6D; p = 0.046). Based on the results from our recycling assay, which indicated that the phosphorylation of GluK2-Ser-846/868 promotes KCC2 recycling, we predicted that in the absence of GluK2-Ser-846/868 phosphorylation, PMA would not hyperpolarize E_{GABA}. To test this prediction, we expressed the phospho-deficient GluK2-S846A/S868A in GluK1/2−/− neurons and repeated the above electrophysiology experiments. We found that PMA treatment did not significantly change E_{GABA} (Fig. 6, C and D; no PMA, −55.6 ± 1.6 mV; PMA, −49.28 ± 2.6; p = 0.09). Lastly, we determined that the re-expression of GluK2 in GluK1/2−/− neurons was sufficient to rescue the
GluK2 regulates KCC2 surface recycling

Discussion

In this study we determined that although both Neto2 and GluK2 increase total KCC2 abundance in hippocampal neurons, GluK2 itself plays a unique role in promoting KCC2 recycling and surface abundance. Through the use of phosphodeficient GluK2, we revealed that PKC-mediated phosphorylation of GluK2 increases KCC2 recycling and surface expression. Increased KCC2 recycling and surface expression culminates in a hyperpolarization of EGABA, which is indicative of increased KCC2-mediated Cl\(^-\) extrusion and augmented GABAergic inhibition.

PKC-mediated phosphorylation is a well established essential post-translational modification of GluK2 (19, 23, 25, 26). At relatively low levels of PKC activation, which can occur in response to metabotropic kainate receptor activation, serine phosphorylation of GluK2 increases GluK2 exocytosis from Rab11-containing recycling endosomes, which results in increased GluK2 surface abundance (25, 27). However, in response to relatively high levels of PKC activity, phosphorylation of membrane expressed GluK2 at Ser-846 can induce endocytosis (24). In our present study using PMA-induced phosphorylation of GluK2, we found an increase in KCC2 recycling, surface expression, and increased KCC2-mediated Cl\(^-\) extrusion, which we attribute to a phosphorylation-dependent increase in GluK2 exocytosis and surface expression. However, it is possible that relatively high PKC activity levels capable of inducing GluK2 endocytosis could promote a decrease in KCC2 surface expression. Thus, it remains to be determined how the activity-dependent regulation of GluK2 phosphorylation will regulate KCC2 surface expression during a range of physiologically relevant brain states.

PKC-mediated phosphorylation is also a well established essential post-translational modification of KCC2 (12, 29). Specifically, PKC phosphorylation of the Ser-940 residue increases KCC2 cell surface stability (by a decrease in internalization) and increases transporter efficacy (30). In the presence of GluK2 (WT neurons and GluK1/2\(^{-/-}\) neurons transfected with GluK2), we propose that PMA has two independent effects that cooperate to result in the hyperpolarization of EGABA; the phosphorylation of GluK2 increases KCC2 recycling and surface expression, which essentially provides more substrate for Ser-940-KCC2 phosphorylation. However, in the case of GluK1/2\(^{-/-}\) neurons transfected with GluK2-S846A/S868A, the lack of GluK2 phosphorylation results in a decrease in KCC2 surface expression and recycling, and therefore there is a significantly smaller effect on Ser-940-KCC2. However, it is curious to note that there is no effect of PMA on EGABA of GluK1/2\(^{-/-}\) neurons transfected with GluK2-S846A/S868A, when we would have expected a hyperpolarization similar to that observed for untransfected GluK1/2\(^{-/-}\) neurons. One possibility is that the GluK2-phosphomutant decreases KCC2 levels below that
observed in GluK1/2−/− neurons alone; however, our results from recycling assays in Fig. 5 do not support this idea. Despite this inconsistency, overall the present results reconcile well with the existing literature on PKC-mediated phosphorylation of Ser-940-KCC2.

