Chloride transporter KCC2-dependent neuroprotection depends on the N-terminal protein domain

A Winkelmann1,2, M Semtner*1,3 and JC Meier*1,2

Neurodegeneration is a serious issue of neurodegenerative diseases including epilepsy. Downregulation of the chloride transporter KCC2 in the epileptic tissue may not only affect regulation of the polarity of GABAergic synaptic transmission but also neuronal survival. Here, we addressed the mechanisms of KCC2-dependent neuroprotection by assessing truncated and mutated KCC2 variants in different neurotoxicity models. The results identify a threonine- and tyrosine-phosphorylation-resistant KCC2 variant with increased chloride transport activity, but they also identify the KCC2 N-terminal domain (NTD) as the relevant minimal KCC2 protein domain that is sufficient for neuroprotection. As ectopic expression of the KCC2-NTD works independently of full-length KCC2-dependent regulation of Cl− transport or structural KCC2 C-terminus-dependent regulation of synaptogenesis, our study may pave the way for a selective neuroprotective therapeutic strategy that will be applicable to a wide range of neurodegenerative diseases.

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Neurodegeneration restricts neuron numbers during development but can become a serious issue in disease conditions such as temporal lobe epilepsy (TLE).1 GABA-activated Cl− channels contribute to activity-dependent refinement of neural networks by triggering the so-called giant depolarizing potentials providing developing neurons with a sense of activity essential for neuronal survival and co-regulation of excitatory glutamatergic and (inhibitory) GABAergic synapses.2 By regulating transmembrane Cl− gradients KCC2 plays a vital role in development and disease.3 In addition, KCC2 plays a protein structural role in spine formation through its C-terminal protein domain (CTD).4,5 Hence, regulation of KCC2 expression and function is relevant for development and disease-specific plasticity of neural networks.6−9

GlyR α3K RNA editing leads to proline-to-leucine substitution (P185L) in the ligand-binding domain and generates gain-of-function neurotransmitter receptors.10−13 GlyR RNA editing is upregulated in the hippocampus of patients with TLE and leads to GlyR α3K185L−dependent tonic inhibition of neuronal excitability associated with neurodegeneration.14 KCC2 expression promotes neuroprotection14,15 but whether this involves regulation of transmembrane Cl− gradient or protein structural role is a matter of debate.14,15

Here, we assessed neuroprotection through several KCC2 variants in two different models of neurodegeneration including chronic neuronal silencing (α3K185L model) and acute neuronal overexcitation (NDMA model).14,15 The results identify a threonine- and tyrosine-phosphorylation-resistant KCC2 variant with increased Cl− transport activity, but they also demonstrate that the N-terminal KCC2 protein domain (NTD) is sufficient for neuroprotection.

Results

We first investigated the mechanisms underlying the neurotoxic effects of the RNA-edited GlyR α3K variant (α3K185L) on primary hippocampal neurons as described.14 Neurons were transfected at day in vitro (DIV) 6 either with non-edited (185P; control) or RNA-edited GlyR α3K (185L) and maintained for 3 days in 10 μM glycine, a concentration that should selectively activate GlyR α3K185L.14 As shown previously,14 ~50% of the GlyR α3K185L−expressing neurons exhibited fragmented dendrites and pyknotic nuclei (Figures 1a and c; Supplementary Table 1), both clear indicators of neurodegeneration, whereas overexpression of non-edited GlyR α3K185P in 10 μM glycine had little effect on neuronal survival (Figures 1b and c). Appearance of fragmented dendrites was not due to rapid internalization of surface-labeled GlyR α3K185L because comparable fluorescent signals were obtained upon cell permeabilization (not shown). To further investigate the possibility that GlyR activation is responsible for neurodegeneration, GlyR α3K185P−expressing neurons were maintained under GlyR-activating conditions (400 μM glycine), and GlyR α3K185P−expressing neurons under non-activating (0 μM glycine)
glycine) conditions. In agreement with different apparent glycine affinities of non-edited GlyR α3K185P and edited GlyR α3K185L, the data in Figure 1c clearly show that glycine-dependent activation of GlyR α3K triggers neurodegeneration.

Expression of KCC2 is inversely correlated to GlyR RNA-editing in TLE hippocampi. Two KCC2 RNA splice variants with different N-termini are known (Supplementary Figure 1A), but neuroprotective effects were demonstrated for KCC2b only. We therefore tested whether KCC2a and KCC2b protect neurons against GlyR α3K-activating conditions (Figure 1c). KCC2a- and KCC2b-positive neurons were identified with 2A self-processing peptide-coupled EGFP (Supplementary Figure 1B). Co-expression of KCC2a and KCC2b rescued the survival of neurons cultured under GlyR α3K185L-activating conditions (Figure 1c; Supplementary Table 1). This result confirms our previous data and furthermore reveals that KCC2-dependent neuroprotection does not depend on alternative splicing of exon 1. KCC2b was henceforth used and referred to as ‘KCC2wt’.

