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Modulation of GABAergic Transmission by Activity via Postsynaptic Ca^{2+}-Dependent Regulation of KCC2 Function

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Summary

Activity-induced modification of GABAergic transmission contributes to the plasticity of neural circuits. In the present work we found that prolonged postsynaptic spiking of hippocampal neurons led to a shift in the reversal potential of GABA-induced Cl^- currents (E_{Cl}) toward positive levels in a duration- and frequency-dependent manner. This effect was abolished by blocking cytosolic Ca^{2+} elevation and mimicked by releasing Ca^{2+} from internal stores. Activity- and Ca^{2+}-induced E_{Cl} shifts were larger in mature neurons, which express the K-Cl cotransporter KCC2 at high levels, and inhibition of KCC2 occluded the shifts. Overexpression of KCC2 in young cultured neurons, which express lower levels of KCC2 and have a more positive E_{Cl}, resulted in hyperpolarized E_{Cl} similar to that of mature cells. Importantly, these young KCC2-expressing neurons became responsive to neuronal spiking and Ca^{2+} elevation by showing positive E_{Cl} shifts. Thus, repetitive postsynaptic spiking reduces the inhibitory action of GABA through a Ca^{2+}-dependent downregulation of KCC2 function.

Introduction

Activity-induced synaptic modification is critical for the development and proper functioning of the nervous system. Much is known about the cellular mechanisms by which activity affects the formation, maintenance, and regulation of excitatory synapses, but those underlying activity-induced modification of inhibitory synapses are only beginning to be elucidated (Gaiarsa et al., 2002). The principal inhibitory neurotransmitter in the CNS, γ-aminobutyric acid (GABA), exerts fast postsynaptic actions by activating $GABA_A$ receptors, which predominantly conduct chloride ions. Activity may modify the efficacy of GABAergic transmission in multiple ways. In addition to modification of presynaptic GABA release and number/property of postsynaptic $GABA_A$ receptors, activity may also alter the transmembrane Cl^- gradient, which determines the nature of GABAergic transmission—whether it is excitatory or inhibitory. In mature neurons, activation of $GABA_A$ receptors elicits Cl^- influx and membrane hyperpolarization. This well-known inhibitory action of GABA is due to a low intracellular Cl^- concentration ($[Cl^-]_i$), which sets the Cl^- reversal potential (E_{Cl}) at a level lower than the resting potential. However, in immature or injured neurons, where $[Cl^-]_i$ is relatively high and the E_{Cl} is more positive than the resting potential, GABA responses are depolarizing and can be excitatory (Ben-Ari, 2002; Payne et al., 2003). Therefore, changes in E_{Cl} may constitute a postsynaptic mechanism by which GABAergic transmission is regulated (Ling and Benardo, 1995; McCarren and Alger, 1985).

In neonatal animals, neurons accumulate Cl^- through the Na-K-Cl cotransporter NKCC1, resulting in a depolarized E_{Cl} and the excitatory action of GABA (Plotkin et al., 1997). Later in development, however, increased expression of the neuron-specific K-Cl cotransporter KCC2 results in Cl^- extrusion and a negative shift in E_{Cl} (Rivera et al., 1999), leading to the inhibitory action of GABA (Owens et al., 1996). This upregulation of KCC2 expression depends on neuronal activity (Ganguly et al., 2001; Rivera et al., 2002, 2004). Thus, it is of interest to know whether KCC2 function can be regulated also in more mature neurons (expressing higher levels of KCC2) by neuronal activity, thereby conferring activity-dependent plasticity on GABAergic transmission.

In the present study, we examined how postsynaptic spiking affects the efficacy of GABAergic synapses in hippocampal neurons. Whole-cell patch recording from postsynaptic neurons in both cultures and acute slices was performed to monitor GABAergic transmission, using gramicidin perforation to preserve endogenous $[Cl^-]_i$. We found that repetitive postsynaptic spiking of mature neurons led to an immediate and persistent modification of the efficacy of GABAergic synapses. This modification involved a positive shift in E_{Cl} due to a Ca^{2+}-dependent downregulation of KCC2 function. Activity-dependent regulation of KCC2 may thus contribute to the plasticity of inhibitory synapses under physiological or pathological conditions.

Results

Repetitive Postsynaptic Spiking Alters GABAergic Transmission

We first examined whether repetitive spiking of postsynaptic neurons affected the efficacy of GABAergic transmission. Whole-cell gramicidin perforated-patch recordings were made from pairs of neurons in low-density rat hippocampal cultures 14–15 days in vitro (DIV 14–15, Figure 1A). Gramicidin forms membrane pores permeable to monovalent cations and small uncharged molecules, but not to Cl^- (Kyrozis and Reichling, 1995), thereby leaving $[Cl^-]_i$ undisturbed and permitting reliable recording of GABAergic postsynaptic currents (GPSCs) and E_{Cl}. To block glutamatergic transmission and to isolate inhibitory monosynaptic responses, experiments were performed in the presence of 6-cyano-7-nitro-quinoxaline-2, 3-dione (CNQX, 10 μM), an antagonist of the AMPA subtype of glutamate receptors. Connectivity and synaptic responses were assessed by stimulating each neuron at a low frequency (0.05 Hz) with step-depolarizations (+100 mV, 1.5 ms)

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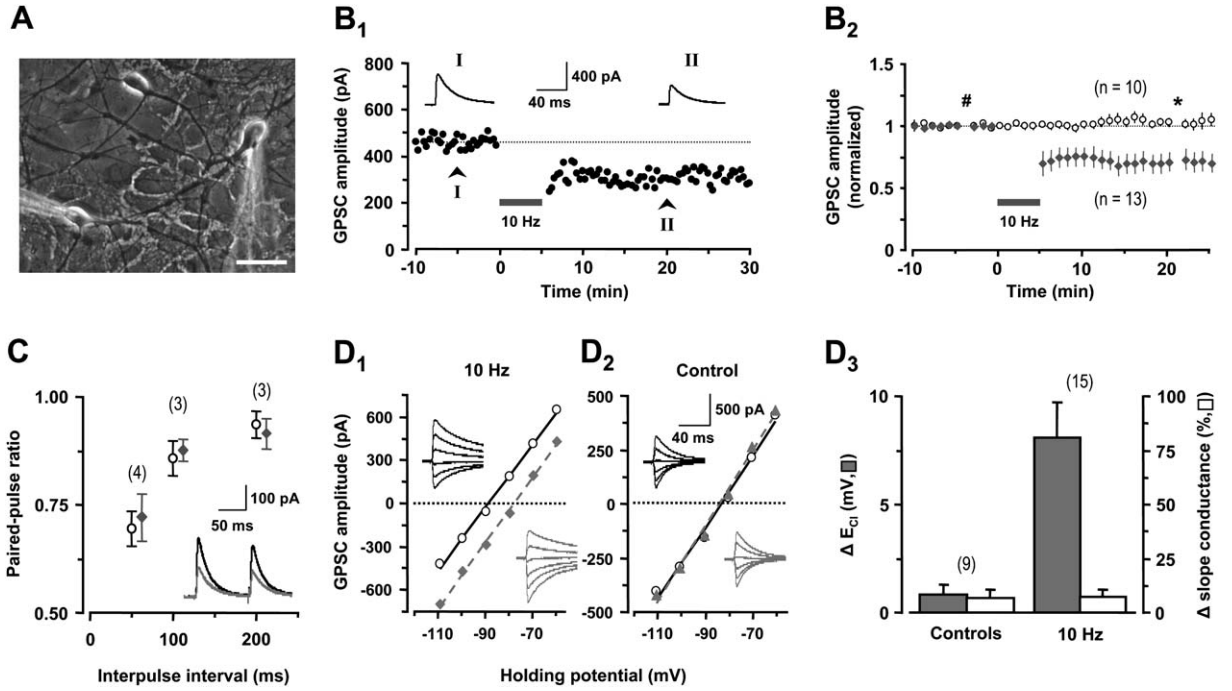

Figure 1. Repetitive Postsynaptic Spiking Decreases the Amplitude of GPSCs and Induces a Positive Shift in E_{Cl}

(A) Image of a pair of connected neurons in a DIV 15 hippocampal culture. Scale bar, 20 μm.

(B_1) Recording example of GPSCs before and after repetitive postsynaptic spiking (10 Hz, 5 min). Data points depict the peak GPSC amplitude. The dotted line represents the average GPSC amplitude during the baseline period. Insets: sample traces (averages of five events, at the times marked as I and II).

(B_2) Summary of all experiments of the effect of repetitive postsynaptic spiking. The GPSC amplitude was normalized to the mean value of the baseline period for each experiment before averaging. Data represent the mean ± SEM before and after (diamonds) or in the absence of (circles) repetitive postsynaptic spiking. The two data sets were significantly different 10–20 min after spiking ($p = 0.0013$, Student's t test). # and *, times of E_{Cl} measurements; n, the total number of cells recorded.

(C) Mean paired-pulse ratios (±SEM) of GPSC amplitudes (second/first) for different interpulse intervals. Shown are mean values over the baseline period (circles) and 10–20 min after repetitive spiking (diamonds, right-shifted for clarity). Inset: sample traces for the interval of 100 ms (averages of five sweeps) 7 min before (black) and 15 min after (gray) repetitive spiking (10 Hz, 5 min).

(D_1 and D_2) Examples of the I-V relation of GPSCs recorded from pairs of neurons 5 min before (circles/black line) and 20 min after (diamonds/dashed line) repetitive spiking (D_1) or in the absence of repetitive spiking (D_2). Insets in (D_1): traces illustrating GPSCs 5 min before (black) and 20 min after (gray) repetitive spiking at various V_cs. Insets in (D_2): traces times similar to those in (D_1).

(D_3) Summary of changes in E_{Cl} (gray) and synaptic slope conductance (white). Data represent the mean ± SEM. The change in E_{Cl} was significantly different between the two data sets ($p < 0.001$, Student's t test).

while the neurons were voltage-clamped at −60 mV. Under our experimental conditions, GPSCs were outward currents, with a relatively long duration and an E_{Cl} of −70 to −110 mV, and were abolished by the $GABA_A$ receptor antagonist bicuculline methiodide (10 μM). After stable GPSCs were recorded for at least 10 min, the postsynaptic neuron was current-clamped, and depolarizing current pulses (2 nA, 1.5 ms) were injected to fire action potentials at 10 Hz for 5 min. We found a prolonged reduction in the amplitude of GPSCs after repetitive postsynaptic spiking (Figure 1B). This reduction persisted for as long as stable recording was maintained (up to 30 min). At 10–20 min after spiking, the amplitude was reduced to 71.8% ± 7.5% (mean ± SEM) of the average baseline value before spiking. In control experiments in which no repetitive postsynaptic stimulation was applied, no change in the GPSC amplitude was observed over the same duration (102.5% ± 2.7%, Figure $1B_2$).

Postsynaptic Spiking Alters E_{Cl}

The change in the GPSC amplitude could result from alteration in either presynaptic transmitter release or postsynaptic GABA-induced responses. Measurement of GPSCs elicited by paired-pulse stimulation showed that the paired-pulse ratio (PPR) of the GPSC amplitude, which reflects the presynaptic release probability, was not significantly different before and after repetitive spiking (10 Hz, 5 min) at all intervals tested (Figure 1C). In control experiments in which no repetitive postsynaptic stimulation was applied, the PPR remained unchanged over the same duration (data not shown). Therefore, changes in the GPSC amplitude after repetitive spiking are unlikely to result from presynaptic alteration in transmitter release probability.

