Neto2 is a KCC2 interacting protein required for neuronal Cl^- regulation in hippocampal neurons

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KCC2 is a neuron-specific K^+–Cl^- cotransporter that is essential for Cl^- homeostasis and fast inhibitory synaptic transmission in the mature CNS. Despite the critical role of KCC2 in neurons, the mechanisms regulating its function are not understood. Here, we show that KCC2 is critically regulated by the single-pass transmembrane protein neuropilin and tolloid like-2 (Neto2). Neto2 is required to maintain the normal abundance of KCC2 and specifically associates with the active oligomeric form of the transporter. Loss of the Neto2:KCC2 interaction reduced KCC2-mediated Cl^- extrusion, resulting in decreased synaptic inhibition in hippocampal neurons.

chloride | GABA

In the mature CNS, inhibition primarily occurs via $GABA_A$ receptor ($GABA_AR$)-mediated synaptic transmission. The action of GABA depends on the concentration of intracellular Cl^- ($[Cl^-]_i$): when the $[Cl^-]_i$ is high during embryonic development, $GABA_AR$ activation results in Cl^- efflux, membrane depolarization, and excitatory synaptic transmission; whereas low $[Cl^-]_i$ results in Cl^- influx, membrane hyperpolarization, and thus inhibitory synaptic transmission (1–3). The low $[Cl^-]_i$ that makes fast $GABA_AR$-mediated synaptic inhibition possible is maintained by the neuron-specific K^+–Cl^- cotransporter KCC2 (4), a member of the cation-chloride cotransporter SLC12 gene family (5). KCC2 is essential for survival, as KCC2 knockout mice die immediately at birth due to respiratory failure (6). In the adult nervous system, decreased KCC2 expression correlates with neuropathic pain, spasticity following spinal cord injury, and epileptic seizures (5, 7–11).

Based on the critical importance of KCC2 in the brain, an understanding of the mechanisms that promote and maintain KCC2 expression and efficacy is essential for designing therapeutic strategies to treat neurological disorders characterized by KCC2 dysfunction and the subsequent loss of inhibitory synaptic transmission. A major limitation in the development of these strategies is a lack of understanding regarding the cellular mechanisms regulating KCC2. In particular, KCC2 interacting proteins required for KCC2 transport and function in the mature CNS have not been identified, which represents a large gap in our fundamental knowledge regarding neuronal Cl^- regulation and inhibitory synaptic transmission.

Here, we identify neuropilin and tolloid like-2 (Neto2) as a KCC2 interacting protein in vivo and demonstrate that this interaction is required for normal neuronal Cl^- homeostasis. Neto2 is a complement C1r/C1s, Uegf, Bmp1 (CUB) domain-containing transmembrane protein abundantly expressed in neurons (12), which is important for proper neurological function (13). CUB domains are evolutionarily conserved protein domains (14) that participate in protein–protein interactions (15, 16). In the present study, we performed an unbiased proteomic screen and discovered that Neto2 interacts with KCC2. We elucidated the role of this association and characterized its importance in the normal neurophysiological function of KCC2. Because Neto proteins were previously identified as auxiliary subunits of ionotropic glutamate receptors, including kainate and NMDA receptors (17–21), this study further extends the role of the Neto proteins to inhibitory synapses.

Results

Neto2 and KCC2 Interact in Vivo. Recent reports have demonstrated that Neto2 and its homologous protein Neto1 have important functions at excitatory synapses. Neto1 interacts with both the *N*-methyl D-aspartate receptor (NMDAR) and kainate receptor complexes (17, 20, 22), whereas Neto2 was shown to be an auxiliary subunit of the neuronal kainate receptor (18, 21–23). As the Neto proteins appear to have multiple functions in the nervous system, we chose to explore additional roles these proteins might have by conducting an unbiased screen to identify proteins that interact with the Neto proteins. We performed a GST pull-down experiment on adult mouse brain membrane fractions using a GST-$Neto2_{cyto}$ fusion protein as bait, followed by liquid chromatography/mass spectrometry. A 145-kDa band was enriched in the GST-$Neto2_{cyto}$ lane compared with the GST lane; tryptic digest and peptide mapping of this band identified two peptides of the KCC2 protein (Fig. 1*A*). To confirm this putative interaction, we tested whether Neto2 and KCC2 associate using a coimmunoprecipitation approach. Using whole-brain membrane preparations, we found that anti-Neto2 antibodies coimmunoprecipitated KCC2 protein from wild-type but not from Neto2-null mice (Fig. 1*B*). In contrast, we did not detect a coimmunoprecipitation between Neto2 and the other major regulator of neuronal chloride homeostasis, NKCC1 (8), or the other KCC family member abundantly expressed in neurons, KCC3 (24). Reciprocally, anti-KCC2 antibodies coimmunoprecipitated Neto2 from wild-type membrane fraction protein preparations (Fig. 1*C*). These results demonstrated that Neto2 is a KCC2 interacting protein in vivo.

