Altered Synaptic Dynamics and Hippocampal Excitability but Normal Long-Term Plasticity in Mice Lacking Hyperpolarizing GABA<sub>A</sub> Receptor-Mediated Inhibition in CA1 Pyramidal Neurons

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Riekki R, Pavlov I, Tornberg J, Lauri SE, Airaksinen MS, Taira T. Altered synaptic dynamics and hippocampal excitation but normal long-term plasticity in mice lacking hyperpolarizing GABA<sub>A</sub> receptor-mediated inhibition in CA1 pyramidal neurons. J Neurophysiol 99: 3075–3089, 2008. First published April 24, 2008; doi:10.1152/jn.00606.2007. GABA<sub>A</sub> receptor (GABA-AR)-mediated inhibition is critical for proper operation of neuronal networks. Synaptic inhibition either shifts the membrane potential farther away from the action potential firing threshold (hyperpolarizing inhibition) or via increase in the membrane conductance shunts the excitatory currents. However, the relative importance of these different forms of inhibition on the hippocampal function is unclear. To study the functional consequences of the absence of hyperpolarizing inhibition, we have used KCC2-deficient mice (KCC2<sup>hy/null</sup>) maintaining only 15–20% of the neuron-specific K-Cl-cotransporter. Gramicidin-perforated patch-clamp recordings in hippocampal CA1 pyramidal cells revealed that the reversal potential of the GABA-AR-mediated postsynaptic currents (E<sub>GABA-A</sub>) was −20 mV more positive in KCC2<sup>hy/null</sup> mice than in wild-type (WT) animals. The basic glutamatergic transmission appeared unaltered in the KCC2<sup>hy/null</sup> mice, yet they displayed lowered threshold for stimulation-induced synchronous afterdischarges in the CA1 area. Also fatigue of field excitatory postsynaptic potentials/excitatory postsynaptic currents in response to repetitive stimulation was smaller in KCC2<sup>hy/null</sup> mice, indicating altered synaptic dynamics. Interestingly, this effect was present also under blockade of GABA-ARs and was dependent on the extracellular K<sup>+</sup> concentration. Moreover, there were no differences in the levels of either long-term potentiation or long-term depression between the genotypes. The local hippocampal CA1 network can in several aspects maintain its functional viability even in the absence of hyperpolarizing inhibition in pyramidal cells. Our results underscore the central role of shunting type of inhibition in controlling the neuronal excitation/inhibition balance. Moreover, our data demonstrate a novel, unexpected role for the KCC2, namely the modulation of properties of glutamatergic transmission during repetitive afferent activity.

INTRODUCTION

GABA<sub>A</sub> receptor (GABA-AR)-mediated fast inhibition is required for proper operation of neuronal networks. Via fast postsynaptic inhibition GABAergic interneurons orchestrate neuronal activities by pacing, timing, and synchronizing neural circuits. The two modes by which the phasic fast GABA-AR-mediated inhibition exerts its actions are hyperpolarizing and shunting inhibition. In hyperpolarizing inhibition, which requires powerful neuronal chloride extrusion mechanisms, opening of the GABA-AR-linked anion channels leads to a chloride influx and a consequent membrane hyperpolarization thus decreasing the probability of action potential firing. In shunting inhibition, which can be associated to both hyperpolarizing and depolarizing conductances, the GABA-AR-mediated increase in membrane conductance shunts the incoming excitatory currents resulting in silencing of the postsynaptic cell (Lamsa et al. 2000; Staley and Mody 1992).

The specific physiological significance of these different types of inhibition has remained relatively poorly characterized, partially because of the lack of feasible experimental approaches. Due to the effective Cl<sup>-</sup> extrusion mechanisms in neurons, manipulations of transmembrane Cl<sup>-</sup> gradients (and consequently, of E<sub>GABA-A</sub>) are difficult to perform. Pharmacological dissection of hyperpolarizing and shunting inhibition is a complicated task because direct manipulations of the GABA-AR-linked conductance will alter both modes of action. Further, e.g., inhibitors of co-transporters that can be used to produce positive shift in chloride equilibrium potential can have serious unspecific effects. Both transient and long-lasting depolarizing shifts in E<sub>GABA-A</sub> have been associated with several experimental models, in particular those addressing the mechanisms of epileptogenesis. The depolarizing shift in E<sub>GABA-A</sub> produced, e.g., by tetanic stimulation results from a massive GABA release and is accompanied by a large decrease in input resistance and changes in field effects (Bracci et al. 1999) Therefore the significance of the sole liability of the GABA-AR-mediated responses on the hippocampal network behavior is difficult to deduce from experiments of this kind. The controversy on the role of depolarizing GABA-AR-mediated responses in various physiological and pathophysiological phenomena may at least partially rise from the complications linked to the interpretation of the results obtained using the tetanic model.