To investigate whether alteration of the postsynaptic $GABA_A$ conductance and/or change in E_{Cl} were involved, we measured GPSCs from connected cell pairs at different holding potentials (V_cs) and computed both E_{Cl} and slope conductance at various times before and after repetitive postsynaptic spiking (10 Hz, 5 min). As shown in the example in Figure $1D_1$, postsynaptic spiking resulted in a shift in E_{Cl} toward more depolarizing values relative to the value found during the baseline period (10 min), with no obvious change in slope

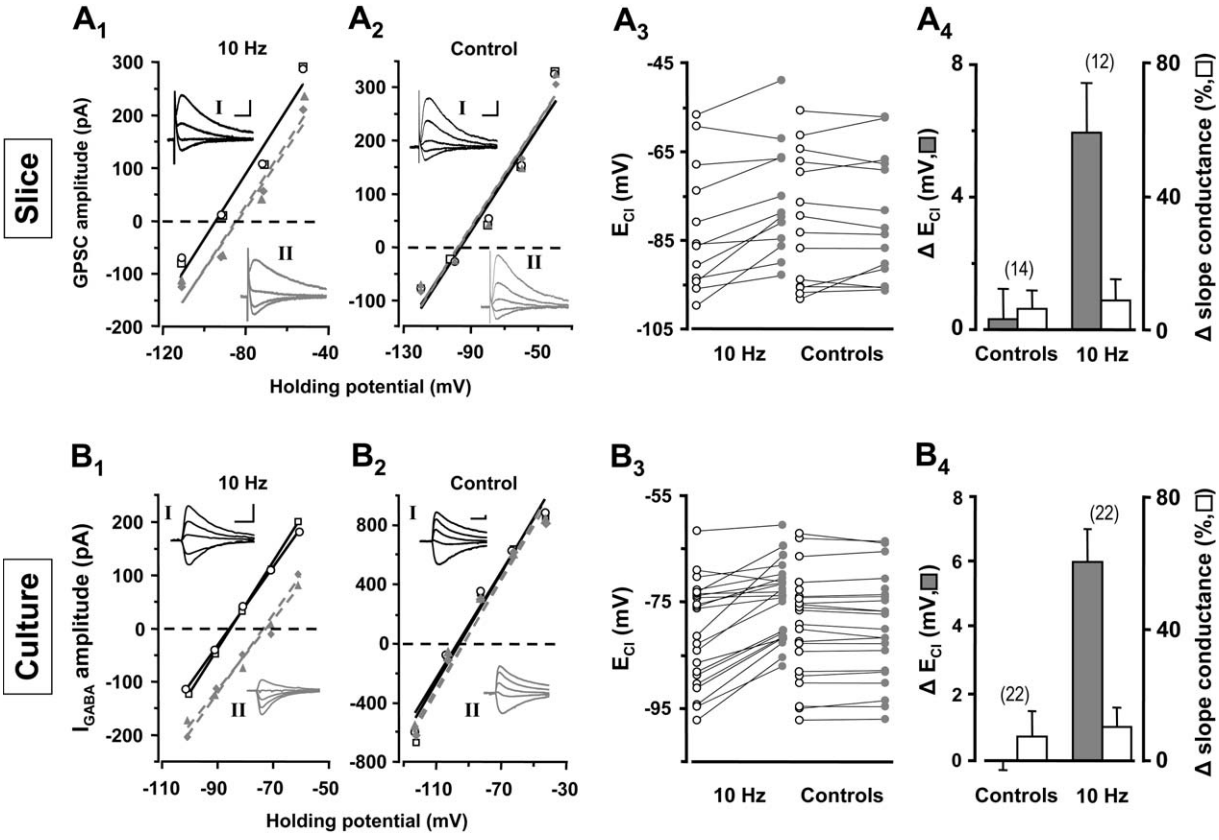

Figure 2. Repetitive Postsynaptic Spiking Induces a Positive Shift in E_{Cl} in Both Acute Hippocampal Slices and Cultures

(A_1) Examples of I-V relations for responses elicited by electrical stimulation of *stratum radiatum* in acute hippocampal slices, before (open symbols/black lines, two repeats at 5 min intervals) and after (filled symbols/dashed lines, two repeats at 5 and 10 min poststimulation) repetitive postsynaptic spiking (10 Hz, 5 min). Insets: sample traces of GPSCs at various V_cs before (I) and after (II) repetitive postsynaptic stimulation. Stimulus artifacts were truncated for clarity. Scale bar, 25 ms, 100 pA.

(A_2) Recording example of a control neuron in the absence of repetitive spiking.

(A_3) Summary plot showing E_{Cl} values measured before (open circles) and after (filled circles) repetitive spiking (10 Hz, 5 min) and those values measured in cells not exposed to stimulation. Data from the same cell are connected by a line. The difference in E_{Cl} before and after 10 Hz stimulation was significant ($p = 0.002$, paired Student's t test).

(A_4) Summary of average changes (±SEM) in E_{Cl} and normalized slope conductance for the same data set as used in (A_3).

(B_1–B_4) Data obtained from cultured neurons, as in (A_1)–(A_4), except that GABA puffing was used for E_{Cl} and slope conductance measurements. In (B_3), the paired Student's t test showed a significant difference in E_{Cl} before and after 10 Hz stimulation ($p < 0.001$).

conductance. Thus, the decrease in GPSC amplitudes was due to a change in E_{Cl} rather than in $GABA_A$ conductance. In control experiments in which no postsynaptic spiking was elicited, we found no modification in either E_{Cl} or slope conductance over the same duration (Figure 1D_2). Results from all experiments showed an average of 8.1 ± 1.6 mV positive shift in E_{Cl} after postsynaptic spiking, but no significant change in the normalized slope conductance (Figure 1D_3).

Postsynaptic Spiking Alters E_{Cl} Also in Acute Hippocampal Slices

In order to determine whether modulation of GABAergic inhibition by postsynaptic spiking also occurs in a more intact preparation, we stimulated presynaptic GABAergic neurons (0.05 Hz) with a bipolar stimulating electrode placed in the stratum radiatum of acute hippocampal slices (postnatal days 16 to 19 [P16–P19]), while gramicidin perforated-patch whole-cell recordings were made from CA1 neurons. GABAergic synaptic currents were isolated by applying CNQX (15 μM) and D-AP5 (25 μM), which block AMPA and NMDA receptors, respectively. Current-voltage plots were determined before and after (two repeats each) repetitive postsynaptic spiking (10 Hz, 5 min, Figure 2A_1) or in the absence of stimulation over the same time period (Figure 2A_2). As summarized for individual experiments (Figure 2A_3) and averages (Figure 2A_4), we found a significant positive shift in E_{Cl} (6.0 ± 1.5 mV), with magnitudes similar to those found in cultures, and no significant change in slope conductance (9.6% ± 6.2% versus 10.5% ± 5.8% in controls). Therefore, in hippocampal slice preparations, prolonged postsynaptic spiking also modifies GABAergic synapses by inducing a positive shift in E_{Cl} without affecting $GABA_A$ receptor conductance, effectively reducing GABAergic inhibition.

Repetitive Spiking Induces a Global Shift in E_{Cl}

To further confirm that repetitive postsynaptic spiking can induce a shift in E_{Cl} in the absence of presynaptic activation and to assay whether there is a global change in E_{Cl}, we recorded whole-cell currents (I_{GABA}) in cultured

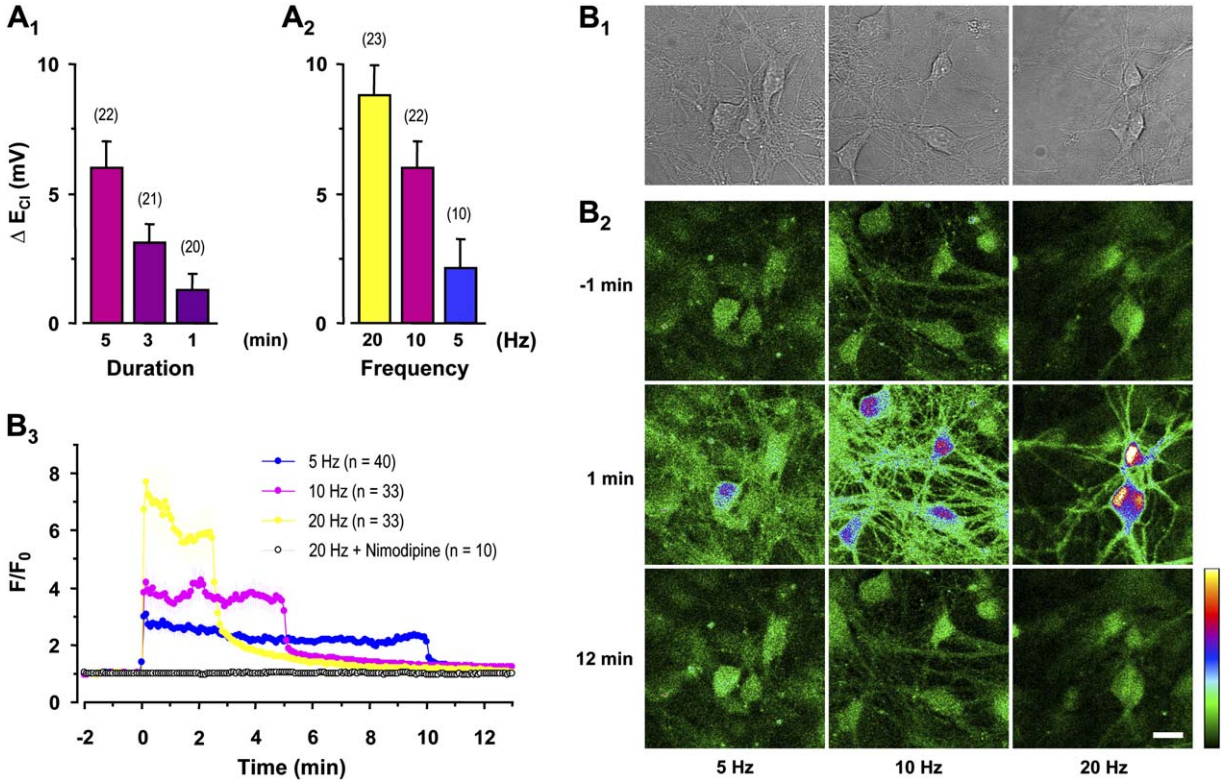

Figure 3. The E_{Cl} Shift and $[Ca^{2+}]_i$ Elevation Depend on the Spiking Pattern

(A_1 and A_2) Dependency of the E_{Cl} shift on the duration and frequency of repetitive stimulation. Data represent the mean ± SEM. The difference was significant among values of the E_{Cl} shift after 5, 3, and 1 min of stimulation at 10 Hz ($p < 0.001$, two-way ANOVA; $p < 0.05$, post hoc Holm-Sidak method) and among those after stimulation at 20 Hz (2.5 min), 10 Hz (5 min), and 5 Hz (10 min) ($p < 0.05$), but not significant between data for the unstimulated controls (data not shown) and those neurons stimulated for 1 min (10 Hz) or 5 Hz (10 min) ($p > 0.05$).