Both CUB1 and CUB2 Domains of Neto2 Are Required for Interaction with KCC2. Neto2 has two CUB domains, a low-density lipoprotein-α (LDLα) domain, a transmembrane region, and a cytoplasmic tail (Fig. 2*A*). To determine the portion of Neto2 required for interaction with KCC2, we used a series of Neto2 deletion proteins in HEK-293 cells (Fig. 2*A*). Neto2 lacking the entire cytoplasmic region was still able to coimmunoprecipitate with KCC2 (Neto2-Δcyto; Fig. 2*B*, lane 2), suggesting that this domain is not required for binding to the transporter. In contrast, no interaction with KCC2 was observed when the entire ectodomain of Neto2

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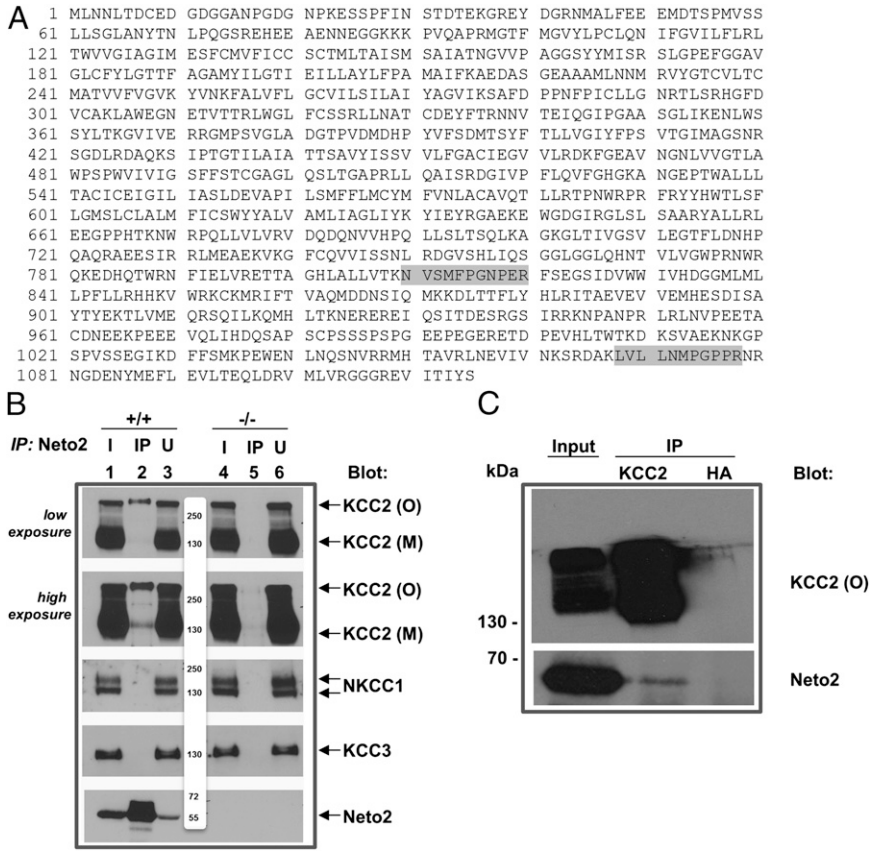

Fig. 1. Neto2 associates with KCC2 in vivo. (*A*) Peptides of the KCC2 protein identified by mass spectrometry. Tryptic digest and peptide mapping of the 145-kDa band, enriched in the GST-Neto2$_{cyto}$ lane compared with the GST lane identified two peptides of the KCC2 protein (highlighted). (*B*) Immunoblot of immunoprecipitates from adult wild-type (+/+) and Neto2-null (–/–) brain membrane fractions. Blot, antibody used for immunoblot analysis; I, Input; IP, Immunoprecipitate; U, Unbound fraction. Both the Input and the Unbound fraction for the immunoblot is 2% of the volume of sample used in the immunoprecipitation experiment. KCC2 (O) and KCC2 (M) represent KCC2 oligomeric and monomeric forms, respectively. The nonspecific band (lane 2) that migrates higher than the oligomeric form of KCC2 can be detected using the secondary antibody alone. (*C*) Immunoblot of immunoprecipitate from wild-type brain membrane fractions using either anti-KCC2 or an unrelated anti-HA antibody. The input for the immunoblot is 2% of the volume of sample used in the immunoprecipitation experiment.

was removed (Neto2-Δecto; Fig. 2 *B*, lane 3, and *C*, lane 2). Removal of the LDLα domain did not abolish the Neto2:KCC2 interaction (Neto2-ΔLDLα; Fig. 2*C*, lane 3), whereas removal of the first or second CUB domain markedly reduced the amount of KCC2 that coimmunoprecipitated with the deletion proteins (Neto2-ΔCUB1 and Neto2-ΔCUB2; Fig. 2*C*, lanes 4 and 5). Taken together, our results demonstrate that the Neto2:KCC2 interaction depends upon CUB1 and CUB2 domains of Neto2.

Neto2-Null Mice Have Depolarized E_{GABA} Compared with Wild-Type Mice. Having established that Neto2 interacts with KCC2, we next asked whether Neto2 regulates KCC2 function. Based on the critical role of KCC2 in extruding neuronal Cl^-, we examined KCC2 function by recording the reversal potential of GABAergic transmission (E_{GABA}), which is principally determined by $[Cl^-]_i$. Using the gramicidin perforated patch-clamp technique to record E_{GABA} without altering $[Cl^-]_i$, we found that hippocampal neurons cultured from Neto2-null mice had significantly depolarized E_{GABA}, in comparison with wild type (Fig. 3*A*; wild type: –75.2 ± 2.1 mV, $n = 14$; *Neto2*$^{-/-}$: –53.7 ± 2.4 mV, $n = 11$; $P < 0.001$). Remarkably, in some neurons, the depolarization of E_{GABA} reached levels above the threshold for action potentials, thus rendering GABA excitatory (2 of 11 neurons). The depolarization of E_{GABA} in *Neto2*$^{-/-}$ neurons, relative to wild-type neurons, was not accompanied by significant changes in any other neuronal property that we examined electrophysiologically, including resting membrane potential, input resistance (IR), action potential (AP) amplitude, or AP threshold (Table S1) except AP frequency (Table S2); nor were there significant differences in spontaneous neuronal activity (Fig. S1). Taken together, these results indicate that the loss of Neto2 depolarizes E_{GABA} relative to wild-type neurons.