Thus to assess the role of hyperpolarizing inhibition under more physiological conditions, alternative approaches should be taken. Here we have taken an advantage of the mice deficient of the major neuronal chloride exchanger, the chloride-extruding potassium–chloride cotransporter KCC2 (Mecado et al. 2004; Payen 1997; Rivera et al. 1999) to study the hippocampal function under conditions in which the phasic GABA-AR-mediated responses are depolarizing. As shown previously, these KCC2-deficient mice (KCC2<sup>hy/null</sup>) mice retain 15–20% of normal KCC2 protein levels in the CNS. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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(Tornberg et al. 2005). We have recently shown that these animals show various behavioral deficits (Tornberg et al. 2005, 2006) but unaltered tonic GABA-AR-mediated conductance in hippocampal pyramidal cells. Using conventional and gramicidin-perforated whole cell patch-clamp techniques as well as field potential recordings, we have now characterized the properties of glutamatergic synaptic transmission and plasticity in the KCC2hy/null mice. We focus on the synaptic behaviors known to be sensitive to changes in the efficacy of phasic GABAergic inhibition; synaptic dynamics, long-term plasticity of glutamatergic transmission [long-term depression (LTD) and long-term potentiation (LTP)] as well as the synchronous activity of the CA1 hippocampal network.

METHODS

Preparation and solutions

Eighteen- to 30-day-old wild-type (WT) and KCC2hy/null mice were decapitated under deep pentobarbital (30–40 mg/kg) anesthesia. Transverse slices (400 μm) were cut from the hippocampi using a vibratome. Whole cell experiments were carried out under infrared visual guidance in a submerged chamber at 32°C [recordings of miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs)] or room temperature (evoked EPSCs). Standard extracellular solution contained (in mM): 124 NaCl, 3.0 KCl, 2.0 CaCl2, 2.5 NaHCO3, 1.1 NaH2PO4, 1.0 MgSO4, and 10 glucose (pH 7.4, equilibrated with 95% O2-5% CO2). For the field potential recordings, an interface-type of chamber was used, and all recordings were at 32°C.

Drugs

6-cyano-7-nitroquinazoline-2,3-dione (CNQX, 20 μM; Tocris Cookson), d-2-amino-5-phosphonopentanoic acid (AP-5, 40 μM; Tocris Cookson), CGP55845 (1 μM; Tocris Cookson), and picrotoxin (PITX, 100 μM; Sigma) were bath applied.

Animals

Generation and genotyping of KCC2hy/null mice has been described previously (Tornberg et al. 2005). The KCC2hy/null mice are compound-heterozygous for KCC2 null and hypomorphic alleles and retain 15–20% of normal KCC2 protein levels in the brain. In the KCC2 null allele, a neo-cassette in exon 4 of the KCC2 gene disrupts the reading frame, whereas in the KCC2 hydroporphic allele, a neo-cassette lies within intron 3 in the opposite orientation relative to the KCC2 gene. KCC2hy/null and WT littermates in a (C57BL/6J × 129S2)F1 hybrid background were used in all the experiments described in this study. Animal experiments were approved by the ethics committee for animal research at the University of Helsinki.

Electrophysiology

All recordings were made by using Multiclamp 700A or Axoclamp 200B amplifier (Axon Instruments, Foster City, CA). Whole cell recordings were made from CA1 pyramidal cells with patch electrodes (2–5 MΩ) filled with a solution containing (in mM) 130 CsMeSO4, 10 HEPES, 0.5 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 5 QX-314, and 8 NaCl, 285 mosM, pH 7.2. For the recordings of mEPSCs, the NaCl concentration was increased to 20 mM. Perforated patch-clamp recordings were made with high-resistance electrodes (12–14 MΩ) to prevent spontaneous rupture of the seal. The tip of the electrodes were filled with a solution containing (in mM) 135 K-gluconate, 10 HEPES, 5 EGTA, 2 KCl, and 2 Ca(OH)2, 290 mosM, pH 7.2, and then backfilled with the solution containing gramicidin (100 μg/ml, Sigma). Recordings were started when an access resistance <150 MΩ was reached. Electrodes filled with 150 mM NaCl were used for field-potential recordings. LTP230D (Anderson and Collingridge 2001; www.ltp-program.com), Axoscope and Clampfit 9.2 programs (Axon Instruments, Foster City, CA), and Mini Analysis Program 5.6.6. (Synaptosoft) were used for data acquisition and analysis. 

\( E_{GABA} \) was determined from the reversal potential of evoked IPSCs obtained at voltages ranging from −90 to −20 mV. Only cells with resting membrane potential more negative than −58 mV were accepted.