(B_1 and B_2) Calcium elevation triggered by field stimulation at different frequencies. Bright-field (B_1) and Fluo-4 fluorescence images, representing typical $[Ca^{2+}]_i$ in neurons before (−1 min), during (1 min after the onset), and after (12 min) field stimulation at 5, 10, and 20 Hz (B_2). The fluorescence intensity is coded by the linear color scale. Scale bar, 20 μm.

(B_3) Average changes (±SEM) in fluorescence intensity of all recorded neurons in response to 3000 pulses of field stimulation at 5 Hz (blue), 10 Hz (pink), 20 Hz (yellow), and 20 Hz in the presence of 15 μM nimodipine (open circles).

hippocampal neurons in response to exogenous GABA pulses applied at the soma ("GABA puffing"). The peak amplitude of GABA-induced currents observed at various V_cs was used to determine E_{Cl}. As shown by the example in Figure 2B_1, E_{Cl} measurements obtained by GABA puffing (two repeats, each before and after spiking, 10 Hz, 5 min) showed a significant positive shift in E_{Cl} without changing the slope conductance, whereas in the control experiment (without spiking) no change in E_{Cl} was observed over the same period (Figure 2B_2). As summarized for individual experiments (Figure 2B_3) and averages (Figure 2B_4), E_{Cl} underwent a significant shift toward more positive levels of 6.0 ± 1.0 mV after repetitive spiking, but not in the absence of stimulation (−0.1 ± 0.2 mV). These results largely agree with the changes in E_{Cl} determined by GPSCs in both culture and acute slice preparations, and they demonstrate that GABA puffing is a convenient assay for studying activity-induced changes in E_{Cl}.

The E_{Cl} Shift and $[Ca^{2+}]_i$ Elevation Depend on the Spiking Pattern

The dependence of the activity-induced E_{Cl} shift on the pattern of neuronal spiking was examined in cultured neurons by varying the duration and frequency of repetitive spiking. We first stimulated neurons at 10 Hz for three different durations (5, 3, and 1 min) to vary the total number of pulses, and changes in E_{Cl} were determined by GABA puffing before and after repetitive spiking. We found that the extent of the shift in E_{Cl} depended on the total number of pulses, with significant shifts observed after 5 min and 3 min (Figure 3A_1), in comparison with nonstimulated controls (data not shown), but not after 1 min of spiking. When the neurons were stimulated at three different frequencies (20, 10, and 5 Hz) for different durations (2.5, 5, and 10 min, respectively), keeping the total number of pulses constant, the extent of the E_{Cl} shift was frequency-dependent, with significant shifts observed at 20 Hz and 10 Hz, but not at 5 Hz stimulation (Figure 3A_2). Thus, the magnitude of the E_{Cl} shift induced by activity depends on the pattern of spiking.

A direct consequence of neuronal spiking is the elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which is known to depend on the frequency and duration of spiking (Wan and Poo, 1999). Fluorescence Ca^{2+} imaging was used to determine whether the patterns of stimulation used above induced different degrees of $[Ca^{2+}]_i$ elevation. Cultured hippocampal neurons were preloaded with the Ca^{2+}-sensitive dye Fluo-4, and field stimulation was used to induce spiking of a large population of

neurons. Optimal stimulation parameters to elicit action potentials were determined by whole-cell recording from individual neurons in sister cultures (see the Experimental Procedures). We found that the extent of $[Ca^{2+}]_i$ elevation depended on the frequency of stimulation, and the higher frequencies (10 and 20 Hz) that induced higher levels of $[Ca^{2+}]_i$ elevation corresponded to those found to induce a significant shift in E_{Cl} (Figure 3B). This correlation suggests that $[Ca^{2+}]_i$ elevation may be responsible for inducing the E_{Cl} shift and that this elevation must exceed a threshold to trigger the shift.

Ca^{2+} Elevation Is Required for the Activity-Induced E_{Cl} Shift

The importance of $[Ca^{2+}]_i$ elevation was further studied by chelating intracellular Ca^{2+} with pretreatment of BAPTA-AM (25 μM for 40 min, followed by a 15 min wash to allow de-esterification in the cytoplasm). Measurement of E_{Cl} by GABA puffing at different times before and after repetitive spiking (10 Hz, 5 min) showed that BAPTA-AM pretreatment completely prevented the E_{Cl} shift induced by stimulation (Figures 4A and 4B), indicating that $[Ca^{2+}]_i$ elevation is necessary for the activity-induced E_{Cl} shift.

The source of Ca^{2+} responsible for the activity-dependent E_{Cl} shift was also examined. Pretreatment (10–20 min) of cultured neurons and the continuous presence of the inhibitor of L-type voltage-gated Ca^{2+} channels, nimodipine (15 μM), completely eliminated $[Ca^{2+}]_i$ elevation (Figure $3B_3$) and prevented the E_{Cl} shift (Figures 4A and 4B) induced by repetitive spiking (10 Hz, 5 min), suggesting that Ca^{2+} influx through L-type Ca^{2+} channels was necessary for the activity-induced E_{Cl} shift. In addition, depletion of internal Ca^{2+} stores by pretreatment with thapsigargin, which inhibits Ca^{2+} reuptake by an endoplasmic reticulum Ca^{2+} pump, also abolished the spiking-induced E_{Cl} shift (Figure 4). The thapsigargin pretreatment by itself had no effect on E_{Cl} (data not shown). Furthermore, pretreatment of cultured neurons with ryanodine (10–20 min), which at a high concentration (100 μM) inhibits Ca^{2+} release through ryanodine receptor channels (Zucchi and Ronca-Testoni, 1997), also prevented the E_{Cl} shift induced by repetitive stimulation (Figure 4). Therefore, $[Ca^{2+}]_i$ elevation is required for the activity-dependent E_{Cl} shift, and both external Ca^{2+} influx through voltage-gated Ca^{2+} channels and Ca^{2+} release from internal stores are essential for the effect. Hence, Ca^{2+}-induced Ca^{2+} release appears to be essential for the spiking-dependent E_{Cl} shift.

Ca^{2+} Release from Internal Stores Is Sufficient to Induce a Shift in E_{Cl}

We next examined whether Ca^{2+} release from internal stores is by itself sufficient to trigger a shift in E_{Cl}. Pairs of connected cultured neurons were used to determine GPSC amplitudes at various V_cs, and both E_{Cl} and GPSC slope conductance were computed at different times before and after application of caffeine (10 mM), a drug known to induce Ca^{2+} release from internal stores (Zucchi and Ronca-Testoni, 1997). That caffeine elicits $[Ca^{2+}]_i$ elevation in our cultures was confirmed by fluorescence Ca^{2+} imaging: A majority of neurons (35/38) exhibited a rapid and transient $[Ca^{2+}]_i$ increase following caffeine application (Figure 5A). Measurements of E_{Cl} showed a shift toward positive levels and a reduction of slope conductance after caffeine treatment (Figure $5B_1$), whereas no change was observed in a control cell pair (Figure $5B_2$). The average E_{Cl} shift (8.1 ± 1.8 mV; Figure $5B_3$) after the caffeine treatment was comparable

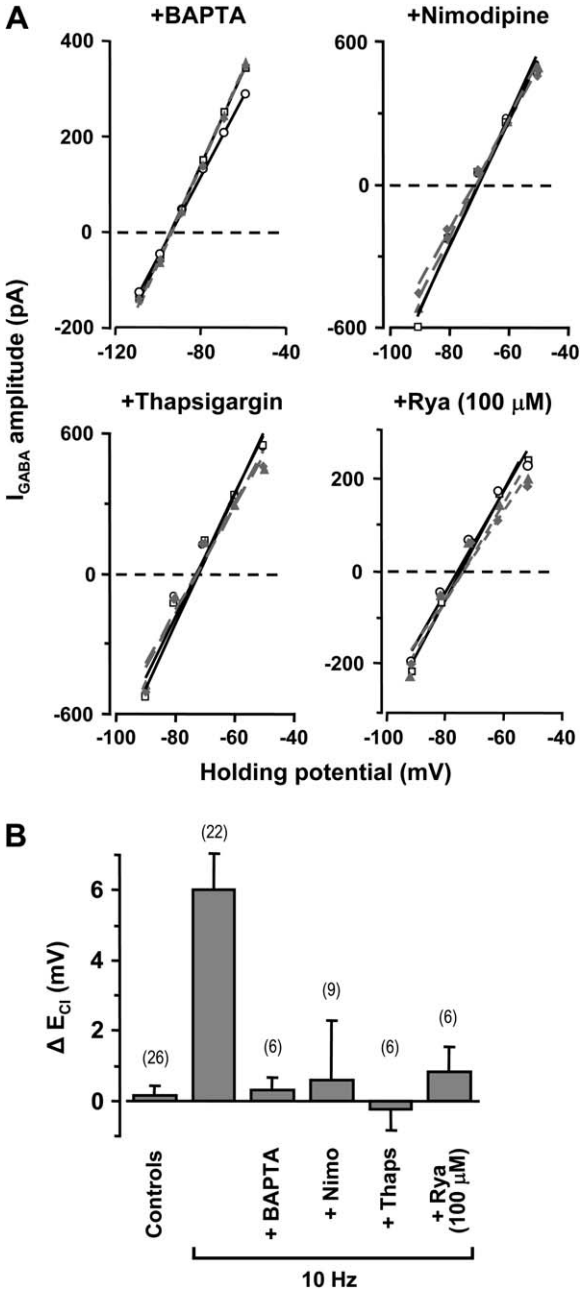

Figure 4. $[Ca^{2+}]_i$ Elevation Is Required for the Spiking-Induced E_{Cl} Shift

(A) Examples of the I-V relation for GABA responses elicited by GABA puffing before (open symbols/black lines; two repeats at 5 min intervals) and after (filled symbols/dashed lines, two repeats at 5 min and at 10 min poststimulation) repetitive spiking (10 Hz, 5 min) in the presence of BAPTA-AM, nimodipine, thapsigargin, or ryanodine.

(B) Summary of E_{Cl} shifts recorded under the various conditions described in (A). The E_{Cl} shift induced by repetitive spiking only was significantly different from that found under all other conditions ($p < 0.001$, Kruskal-Wallis one-way ANOVA on ranks; $p < 0.05$, post hoc Dunn's Method).