Depolarized E_{GABA} in Neto2-Null Mice Is KCC2 Dependent. If the depolarization of E_{GABA} in Neto2-null mice, relative to wild-type mice, is due to the dysfunction of KCC2 resulting from the loss of the Neto2:KCC2 interaction, then suppressing KCC2 function should produce a significantly smaller depolarization of E_{GABA} in Neto2-null mice compared with wild-type mice. To test this prediction, we compared the change in E_{GABA} between wild-type and *Neto2*$^{-/-}$ neurons, in response to both the specific KCC2 antagonist VU0240551 (25 μM) (25) and siRNA-mediated silencing of KCC2. As we expected, pharmacological inhibition of KCC2 with VU0240551 produced a significantly larger change in E_{GABA} in wild-type neurons compared with *Neto2*$^{-/-}$ neurons (Fig. 3B_i; $P < 0.001$). siRNA-mediated silencing of KCC2 also produced a significantly larger change in E_{GABA} in wild-type neurons compared with *Neto2*$^{-/-}$ neurons (Fig. 3B_{ii}; $P < 0.05$). Importantly, transfecting wild-type and *Neto2*$^{-/-}$ neurons with a nonsilencing control plasmid produced no significant change in E_{GABA} ($P < 0.05$). Taken together, suppressing KCC2 function, both pharmacologically and using a siRNA interference approach, revealed a significantly smaller effect on E_{GABA} in Neto2-null mice, suggesting that the absence of the Neto2:KCC2 interaction occluded the depolarizing effect of KCC2 suppression on E_{GABA} that occurred in wild-type neurons.

The other major cation-chloride cotransporter in neurons that functions under isotonic conditions is the Na^+–K^+–$2Cl^-$ cotransporter (NKCC1). Although NKCC1 is expressed only at low levels in mature neurons, it was conceivable that the loss of Neto2 altered E_{GABA} through a regulation of NKCC1. To test this prediction, we inhibited NKCC1 with 10 μM bumetanide, and again compared the change in E_{GABA} between wild-type and *Neto2*$^{-/-}$ neurons. As expected, there was no significant difference in the amount of E_{GABA} hyperpolarization produced by NKCC1 inhibition (Fig. 3B_{iii}; $P = 0.113$); however, the hyperpolarization in wild-type and *Neto2*$^{-/-}$ neurons reflects the presence of some functional NKCC1 in both membranes. Taken together, these experiments demonstrate that the decrease in neuronal Cl^--regulation and subsequent depolarization of E_{GABA} in neurons from Neto2-null mice, compared with wild types, is dependent on

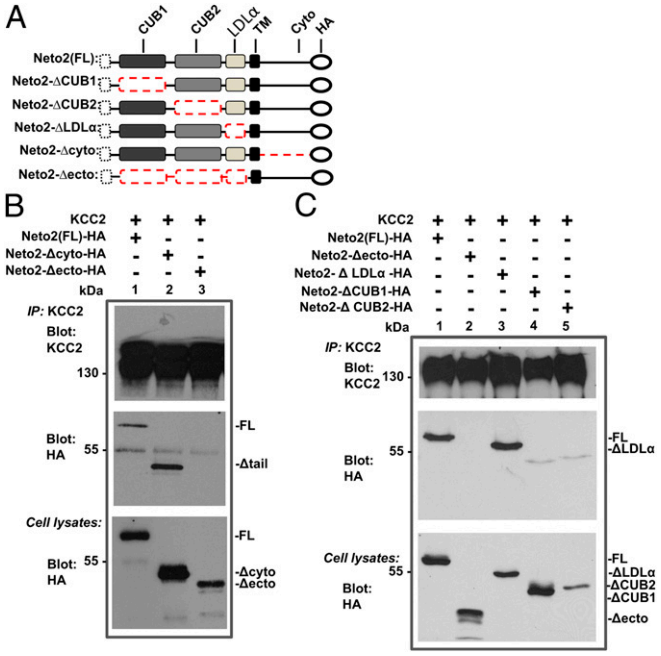

Fig. 2. CUB1 and CUB2 domains of Neto2 are required for the interaction with KCC2. (*A*) Cartoon diagram of deletion proteins: the dashed-line boxes represent the domain that has been deleted from the full-length protein. (*B* and *C*) Immunoblot of immunoprecipitates from transfected HEK-293 cell lysates. The identities of the transfected cDNAs are indicated above each lane. Blot, antibody used for immunoblot analysis; HA, anti-hemagglutinin antibody; IP, antibody used for immunoprecipitation. Similar results were observed in each of three experiments.

KCC2. This result is consistent with the lack of interaction between Neto2 and NKCC1 demonstrated in Fig. 1*B*.