In the present study we primarily utilized cultured hippocampal neurons, which lack kainate receptor-mediated transmission but abundantly express the kainate receptor subunit GluK2 (31). Kainate receptors are ionotropic receptors that can also signal through G-proteins (32, 33), thus raising the intriguing possibility that this non-canonical metabotropic activity may trigger a signal transduction cascade, leading to the regulation of KCC2 function. This possibility is particularly interesting given that the metabotropic activity of kainate receptors can lead to an activation of PKC (34), a kinase that is well known to regulate KCC2 surface expression (12, 29). It is also possible that activation of the ionotropic current could lead to a change in local intracellular osmolarity, which could regulate KCC2 function because this transporter is sensitive to changes in isotonic osmolarity (35). However, the activity-dependent kainate receptor-mediated regulation of KCC2 function awaits future testing, which will be significantly aided by the development of specific inhibitors of the metabotropic signaling pathways downstream of kainate receptors.

Previous studies have demonstrated that Neto2 expression is dependent on GluK2 and that GluK1/2−/− neurons have reduced Neto2 protein abundance (9, 16). It is therefore possible to interpret that the observed diminution in KCC2 surface expression in GluK1/2−/− neurons may be indirectly mediated by a reduction in total Neto2. However, two of our present findings suggest that this interpretation is incorrect. First, we found that the overexpression of Neto2 alone was not sufficient to increase KCC2 surface expression in GluK1/2−/− neurons. Second, GluK2 overexpression alone was sufficient to increase KCC2 surface abundance in GluK1/2−/− cells. Thus, GluK2 plays a specific role in mediating KCC2 surface expression. However, this finding that GluK2 is sufficient to increase KCC2 surface expression does not preclude the important synergistic role that occurs when GluK2 is co-expressed with Neto2; in this scenario KCC2 surface abundance increases significantly compared with when GluK2 is expressed alone. This synergistic role of GluK2 and Neto2 in regulating KCC2 expression is important because all three proteins (GluK2, Neto2, and KCC2) commonly exist in a macromolecular complex in hippocampal neurons (9). Based on the individual and synergistic roles of GluK2 and Neto2 in regulating KCC2 expression, we propose the following working model: (i) GluK2 regulates Neto2 total abundance (9, 16); (ii) GluK2 and Neto2 together increase KCC2 abundance (Figs. 2 and 3); and (iii) GluK2 regulates KCC2 surface expression (Figs. 3 and 4). This complex co-regulation of transmembrane proteins within a macromolecular complex is similar to other recent discoveries that demonstrated how diverse classes of multimeric proteins can regulate each other within a protein complex (36–38).

Not all neuron types express GluK2 and/or Neto2, which raises the question of how KCC2 is recycled to the surface membrane in those cell types. For example, Purkinje cells in the cerebellum do not express Neto2 or GluK2 (17, 39) but still express KCC2 (40). This observation would argue that the mechanism reported here may not be universal throughout the nervous system and may be restricted to distinct brain regions and/or cell types that co-express these proteins. Moreover, given that we observed GluK1/2−/− neurons still recycled KCC2 to the surface, albeit at significantly reduced abundance, there may be additional proteins critical for KCC2 recycling. To investigate additional proteins involved in KCC2 recycling, it would be beneficial to perform an unbiased characterization of KCC2 interacting proteins. Such a proteomic analysis of the KCC2-interactome may reveal key proteins with known roles in transmembrane protein recycling.

The loss of KCC2 membrane expression underlies neurological and psychiatric disorders including epilepsy (41–44), stress (45), autism spectrum disorder (46, 47), schizophrenia (48), motor spasticity (49), and neuropathic pain (50). Thus, it is essential to elucidate the pathways and mechanisms regulating KCC2 function to develop therapeutic strategies to restore KCC2 expression and synaptic inhibition. Here we have discovered that promoting the interaction between GluK2 and KCC2 leads to an increase in KCC2 surface abundance, which can be further enhanced by promoting phosphorylation of GluK2.

Thus, we describe how components of excitatory synaptic transmission regulate KCC2 function in hippocampal neurons and, in doing so, reveal a new molecular target that may be exploited to enhance KCC2 function.