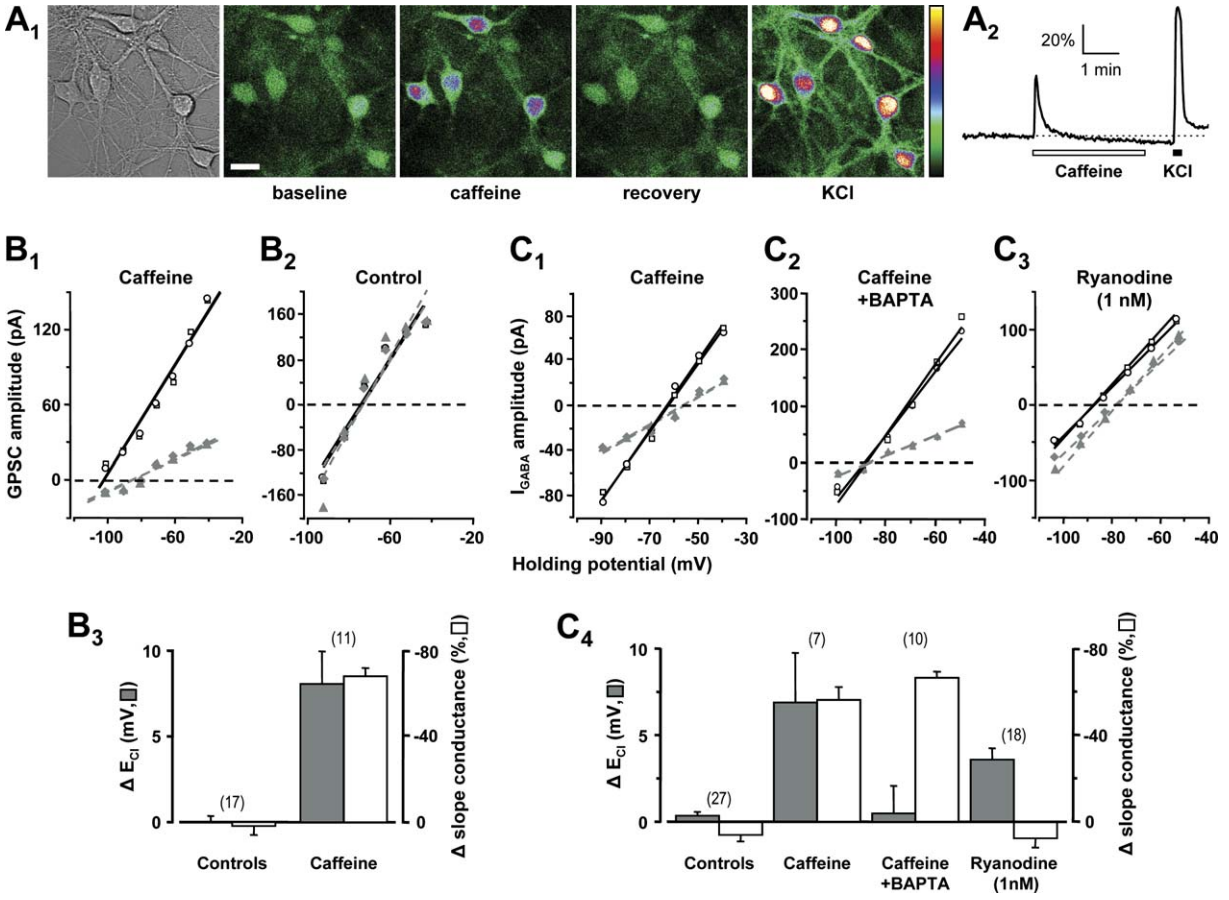

Figure 5. Ca^{2+} Release from Internal Stores Is Sufficient to Induce E_{Cl} Shift

(A_1) Representative images (bright-field and Oregon Green BAPTA-1 fluorescence) from Ca^{2+} imaging experiments. The images depict $[Ca^{2+}]_i$ before, during, after caffeine (10 mM) treatment, and during application of a pulse of KCl (20 mM). The fluorescence intensity is coded by the linear color scale. Scale bar, 20 μm.

(A_2) Average change in the fluorescence intensity of 12 randomly sampled neurons in response to caffeine and KCl.

(B_1 and B_2) Examples of the I-V relation of GPSCs recorded from a pair of connected neurons before (open symbols/black lines) and after (filled symbols/dashed lines) exposure to caffeine (B_1) and saline alone (B_2).

(B_3) Summary of the effects of caffeine on E_{Cl} and normalized slope conductance for GPSCs. Data represent the mean ± SEM. Changes in E_{Cl} and in slope conductance induced by caffeine treatment were significantly different from those found in control (ΔE_{Cl}: $p < 0.001$, Mann-Whitney rank sum test; Δ slope conductance: $p < 0.001$, Student's t test).

(C_1–C_4) Similar measurements as in (B_1)–(B_3), except that GABA puffing was used to determine E_{Cl} and slope conductance and cells were treated with caffeine in the absence or presence of preincubation with BAPTA-AM or with a low concentration of ryanodine. Changes in E_{Cl} induced by caffeine or ryanodine were significantly different from those in controls ($p < 0.001$, Kruskal-Wallis one-way ANOVA on ranks; $p < 0.05$, post hoc Dunn's Method). BAPTA-AM pretreatment completely abolished the effect of caffeine on E_{Cl} ($p < 0.05$). Only the caffeine treatment (with or without BAPTA-AM preincubation) induced a significant change in slope conductance ($p < 0.05$).

to that induced by repetitive spiking. Experiments using GABA puffing showed a similar caffeine-induced E_{Cl} shift and reduction of slope conductance (Figures 5C_1 and 5C_4). That the positive shift induced by caffeine was due to $[Ca^{2+}]_i$ elevation rather than other caffeine effects was shown by the finding that pretreatment with BAPTA-AM (25 μM) completely abolished the shift in E_{Cl} without affecting the reduction in slope conductance (Figures 5C_2 and 5C_4). The latter effect may reflect the action of caffeine on $GABA_A$ receptors (Taketo et al., 2004).

To further examine the effect of Ca^{2+} release from internal stores on E_{Cl}, we treated neurons with ryanodine at a low concentration known to open ryanodine receptor channels (Zucchi and Ronca-Testoni, 1997), allowing Ca^{2+} to be released from internal stores (Hong et al., 2000). As shown by the example in Figure 5C_3, ryanodine treatment resulted in a shift in E_{Cl} toward a more positive level, as measured by GABA puffing. Interestingly, unlike the change found after caffeine treatment, no change in slope conductance was observed after ryanodine application. As summarized in Figure 5C_4, after caffeine treatment the magnitude of the E_{Cl} shift was 6.9 ± 2.8 mV (similar to that determined by GPSCs), and a smaller shift in E_{Cl} (3.6 ± 0.6 mV) was found after ryanodine application. This difference may reflect distinctive patterns and extents of Ca^{2+} elevation induced by these two drugs. Taken together, our results demonstrate that intracellular Ca^{2+} release from internal stores is sufficient to induce a shift in E_{Cl} toward more positive values, similar to that induced by spiking activity.

KCC2 Sets the Level of E_{Cl}

During development or under pathological conditions, changes in the expression of Cl^- transporters, in

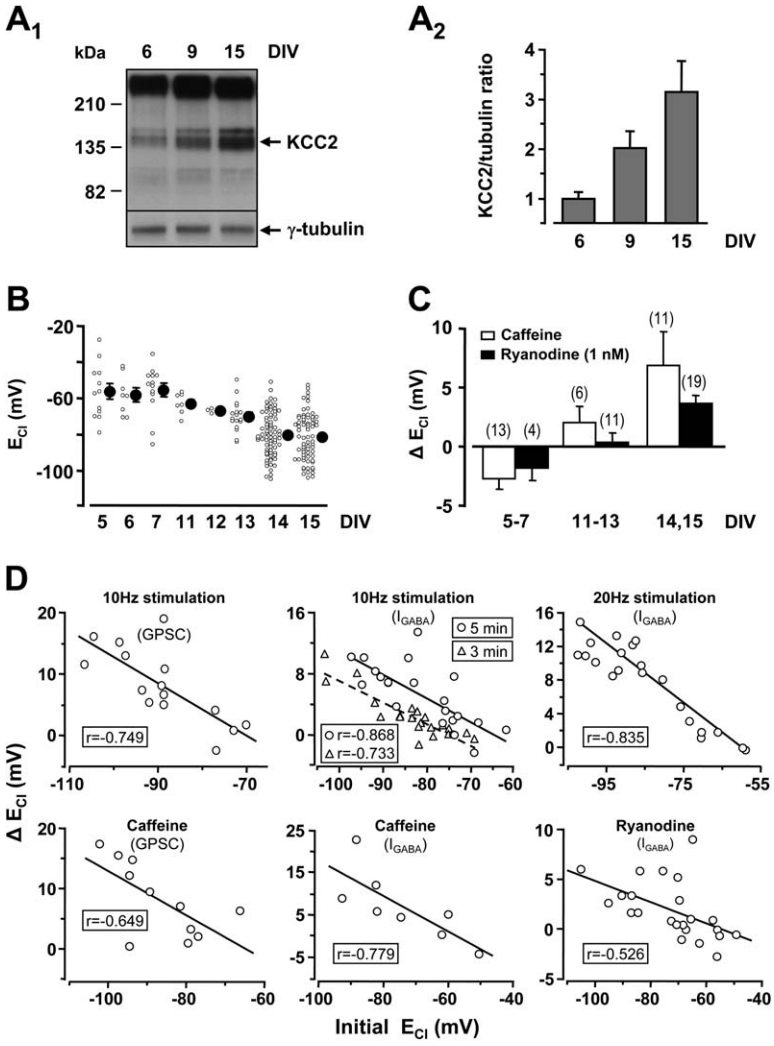

Figure 6. KCC2 Expression Is Correlated with the E_{Cl} Shift Induced by Spiking and $[Ca^{2+}]_i$ Elevation.

(A_1) Immunoblot with anti-KCC2 antibody, showing developmental upregulation of KCC2 protein (~140–150 kDa) in hippocampal neurons. The blot was reprobed with anti-γ-tubulin antibodies to estimate loading.

(A_2) Quantitative analysis of KCC2 protein levels for various DIVs. Data represent the mean ± SEM (n = 3) densitometric intensity of KCC2 bands, normalized by that of tubulin.

(B) Developmental changes in E_{Cl} determined by GABA puffing. Each open symbol represents the results from one cell, and filled circles depict averages (±SEM). Data for DIV 14 and DIV 15 are significantly different from those for DIV 5 and DIV 6 ($p < 0.001$, one-way ANOVA; $p < 0.05$, post hoc Holm-Sidak method).

(C) The effect of internal Ca^{2+} release on the E_{Cl} shift depends on DIV. Data represent mean ± SEM. The difference between controls (saline alone) (data not shown) and either caffeine or ryanodine treatment is significant at DIV 5–7 and DIV 14–15 ($p < 0.001$, two-way ANOVA for each treatment; $p < 0.05$, post hoc Holm-Sidak method), but not between controls and drug-treated cells at DIV 11–13 ($p > 0.05$).