Reintroduction of Neto2 in *Neto2*$^{-/-}$ Neurons Rescues E_{GABA}. If the depolarized E_{GABA} in Neto2-null mice was directly due to the loss of Neto2, then transfecting cultured *Neto2*$^{-/-}$ hippocampal neurons with a DNA construct containing full-length Neto2 (FL-Neto) should hyperpolarize E_{GABA}. When FL-Neto was introduced into these neurons, E_{GABA} significantly hyperpolarized in comparison with *Neto2*$^{-/-}$ neurons (Fig. 4*A*; *Neto2*$^{-/-}$: −55.8 ± 3.9 mV, $n = 9$; FL-Neto2 rescue: −73.2 ± 3.1 mV, $n = 9$; $P < 0.001$). Transfecting Neto2-null neurons with a control vector produced no significant change in E_{GABA} compared with *Neto2*$^{-/-}$ neurons (Fig. 4A_{ii}; −58.6 ± 2.3 mV, $n = 5$; $P = 0.503$). These results indicate that the depolarization of E_{GABA} observed in Neto2-null mice can be rescued by reintroducing the protein, which confirms that the alterations in E_{GABA} observed in the Neto2-null mice is due to the loss of the Neto2 protein.

In the initial screen in which we identified that KCC2 interacts with Neto2 (Fig. 1*A*), we used the Neto2 cytoplasmic tail as bait. We next asked whether this domain was capable of rescuing the depolarization of E_{GABA} we recorded in *Neto2*$^{-/-}$ neurons. When we transfected *Neto2*$^{-/-}$ neurons with the construct containing the cytoplasmic tail of Neto2, we observed a significant change in E_{GABA} compared with *Neto2*$^{-/-}$ neurons recorded from untransfected neurons in the same culture dishes (Fig. 4*B*; *Neto2*$^{-/-}$: −53.1 ± 3.3 mV, $n = 8$; cyto-Neto2 rescue: −68.2 ± 3.1 mV, $n = 7$; $P = 0.005$). Because the construct containing the cytoplasmic tail did not contain a fluorescent reporter, we visualized transfection by cotransfecting with a GFP-containing construct. As a positive control, we also cotransfected this GFP construct and FL-Neto2, and as we observed above in Fig. 4*A*, FL-Neto2 could rescue E_{GABA} (Fig. 4B_{ii}; *Neto2*$^{-/-}$: −53.1 ± 3.3 mV, $n = 8$; FL-Neto2 rescue: −70.3 ± 2.2 mV, $n = 6$; $P = 0.002$). These results demonstrate that the depolarization of E_{GABA} recorded in *Neto2*$^{-/-}$ neurons can be rescued by reintroducing Neto2 protein devoid of the ectodomain. This observation is important because the cytoplasmic tail of Neto2 was dispensable for the interaction of Neto2 and KCC2 in heterologous cells.

Neto2 Is Required to Maintain Efficient KCC2-Mediated Cl$^-$ Extrusion. We have shown that the loss of Neto2 produces a decrease in KCC2-mediated Cl$^-$ extrusion, but is Neto2 required on an ongoing basis to maintain efficient Cl$^-$ regulation? To address this question, we used a Neto2-specific silencing shRNA. If the sole function of Neto2 was to initially up-regulate KCC2, we would not expect that silencing of Neto2 in neurons already expressing KCC2 would alter E_{GABA}. When mature hippocampal cultured wild-type neurons were transfected with the silencing Neto2 shRNA construct, E_{GABA} depolarized relative to untransfected neurons (Fig. 4*C*; wild type: −66 ± 3.1 mV, $n = 8$; Neto2 silenced: −54.4 ± 2.2 mV, $n = 8$; $P = 0.016$). Transfecting neurons with a scrambled shRNA as a gene silencing control produced no significant change in E_{GABA} (Fig. 4*C*; −64.7 ± 2.9 mV, $n = 5$) compared with wild-type neurons ($P = 0.936$). Thus, Neto2 is required on an ongoing basis to maintain efficient KCC2-mediated Cl$^-$ extrusion.

KCC2 Protein Levels Are Reduced in Neto2-Null Neurons. To determine whether the loss of Neto2 leads to reduced KCC2 protein levels, we examined levels of KCC2 from wild-type and Neto2-null mice. As exemplified in Fig. 5A_i, total KCC2 levels are significantly reduced in neurons from Neto2-null mice compared with wild-type neurons ($P = 0.002$, $n = 6$; Fig. 5A_{iv}). This reduction in total KCC2 levels resulted from significant reductions in both monomeric ($P = 0.029$, $n = 4$; Fig. 5A_{iii}) and oligomeric ($P = 0.029$, $n = 4$; Fig. 5A_{ii}) KCC2. There was no significant change in either NKCC1 or ATP1A3 ($P > 0.05$, $n = 4$; Fig. 5A_i). To investigate whether the decrease in KCC2 protein levels was due to transcriptional regulation, we next determined relative *KCC2* mRNA abundance using quantitative real-time PCR. No significant differences were observed between wild-type and Neto2-null samples ($P = 0.67$,