**Experimental procedures**

**Cultured hippocampal neurons and cell lines**

All animal procedures were performed in accordance with the University of Toronto animal care committee’s regulations. Low density cultures of dissociated mouse hippocampal neurons were prepared by removing hippocampi from postnatal day 0–1 mouse brains. Both male and female mouse pups were used to prepare cultures; pups were obtained from either wild type (C57Bl6) or GluK1/2−/− mice (129SV/C57Bl6). Hippocampi were treated with trypsin for 15 min at 37 °C, followed by gentle trituration. The dissociated cells were plated at a density of ~50,000 cells/ml on poly-d-lysine-coated 25-mm glass coverslips and placed in 35-mm Petri dishes. The cells were plated in Neurobasal medium (Invitrogen) containing 0.1% penicillin/streptomycin and 1% FBS. Twenty-four hours after plating, one-third of the medium was replaced with original plating medium and then changed again every 7 days.

**Transfection and cDNA constructs**

Neurons were transfected using TransFectin lipid reagent (Bio-Rad) at 8–10 days in culture prior to a significant up-regulation of KCC2 expression. Experiments were performed 24 h following transfection. The following constructs were transfected (noting where they were previously characterized in publication or obtained): Neto2-FL-HA, Neto2-ΔCUB1&2-HA, and Neto2-cyto-HA (10); GluK2(GluR6a)-MYC and GluK5 (Christophe Mulle, University of Bordeaux, France); GluK1-MYC (Dr. Sari Lauri, University of Helsinki, Helsinki, Finland) (51); GluN1-eGFP (Dr. Mike Salter, Sick Kids Research Institute, Toronto, Canada); GluK2-S846A/S868A-
Gluk2 regulates KCC2 surface recycling

MYC-YFP (Jeremy Henley, Bristol University, Bristol, UK) (24); KCC2-HA (35); and KCC2-FLAG.

To generate KCC2-FLAG construct containing FLAG tag in the third extracellular loop of KCC2b isoform, we used rat KCC2b cDNA subcloned previously (52) into XbaI and HindIII restriction sites of pcDNA3.1(−) expression vector. Two short oligonucleotides (kcc2_FLAG_sense [5′-CGA TTA CAA GGA TGA CGA TAA GGG TAG-3′] and kcc2_FLAG_antisense [5′-CCT TAT CTT CAT CCT TGT ATT AAT CGG TAC-3′]) were synthesized and annealed to each other. As a result, a short DNA duplex carrying 4-bp protruding 3′ ends (GTAC) on both sides and encoding FLAG tag peptide (DYKDDDDK) was formed. KCC2b-pcDNA3.1(−) plasmid vector, which is known to contain a unique KpnI restriction site in a sequence corresponding to the third extracellular loop of KCC2b, was precip with KpnI, dephosphorylated, and ligated with the preannealed FLAG tag encoding DNA duplex. Several KCC2-FLAG clones were sequenced, and those that contained FLAG tag in a correct orientation were overexpressed in HEK293 cells and subsequently analyzed by Western blotting using anti-FLAG antibodies.

Recombinant KCC2 total expression analysis

For Fig. 1, COS-7 cells were transfected with 125 ng of KCC2, in combination with increasing concentrations (corresponding to 0, 60, 125, 250, and 500 ng) of Neto2-HA full-length/Neto2-ΔCUB1&2 HA/Neto2-Δcytoplasmic tail HA/GluK2-MYC/GluK1-MYC/GluN1-eGFP using Lipofectamine (Invitrogen) at ~70% confluency. For Fig. 2A, 125 ng of KCC2 was transfected in combination with 125 ng of GluK1/GluK2/GluK5/Neto2 or 125 ng each of GluK1+GluK2/125 ng each of GluK5+GluK2/125 ng each of Neto2+GluK2. Thirty-six hours after transfection, the cells were washed with ice-cold 1× PBS and lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% DOC, and protease inhibitors; Roche). Lysed cells were incubated on ice for 30 min and were centrifuged at 15,000 ×g for 15 min at 4 °C. Cell lysates were mixed with SDS sample buffer containing 100 mM DTT, denatured at 37 °C for 1 h, and subjected to SDS/PAGE and immunoblotted.