(D) Scatter plots showing negative correlation between the initial E_{Cl} and the magnitude of the E_{Cl} shift found in experiments shown in Figures 1, 2B, 3A, 5B, and 5C. All lines represent linear regression (boxed r value: Pearson product moment correlation coefficient, $p < 0.05$).

particular, the neuron-specific K-Cl cotransporter KCC2, have been shown to affect E_{Cl} (Ganguly et al., 2001; Nabekura et al., 2002; Rivera et al., 2004; Toyoda et al., 2003). We thus investigated whether KCC2 mediated the changes of E_{Cl} induced by spiking activity and $[Ca^{2+}]_i$ elevation. Immunoblotting showed that the KCC2 protein level in cultured hippocampal neurons was low at DIV 6, but increased markedly at DIV 9 and DIV 15 (Figure 6A). Measurements of E_{Cl} by GABA puffing showed that E_{Cl} was significantly less hyperpolarized in young neurons (−55.2 ± 4.4 mV; DIV 5), gradually became more hyperpolarized, and reached a plateau value in more mature neurons (−81.3 ± 1.5 mV; DIV 15 (Figure 6B). Since KCC2 expression and its effect on E_{Cl} vary with development, we also examined whether the magnitude of the E_{Cl} shift induced by $[Ca^{2+}]_i$ elevation correlated with neuronal maturation. We found that the E_{Cl} shift induced by either caffeine or ryanodine showed an age-dependency, with magnitudes largely correlated with the culture age (Figure 6C): While more mature (DIV 14–15) neurons with hyperpolarized E_{Cl} exhibited significant shifts toward depolarized levels (caffeine, 6.9 ± 2.8 mV; ryanodine, 3.6 ± 0.7 mV), DIV 10–13 neurons exhibited no shift, and DIV 5–7 neurons showed small shifts toward hyperpolarized levels (caffeine, −2.7 ± 0.8 mV; ryanodine, −1.7 ± 1.0 mV). These negative shifts found in young neurons may be attributed to a downregulation of NKCC1 activity, which is known to be expressed at high levels in these neurons, but exerts an opposite action on Cl^- flux (Plotkin et al., 1997). That the hyperpolarized E_{Cl} of more mature neurons was due to a higher expression of KCC2 in these cells was supported by the strong correlation between the initial value of E_{Cl} and the magnitude of the E_{Cl} shift induced by repetitive spiking or caffeine and ryanodine in DIV 14–15 cultures (Figure 6D).

Blocking KCC2 Function Abolishes the Activity/Ca^{2+}-Induced E_{Cl} Shift

The correlations among the culture age, the initial E_{Cl} value, and the extent of the activity/Ca^{2+}-induced E_{Cl} shift prompted us to examine whether KCC2 directly mediates the E_{Cl} shifts induced by spiking and $[Ca^{2+}]_i$ elevation. We treated DIV 14–15 cultured neurons with furosemide (100 μM), a loop diuretic that inhibits K-Cl cotransporters (Thompson and Gahwiler, 1989) and found that this treatment by itself caused a marked shift in E_{Cl} toward more positive levels. An example of the current-voltage relation of I_{GABA} before and 10 min after furosemide application for a single neuron is shown in

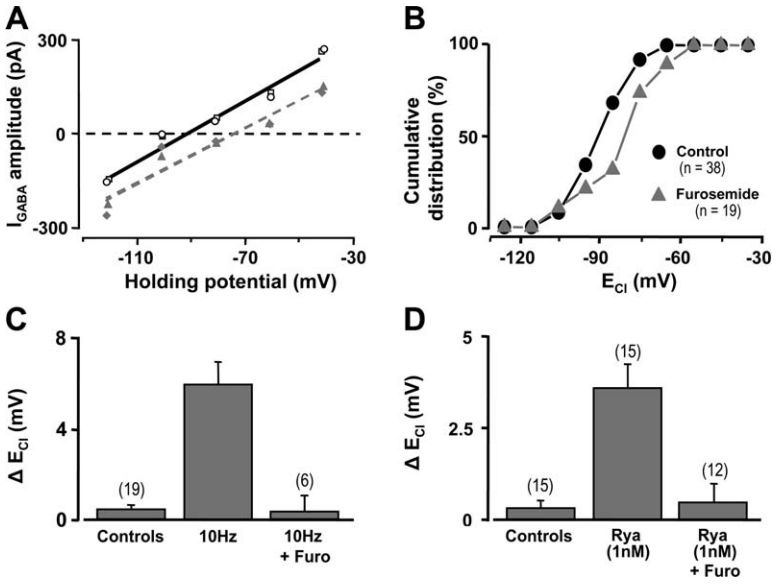

Figure 7. Blocking KCC2 Activity Abolishes the E_{Cl} Shift Induced by Spiking and $[Ca^{2+}]_i$ Elevation

(A) Example of the current-voltage relation of I_{GABA} before (open symbols/black lines) and 10 min after (filled symbols/dashed lines) furosemide application.

(B) Cumulative distribution of E_{Cl} for all furosemide-treated neurons and nontreated control neurons. E_{Cl} values were averaged over 10 mV bins. The two data sets are significantly different ($p < 0.05$, Kolmogorov-Smirnov test).

(C and D) Summary of all experiments, showing that the E_{Cl} shift induced by repetitive spiking (C) or by a low concentration of ryanodine (D) was abolished in the presence of furosemide ($p < 0.001$, Kruskal-Wallis one-way ANOVA on ranks; $p < 0.05$, post hoc Dunn's Method). Data represent the mean ± SEM.

Figure 7A. The distribution of E_{Cl} observed for a population of nontreated control cells and for furosemide-treated cells is summarized in Figure 7B. In the presence of furosemide, repetitive spiking (10 Hz, 5 min) or ryanodine treatment resulted in no additional shift in E_{Cl} (Figures 7C and 7D). This occlusion by furosemide is consistent with the requirement of KCC2 for the E_{Cl} shift induced by activity or $[Ca^{2+}]_i$ elevation.

KCC2 Overexpression in Young Neurons Leads to Hyperpolarized E_{Cl}

In young neurons expressing KCC2 at a low level and exhibiting a depolarized E_{Cl}, GABA is depolarizing and capable of activating NMDA receptors and voltage-dependent Ca^{2+} channels (Leinekugel et al., 1997), leading to $[Ca^{2+}]_i$ elevation (Owens et al., 1996). To directly demonstrate that regulation of KCC2 underlies the activity/Ca^{2+}-induced E_{Cl} shift, we expressed KCC2 in immature hippocampal neurons at an early developmental stage (DIV 5–7) when it is normally expressed at a low level, and we examined whether these neurons became responsive to spiking activity and $[Ca^{2+}]_i$ elevation as more mature neurons do. We prepared bicistronic constructs encoding KCC2 and either GFP or DsRed, which allowed us to distinguish KCC2-expressing cells from nonexpressing cells (Figure S1). Exogenous application of GABA (100 μM, 10 s pulse) elicited a rapid and reversible increase in $[Ca^{2+}]_i$ in untransfected or pIRES-DsRed cells (74/85, untransfected; 10/10, pIRES-DsRed), whereas the GABA-induced $[Ca^{2+}]_i$ elevation was absent in 19 of 20 neurons overexpressing KCC2 (Figure 8A). These data demonstrate that the exogenous KCC2 expressed in hippocampal neurons was functional.

Support for the critical role of KCC2 in setting the E_{Cl} was provided by the finding that heterologous expression of KCC2 in young neurons, which normally have a depolarized E_{Cl}, resulted in hyperpolarized E_{Cl} values similar to those of mature neurons. We transfected cells at DIV 4–5 with KCC2-IRES-GFP or IRES-GFP and assessed E_{Cl} at DIV 5–7. As shown in Figure 8B, in nontransfected or IRES-GFP-expressing neurons, the E_{Cl} values were −53. 9 ± 3.2 mV and −55.9 ± 2.6 mV, respectively, whereas in KCC2-overexpressing neurons the value was −90.2 ± 2.6 mV, similar to that found in mature neurons (see Figure 6B for comparison). Thus, KCC2 exogenously expressed in immature neurons appears to be physiologically relevant, and expression of KCC2 by itself is sufficient to induce the developmental switch of GABAergic responses from excitation to inhibition, consistent with findings in recent reports (Chudotvorova et al., 2005; Lee et al., 2005).

KCC2 Expression Endows Young Neurons with the Positive E_{Cl} Shifts by Activity/Ca^{2+}

Finally, we examined whether KCC2-overexpressing young neurons became responsive to neuronal spiking or drug-induced $[Ca^{2+}]_i$ elevation in a manner similar to that of mature neurons. In nontransfected or GFP-expressing young (DIV 5–7) neurons, both repetitive spiking and caffeine application induced a small negative shift in E_{Cl} (−3.6 ± 1.5 mV and −2.7 ± 0.9 mV, respectively). However, in young neurons overexpressing KCC2, repetitive spiking (10 Hz, 5 min) or caffeine treatment induced a positive shift in E_{Cl} (7.8 ± 2.3 mV and 6.8 ± 2.1 mV, respectively), similar to the shift obtained in DIV 14–15 mature neurons (Figure 8C and see Figure 6C for comparison). Hence, expression of KCC2 in young cultured neurons confers these neurons with the sensitivity for the positive E_{Cl} shift in response to neuronal activity or $[Ca^{2+}]_i$ elevation, demonstrating that KCC2 is essential in mediating these effects.

PKC Activity Is Required for the Spiking-Induced E_{Cl} Shift

The activity of cation-chloride cotransporters may be modulated by kinases and phosphatases (Adragna et al., 2004). To investigate the downstream mechanism by which spiking-induced $[Ca^{2+}]_i$ elevation leads to the KCC2-dependent shift in E_{Cl}, we treated mature cultured neurons with inhibitors of a variety of Ca^{2+}-dependent kinases and used GABA puffing to monitor E_{Cl} before and after repetitive spiking (10 Hz, 5 min). We first tested