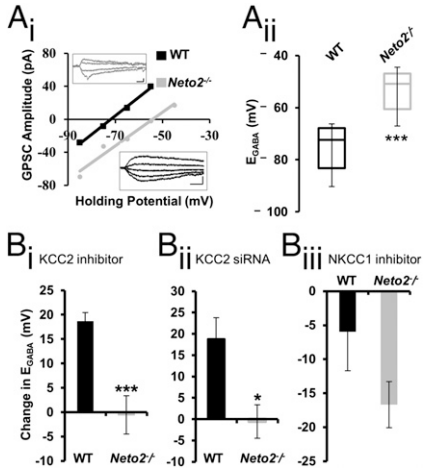

Fig. 3. Neto2-null mice have depolarized E_{GABA} compared with wild-type mice. (A_i) Example of gramicidin perforated patch-clamp recordings obtained from neurons in dissociated culture prepared from hippocampus of Neto2-null mice (gray) and wild-type mice (black). The GABAergic postsynaptic current (GPSC) amplitude was plotted against the holding (membrane) potential of the postsynaptic membrane, and the intercept of this curve with the *x*-axis was taken as E_{GABA}. (*Insets*) Traces of GPSCs for the examples shown. (Scale bars: 20 pA, 50 ms.) (A_{ii}) Summary of all experiments similar to A_i. (B_i) Bar graph illustrating the change in E_{GABA} in response to 25 μM VU0240551 obtained from wild-type and *Neto2*$^{-/-}$ neurons. (B_{ii}) Bar graph illustrating the change in E_{GABA} in response to siRNA-targeted to KCC2 obtained from wild-type and *Neto2*$^{-/-}$ neurons. (B_{iii}) Bar graph illustrating the change in E_{GABA} in response to 10 μM bumetanide obtained from wild-type and *Neto2*$^{-/-}$ neurons. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

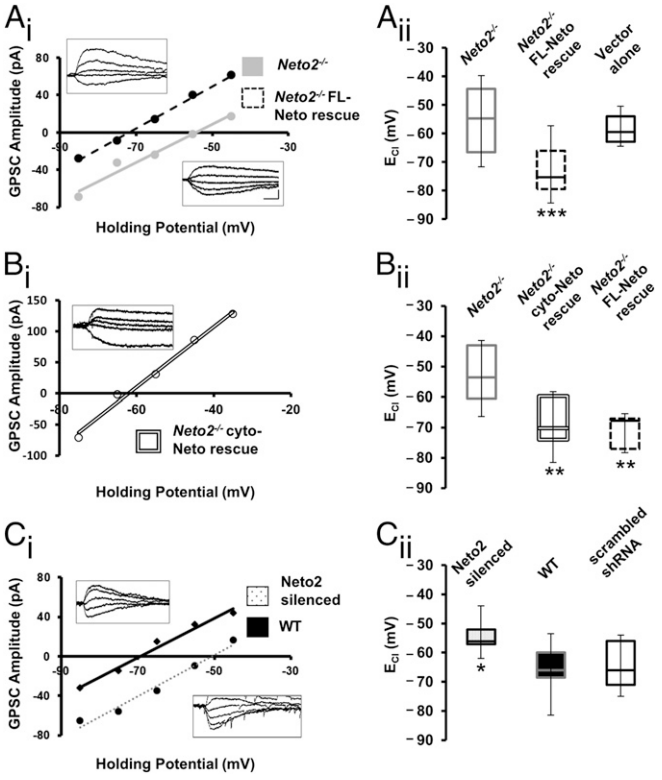

Fig. 4. Reintroduction of Neto2 in *Neto2*$^{-/-}$ neurons rescues E_{GABA}. (A_i) Example of gramicidin perforated patch-clamp recordings of E_{GABA} obtained from *Neto2*$^{-/-}$ neurons (gray) and *Neto2*$^{-/-}$ neurons transfected with full-length Neto2 (*Neto 2*$^{-/-}$ FL-Neto rescue; dashed line). (*Insets*) Traces of GPSCs for the examples shown. (Scale bar: 40 pA, 100 ms.) (A_{ii}) Summary of all experiments similar to A_i. (B_i) Example of E_{GABA} recordings (similar to A_i) obtained from *Neto2*$^{-/-}$ neurons transfected with the cytoplasmic tail of Neto2 (*Neto2*$^{-/-}$ cyto-Neto rescue; double line). (Scale bars: 30 pA, 50 ms.) (B_{ii}) Summary of all experiments similar to B_i. (C_i) Example of E_{GABA} recordings (similar to A_i) obtained from wild-type neurons (black) and neurons transfected with a Neto2 shRNA (Neto2 silenced; stippled). (Scale bars: 30 pA, 50 ms.) (C_{ii}) Summary of all experiments similar to C_i. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

$n = 4$), suggesting that posttranscriptional mechanisms are responsible for the KCC2 protein decrease in *Neto2* $^{-/-}$ neurons.

To further investigate whether an overall neuronal distribution of KCC2 differed between wild-type and *Neto2*$^{-/-}$ neurons, we performed immunofluorescent staining of cultured hippocampal neurons with anti-KCC2 antibody. The pattern of KCC2 immunoreactivity differed between the wild-type and *Neto2*$^{-/-}$ neurons (Fig. 5*B*). We quantified both the peak KCC2 fluorescence intensity, which occurred at the somatic periphery, as well as the intensity throughout the soma. Compared with wild-type neurons, *Neto2*$^{-/-}$ neurons had a 27% decrease in fluorescence intensity in the soma (Fig. 5C_{ii}; $P = 0.009$, $n = 13$), and a 53% decrease in the peak fluorescence intensity (Fig. 5C_i; $P < 0.001$, $n = 13$). Fig. S2 provides an example of the immunofluorescence analysis. Taken together, our immunoblot and immunofluorescence results demonstrate that Neto2-null mice have decreased KCC2 levels and altered KCC2 distribution throughout the neurons.