Immunocytochemistry

For live imaging of transfected neurons and COS-7 cells, coverslips were incubated in culture medium on ice for 10 min to stop membrane trafficking. The cells were then rinsed in chilled PBS and incubated in mouse anti-FLAG or mouse anti-HA primary antibody (1 μg/ml) for 40 min. The cells were subsequently incubated in Alexa Fluor 488- or Alexa Fluor 555-conjugated rabbit anti-mouse secondary antibody to label surface expressed KCC2. To further assess total KCC2 abundance, the cells were rinsed in chilled PBS and then fixed in 4% paraformaldehyde for 12 min followed by blocking and permeabilization in 10% goat serum containing 0.1% Triton X-100 for 30 min at room temperature. Lastly, the cells were incubated in primary rabbit anti-KCC2 antibody to label the total expressed KCC2 protein, and subsequently in Alexa Fluor 555-conjugated goat anti-rabbit secondary antibody each for 45 min at room temperature. All cells were mounted in ProLong Gold Antifade (Invitrogen/Life Technologies, Ontario, Canada). Quantification of blot intensities was only performed on data obtained within a linear range of exposure.

Confocal microscopy and image analysis

Confocal images (2048 × 2048 pixels) were acquired with a Leica TCS SP8 confocal system with a Leica DMI 6000 inverted microscope using standard Leica imaging software. Images were obtained using a 40×/1.4 NA oil immersion objective by laser excitation (488-, 568-, and 647-nm wavelengths) and a 3× zoom, a pixel resolution of 100-nm xy and a z step of 0.29 μm. We analyzed fluorescence in the somatodendritic compartment (soma and the first 50 μm of the proximal dendrites). For experiments where neurons were transfected with GFP-containing constructs: neurons were selected for image acquisition if they were positive for both GFP fluorescence (indicative of transfection) as well as surface labeling and displayed a typical neuronal somatic shape and dendritic branching. For experiments lacking GFP transfection (e.g. Fig. 3), neurons were selected for image acquisition if they had KCC2-FLAG immunostaining and surface labeling and displayed a typical neuronal somatic shape and dendritic branching. COS-7 cells were selected for image acquisition if they were positive for both GFP fluorescence and surface labeling, and based on phenotypic characteristics to avoid dividing cells. Image stacks were acquired from the top to the bottom of the transfected cell. Image names were blinded and analyzed by importing acquired z stacks into Imaris 7.6.5 (Bitplane Scientific Software) where an isosurface representing the computational volume of fluorescence was generated for each image (53). These rendered isosurfaces were used for fluorescence intensity quantification and volumetric measurements of the neuronal soma and proximal dendrites included in the analysis. These values were exported to Excel, where they were used to calculate the fluorescence intensity per volume of each cell. Neuronal intensity per volume measurements for each experimental condition was normalized to neurons expressing KCC2-FLAG alone.

Recycling immunofluorescence

Recycling of internalized KCC2 co-transporters was assessed as previously described by Manna et al. (28) with minor modifications. Neurons were incubated with mouse anti-FLAG antibody (1 μg/ml) for 1.5 h at 37 °C, and COS-7 cells were incubated for 12 h at 37 °C to allow antibody labeling of all KCC2 inserted into the surface membrane during that period. The cells were rinsed in chilled PBS and washed with ice-cold acetic acid buffer (0.5 M NaCl, 0.5% acetic acid, pH 2.4). The cells were then incubated at 37 °C for 2 h to allow for membrane recycling of antibody labeled KCC2. The cells were chilled for 10 min on ice to stop membrane recycling, and incubated with Alexa Fluor 488-conjugated rabbit anti-mouse antibody (1:350; Invitrogen). The cells were then fixed with 4% paraformaldehyde and permeabilized, and all antibody-labeled KCC2 was then labeled with Alexa Fluor 555-conjugated rabbit anti-mouse secondary antibody to stain the non-recycled KCC2 protein. Fluorescence quantification and volume measurements were made as indicated above using Imaris 7.6.5.
COS-7 cells were transfected with KCC2-HA, and 24 h posttransfection, the cells were washed in PBS prior to incubating them with mouse monoclonal anti-HA antibody (1:350) at 37 °C for 20 min. The cells were washed in PBS and incubated in secondary (Alexa Fluor 555 goat anti-mouse secondary, 1:350) for 10 min. Transfected cells were detected by GFP expression, and live images were acquired using TIRF microscopy at a controlled temperature of 37 °C on an Olympus IX81 inverted microscope. Images were taken with a Hamamatsu C9100–13 EM-CCD camera and a 60 × 1.49 NA oil immersion objective using 561-nm excitation wavelength at a penetration depth of 100 nm. Velocity (PerkinElmer Life Sciences) was used for both image acquisition and analysis. GFP fluorescence was used to measure the cell area. Anti-FLAG immunofluorescence was identified on transfected cells, and using the spots identification tool in the Velocity software, the number of puncta/area was determined.