Role of spontaneous neural network activity for GlyR-dependent neurodegeneration. Spontaneous neuronal activity at the beginning of experimental GlyR α3K185L expression period is mainly dependent on GABA_A activation (Supplementary Figure 2), suggesting that inability of chronically silent neurons with enduring GlyR α3K185L activation to participate in spontaneous neural network activity underlies neurodegeneration. To address this possibility, we blocked either synaptic or synaptic and non-synaptic GABA_A receptors, as described, using 0.2 μM or 3 μM of the competitive GABA_A antagonist GABA_zine. However, neither 0.2 μM nor 3 μM GABA_zine rescued chronically silent neurons, and KCC2wt-dependent neuroprotection did not require GABA_A activation (Figure 2a; Supplementary Table 1). Furthermore, neurodegeneration/neuroprotection did not require action-potential-dependent synaptic transmission because tetrodotoxin (TTX) had no effect on survival (Figure 2b; Supplementary Table 1). These results suggest that intrinsic cellular mechanisms are responsible for neurodegeneration rather than inability of chronically silent neurons to participate in spontaneous neural network activity.

Role of intracellular Ca^2+ concentration for GlyR-dependent neurodegeneration. Activation of non-synaptic NMDA receptors (NMDARs) and Ca^2+ -activated Cl^- channel Clca1 were implicated in neurodegeneration, and changes in resting intracellular Ca^2+ concentration ([Ca^2+]_i) are involved in excitotoxicity. Thus, GlyR α3K185L-dependent neuronal intrinsic mechanisms of neurodegeneration may involve changes in intracellular Ca^2+ homeostasis. To investigate this possibility, [Ca^2+]_i of neurons expressing GlyR α3K185L or GlyR α3K185P in 10 μM glycine (i.e., receptor-activating and non-activating conditions) were determined using fura-2, as described by Jung et al. (Figure 3a). However, [Ca^2+]_i was comparable...
in all conditions as [Ca$^{2+}$] in neurons with activated GlyR \( \alpha_{3K185L} \) (78.9 ± 19.2 nM, \( n=13 \)) were similar to those in neurons with non-activated GlyR \( \alpha_{3K185P} \) (69.4 ± 11.1 nM, \( n=10; P=0.7125 \)) or untransfected neurons (80.6 ± 6.2 nM, \( n=194; P=0.9280 \); Figure 3b). These results rule out increased [Ca$^{2+}$] as a cause of GlyR \( \alpha_{3K185L} \) activation-dependent neurodegeneration.

**Enduring GlyR \( \alpha\)-activation persistsently changes neuronal intrinsic membrane properties.** We next investigated neuronal intrinsic membrane properties including membrane resistance (\( R_N \)) and the reversal potential of all membrane conductances, henceforth termed ‘membrane reversal potential’ (\( V_{rev} \)), a value of transmembrane voltage at which ionic diffusive and electrical forces counterbalance and no net transmembrane current is observed. We used gramicidin-perforated patch clamp because under this condition the intracellular milieu is not dependent on the recording pipette solution but on ion channels open at a given time of recording. We used voltage-clamp configuration and voltage pulses (−5 mV) starting from the holding potential (−50 mV) before determining \( R_N \). Voltage ramps (from −100 to −30 mV, 0.5 mV/ms; 140 ms total duration) were used to determine \( V_{rev} \) as accurately as possible according to reversal of current–voltage (IV) relationships. To determine \( V_{rev} \) of GABA$\alpha$R-dependent currents (\( E_{\text{GABA}} \)) IV curves measured in the presence and absence of GABA were subtracted. The fluorescent dye lucifer yellow and high Cl$^-$ concentration in the pipette solution allowed monitoring stability of the perforated patch configuration (Supplementary Figure 3A).

Neurons with GlyR \( \alpha_{3K185L} \) expression were identified according to co-expression of mCherry fluorescent protein (Supplementary Figure 3A).

We first analyzed IV relationships in the absence of GABA, that is, under basal conditions (Figure 4a, ‘IV$\text{bas}$’), in control neurons (mCherry only) and in neurons with activated GlyR

### Figure 3
Continuous GlyR \( \alpha_{3K185L} \) activation does not affect resting [Ca$^{2+}$]. Intracellular Ca$^{2+}$ concentration at rest ([Ca$^{2+}$]$^r$) was determined according to the procedure described by Jung et al.22 (a) Neurons were loaded 2–3 days after transfection with fura-2. Red and gray traces correspond to signals obtained from a GlyR \( \alpha_{3K185L} \)-positive neuron (red) and non-transfected neurons (gray) in the neighborhood of the transfected neuron in the same viewfield. A solution with 50 mM KCl was applied to monitor the viability of the cells according to their response with regard to fura-2 signals. Cells that did not respond to 50 mM KCl with changes in the F$340$/F$380$ ratio were not included in the determination of resting [Ca$^{2+}$]. Resting [Ca$^{2+}$] was determined within the 10 s (marked with a yellow bar) prior to the application of 50 mM KCl. To obtain minimal (0 mM) and maximal (10 mM) Ca$^{2+}$ signals for calibration, cells were permeabilized with either 10 \( \mu \)M ionomycin or 10 \( \mu \)M 4-bromo-antibiotic A23187. 2 mM Mn$^{2+}$ were applied to quench the signal at the end of each experiment and to obtain background fluorescence that was subtracted from all F340 and F380 values. (b) GlyR \( \alpha_{3K185L} \) expression and activation in the presence of 10 \( \mu \)M glycine does not affect resting [Ca$^{2+}$]. Numbers in the bar graphs indicate the number of neurons analyzed.
α3K185L and determined $V_{\text{rev}}$. In neurons with enduring GlyR α3K185L activation, $V_{\text{rev}}$ was significantly more depolarized than in control neurons (Figures 4a and c; $-52.1 \pm 1.1 \text{ mV}$, $n=12$ versus $-63.6 \pm 2.7 \text{ mV}$, $n=12$; $P=0.0005$). Furthermore, $R_N$ of neurons with enduring GlyR α3K185L activation was significantly decreased compared to control neurons (Figure 4d; $108 \pm 31 \text{ MΩ}$, $n=12$ versus $429 \pm 65 \text{ MΩ}$, $n=12$; $P=0.0012$), indicating presence of open GlyR α3K185L channels in the plasma membranes of neurons exposed to 10μM glycine.