Neto2 Interacts with Oligomeric KCC2 and Regulates Cl$^-$ Extrusion Efficacy. We still observed KCC2 in the membrane of *Neto2*$^{-/-}$ neurons (Fig. 5), so we next asked whether this residual protein was still capable of extruding Cl$^-$. Functional changes in the Cl$^-$ extrusion capacity of KCC2 can be examined in the presence of an ionic load (8, 26, 27). We examined the function of KCC2 in Neto2-null mice by loading the neuronal soma with 50 mM Cl$^-$ (using a patch pipette). Neurons from wild-type mice loaded with Cl$^-$ had a measured E_{GABA} of −30.8 ± 4.6 mV ($n = 7$), whereas *Neto2*$^{-/-}$ neurons showed a depolarized E_{GABA} of −0.18 ± 7.9 mV ($n = 9$). When KCC2 was pharmacologically inhibited with VU02450551 (25 μM), we found a significant difference in the change in E_{GABA} between wild-type and *Neto2*$^{-/-}$ neurons (Fig. 6A_i; wild type: 23.2 ± 5 mV, $n = 10$; *Neto2*$^{-/-}$: −4.9 ± 4.9 mV, $n = 14$; $P = 0.001$). Next, we verified the effect of pharmacological inhibition of KCC2 on E_{GABA} using KCC2 siRNA, and found a significant difference in the change in E_{GABA} between wild-type and *Neto2*$^{-/-}$ neurons (Fig. 6A_{ii}; wild type: 19.7 ± 4.6 mV, $n = 8$; *Neto2*$^{-/-}$: −4.4 ± 2.7 mV, $n = 7$; $P < 0.001$). Taken together, the suppression of KCC2 by both pharmacological inhibition and siRNA interference, produced a significantly smaller change in E_{GABA} in *Neto2*$^{-/-}$ neurons compared with wild-type neurons, consistent with a lack of functional KCC2 in the membrane of *Neto2*$^{-/-}$ neurons. The E_{GABA} values recorded in the presence of the KCC2 antagonist are more depolarized than the Nernst equation would predict (~28 mV). However, the predicted value does not take into consideration transporter activity. When the Cl$^-$ loading experiments were performed in the presence of the NKCC1 antagonist bumetanide (10 μM), the significant difference in the change in E_{GABA} between WT and *Neto2*$^{-/-}$ neurons was still present (Fig. 6A_{iii}; WT bumetanide: −6.5 ± 1.6 mV, $n = 6$; *Neto2*$^{-/-}$ bumetanide: –23.7 ± 3.5 mV, $n = 7$; $P = 0.002$). These experiments demonstrate that loss of Neto2 leads to a reduction in the Cl$^-$ extrusion efficacy of the remaining KCC2.

Efficient KCC2-mediated Cl$^-$ extrusion occurs when KCC2 exists in the oligomerized state (28–30). We, therefore, tested whether Neto2 could associate with the active oligomeric fraction of KCC2. Under coimmunoprecipitation conditions preserving native complexes, the majority of Neto2 coimmunoprecipitated with KCC2 that was bound to the oligomeric form of the transporter (Fig. 6*B*). Taken together with our electrophysiological recordings, these data suggest that association of Neto2 with

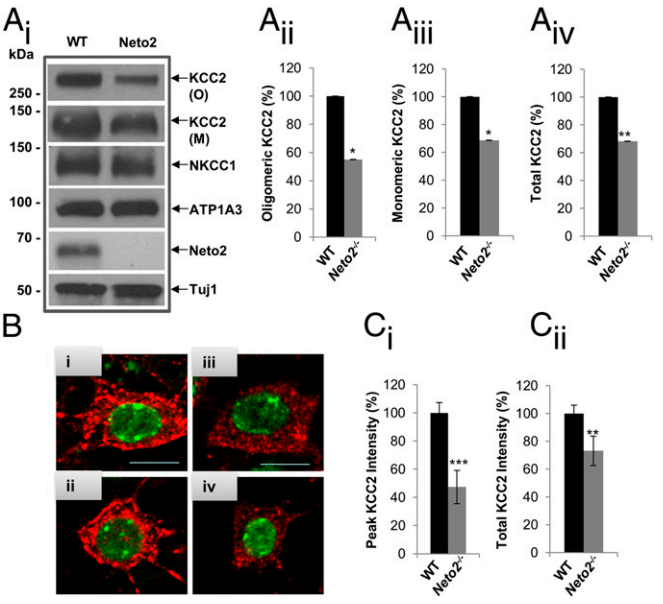

Fig. 5. KCC2 protein levels are decreased in Neto2-null mice. (A_i) Immunoblots of lysates prepared from wild-type (+/+) and Neto2-null (–/–) hippocampi. (A_{ii}–A_{iv}) Quantification of all experiments similar to A_i, for oligomeric, monomeric, and total KCC2 levels. (*B*) Confocal microscopic immunofluorescent images of cultured hippocampal neurons from wild-type (B_i and B_{ii}) and Neto2-null (B_{iii} and B_{iv}) mice stained with anti-KCC2 antibody (red) and DAPI (green). (Scale bars: 12.5 μm.) Summary of the average peak KCC2 intensity (C_i) and the average total KCC2 intensity (C_{ii}) for all neurons similar to those in *B*. The average peak and total KCC2 intensities of wild-type neurons was taken as 100%. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