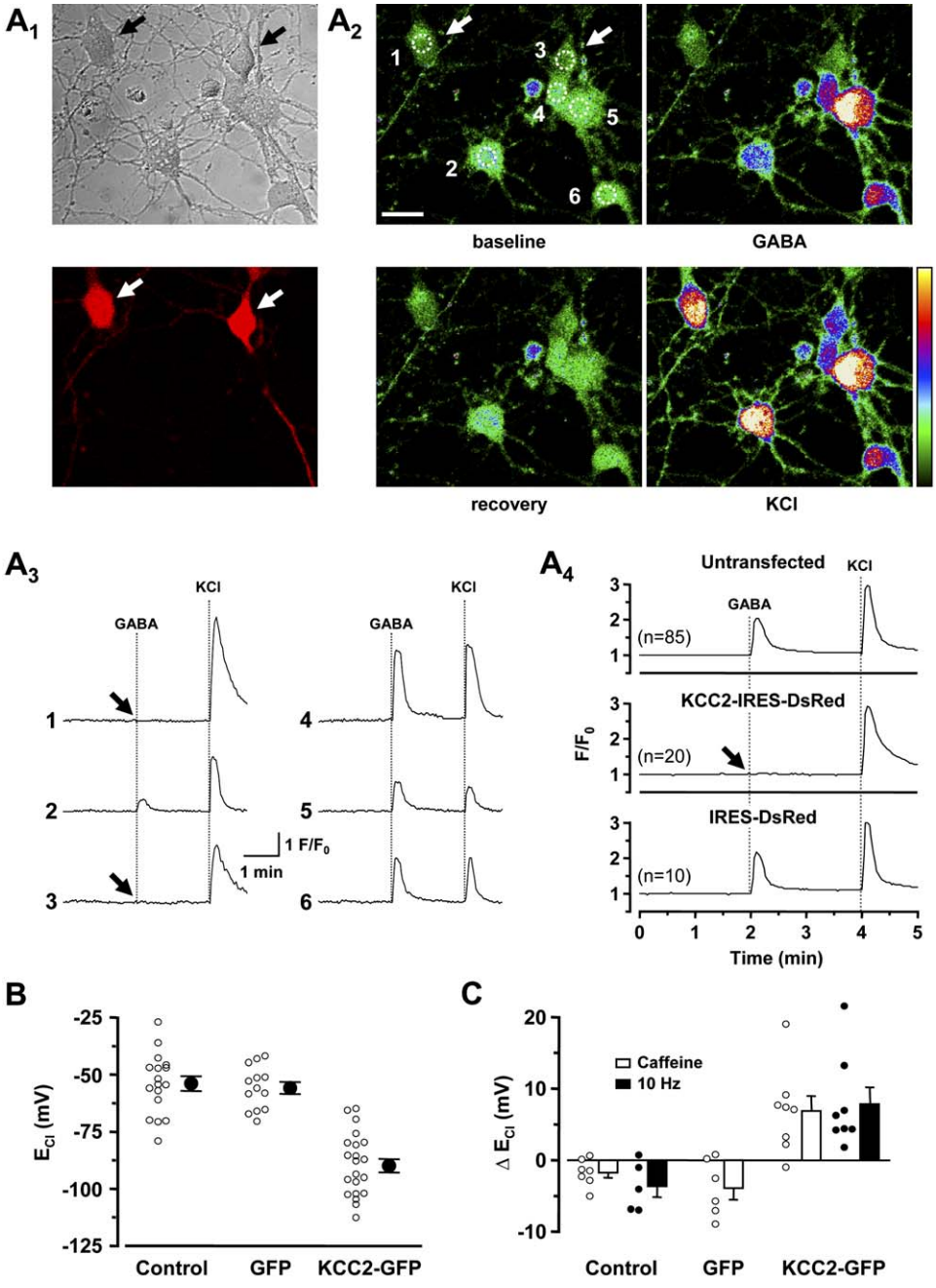

Figure 8. Expression of Exogenous KCC2 Renders Young Neurons Responsive to Spiking and $[Ca^{2+}]_i$ Elevation

(A_1) DIV 6 cultures transfected with pKCC2-IRES-DsRed, showing KCC2 expression in a subpopulation of neurons (marked by arrows).

(A_2) Pseudocolor images of $[Ca^{2+}]_i$ of the same neurons as in (A_1) before (baseline), during (GABA), after (recovery) bath application of GABA (100 μM, 10 s), and during subsequent application of KCl (20 mM, 10 s). GABA-induced $[Ca^{2+}]_i$ elevation was found only in cells (labeled 2 and 4–6) not overexpressing KCC2.

(A_3) Time course analysis of Ca^{2+} fluorescence intensities (sampled at circles shown in A_2) in response to GABA and KCl. Arrows indicate the absence of GABA-induced $[Ca^{2+}]_i$ elevation in pKCC2-IRES-DsRed-expressing neurons.

(A_4) Average traces of normalized fluorescence intensity for all neurons analyzed.

(B) E_{Cl} values determined by GABA puffing for DIV 5–7 neurons untransfected or transfected with pIRES-GFP or pKCC2-IRES-GFP. Shown are the results from single neurons (open circles) and averages (±SEM, filled circles). Data for pKCC2-IRES-GFP-transfected cells are significantly different from those of untransfected (control) or pIRES-GFP-transfected cells ($p < 0.001$, one-way ANOVA; $p < 0.05$, post hoc Holm-Sidak method).

(C) Effect of KCC2 overexpression in DIV 5–7 neurons on the changes in E_{Cl} induced by repetitive spiking (10 Hz, 5 min) and caffeine treatment. Data represent the mean ± SEM. Data from pKCC2-IRES-GFP-transfected cells are significantly different from those of untransfected (control) or pIRES-GFP-transfected cells ($p < 0.001$, one-way ANOVA; $p < 0.05$, post hoc Holm-Sidak method).

staurosporine (100 nM), a broad-spectrum inhibitor of protein kinases, and found that treatment with this drug prevented the spiking-induced E_{Cl} shift (Figure S2). At the concentration we used, staurosporine blocks several kinases, including Ca^{2+}/calmodulin kinase (CaMK), protein kinase A (PKA), and protein kinase C (PKC). We thus performed additional experiments using KN-93, KT5720, and Gö 6976, specific inhibitors of CaMKII, PKA, and Ca^{2+}-dependent PKC isozymes, respectively. We found that Gö 6976 (1 μM) treatment blocked the E_{Cl} shift induced by repetitive spiking, while treatment with KN-93 (10 μM) or KT5720 (1 μM) did not affect the shift. This indicates that PKC is required for the E_{Cl} shift and suggests that repetitive spiking-induced $[Ca^{2+}]_i$ elevation may act through PKC to phosphorylate either KCC2 directly or other KCC2 regulatory proteins, leading to modification of GABAergic transmission.

Since tyrosine kinases are known to modulate KCC2 function in hippocampal neurons (Kelsch et al., 2001), and serine/threonine phosphatase inhibitors block KCC2 activity in oocytes (Strange et al., 2000), we also tested the effect of tyrosine kinase or serine/threonine phosphatase inhibitors. We treated neurons with the general tyrosine kinase inhibitor lavendustin A (10 μM), the calcineurin inhibitor cyclosporine A (1–10 μM), or the protein phosphatase 1/2A inhibitor calyculin A (1 μM) and examined their effect on the activity-induced shift. None of these drugs was found to affect the magnitude of the E_{Cl} shift after repetitive spiking (Figure S2). Therefore, activation of protein tyrosine kinases and phosphatases, including the Ca^{2+}-dependent phosphatase calcineurin, is not required for the activity-induced E_{Cl} shift. In these pharmacological studies, all drug treatments by themselves did not produce a detectable effect on E_{Cl}. In the case of cyclosporine A treatment, we observed a drop in the slope conductance, consistent with the effect of calcineurin activity on $GABA_A$ receptors reported previously (Wang et al., 2003).

Discussion

The main finding of this work is that repetitive postsynaptic spiking induced a Ca^{2+}-dependent downregulation of KCC2 function, reducing the inhibitory action of GABA by a positive shift of E_{Cl} in both hippocampal cultures and acute slices. This effect depended on the pattern of spiking activity, with larger shifts for repetitive spiking of higher frequency or longer duration. Moreover, the extent of $[Ca^{2+}]_i$ elevation also correlated with the frequency of spiking. Both Ca^{2+} influx through voltage-gated Ca^{2+} channels and Ca^{2+} release from internal stores were necessary for the spiking-induced E_{Cl} shift, and internal Ca^{2+} release was sufficient by itself to cause the shift. Our conclusion that the Ca^{2+}-dependent regulation of KCC2 function is responsible for the E_{Cl} shift is based on three lines of evidence: (1) Pharmacological blockade of K-Cl cotransporter with furosemide abolished the E_{Cl} shift induced by activity or $[Ca^{2+}]_i$ elevation; (2) The magnitude of the E_{Cl} shifts correlated with the initial level of E_{Cl}, which was determined primarily by the level of KCC2 expression; and (3) Young neurons expressing a low level of KCC2 did not exhibit a positive shift in response to spiking activity or $[Ca^{2+}]_i$ elevation, but showed a shift comparable to that of mature neurons upon expression of exogenous KCC2. These findings shed new light on the mechanisms by which prolonged spiking activity may modify neuronal Cl^- transport activity, which, in turn, reduces the inhibitory action of GABAergic inputs.

Two Forms of Activity-Dependent Plasticity of GABAergic Transmission

The efficacy of GABAergic transmission depends on the extent of synaptic activation of $GABA_A$ receptors and depends strongly on the driving force for Cl^- currents through the activated receptors, because postsynaptic $[Cl^-]_i$ is highly susceptible to modulation. Since KCC2 expression is low and NKCC1 expression is high early in development, $[Cl^-]_i$ is elevated in young neurons and GABAergic transmission is depolarizing and excitatory (Ben-Ari, 2002). Later in development, upregulation of KCC2 and downregulation of NKCC1 lower $[Cl^-]_i$, which causes a shift in E_{Cl} toward more negative levels, converting GABAergic transmission from excitatory to inhibitory (Ben-Ari, 2002). The depolarizing action of GABA itself appears to promote the developmental upregulation of KCC2 through a Ca^{2+}-dependent transcriptional regulation (Ganguly et al., 2001).

In the present study of mature neurons, we found another form of activity-induced and Ca^{2+}-dependent modulation of E_{Cl} that has an opposite effect, i.e., a positive shift in E_{Cl}. The two opposite effects of neuronal activity represent very different aspects of KCC2 regulation. The developmental effect occurs at a time when KCC2 expression is low, requires prolonged activity over a period of days, and involves transcriptional activation of KCC2 expression. In contrast, the effect reported here was present in mature neurons expressing a high level of KCC2 and it supposedly occurred within minutes after stimulation and involved mechanisms independent of protein synthesis. It is interesting to note that the acute downregulation of KCC2 function shown here may potentially counteract the effect of developmental upregulation of KCC2 expression. However, it remains to be determined whether the acute effect of activity is long-lasting (beyond the 40 min tested in the present study) and whether the in vivo spiking pattern of developing neurons is sufficient to modulate KCC2 function. Nevertheless, acute activity-dependent modulation of KCC2 may provide a useful mechanism for a partial reversal of the excitation-to-inhibition transition of GABAergic transmission once KCC2 expression has attained mature levels. Indeed, both transcriptional regulation of KCC2 expression and posttranslational modification of KCC2 function may contribute to the plasticity of the GABAergic system.

Plasticity of GABAergic Transmission Mediated by Changes in E_{Cl}

Plasticity of GABAergic transmission is found in many brain regions, where the efficacy of GABAergic synapses may undergo bidirectional long-term changes following repetitive stimulation (Aizenman et al., 1998; Gaiarsa et al., 2002; Kano et al., 1992; McLean et al., 1996).

It is not clear whether in some previous studies these synaptic changes resulted from GABAergic activity or indirectly from postsynaptic spiking induced by costimulation of converging excitatory inputs. Modification

of GABAergic synapses is usually attributed to changes in synaptic conductance, caused by alteration in either presynaptic transmitter release or the number/property of postsynaptic receptors. However, changes in $[Cl^-]_i$ can alter E_{Cl}, thus modifying GABAergic transmission (Ben-Ari et al., 1981; Ling and Benardo, 1995; McCarren and Alger, 1985; Thompson and Gahwiler, 1989). While some earlier studies could not address this possibility due to the use of the whole-cell recording method, which does not preserve endogenous $[Cl^-]_i$, our present findings (using gramicidin perforated-patch recording) directly showed that postsynaptic spiking can modify GABAergic synapses through a mechanism that alters the driving force for Cl^- currents.

Ca^{2+} Elevation and E_{Cl} Modulation

A direct consequence of spiking activity is the elevation of $[Ca^{2+}]_i$. We observed that the effect of postsynaptic spiking on E_{Cl} and $[Ca^{2+}]_i$ elevation depended on the frequency of repetitive spiking (Figure 3A). We propose that the dependence of the E_{Cl} shift on the pattern of spiking simply reflects the dependence on $[Ca^{2+}]_i$ and that a threshold of elevated $[Ca^{2+}]_i$ is required for triggering the activity-induced E_{Cl} shift. That the spiking activity acts through $[Ca^{2+}]_i$ elevation was further shown by the finding that preventing $[Ca^{2+}]_i$ elevation with intracellular loading of BAPTA, blocking Ca^{2+} influx through voltage-gated L-type Ca^{2+}channels, or reducing Ca^{2+} release from internal stores all abolished the effect of activity on E_{Cl}. The critical importance of $[Ca^{2+}]_i$ elevation was also supported by the finding that caffeine- or ryanodine-induced $[Ca^{2+}]_i$ elevation was by itself sufficient to induce a positive shift in E_{Cl}.