We next determined $E_{\text{GABA}}$ by subtracting IV curves measured in the presence (IV$_{\text{GABA}}$) and absence (IV$_{\text{bas}}$) of 100 μM GABA (Figures 4a, b and e$^2$). $E_{\text{GABA}}$ values should be a measure of $V_{\text{rev}}$ of Cl$^-$ currents because GABAA Rs are like GlyRs permeable for Cl$^-$ (GABA$A$ Rs are also permeable for HCO$_3^-$ ($P_{\text{HCO}_3}/P_{\text{Cl}} \sim 0.5$–0.6$^{2,22,23}$), but as our solutions were not based on CO$_2$/HCO$_3^-$, the determined $E_{\text{GABA}}$ values primarily reflect $V_{\text{rev}}$ of Cl$^-$, that is $E_{\text{Cl}}$. Most of the control neurons displayed hyperpolarizing currents at $-50 \text{ mV}$ in 100 μM GABA (Figure 4a, left hand), which reversed at $-58.7 \pm 2.3 \text{ mV}$ (n=12; Figures 4b). In contrast, in neurons with enduring GlyR α3K185L activation, GABA responses at the holding potential (−50 mV) were shunted (Figure 4a right hand; Supplementary Figure 3B right hand), and IV$_{\text{GABA}}$ reversed close to that holding potential ($-52.5 \pm 1.1 \text{ mV}$, n=12, $P=0.0195$ compared to control, Figure 4b right hand, $E_{\text{GABA}}$ left hand).
Consequently, the calculated driving force of GABA responses was $4.9 \pm 2.9$ mV in control neurons ($n = 12$; Figure 5d), whereas it remained close to $0$ mV ($−0.4 \pm 0.9$ mV, $n = 12$; Figure 5e) in neurons with enduring GlyR $α3K^{185L}$ activation. Together with the strongly decreased $R_N$ (Figure 4d), we conclude that enduring GlyR $α3K^{185L}$ activation leads to large permanent $Cl^−$ conductance that shunts GABA-dependent currents due to adaptation of transmembrane $Cl^−$ gradient according to imposed membrane potential.

We next asked whether acute glycine washout during recording of neurons with activated GlyR $α3K^{185L}$ would have effects on $V_{rev}$ and $E_{GABA}$ (Figures 4c–e). However, $V_{rev}$ and $E_{GABA}$ remained at more depolarized potentials compared to control neurons ($−49.2 \pm 2.4$ mV for $V_{rev}$, $−50.8 \pm 1.5$ mV for $E_{GABA}$, $n = 11$), and $R_N$ recovered only partially (Figure 4d; $107.8 \pm 30.5$ M$Ω$, $n = 12$ in $10 \mu M$ glycine versus $203.9 \pm 47.2$ M$Ω$, $n = 11$ after glycine washout; $P = 0.0006$). As $GABA_A$-R-dependent currents were observed upon glycine washout (Supplementary Figure 3B, right hand) $GABA_A$R downregulation as a reason for observed effects regarding $E_{GABA}$ can be ruled out. Thus, enduring GlyR $α3K^{185L}$ activation during 3 days induced long-lasting changes in neuronal intrinsic membrane properties, which might involve changes in permeability for different ions including, besides $Cl^−$, also $K^+$, a well known major driving force of resting membrane potential.

Enduring GlyR $α3K^{185L}$ activation persistently changes resting membrane potential. We finally investigated resting membrane potential ($V_m$) in current-clamp configuration (Supplementary Figures 3C and D). We clamped current at $0$ pA and determined apparent $V_m$ of neurons with continuous GlyR $α3K^{185L}$ activation in $10 \mu M$ glycine. Actually, $V_m$ of GlyR $α3K^{185L}$-expressing neurons in $10 \mu M$ glycine required 2–3 min to stabilize after switching from voltage- to current-clamp configuration, and $V_m$ slowly depolarized under these conditions from values around $−50$ mV (i.e., the formerly
imposed holding potential) to values determined in current-clamp configuration reflecting apparent \(V_m\) (Supplementary Figure 3C). These slow adaptations of membrane potential consistently occurred only in 10 \(\mu\)M glycine and in GlyR \(\alpha3K185L\)-expressing neurons indicating that outwardly directed Cl\(^-\) currents through GlyR \(\alpha3K185S\) slowly decrease [Cl\(^-\)], and thereby depolarize \(V_m\). Hence, these experiments revealed that \(V_m\) was significantly more depolarized in neurons with activated GlyR \(\alpha3K185L\) compared to control neurons (Supplementary Figure 3D; \(-35.9 \pm 2.6\) mV, \(n = 5\) versus \(-51.2 \pm 2.4\) mV, \(n = 9\); \(P = 0.0104\)). As acute glycine washout significantly influenced \(V_m\) (Supplementary Figure 3D; \(-35.9 \pm 2.6\) mV, \(n = 5\) in 10 \(\mu\)M glycine versus \(-44.8 \pm 2.2\) mV, \(n = 10\) upon glycine washout; \(P = 0.0026\)), while no differences were observed in control neurons (Supplementary Figure 3D; \(-52.6 \pm 2.4\) mV, \(n = 8\) in 10 \(\mu\)M glycine versus \(-51.2 \pm 2.4\) mV, \(n = 7\) upon glycine washout; \(P = 0.6953\)), these data suggest that enduring GlyR \(\alpha3K185L\) activation shifts \(V_m\) toward \(E_C\). After glycine washout, \(V_m\) remained more depolarized in GlyR \(\alpha3K185S\)-expressing neurons compared to control neurons (\(P = 0.0306\)), and hence as discussed above, these results consolidate conclusion that enduring GlyR \(\alpha3K185L\) activation induced long-lasting changes of neuronal intrinsic membrane properties. A priori, these results suggest that KCC2 expression may prevent persistent changes in intrinsic membrane properties and possibly involve Cl\(^-\) transport in neuroprotection, as suggested earlier.\(^{14,15}\)