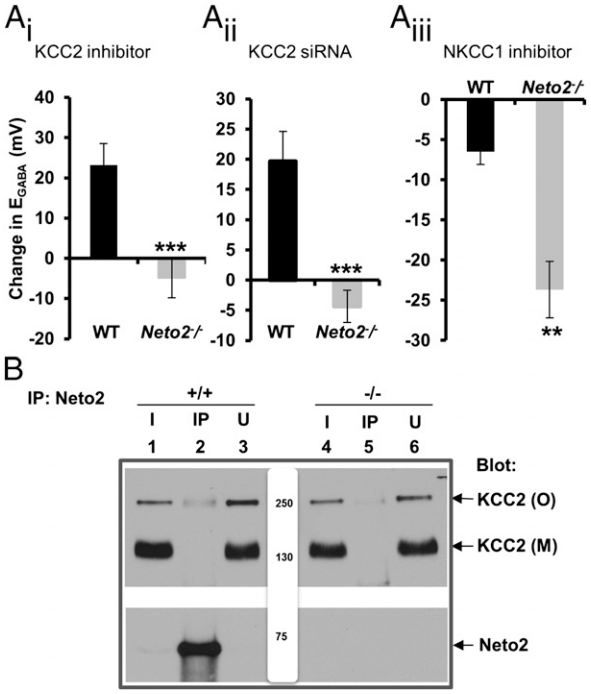

Fig. 6. Neto2 increases Cl^- transport efficacy through association with active oligomeric form of KCC2. (*A*) Summary of all whole-cell recordings obtained from neurons loaded at the soma with 50 mM Cl^-. Recordings were made from wild-type neurons (black), and *Neto2*$^{-/-}$ neurons (gray) in the presence of (*A*$_i$) 25 μM VU0240551, (*A*$_{ii}$) transfected with siRNA KCC2, and (*A*$_{iii}$) 10 μM bumetanide. (*B*) Neto2 predominantly associates with the oligomeric form of KCC2. Immunoblots of immunoprecipitates from adult wild-type (+/+) and Neto2-null (–/–) brain membrane fractions prepared under conditions to preserve oligomeric structure of membrane proteins. Blot, antibody used for immunoblot analysis; I, Input; IP, Immunoprecipitate; U, Unbound fraction. Both the Input and the Unbound fraction for the immunoblot is 2% of the volume of sample used in the immunoprecipitation experiment. KCC2 (O) and KCC2 (M) represent oligomeric and monomeric forms of the transporter. Similar results were obtained from three separate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the active oligomeric form of KCC2 results in the increase in the efficacy of the transporter.

Our results demonstrate that the functional significance of the Neto2–KCC2 interaction is to promote proper KCC2-mediated Cl^- extrusion in hippocampal neurons. However, KCC2 also plays an additional role in regulating neuronal morphology (5, 31, 32). We tested the possibility that disrupting the Neto2–KCC2 interaction also affects neuronal morphology by performing a Scholl analysis, and found no significant difference between the number of dendritic intersections between WT and *Neto2*$^{-/-}$ neurons (Fig. S3*A*; $P = 0.6$). In addition, we also examined soma size, neuronal density, and cell death (using propidium iodide); however, we found no significant differences in any of these parameters between wild-type and *Neto2*$^{-/-}$ neurons (soma size: Fig. S3*A*, $P = 0.315$; neuronal density: Fig. S3*B*, $P = 0.96$; cell death: Fig. S3*C*, $P = 0.07$). In summary, disruption of the Neto2–KCC2 interaction produced no significant differences in neuronal survival or morphology, leaving the main functional significance of this study that the Neto2–KCC2 interaction is primarily important for the maintenance of proper KCC2-mediated Cl^- extrusion.

Discussion

We have discovered that Neto2 is a KCC2 interacting protein that is required for proper KCC2-mediated Cl^- extrusion in hippocampal neurons. Neto2 regulates KCC2 in a twofold manner, by maintaining its abundance, and by enhancing its efficacy to extrude Cl^- through binding to the active oligomeric form of KCC2. Consequently, the Neto2–KCC2 interaction is required for proper inhibition. Our study identifies a KCC2 interacting protein that is required for normal neuronal Cl^- homeostasis in the brain.

The identification of the Neto2–KCC2 interaction represents the discovery of a protein that interacts with KCC2 in vivo and is critically required for normal neuronal Cl^- homeostasis in the mature CNS. Before this, only a few KCC2 interactions have been determined, and most of these were demonstrated in either nonneuronal cells or were shown to be nonspecific to KCC2: (*i*) brain-type creatine kinase was shown to associate with KCC2 in vitro and increase KCC2 activity in heterologous HEK-293 cells (33, 34); (*ii*) protein associated with Myc (35); and *iii*) cation-chloride cotransporter interacting protein 1 (CIP1) (36) were determined to interact with KCC2 by measuring $^{86}Rb/K^+$ uptake assays in heterologous HEK-293 cells, and in the case of CIP1 the interaction was not limited to KCC2 (36). KCC2 also interacts with a member of the Na^+-K^+-ATPase family, Atp1a2 (37); however, this regulation may be limited to the respiratory center of the brainstem (6), because neuronal expression of Atp1a2 dramatically decreases during maturation of the CNS (38). Last, KCC2 interacts to the cytoskeleton-associated protein 4.1N, but this interaction does not regulate KCC2-mediated Cl^- extrusion, but rather functions to promote spine development (32). Thus, our data demonstrate the existence of a KCC2 interacting protein that is required for proper neuronal Cl^- regulation in the mature CNS.