Local versus Global Modulation of KCC2 by Activity

A recent study has shown that coincident presynaptic and postsynaptic spiking in hippocampal neurons resulted in a reduction of GABAergic inhibition that was attributed to a local positive shift in E_{Cl} (Woodin et al., 2003). This shift was blocked by furosemide, suggesting that KCC2 may be involved. In the present study, we provided direct evidence that postsynaptic spiking can lead to a global positive shift in E_{Cl} by downregulating KCC2 activity. A positive shift in E_{Cl} elicited by repetitive spiking or caffeine was observed in young neurons only when KCC2 was overexpressed in these cells. We note that Woodin et al. (2003) did not observe an E_{Cl} shift when they stimulated only the postsynaptic cell at a low frequency (5 Hz) for a brief duration (30 s). This is consistent with our finding that no significant E_{Cl} shift was observed following stimulation at 5 Hz, which was correlated with a low level of global $[Ca^{2+}]_i$ elevation. Woodin et al. (2003) showed that coincident presynaptic and postsynaptic activation at 5 Hz was required in inducing the local positive E_{Cl} shift. Here, we found that prolonged postsynaptic spiking alone at higher frequencies was sufficient by itself to cause a global E_{Cl} shift. Since younger neurons with a more positive E_{Cl} were used in the study by Woodin et al., coincident GABA release presumably provided local depolarization and resultant Ca^{2+} influx in addition to that induced by postsynaptic spiking, leading to high local $[Ca^{2+}]_i$ elevation and consequent KCC2 modulation. The dependence of the E_{Cl} shift on L-type Ca^{2+} channels found in both studies further supports the notion that downregulation of KCC2 function by $[Ca^{2+}]_i$ elevation may account for both the local synaptic E_{Cl} modulation found in Woodin et al. (2003) and the global E_{Cl} modulation reported here.

Potential Mechanisms of the Effects of Ca^{2+} Action on KCC2

The effects of postsynaptic spiking or $[Ca^{2+}]_i$ elevation on KCC2 function were observed within minutes following stimulation, consistent with alteration of membrane trafficking or posttranslational modification of KCC2, rather than changes in gene transcription or protein synthesis. Preliminary studies using surface biotinylation after treatment of our hippocampal cultures with caffeine or high K^+ showed that the surface pool of KCC2 remained unchanged (data not shown), suggesting that the downregulation of KCC2 activity observed here is not due to internalization of KCC2.

Rapid regulation of KCC2 function may also be achieved by changing the status of phosphorylation at consensus sites for protein kinase C or tyrosine kinase (Payne et al., 1996). Serine/threonine phosphorylation has been shown to regulate KCC2 activity in oocytes (Strange et al., 2000), whereas in hippocampal neurons receptor tyrosine kinases activate KCC2 during development (Kelsch et al., 2001) and downregulate KCC2 function after interictal-like activity (Rivera et al., 2004). In the present study, we found that inhibitors of Ca^{2+}-dependent PKC abolished the spiking-induced E_{Cl} shift, while inhibitors of several other kinases and phosphatases did not. Therefore, PKC-dependent phosphorylation of KCC2 or its regulatory proteins may be responsible for the regulation of KCC2 function by $[Ca^{2+}]_i$ elevation.

Our experimental conditions allowed us to perform stable recordings for up to 50 min. The effect of spiking and $[Ca^{2+}]_i$ elevation on E_{Cl} was persistent for as long as the recording was made. It is possible that after the initial downregulation of existing KCC2 activity, protein synthesis is involved in further regulation of KCC2. Indeed, Rivera et al. (2004) showed a fast turnover of the plasmalemmal pool of KCC2 that could provide a mechanism for rapid downregulation of KCC2 activity via transcriptional regulation. The latter study also showed that interictal-like enhanced synaptic activity reduced the transcription and synthesis of KCC2, leading to an increased net removal of the plasmalemmal KCC2 pool and a reduced KCC2 activity. Thus Ca^{2+}-dependent transcriptional regulation and posttranslational modification can both serve for the activity-induced modulation of KCC2.

KCC2 in the Pathophysiology of GABAergic Transmission

The balance between excitation and inhibition is essential for developmental refinement and integrative function of neural circuits, and gross imbalance leads to pathological consequences. There is evidence that KCC2 plays a role in epilepsy, a condition characterized by depolarizing GABAergic signals that cause seizures by inducing hyperexcitability and highly synchronized electrical activity (Cohen et al., 2002; Hochman et al., 1995). In homozygous KCC2-deficient mice (with 5% of wild-type KCC2 level), frequent seizure activity and

severe brain injury are observed, whereas heterozygous mice are characterized by increased susceptibility for epileptic seizure and resistance to anticonvulsants (Woo et al., 2002). Furthermore, hippocampal kindling-induced seizures in vivo and interictal-like activity in slices lead to downregulation of KCC2 mRNA in rodents (Rivera et al., 2002, 2004). Interestingly, spontaneous network activity associated with depolarizing GABAergic transmission is found in the temporal lobe tissue from epileptic patients (Cohen et al., 2002). It is an attractive hypothesis that the depolarizing effect of GABA could be due in part to reduced KCC2 activity, triggered by a mechanism similar to that shown here. Consistent with our observation that larger E_{Cl} shifts were induced by spiking with higher frequency and longer duration, abnormal high-level spiking may result in a substantial reduction of inhibition in neural circuits, leading to epileptic discharges via a positive feedback mechanism.

Brain injury is known to cause $[Ca^{2+}]_i$ elevation in neurons, due to excessive glutamate release (Lipton, 1999), and to engender depolarizing GABA-mediated responses that appear to result from an increased $[Cl^-]_i$ (Nabekura et al., 2002; Toyoda et al., 2003; Van Den Pol et al., 1996). Our results provide direct causal links between a sequence of events consisting of $[Ca^{2+}]_i$ elevation, KCC2 downregulation, increased $[Cl^-]_i$, and depolarizing GABA action. During epilepsy and following neural injury, excessive postsynaptic spiking activity may induce a large E_{Cl} shift, leading to substantial GABA-induced depolarization and Ca^{2+} elevations resembling those seen in embryonic neurons. Such "reversal" to the embryonic state may allow activation of developmental programs for $[Ca^{2+}]_i$-dependent gene expression, sprouting, and neuronal apoptosis, which have been observed under these pathological conditions.

Concluding Remarks

We have shown that GABAergic inputs can be modulated via repetitive postsynaptic spiking, a condition that mimics activity induced by convergent excitatory inputs in vivo. Since excitatory inputs that cause repetitive postsynaptic spiking are likely to undergo long-term potentiation (LTP) themselves, LTP of excitatory inputs may be accompanied by a positive E_{Cl} shift, resulting in a reduced GABAergic inhibition and an enhanced net excitation of the postsynaptic neuron. This might provide a condition for further enhancing activity-dependent synaptic modification. Lower levels of inhibition are known to facilitate induction and maintenance of LTP in the hippocampus (Stelzer et al., 1994); the postsynaptic spiking-induced reduction of inhibition shown here may thus be an integral part of activity-dependent modification of neural circuits.

Experimental Procedures

Cell Cultures, Expression Vectors, and Transfection

Cultures of dissociated embryonic rat hippocampal neurons were prepared as previously described (Woodin et al., 2003). Transfections were carried out 4 to 5 days after plating, using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendations. Full-length coding sequence of KCC2 was obtained by RT-PCR of total RNA from adult rat hippocampus using two synthetic oligonucleotide primers (5′-AGACTCTCGAGCACCATGCTC AACAACCTGACGGACT-3′ and 5′-AGACTGTCGACTCAGGAGTAGA TGGTGATGAC-3′) based on the rat KCC2 sequence (GenBank U55816), with a restriction site added to the 5′ end of each primer. An XhoI-SalI-fragment of the PCR products was then subcloned into the pIRES2-EGFP and pIRES2-DsRed2 vectors (Clontech). The identity of the inserted fragments was confirmed by sequencing.

Electrophysiology

Hippocampal Cultures

All experiments were performed on pairs of connected or single cultured neurons by whole cell gramicidin perforated-patch recording. Pipettes were filled with an internal solution of 150 mM KCl and 10 mM HEPES (ICS; ~300 mOsmol [pH, 7.3]) containing 2.5–5 μg/ml gramicidin A (Sigma; diluted from a stock solution of 5 mg/ml in DMSO). The external bath solution was a HEPES-buffered saline solution (HBS) containing: 150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, and 0.01 mM CNQX (~315 mOsmol, [pH, 7.3]). Cells were constantly perfused with fresh recording medium (1–2 ml/min) at 22ºC. Data, filtered at 5 kHz and sampled at 10 kHz, were acquired with patch-clamp amplifiers (Axopatch 200B; Axon Instruments) and analyzed using pClamp 8.0 software (Axon Instruments). Pipette capacitance was minimized and series resistance, which was typically 15–25 MΩ, was not compensated, but monitored throughout the experiment. It was estimated from capacitive currents elicited by a 5 mV hyperpolarization pulse (50 ms) before each sweep. Recordings were terminated and data were discarded when the series resistance varied by >15% or when the perforated-patch spontaneously ruptured into whole-cell configuration as evidenced by very large depolarizing currents due to an E_{Cl} of ~0 mV. For each recording, after the access resistance had dropped and stabilized (15–30 min), a minimum of 10 min of baseline recording with at least one E_{Cl} measurement was performed prior to the induction protocol, which consisted of either drug application or repetitive stimulation of the postsynaptic neuron (current-clamped) with depolarizing current pulses (2 nA, 1.5 ms). Experiments were included in the data analysis only when a stable baseline of the GPSC amplitude and E_{Cl} were observed before the induction protocol. The synaptic E_{Cl} was determined by varying the holding potential of the postsynaptic cell in 10 mV increments and measuring the resulting GPSC amplitude. Linear regression was used to calculate a best-fit line for the voltage dependence of GPSCs, and the interpolated intercept of this line with the abscissa was taken as the E_{Cl} value. The slope of this line was used as the corresponding slope conductance. Determination of E_{Cl} at the soma was made in a manner similar to that described for the synapse, except that GABA was puffed at the soma in 15 s intervals (100 μM, 20 ms, 5 psi) through a micropipette (2–3 MΩ) controlled by a pressure valve (Picospritzer II; Parker Instrumentation).