**KCC2 Cl\(^-\) transport activity is not relevant for neuroprotection.** Gramicidin-perforated patch clamp was used again to analyze the effects of co-expression of KCC2wt on membrane properties of neurons with enduring GlyR \(\alpha3K185L\) activation. KCC2-positive neurons were identified according to 2A-self-clearing peptide-mCherry (Supplementary Figure 1B). KCC2wt was not able to substantially change neuronal intrinsic membrane properties of GlyR \(\alpha3K185L\)-expressing neurons (Figure 5). Similar to mCherry/GlyR \(\alpha3K185L\)-expressing neurons in 10 \(\mu\)M glycine (Figure 4), \(R_N\) remained significantly decreased (77.6 \(\pm\) 12.8 M\(\Omega\), \(n = 9\), Figure 5a), and \(V_m\) and \(E_{GABA}\) also shifted toward imposed holding potential of \(-50\) mV when KCC2wt was co-expressed (\(-53.4 \pm 1.4\) mV and \(-53.7 \pm 2.5\) mV, respectively, \(n = 8\), Figures 5b and c; calculated driving forces for GABA\(\text{AR}\) responses, Figure 5e). This suggests that KCC2wt Cl\(^-\) transport activity was not sufficient to overcome GlyR \(\alpha3K185L\)-mediated Cl\(^-\) conductance. Moreover, upon acute glycine washout, \(V_m\) and \(E_{GABA}\) largely remained unchanged (Figures 5b and c), and \(R_N\) recovered only partially (Figure 5a), similar to neurons with enduring GlyR \(\alpha3K185L\) activation in the absence of KCC2wt (Figure 4d). Thus, co-expression of KCC2wt was not able to prevent persistent changes in intrinsic membrane properties of neurons with enduring GlyR \(\alpha3K185L\) activation. For control purpose, we checked KCC2 Cl\(^-\) transport functionality and recorded KCC2wt-positive neurons in the absence of GlyR \(\alpha3K185L\) expression (Supplementary Figure 4). Surprisingly, no significant differences in \(E_{GABA}\) were found between control neurons (\(E_{GABA}\): \(-58.7 \pm 2.3\) mV, \(n = 12\)) and those expressing KCC2wt (\(E_{GABA}\): \(-55.4 \pm 2.8\) mV, \(n = 7\); \(P = 0.3920\); Supplementary Figure 4B and Figure 5d). However, a mutant variant of KCC2 (KCC2pr; Supplementary Figure 1A), which should be resistant to phosphorylation-dependent downregulation of Cl\(^-\) transport activity,\(^{24,25}\) shifted \(E_{GABA}\) in neurons without GlyR \(\alpha3K185L\) co-expression (KCC2pr; \(-65.8 \pm 1.5\) mV, \(n = 8\); \(P = 0.0325\) versus control; \(-58.7 \pm 2.3\) mV, \(n = 12\); \(P = 0.0049\) versus KCC2wt; \(-55.4 \pm 2.8\) mV, \(n = 7\); Supplementary Figures 4A and B; Figure 5d). These results were confirmed using recording of intracellular Ca\(^{2+}\) dynamics with Oregon Green 488 (Life Technologies, Darmstadt, Germany) in response to GABA application in more immature hippocampal neurons at DIV 6–7 (Supplementary Figure 5). Again, KCC2wt was not able to prevent GABA-induced increases in intracellular Ca\(^{2+}\) signals in hippocampal neurons without GlyR \(\alpha3K185L\) co-expression (\(F_{GABA}/F_{KCl}\): \(0.21 \pm 0.04\), \(n = 29\) for KCC2wt versus \(0.23 \pm 0.02\), \(n = 114\) for untransfected neurons, \(P = 0.7666\); Supplementary Figure 5C), whereas the KCC2pr mutant was effective (\(F_{GABA}/F_{KCl}\): \(0.12 \pm 0.03\), \(n = 29\) for KCC2pr versus \(0.22 \pm 0.02\), \(n = 110\) for untransfected neurons, \(P = 0.0107\); Supplementary Figure 5C). Importantly, KCC2wt and KCC2pr Cl\(^-\) transport activities were apparent in primary cortical neurons under these conditions (\(F_{GABA}/F_{KCl}\): \(0.11 \pm 0.03\), \(n = 36\) for KCC2wt versus \(0.40 \pm 0.03\), \(n = 158\) for untransfected neurons, \(P < 0.001\); \(F_{GABA}/F_{KCl}\): \(0.08 \pm 0.03\), \(n = 44\) for KCC2pr versus \(0.50 \pm 0.03\), \(n = 188\) for untransfected neurons, \(P < 0.001\); Supplementary Figure 5D). Hence, KCC2wt is a functional Cl\(^-\) transporter, and cortical and hippocampal neurons reveal phosphorylation-dependent differences in the regulation of KCC2 Cl\(^-\) transport activity. However, because KCC2wt protected hippocampal neurons against GlyR \(\alpha3K185L\) activation-dependent neurodegeneration (Figure 1), these results also suggest that Cl\(^-\) extrusion is not relevant to KCC2-dependent neuroprotection. Indeed, block of Cl\(^-\) import activity through NKCC1 using bumetanide (10 \(\mu\)M) also failed to rescue neuronal survival (Figure 6a).