**Antibodies**

The following antibodies were used at the dilutions indicated: anti-KCC2 (rabbit, Millipore 07-432, Western blot dilution 1:1000, immunofluorescence 1 μg/ml); anti-KCC2 (mouse, UC Davis/NIMH/NeuroMab Facility, Clone N1/12, Western blot dilution 1:1000); anti-FLAG (mouse, Sigma F3165, immunofluorescence dilution 3:1000); anti-GluN1 (mouse, Millipore 05-432, Western blot dilution 1:1000); anti-HA.11 (mouse, Covance, immunofluorescence 1 μg/ml); and anti-transferrin (mouse, Invitrogen 13-6800, Western blot dilution 1:1000). Secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 555 were used for visualization at a concentration of 1:500 for live and fixed immunocytochemistry and 1:350 for the recycling assay (Life Technologies).

**Electrophysiology**

Electrophysiology was performed on neurons between 10 and 13 days in culture. Gramicidin (50 μg/ml) perforated patch clamp recordings were performed with −5 to −7 MΩ glass pipettes using an Axon Instruments Multiclamp 700B and Clampex 9.2 (Molecular Devices, Sunnyvale, CA). Cultured neurons were continuously perfused with standard extracellular solution composed of 150 mM NaCl, 3 mM KCl, 3 mM CaCl₂, 2H₂O, 2 mM MgCl₂, 6H₂O, 10 mM HEPES, 5 mM glucose, pH 7.4, osmolarity = 307–315 mOsmol. Intracellular recording solution was composed of 150 mM KCl, 10 mM HEPES, and 50 μg/ml gramicidin, pH 7.4, osmolarity = 300 mOsmol. Recordings started when the series resistance dropped below 50 MΩ. I-V curves were made by depolarizing the membrane potential in steps while simultaneously stimulating GABAergic transmission. A 10 μM GABA puff was applied to the soma using a PicoSpritzer (Parker Hannifin, Hollis, NH). A linear regression of the IPSC/amplitude was calculated to determine the reversal potential of GABA (EGABA) and the slope of that linear regression was taken as the synaptic conductance. Electrophysiological values have not been corrected for the liquid junction potential of ~7 mV.

**Statistics**

The results are given as means ± S.E. The n values represent number of neurons and are stated in the figure legends. For biochemical experiments in Figs. 1 and 2, the n values were obtained from a minimum of three independent experiments. For electrophysiology, immunocytochemistry and recycling assays, the n values were obtained from a minimum of three experiments (independently cultured coverslips); in the majority of cases, only one n value was obtained per coverslip. Statistical significance was determined using either SigmaStat or GraphPad Prism (version 5.01) software and noted as follows: *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

**Author contributions**—J. C. P. and V. M. conceived of the project, designed and conducted the experiments, analyzed and interpreted the data, and drafted the paper; C. S. K., Z. D., J. C., W. Y., M. H., A. K. C., S. J. P., and P. U. acquired and analyzed the data; M. S. A. contributed important intellectual content to experimental design; and M. A. W. drafted and revised the article critically for important intellectual content. All authors approved the final version of the manuscript.