Acute Hippocampal Slices

Hippocampal slices were prepared from P16–P19 Sprague-Dawley rats, as previously described (Woodin et al., 2003). A bipolar stimulating electrode (Rhodes Medical Instruments) placed in the stratum radiatum was used to stimulate interneurons (30–100 μs, 5–20 V). Whole-cell perforated-patch recordings using gramicidin were made from CA1 neurons. Electrodes (glass capillaries; VWR) with a tip resistance of ~5 MΩ were tip filled with ICS and then backfilled with the same solution containing 40 μg/μl gramicidin A (diluted from a stock solution of 40 mg/ml in DMSO). The external bath solution consisting of mM 124 mM NaCl, 2.8 mM KCl, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 12 mM D-Glucose (~310 mOsmol, [pH, 7.4]), 10 μM CNQX, and 25 μM D-AP5 was oxygenated with 95% O_2/5% CO_2 and continually perfused (1.5 ml/min).

Calcium Imaging

Cultured neurons were loaded with AM-ester derivatives of the Ca^{2+}-sensitive fluorescent dyes Fluo-4 or Oregon Green BAPTA-1 (Molecular Probes) for 20–40 min at 22ºC in HBS containing 5 μM fluorescent dye dissolved in a DMSO solution containing 10% Pluronic F-127. Cells were rinsed with HBS and incubated for 15–30 min to allow de-esterification of intracellular AM-esters. Solutions were exchanged using a multibarreled perfusion system (flow-rate, 1–2 ml/min). Imaging was performed using a confocal laser scanning microscope (Leica DM IRBE) and frames were collected every 2–5 s using a 40× oil-immersion objective (NA 1.28) with the pinhole fully open. For Fluo-4/Oregon Green BAPTA-1 and DsRed, excitation was

performed at 488 nm and 568 nm and emission signals were collected at 500–550 nm and 613–700 nm, respectively. A total of 4–12 neurons was examined in each experiment. Transmitted and fluorescent images reflecting Ca^{2+} dynamics were acquired (512 × 512 pixels; voxel size: 488 nm) using Leica confocal software (version 2.0), and data were analyzed using Excel Software. Average fluorescence intensities of an ~50 pixel area taken at the soma were used for quantification. The criterion defining the responsiveness of neurons to various drug applications was the same as previously described (Ganguly et al., 2001). For field stimulation, neurons were loaded with Fluo-4 as described above and imaged in a chamber containing two parallel platinum wire electrodes (Warner Instruments). The intensity and pattern of stimuli were controlled by an S48 stimulator (Grass Instruments). Optimal stimulation parameters were determined from five randomly located neurons in sister cultures using whole-cell gramicidin perforated-patch recording (data not shown). Electric pulses of 25 V/cm (1.5 ms) were necessary to fire action potentials in all neurons for each discharge at all frequencies tested (20, 10, and 5 Hz). Action potentials under these conditions were blocked by tetrodotoxin (1 μM).

Supplemental Data

Supplemental Data include two figures and Supplemental Experimental Procedures and can be found with this article online at http://www.neuron.org/cgi/content/full/48/5/773/DC1/.

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References

Adragna, N.C., Fulvio, M.D., and Lauf, P.K. (2004). Regulation of K-Cl cotransport: from function to genes. J. Membr. Biol. *201*, 109–137.

Aizenman, C.D., Manis, P.B., and Linden, D.J. (1998). Polarity of long-term synaptic gain change is related to postsynaptic spike firing at a cerebellar inhibitory synapse. Neuron *21*, 827–835.

Ben-Ari, Y. (2002). Excitatory actions of GABA during development: the nature of the nurture. Nat. Rev. Neurosci. *3*, 728–739.

Ben-Ari, Y., Krnjevic, K., Reiffenstein, R.J., and Reinhardt, W. (1981). Inhibitory conductance changes and action of gamma-aminobutyrate in rat hippocampus. Neuroscience *6*, 2445–2463.

Chudotvorova, I., Ivanov, A., Rama, S., Hubner, C.A., Pellegrino, C., Ben-Ari, Y., and Medina, I. (2005). Early expression of KCC2 in rat hippocampal cultures augments expression of functional GABA synapses. J. Physiol. *566*, 671–679.

Cohen, I., Navarro, V., Clemenceau, S., Baulac, M., and Miles, R. (2002). On the origin of interictal activity in human temporal lobe epilepsy in vitro. Science *298*, 1418–1421.

Gaiarsa, J.L., Caillard, O., and Ben-Ari, Y. (2002). Long-term plasticity at GABAergic and glycinergic synapses: mechanisms and functional significance. Trends Neurosci. *25*, 564–570.

Ganguly, K., Schinder, A.F., Wong, S.T., and Poo, M. (2001). GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell *105*, 521–532.

Hochman, D.W., Baraban, S.C., Owens, J.W., and Schwartzkroin, P.A. (1995). Dissociation of synchronization and excitability in furosemide blockade of epileptiform activity. Science *270*, 99–102.

Hong, K., Nishiyama, M., Henley, J., Tessier-Lavigne, M., and Poo, M. (2000). Calcium signalling in the guidance of nerve growth by netrin-1. Nature *403*, 93–98.

Kano, M., Rexhausen, U., Dreessen, J., and Konnerth, A. (1992). Synaptic excitation produces a long-lasting rebound potentiation of inhibitory synaptic signals in cerebellar Purkinje cells. Nature *356*, 601–604.

Kelsch, W., Hormuzdi, S., Straube, E., Lewen, A., Monyer, H., and Misgeld, U. (2001). Insulin-like growth factor 1 and a cytosolic tyrosine kinase activate chloride outward transport during maturation of hippocampal neurons. J. Neurosci. *21*, 8339–8347.

Kyrozis, A., and Reichling, D.B. (1995). Perforated-patch recording with gramicidin avoids artifactual changes in intracellular chloride concentration. J. Neurosci. Methods *57*, 27–35.

Lee, H., Chen, C.X., Liu, Y.J., Aizenman, E., and Kandler, K. (2005). KCC2 expression in immature rat cortical neurons is sufficient to switch the polarity of GABA responses. Eur. J. Neurosci. *21*, 2593–2599.

Leinekugel, X., Medina, I., Khalilov, I., Ben-Ari, Y., and Khazipov, R. (1997). Ca2+ oscillations mediated by the synergistic excitatory actions of GABA(A) and NMDA receptors in the neonatal hippocampus. Neuron *18*, 243–255.

Ling, D.S., and Benardo, L.S. (1995). Activity-dependent depression of monosynaptic fast IPSCs in hippocampus: contributions from reductions in chloride driving force and conductance. Brain Res. *670*, 142–146.

Lipton, P. (1999). Ischemic cell death in brain neurons. Physiol. Rev. *79*, 1431–1568.

McCarren, M., and Alger, B.E. (1985). Use-dependent depression of IPSPs in rat hippocampal pyramidal cells in vitro. J. Neurophysiol. *53*, 557–571.

McLean, H.A., Caillard, O., Ben-Ari, Y., and Gaiarsa, J.L. (1996). Bidirectional plasticity expressed by GABAergic synapses in the neonatal rat hippocampus. J. Physiol. *496*, 471–477.

Nabekura, J., Ueno, T., Okabe, A., Furuta, A., Iwaki, T., Shimizu-Okabe, C., Fukuda, A., and Akaike, N. (2002). Reduction of KCC2 expression and GABAA receptor-mediated excitation after in vivo axonal injury. J. Neurosci. *22*, 4412–4417.

Owens, D.F., Boyce, L.H., Davis, M.B., and Kriegstein, A.R. (1996). Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. J. Neurosci. *16*, 6414–6423.

Payne, J.A., Stevenson, T.J., and Donaldson, L.F. (1996). Molecular characterization of a putative K-Cl cotransporter in rat brain. A neuronal-specific isoform. J. Biol. Chem. *271*, 16245–16252.

Payne, J.A., Rivera, C., Voipio, J., and Kaila, K. (2003). Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci. *26*, 199–206.

Plotkin, M.D., Snyder, E.Y., Hebert, S.C., and Delpire, E. (1997). Expression of the Na-K-2Cl cotransporter is developmentally regulated in postnatal rat brains: a possible mechanism underlying GABA's excitatory role in immature brain. J. Neurobiol. *33*, 781–795.

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.

Rivera, C., Li, H., Thomas-Crusells, J., Lahtinen, H., Viitanen, T., Nanobashvili, A., Kokaia, Z., Airaksinen, M.S., Voipio, J., Kaila, K., and Saarma, M. (2002). BDNF-induced TrkB activation down-regulates the K+-Cl- cotransporter KCC2 and impairs neuronal Cl- extrusion. J. Cell Biol. *159*, 747–752.

Rivera, C., Voipio, J., Thomas-Crusells, J., Li, H., Emri, Z., Sipila, S., Payne, J.A., Minichiello, L., Saarma, M., and Kaila, K. (2004). Mechanism of activity-dependent downregulation of the neuron-specific K-Cl cotransporter KCC2. J. Neurosci. *24*, 4683–4691.

Stelzer, A., Simon, G., Kovacs, G., and Rai, R. (1994). Synaptic disinhibition during maintenance of long-term potentiation in the CA1 hippocampal subfield. Proc. Natl. Acad. Sci. USA *91*, 3058–3062.

Strange, K., Singer, T.D., Morrison, R., and Delpire, E. (2000). Dependence of KCC2 K-Cl cotransporter activity on a conserved carboxy terminus tyrosine residue. Am. J. Physiol. Cell Physiol. *279*, C860–C867.

Taketo, M., Matsuda, H., and Yoshioka, T. (2004). Calcium-independent inhibition of GABA(A) current by caffeine in hippocampal slices. Brain Res. *1016*, 229–239.

Thompson, S.M., and Gahwiler, B.H. (1989). Activity-dependent disinhibition. II. Effects of extracellular potassium, furosemide, and membrane potential on ECl- in hippocampal CA3 neurons. J. Neurophysiol. *61*, 512–523.

Toyoda, H., Ohno, K., Yamada, J., Ikeda, M., Okabe, A., Sato, K., Hashimoto, K., and Fukuda, A. (2003). Induction of NMDA and GABAA receptor-mediated Ca2+ oscillations with KCC2 mRNA downregulation in injured facial motoneurons. J. Neurophysiol. *89*, 1353–1362.

Van Den Pol, A.N., Obrietan, K., and Chen, G. (1996). Excitatory actions of GABA after neuronal trauma. J. Neurosci. *16*, 4283–4292.

Wan, J., and Poo, M. (1999). Activity-induced potentiation of developing neuromuscular synapses. Science *285*, 1725–1728.

Wang, J., Liu, S., Haditsch, U., Tu, W., Cochrane, K., Ahmadian, G., Tran, L., Paw, J., Wang, Y., Mansuy, I., et al. (2003). Interaction of calcineurin and type-A GABA receptor gamma 2 subunits produces long-term depression at CA1 inhibitory synapses. J. Neurosci. *23*, 826–836.

Woo, N.S., Lu, J., England, R., McClellan, R., Dufour, S., Mount, D.B., Deutch, A.Y., Lovinger, D.M., and Delpire, E. (2002). Hyperexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. Hippocampus *12*, 258–268.

Woodin, M.A., Ganguly, K., and Poo, M.M. (2003). Coincident pre- and postsynaptic activity modifies GABAergic synapses by postsynaptic changes in Cl- transporter activity. Neuron *39*, 807–820.

Zucchi, R., and Ronca-Testoni, S. (1997). The sarcoplasmic reticulum Ca2+ channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. Pharmacol. Rev. *49*, 1–51.