The KCC2-NTD mediates neuroprotection in the GlyR \(\alpha3K185L\) model of neurodegeneration. KCC2 was previously shown to have a protein structural role for synaptogenesis by interaction with cytoskeleton-associated protein 4.1N.\(^{5,26}\) This finding encouraged us to clarify if cytoskeletal signaling is involved in neuroprotection, but KCC2-C568A mutant which is unable to interact with protein 4.1N and Cl\(^-\) transport-deficient\(^{5,26}\) rescued survival of neurons with continuous GlyR \(\alpha3K185L\) activation as well as KCC2wt (Figure 6b; Supplementary Table 1). Furthermore, KCC2pr-C568A rescued neuronal survival indicating that KCC2-dependent neuroprotection is independent of phosphorylation of Y903, T906, T1007 and Y1087 in the KCC2-CTD (Supplementary Figure 1A). These results reveal that KCC2-dependent neuroprotection is independent of protein 4.1N-dependent cytoskeletal signaling and further strengthen our conclusion that KCC2-dependent neuroprotection does not rely on Cl\(^-\) transport. They also suggest that it is not the KCC2-CTD, which mediates neuroprotection. Indeed, deletion of NTD, not of CTD, abolished KCC2-dependent neuroprotection (Figure 6b; Supplementary Table 1). Reciprocally, co-expression of the KCC2-NTD, not of KCC2-CTD,
rescued survival of neurons with enduring GlyR α3K<sup>185L</sup> activation (Figure 6b). That KCC2-ΔNTD or KCC2-CTD failed to protect neurons was not due to poor protein expression (Supplementary Figure 6). These results identify KCC2-NTD as relevant neuroprotective signaling domain.

**KCC2-NTD mediates neuroprotection in the NMDA-dependent model of excitotoxicity.** To sustain the finding that KCC2-NTD plays a pivotal role in neuroprotection, we investigated whether neuroprotective function of KCC2-NTD holds in another model of neurodegeneration. In contrast to our GlyR α3K<sup>185L</sup>-dependent model of neurodegeneration, which uses chronic silencing of neuronal activity, the NMDA-dependent excitotoxicity model relies on neuronal over-excitation. Actually, KCC2 Cl<sup>−</sup> transport activity was recently postulated to mediate neuroprotection in the NMDA model of neurodegeneration. Therefore, we tested KCC2<sub>wt</sub> and mutant KCC2 variants coupled to EGFP via 2A peptides (Supplementary Figure 1) in the NMDA-dependent model of neurodegeneration. Neurons were transfected at DIV 6 with non-edited GlyR α3K<sup>185L</sup> (to assess neuronal morphology in addition to appearance of pyknotic nuclei for quantification of neurodegeneration, Figures 7a and b), kept in culture for two days (in non-GlyR-activating conditions), and then incubated for 30 min in 40 μM NMDA in the absence or presence (10 μM) of glycine before maintaining the culture for 24 h as described by Pellegrino et al. Glycine is a co-agonist of GABA<sub>AR</sub> activation, which provides a major driving force for spontaneous neuronal activity at this developmental stage and resulting impairment of intracellular ion homeostasis were recently associated with the pathophysiology of Alzheimer's disease. This is possibly due to inactivation of, or changes in, expression of voltage-gated Ca<sup>2+</sup> channels as a consequence of decreased V<sub>m</sub> in neurons with enduring GlyR α3K<sup>185L</sup> activation.
Figure 7  Protein structural aspects of KCC2-dependent neuroprotection in the NMDA model of neurodegeneration. (a and b) Images of control neurons (a) and neurons treated for 30 min with 40 μM NMDA 24 h prior to fixation (b) are shown. GlyR α3K185P was expressed under non-receptor-activating conditions and surface stained in order to assess neurodegeneration in the same way as in the GlyR-dependent model of neurodegeneration (Figure 1). Degenerated neurons could be identified by their fragmented dendrites and pyknotic nuclei (b). (c) Quantification of the fraction of surviving neurons revealed that co-expression of KCC2wt or KCC2pr also protects NMDA-treated neurons. Again, Cl⁻ transport-deficient KCC2wt/C568A also rescued neuronal survival. N-terminally truncated KCC2-ΔNTD or the isolated KCC2-CTD was not able to mediate neuroprotection. Note that the isolated KCC2-NTD was sufficient to mediate full rescue of neuronal survival. (d) Quantification of the effects of glycine (10 μM) on NMDA-dependent excitotoxicity and KCC2-dependent neuroprotection. Numbers in the bar graphs indicate the number of cultures analyzed. For values, see Supplementary Table 1. Statistical significance is indicated with ***P < 0.001.
In the hippocampus, RNA-edited GlyR α3K185L contributes to tonic inhibition of cells with low GlyR β subunit protein expression.14,29–34 RNA splicing generates the long variant GlyR α3L185L which is preponderantly expressed in the hippocampus and operates at presynaptic sites by contributing to regulation of neurotransmitter release.12,29,35,36 However, as the ratio between α3K and α3L is increased in TLE patients with hippocampal sclerosis (i.e., neurodegeneration),29 our results regarding mechanisms of GlyR α3K185L-dependent neurodegeneration and KCC2-mediated neuroprotection are relevant to the understanding of the pathophysiology of TLE. RNA splicing and its regulatory impact on subcellular distribution of ligand-gated GlyRs and GABA_A receptors has also been implicated by other groups in neuronal dysfunction in TLE,29,37–39 and non-synaptic Cl− channels have also been implicated by other groups in neuronal cell death. A recent study even provides a link between non-synaptic NMDAR activation and excitotoxicity through Ca2+–activated Cl− channel Cica1.18 With regard to neurodegeneration, alteration of non-synaptic Cl− channel function thus represents an important determinant of cellular programs that elicit cell death, and for this reason, care should be taken when considering the use of glycine for neuroprotective purposes.40–42