The ability of KCC2 to interact with the cytoskeleton-associated protein 4.1N (31, 32), and the requirement for KCC2 in the morphological maturation of cortical neurons (39), indicates that KCC2 plays important roles in neuronal morphology. However, these roles may be independent of KCC2's ability to extrude Cl^- (31, 32). In the present study, we found Neto2-deficiency did not affect basic neuronal morphology (Fig. S3), suggesting that the primary role of Neto2 in regulating KCC2 is restricted to its Cl^- extrusion function, and is likely independent of its morphological roles mediated by 4.1N.

During embryonic development, NKCC1 is highly expressed in the membrane, and as a result GABA acts as an excitatory neurotransmitter (40, 41). However, during the first few weeks of postnatal development, there is a significant increase in KCC2 expression that results in a hyperpolarizing shift in E_{GABA} (4, 5). Thus, the switch from GABAergic excitation to inhibition results from a postnatal developmental increase in KCC2. For Neto2 to play an important role in KCC2 regulation, we would expect that Neto2 be expressed in a similar time course. In fact, Michishita et al. (12) found that Neto2 mRNA expression was first detected at embryonic day 15, and increased its expression between postnatal day 5 (P5) and P21. This expression pattern closely parallels the expression of KCC2 during the same developmental period.

We have discovered that Neto2 regulates KCC2-mediated Cl^- homeostasis through two essential mechanisms. First, our biochemical and immunofluorescence data reveal that Neto2 is required for maintaining KCC2 in the neuronal membrane. The reduction of KCC2 in the membrane that occurs in Neto2-null mice led us to consider whether the KCC2 that remained was still capable of efficient Cl^- extrusion. We obtained compelling lines of converging evidence in support of decreased Cl^- extrusion in Neto2-null mice. We determined biochemically that Neto2 preferentially interacts with oligomerized KCC2. KCC2 oligomerization, which depends on tyrosine phosphorylation of the C terminus (29), correlates with the maturation of inhibitory neurotransmission in the CNS (28), and is believed to play a major role in the change from the inactive to the active state of this transporter (5, 30). Next, we demonstrated electrophysiologically that Neto2-null neurons were unable to extrude a Cl^- load, indicating that the function of the remaining neuronal KCC2 in Neto2-null is severely compromised. Remarkably, the decrease in KCC2 function in Neto2-null neurons can be such that intracellular Cl^- levels rise to the point where GABAergic transmission becomes excitatory. Thus, the second essential function of Neto2 is binding to the active oligomeric form of KCC2 and allowing KCC2 to extrude Cl^- more efficiently. These two functions of the Neto2–

KCC2 are so fundamental that the loss of Neto2 causes the neuronal Cl^- gradient to collapse. Thus, we have discovered that Neto2 interacts with the key protein that regulates the strength of inhibitory synaptic transmission in the CNS.

We initially identified the Neto2–KCC2 interaction using the Neto2 cytoplasmic tail (Fig. 1), but then later demonstrated that it is the CUB domains and not the cytoplasmic tail that are required for the interaction in heterologous HEK-293 cells (Fig. 2), suggesting that in our initial screen we likely pulled out KCC2 in a multiprotein complex. This possibility was strengthened by our finding that we could rescue the depolarization in E_{GABA} in Neto2-null neurons using the cytoplasmic tail alone (Fig. 4*B*). The identification of the additional protein(s) in this Neto2–KCC2 multiprotein complex is an important direction of future research, given the importance of this complex in regulating inhibitory synaptic transmission.

In summary, we have identified Neto2 as a regulator of KCC2 transporter activity. Moreover, because Neto2 also binds to kainate receptors, we have demonstrated that Neto2 is a multifunctional protein that can modulate the activity of both ion channels and transporters. By regulating proteins involved in both excitatory (kainate receptors) and inhibitory (KCC2) neurotransmission, Neto2 is uniquely positioned to fine-tune synaptic activity on both neuronal and network levels. This finding raises the possibility that other CUB domain-containing proteins may function in a similar manner to regulate more than one class of membrane proteins.

Methods

Wild-type mice were C57BL/6 (Charles River). To generate *Neto2*$^{-/-}$ congenic mice, *Neto2*$^{-/-}$ mice on mixed 129/C57BL/6 background (17) were backcrossed to C57BL/6 mice for 10 generations, and the resulting *Neto2*$^{+/-}$ heterozygous mice were intercrossed to generate Neto2-null mice. All experimental procedures were approved by the University of Toronto and the Hospital for Sick Children animal care committees according to Canadian Council for Animal Care guidelines. Low-density cultures of dissociated mouse hippocampal neurons were prepared [as similarly described (42)] from P0–P1 mice. Perforated-patch recordings were made with pipette resistances of 7–12 MΩ. Additional experimental details for all experiments are available in *SI Methods*.

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