**Acknowledgments**—We thank Dr. Christophe Mulle (Bordeaux University) for the GluK2-myc, GluK5 cDNA, Dr. Sari Lauri (University of Helsinki) for the GluK1-myc cDNA, Dr. Michael Salter (SickKids Research Institute, Toronto, Canada), and Dr. Chris Mc Bain (National Institutes of Health, Bethesda, MD) for the GluK1/2/−/− mice.

**Note added in proof**—In the version of this article that was published as a Paper in Press on February 24, 2017, Wenqing Ye and Michelle Huang were inadvertently omitted as authors. This error has now been corrected.

**References**

1. Kaila, K. (1994) Ionic basis of GABAergic channel function in the nervous system. *Prog. Neurobiol.* 42, 489–537
2. Rivera, C., Voipio, J., Payne, J. A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M., and Kaila, K. (1999) The K⁺/Cl⁻ co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 397, 251–255
3. Blesse, P., Airaksinen, M. S., Rivera, C., and Kaila, K. (2009) Cation-cliochloride cotransporters and neuronal function. *Neuron* 61, 820–838
4. Gulyás, A. I., Sik, A., Payne, J. A., Kaila, K., and Freund, T. F. (2001) The KCI cotransporter, KCC2, is highly expressed in the vicinity of excitatory synapses in the rat hippocampus. *Eur. J. Neurosci.* 13, 2205–2217
5. Chamma, I., Heubl, M., Chey, Q., Renner, M., Moutkine, I., Eugène, E., Poncer, J. C., and Lévi, S. (2013) Activity-dependent regulation of the KCl cotransporter KCC2 membrane diffusion, clustering, and function in hippocampal neurons. *J. Neurosci.* 33, 15488–15503
6. Li, H., Khiroug, S., Cai, C., Ludwig, A., Blesse, P., Kolikova, J., Ažfarov, R., Coleman, S. K., Lauri, S., Airaksinen, M. S., Keinänen, K., Khiroug, L., Saarma, M., Kaila, K., and Rivera, C. (2007) KCC2 interacts with the dendritic cytoskeleton to promote spine development. *Nature* 456, 1019–1033
7. Gauvain, G., Chamma, I., Chey, Q., Cabezaz, C., Irinopoulou, T., Bodrug, N., Carnaud, M., Lévi, S., and Poncer, J. C. (2011) The neuronal K-Cl cotransporter KCC2 influences postsynaptic AMPA receptor content and lateral diffusion in dendritic spines. *Proc. Natl. Acad. Sci. USA.* 108, 15474–15479
8. Chey, Q., Heubl, M., Goutièrre, M., Backer, S., Moutkine, I., Eugène, E., Bloch-Gallego, E., Lévi, S., and Poncer, J. C. (2015) KCC2 gates activity-

**TIRF microscopy**

Gulyás, A. I., Sík, A., Payne, J. A., Kaila, K., and Freund, T. F. (2001) The role of GABAA receptor channel function and the nervous system. *Prog. Neurobiol.* 42, 489–537

**3. Blaesse, P., Airaksinen, M. S., Rivera, C., and Kaila, K. (2009) Cation-cliochloride cotransporters and neuronal function. *Neuron* 61, 820–838
4. Gulyás, A. I., Sik, A., Payne, J. A., Kaila, K., and Freund, T. F. (2001) The KCl cotransporter, KCC2, is highly expressed in the vicinity of excitatory synapses in the rat hippocampus. *Eur. J. Neurosci.* 13, 2205–2217
5. Chamma, I., Heubl, M., Chey, Q., Renner, M., Moutkine, I., Eugène, E., Poncer, J. C., and Lévi, S. (2013) Activity-dependent regulation of the KCl cotransporter KCC2 membrane diffusion, clustering, and function in hippocampal neurons. *J. Neurosci.* 33, 15488–15503
6. Li, H., Khiroug, S., Cai, C., Ludwig, A., Blesse, P., Kolikova, J., Ažfarov, R., Coleman, S. K., Lauri, S., Airaksinen, M. S., Keinänen, K., Khiroug, L., Saarma, M., Kaila, K., and Rivera, C. (2007) KCC2 interacts with the dendritic cytoskeleton to promote spine development. *Nature* 456, 1019–1033
7. Gauvain, G., Chamma, I., Chey, Q., Cabezaz, C., Irinopoulou, T., Bodrug, N., Carnaud, M., Lévi, S., and Poncer, J. C. (2011) The neuronal K-Cl cotransporter KCC2 influences postsynaptic AMPA receptor content and lateral diffusion in dendritic spines. *Proc. Natl. Acad. Sci. USA.* 108, 15474–15479
8. Chey, Q., Heubl, M., Goutièrre, M., Backer, S., Moutkine, I., Eugène, E., Bloch-Gallego, E., Lévi, S., and Poncer, J. C. (2015) KCC2 gates activity-
Glutamate regulates KCC2 surface recycling