Identification of the neuroprotective capacity of the KCC2-NTD in different models of neurodegeneration. KCC2 is a developmentally regulated gene product, which can undergo functional downregulation in the diseased brain.7,9,43,44 Functional downregulation involves phosphorylation of amino acids in the KCC2-CTD.24,25 In our study, KCC2wt-dependent Cl− extrusion was apparent only in cortical neurons (Supplementary Figure 5), which demonstrates functionality of KCC2wt-dependent Cl− extrusion in general, but also identifies neuron type-specific (cortical versus hippocampal neurons) differences in the apparent efficacy of Cl− extrusion through KCC2wt. The discrepancy to other studies in which KCC2wt overexpression was reported to significantly shift E_GABA to hyperpolarized potentials in cultured hippocampal neurons (e.g., Li et al.45 and Chudotvorova et al.46) is probably due to differences in the cell culture preparation (e.g., cell density, duration and strength of KCC2 expression, time point of investigation and percentage of GABAergic interneurons in the culture dish). However, hippocampal neurons in our culture preparations develop normally as they are excitable and spontaneously active due to depolarizing GABA_A signaling (Supplementary Figure 2). Thus, the discrepancy to other studies might involve culture-specific changes in the phosphorylation of the S940 site, which was shown to enhance Cl− extrusion capacity of KCC2wt.46,47 Nevertheless, as Cl− extrusion through the KCC2pr variant (which cannot be phosphorylated at Y903, T906, T1007 and Y1087) was apparent in our hippocampal neuron preparations (Supplementary Figures 4 and 5, and Figure 5d), our results clearly identify a role for these sites in phosphorylation-dependent regulation of KCC2 Cl− extrusion, which is in agreement with mounting evidence for the role of threonine phosphorylation in the downregulation of KCC2-dependent Cl− transport (for review see Kahlé et al.48). Our results furthermore make a clarifying contribution to the controversial discussion of the role of tyrosine phosphorylation in the regulation of KCC2 Cl− extrusion capacity as they show that KCC2pr with unphosphorylatable threonine and tyrosine residues has an increased Cl− extrusion capacity. However, more detailed study is necessary to fully clarify the role of threonine and tyrosine phosphorylation.

Beyond its ‘classical’ function as Cl− transporter that contributes to the developmental switch of GABA action from depolarizing GABA_A receptors to classical Cl− channels associated with hyperpolarization,5,6 KCC2 fulfills a protein structural function, which contributes to co-regulation of glutamatergic and GABAergic synaptic transmission, which is the case in a variety of neurodegenerative conditions and with the relatively compact, 100 amino acid spanning, KCC2-NTD (Supplementary Figure 1A) is a novel suitable but non-exclusive strategy to prevent neurodegeneration in TLE and other neurodegenerative diseases as it would work independently of the KCC2-CTD (involved in regulation of synaptic transmission) or full-length KCC2-dependent regulation of chloride transport. Thus, KCC2 is a moonlighting protein49,50 as it is not only a protein with Cl− transport activity but also harbors several protein domains with synaptogenic and neuroprotective activities (Figure 8).

Materials and Methods
Molecular cloning. A cDNA coding for KCC2b wildtype (designated ‘wt’) was kindly provided by the laboratory of Kai Kaila (Helsinki, Finland) and recently used in one of our studies.14 The KCC2a cDNA was cloned from human postmortem hippocampus RNA pool of 20 healthy Caucasians, Clontech (Palo Alto, CA, USA). By using the 2A-self-processing peptide EGRGSLLTCGD-VEENPS derived from Thosea asigna,51 we generated constructs for expression of KCC2 and mCherry or EFSP from polyctionic mRNA (Supplementary

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DNA sequencing. Molecular cloning of GlyR α from a lentiviral vector (Clontech). All expression constructs were verified with woodchuck posttranscriptional regulatory element (WPRE), which was derived enhancer-human synapsin-1 promoter (hSyn1). All the constructs contain the constructs for neuronal expression are equipped with a cytomegalovirus (CMV) the full-length KCC2 constructs and appropriate primer sets. All the KCC2 μ in the presence or absence of added glycine (0, 10 or 400 μM) for 30 min in the absence or presence of 10 μM glycine and analyzed 24 h after application. For co-transfection, 60 ng of KCC2-coding plasmids were mixed with 240 ng GlyR α3K and GlyR α3K constructs is described in former publications of our laboratory.10

Cell culture and transfection. All animals were killed according to the permit given by the Office for Health Protection and Technical Safety of the regional government of Berlin (LaGeSo, 0122/07) and in compliance with regulations laid down in the European Community Council Directive. Hippocampal cells were isolated from E19 Wistar rats and kept in B27-supplemented Neurobasal medium (Life Technologies) in 24-well plates as described.14 Transfection was carried out on DIV 6 using 300 ng DNA per well in combination with Effectene medium (Life Technologies) in 24-well plates as described.14 Transfection was carried out on DIV 6 with the manufacturer’s protocol as described.14 Either low (165F) or high (185L) affinity receptor types of the short (K) splice variant of GlyR α3L were expressed for the duration of 3 days in the presence or absence of added glycine (0, 10 or 400 μM). For this purpose, a glycine-free minimal essential medium was used.15 In some experiments, neurons were exposed to NMDA (40 μM) for 30 min in the absence or presence of 10 μM glycine and analyzed 24 h after application. For co-transfection, 60 ng of KCC2-coding plasmids were mixed with 240 ng GlyR α3K. For Oregon Green imaging of GABA-elicted Ca2+ signals, neurons were investigated at an earlier time point in cell culture, at DIV 6–7.

Chemicals. HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) and all inorganic salts as well as glycine (G7126), GABA (A2129), NMDA (M2626), gramicidin (G5002), lucifer yellow (L0144) and bicuculline methiodide (14343) were purchased from Sigma-Aldrich (Steinheim, Germany). GABAzine (1262) came from Tocris (Bristol, UK), TTX (6973.1) from Carl Roth (Karlsruhe, Germany), 4-bromo-A-23187 (BML-CA101-0001) and ionomycin (LKT-M001) from ENZO Life Sciences (Lörrach, Germany). Fura-2/AM and Oregon Green 488/AM were purchased from Life Technologies. Stock solutions were made with 100 mM of the compound, glycine and GABA (1 M each), and with DMSO (dimethyl sulfoxide) (SERVA Electrophoresis GmbH, Heidelberg, Germany) in the case of gramicidin, 4-bromo-A-23187 and ionomycin (10 mM each).

Immunocytochemistry. A polyclonal chicken antibody was used to visualize HA-tagged surface GlyR (1: 200, Bethyl Laboratories, Montgomery, TX, USA). The secondary antibody was made in donkey, conjugated with indocarbocyanine (Cy3) and purchased from Jackson ImmunoResearch Laboratories (Suffolk, UK). Surface staining of HA-tagged GlyR α3K was performed for 5 min at 37 °C in cell culture medium, as described.14,25 Prior to fixation with paraformaldehyde (PFA) cells were washed three times with cell culture medium. For fixation with PFA, cells were incubated for 15 min at room temperature (RT) with ice-cold PBS containing 4% PFA and 4% sucrose, followed by three wash steps in PBS. Incubation of fixed cells in freshly made 50 mM NH4Cl for 15 min at RT was used to quench free aldehyde groups from the PFA fixation. Cells were then again washed three times in PBS and blocked with PBS/gelatine (0.1%) prior to permeabilization with 0.12% Triton X-100 (Sigma-Aldrich) in PBS/gelatine for 4 min at RT. Before incubation with first antibodies, coverslips were again washed with PBS/gelatine. In some experiments, KCC2 was stained for 1 h at RT using a rabbit polyclonal antibody (#07-432; Merck Millipore, Germany) diluted 1: 200 in PBS/gelatine. Prior to incubation with secondary antibodies cells were washed three times with PBS/gelatine. For KCC2 stainings, a secondary antibody made in donkey and conjugated with indocarbocyanine (Cy3, Jackson ImmunoResearch Laboratories) was used. After 45 min of incubation at RT cells were again washed three times with PBS/gelatine followed by additional two wash steps in PBS. Stained cell preparations were finally mounted on microscope slides using DAPI-containing vectashield medium (Vector Laboratories, Peterborough, UK).

Electrophysiology. An EPC-7 amplifier and Patchmaster software (HEKA, Lambrecht, Germany) were used for patch-clamp recordings. Patch pipettes, made from borosilicate glass (Science Products, Holzheim, Germany), had resistances of 2–8 MΩ when filled with the intracellular solution containing (in mM) KCl (150), NaCl (5), CaCl2 (0.5), MgCl2 (1), EGTA (5) and HEPES (30). Application of substances was gravity driven. The tip (250 μM) of a perfusion pencil (AutoMate Scientific, Berkeley, CA, USA) was placed close (ca. 100 μM of the recorded neuron to ensure relatively rapid application of substances. Under these conditions, the wash-in duration of lucifer yellow-containing test solution was < 500 ms. For the perforation of the patch, the pipette solution contained additionally 50–100 μM gramicidin and 100 μM lucifer yellow, which allowed monitoring the stability of the perforated patch (Supplementary Figure 3A). In addition, a strong shift of the baseline current due to the high Cl− concentration in the pipette solution indicated membrane rupture upon transition to whole-cell configuration. In this case, the recording was stopped. The standard extracellular solution (E1; pH 7.4) contained (in mM) NaCl (140), KCl (5), MgCl2 (1), CaCl2 (2), HEPES-NaOH (10) and glucose (10). In the voltage-clamp mode, neurons were clamped at a potential of —50 mV. IV relationships were obtained from voltage ramps from —100 to —30 mV with a duration of 140 ms applied every 5 s. Series and input resistances were checked throughout the whole duration of each experiment by applying —5 mV pulses prior to the voltage ramps. All data were acquired with a sampling rate of 10 kHz after filtering at 2.8 kHz. All experiments were performed at RT (20–25 °C).