driven AMPA receptor traffic through cofilin phosphorylation. J. Neurosci. 35, 15772–15786
9. Mahadevan, V., Pressey, J. C., Acton, B. A., Uvarov, P., Huang, M. Y., Chevrier, J., Puchalski, A., Li, C. M., Ivakin, E. A., Airaksinen, M. S., Delpire, E., McNlnes, R. R., and Woodin, M. A. (2014) Kainate receptor coexist in a functional complex with KCC2 and regulate chloride homeostasis in hippocampal neurons. Cell Rep. 7, 1762–1770
10. Ivakin, E. A., Acton, B. A., Mahadevan, V., Ormonde, J., Tang, M., Pressey, J. C., Huang, M. Y., Ng, D., Delpire, E., Salter, M. W., Woodin, M. A., and McNlnes, R. R. (2013) Neto2 is a KCC2 interacting protein required for neuronal Cl– regulation in hippocampal neurons. Proc. Natl. Acad. Sci. U.S.A. 110, 3561–3566
11. Mahadevan, V., Dargaei, Z., Ivakin, E. A., Hartmann, A.-M., Ng, D., Chevrier, J., Ormonde, J., Nothwang, H. G., McNlnes, R. R., and Woodin, M. A. (2015) Neto2-null mice have impaired GABAergic inhibition and are susceptible to seizures. Front. Cell Neurosci. 9, 368
12. Lee, H. H., Deeb, T. Z., Walker, J. A., Davies, P. A., and Moss, S. J. (2011) NMDA receptor activity downregulates KCC2 resulting in depolarizing GABA receptor-mediated currents. Nat. Neurosci. 14, 736–743
13. Deleted in proof
14. Deleted in proof
15. Fisher, M. T., and Fisher, J. L. (2014) Contributions of different kainate receptor subunits to the properties of recombinant homomeric and heteromeric receptors. Neuroscience 278, 70–80
16. Zhang, W., St-Gelais, F., Grabner, C. P., Trinidad, J. C., Sumioka, M., Moroni, M., Lima, W. C., Wende, H., Kirchner, M., Adelfinger, L., Schrenk-Siemens, K., Tapken, D., Kucich, P. H., Gassmann, M., Roggenkamp, D., Brouchoud, C., Burlingan, W., Huang, M. Y., Ng, D., Delpire, E., Salter, M. W., Woodin, M. A. (2012) Hyperpolarizing GABAergic transmission requires the KCC2 C-terminal isoform. J. Neurosci. 32, 8746–8751
17. Schwenk, J., Zolles, G., Turecek, R., Fritzius, T., Bildl, W., Tarusch, J., Chevrier, J., Puchalski, A., Li, C. M., Ivakin, E. A., Airaksinen, M. S., Mount, D. B., and Woodin, M. A. (2012) Genetically encoded impairment of neuronal KCC2 cotransporter function in mice. Science 335, 644–648
18. Bahn, S., Volk, B., and Wisden, W. (1994) Kainate receptor gene expression in the developing rat brain. J. Neurosci. 14, 5525–5547
19. Payne, J. A., Stevenson, T. J., and Donaldson, L. F. (1996) Molecular characterization of putative K-Cl cotransporter in rat brain. J. Biol. Chem. 271, 16245–16252
20. Woo, N.-S., Lu, J., England, R., McCellan, R., Dufour, S., Mount, D. B., Deutch, A. Y., Lovinger, D. M., and Delpire, E. (2002) Hypereexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. Hippocampus. 12, 258–268
21. Kahle, K. T., Merner, N. D., Friedel, P., Sivaprasadarao, A. (2010) Constitutive endocytic recycling and protein kinase C-mediated lysosomal degradation control K(ATP) channel surface density. J. Biol. Chem. 285, 5963–5973
22. Kahl, R. W., Dufour, S., Mount, D. B., Deutch, A. Y., Lovinger, D. M., and Delpire, E. (2002) Hypereexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. Hippocampus. 12, 258–268
23. Konopacki, F. A., Jaafari, N., Rocca, D. L., Wilkinson, K. A., Chamberlain, S., Rubin, P., Kattamneni, S., Mellor, J. R., and Henley, J. M. (2011) Agonist-induced PKC phosphorylation regulates GluK2 SUMOylation and kainate receptor endocytosis. Proc. Natl. Acad. Sci. U.S.A. 108, 19772–19777
24. González-González, I. M., and Henley, J. M. (2013) Postsynaptic kainate receptor recycling and surface expression are regulated by metabotropic autoreceptor signalling. Traffic. 14, 810–822
25. Yan, S., Sanders, J. M., Xu, J., Zhu, Y., Contractor, A., and Swanson, G. T. (2004) A C-terminal determinant of GluR6 kainate receptor trafficking. J. Neurosci. 24, 679–691
26. Pahl, S., Tałpek, D., Haering, S. C., and Hollmann, M. (2014) Trafficking of kainate receptors. Membranes (Basel) 4, 565–595
27. Manna, P. T., Smith, A. J., Taneja, T. K., Howell, G. J., Lippiat, J. D., and Sivaprasadarao, A. (2010) Constitutive endocytic recycling and protein
47. Cellot, G., and Cherubini, E. (2014) GABAergic signaling as therapeutic
target for autism spectrum disorders. *Front. Pediatr.* 2, 70
48. Tao, R., Li, C., Newburn, E. N., Ye, T., Lipska, B. K., Herman, M. M.,
Weinberger, D. R., Kleinman, J. E., and Hyde, T. M. (2012) Transcript-
specific associations of SLC12A5 (KCC2) in human prefrontal cortex with
development, schizophrenia, and affective disorders. *J. Neurosci.* 32,
5216–5222
49. Boulenguez, P., Liabeuf, S., Bos, R., Bras, H., Jean-Xavier, C., Brocard, C.,
Stil, A., Darbon, P., Cattaert, D., Delpire, E., Marsala, M., and Vinay, L.
(2010) Down-regulation of the potassium-chloride cotransporter KCC2
contributes to spasticity after spinal cord injury. *Nat. Med.* 16,
302–307
50. Coull, J. A., Boudreau, D., Bachand, K., Prescott, S. A., Nault, F., Sík, A., De
Koninck, P., and De Koninck, Y. (2003) Trans-synaptic shift in anion gra-
dient in spinal lamina 1 neurons as a mechanism of neuropathic pain.
*Nature* 424, 938–942
51. Vesikansa, A., Sakha, P., Kuja-Panula, J., Molchanova, S., Rivera, C., Hutt-
tunen, H. J., Rauvala, H., Taira, T., and Lauri, S. E. (2012) Expression of
GluK1c underlies the developmental switch in presynaptic kainate recep-
tor function. *Sci. Rep.* 2, 310
52. Uvarov, P., Ludwig, A., Markkanen, M., Pruunsild, P., Kaila, K., Delpire, E.,
Timmusk, T., Rivera, C., and Airaksinen, M. S. (2007) A novel N-terminal
isoform of the neuron-specific K-Cl cotransporter KCC2. *J. Biol. Chem.*
282, 30570–30576
53. Caster, A. H., and Kahn, R. A. (2012) Computational method for calculat-
ing fluorescence intensities within three-dimensional structures in cells. *Cell Logist.* 2, 176–188

**GluK2 regulates KCC2 surface recycling**