Calcium imaging. Prior to ratiometric and non-ratiometric Ca2+ imaging experiments cells were loaded with fura-2/AM or Oregon Green 488/AM, respectively, by incubating the cells in E1 buffer (see above) supplemented with 1–5 μM of fura-2 or Oregon Green for 20 min at 37 °C. Subsequently, cells were incubated for further 20 min in E1 to ensure deesterification. Glass coverslips with the dye-loaded neurons were placed into a recording chamber (ca. 1 ml volume) on
the stage of an Axovent 10 or an Axio Lab.A1 microscope (both Zeiss, Oberkochen, Germany). Cells were submerged with a constant flow of E1 through an infusion pipette, which was placed in close vicinity (ca 200 μm) to the recorded cells to ensure short wash-in/washout durations. Transfected cells were identified by the fluorescence of mCherry, which served as control or was co-expressed using the 2A-self-cleaving peptide in KCC2 constructs (see Supplementary Figure 1B for constructs). Ratiometric measurements were performed with the Polychrome V. A Clara Interline CCD camera (Andor Technology, Belfast, UK) and Live Acquisition software (Till Photonics, Martinsried, Germany) using 340 and 380 nm excitation wavelengths. Excitation and emission light were separated by a 510-nm dichroic mirror. The emitted light was filtered using a 530-nm longpass filter. Exposure times were 20 ms (340 nm) and 5 ms (380 nm), and the rate for [Ca2+]2 measurement was set to one pair of images per 1 sec. 50 mM KOI was applied to check the viability of the cells. To obtain minimal (0 mM) and maximal (10 mM) Ca2+ signals for calibration, cells were perfused with either 10 mM ionomycin or 10 μM 4-bromo-antibiotic A23187. 2 mM Mn2+ were applied at the end of each experiment to quench the signal and thus to obtain background fluorescence that was subtracted from all F340 and F380 values used for [Ca2+]2 calculation according to:

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[Ca^{2+}]_i = K_D \left( \frac{F_{380\text{,min}}}{F_{380\text{,max}}} \right) \left( \frac{R_{\text{min}} - R}{R_{\text{max}} - R} \right),
\]

where KD is the dissociation constant of the fura-2-Ca2+ complex (225 nM 51). F380,min and F380,max are the fluorescence values of free (0 mM min) and Ca2+-bound (10 mM, max) fura-2. R Rmin, Rmax are the fluorescence ratios (F340/F380) at the beginning of the experiment (R) and after permeabilization in 0 mM (R0) and 10 mM (Rmax) Ca2+, respectively. Non-ratiometric experiments were performed with an HBO-100 lamp (OSRAM GmbH, Munich, Germany), an electronic shutter, a Sputz pocket camera and metal morphow (Visitron Systems, Puchheim, Germany). Oregon Green fluorescence was excited and detected using an appropriate filter set (XP22, Omega Opticaís, Olching, Germany) using 100 μm shutter open times at a frequency of three per second. Oregon Green fluorescence signals are expressed as FOA/BFOF22, where FOA and F22 are the fluorescence values in the presence of E1 I supplemented with 50 mM KCl (90 mM instead of 140 mM NaCl) and 100 μM GABA, respectively.

Data analysis and statistics. All numerical data are reported as mean ± S.E.M. Statistical analysis (ANOVA and post hoc Tukey’s test) was performed using the software IGOR Pro 6.3 (WaveMetrics, Lake Oswego, OR, USA). Significance levels are indicated as *P < 0.05, **P < 0.01 and ***P < 0.001.

Quantitative data of neuronal survival is presented as values that are normalized to the number of vital neurons in control conditions. Degenerated and vital neurons were counted from at least three independent hippocampal cell cultures. The number of experiments is indicated in brackets in the bar graphs. The conditions GluR2/CX3BP and α3K185L in the presence of 10 μM ionomycin were evaluated using a T-test and calculated as follows:

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\text{V}_{\text{norm}} \text{ was calculated by determining the voltage at which no net current was observed (zero point of the basal IV curve in the absence of GABA, V}_{\text{GABA}}\text{. The values presented in figures and text are not corrected for the liquid junction potential (}+3.75 \text{ mV})\text{, E}_{\text{GABA}}\text{ was obtained by determining the zero point of the GABA IV relationship that was calculated by subtraction of V}_{\text{max}}\text{ from V}_{\text{GABA}}\text{ (Figure 4). Analysis was performed by a homemade procedure written using IGOR Pro 6.3 (WaveMetrics).}
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Conflict of Interest

The authors declare no conflict of interest.

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