Field excitatory postsynaptic potentials (fEPSPs) or population spikes reflecting synchronous discharging of pyramidal cells were recorded in the CA1 stratum radiatum or s. pyramidale, respectively, and were evoked by stimulation of the Schaffer collateral afferents. Baseline stimulation frequency was 0.05 Hz, pulse duration was 0.1 ms. Stimulus intensity was adjusted to evoke half-maximal fEPSP amplitude. Input-output data obtained at different stimulus strengths was collected. Paired-pulse stimulation at 20-, 40-, 60-, 80-, 100-, 150-, and 200-ms interpulse intervals was used to examine paired pulse facilitation (PPF). After ≥20 min of stable baseline, LTP was induced by 100-Hz tetanic stimulation for 1 s. One-hertz/900-pulse stimulation was used to induce LTD. The slope of fEPSP was used as an indicator of synaptic efficacy and was calculated between 20 and 80% of the maximal amplitude. In experiments addressing the network excitability, the amplitude of the population spike as well as the coastline bursting index (CBI) (Korn et al. 1987) were measured. The level of LTP/LTD was measured as a percentage change of the fEPSP slope.

EPSCs and IPSCs were evoked by stimulation of Schaffer collateral-commissural fibers and recorded from CA1 pyramidal neurons. The amplitude of the evoked synaptic responses was measured as the peak relative to the average baseline level 2–8 ms before the stimulation. For spontaneous mEPSCs and mIPSCs, the amplitude detection threshold was set between 4 and 6 pA (2 times the baseline root mean square noise level), and the detected events were verified visually. Cumulative plots were constructed from ≥10 min of stable recording/cell. All the pooled data are given as means ± SE for the number of cells indicated. For statistical analysis, Student’s two-tailed t-test and ANOVA were used. \( P < 0.05 \) was considered as statistically significant.

RESULTS

Shift in \( E_{GABA,A} \) in KCC2hy/null mice

Using the perforated patch-clamp technique, we first determined the \( E_{GABA,A} \) in WT and KCC2hy/null mice. IPSCs were evoked by afferent stimulation in the presence of CNQX and AP5. In line with the established role of KCC2 in maintaining the transmembrane Cl− concentration gradient, we found that the \( E_{GABA,A} \) in KCC2hy/null mice was −48 ± 5 mV, whereas in WT mice, it was −69 ± 4 mV (n = 5 cells/5 animals and 6/4, respectively; \( P < 0.001 \); Fig 1). Thus with respect to the resting membrane potential which in WT and KCC2hy/null mice was −66 ± 0.5 mV (n = 6) and −65 ± 0.3 mV (n = 5), respectively, the GABA-AR-mediated responses in the KCC2hy/null mice were clearly depolarizing.

Properties of basic synaptic transmission in mice lacking hyperpolarizing GABA-AR-mediated responses

To start to characterize the properties of basal glutamatergic synaptic transmission in the area CA1 in WT and KCC2hy/null mice, we first measured the slope of the field EPSP obtained at different stimulus strengths. The input-output curve showing the ratio of the stimulus intensity and the slope of the fEPSP in the slices from KCC2hy/null mice was indistinguishable from that obtained from the WT animals (Fig 2A). We next studied PPF, a form of short-term plasticity using an interpulse interval (IPI) range of 20–200 ms. PPF reflects an enhanced neurotransmitter release due to an accumulation of Ca\(^{2+}\) in the presynaptic nerve.
terminal following previous stimulus; yet pre- and postsynaptic inhibition may also contribute (Manabe et al. 1993). Whereas there were no differences in PPF between the genotypes with IPIs <100 ms, the PPF appeared to be more accentuated in KCC2hy/null mice at 150- and 200-ms intervals (Fig. 2B).

**Altered synaptic dynamics in KCC2hy/null mice**

We next used repetitive stimulation using 100 pulses given at 1, 10, and 100 Hz to assess the dynamic properties of the Schaffer collateral-CA1 synapses in WT and hypomorphic mice. Both genotypes displayed the characteristic 150–200% short-term facilitation during the first 5–10 pulses at all frequencies studied (Fig. 3, A–C), the initial facilitation being bigger with higher frequencies. However, whereas in WT mice the fEPSPs rapidly decayed back to the baseline level or even below during the stimulation, in KCC2hy/null mice, the responses showed much less fatigue. This difference was most striking when 1-Hz stimulation was used. Here, the KCC2hy/null fEPSPs showed little or no fatigue following initial facilitation (fEPSP slope values at the end of the stimulation 130 ± 15 and 108 ± 10% in KCC2hy/null mice and WTs, respectively; *P < 0.05, Fig. 3A). With 10- and 100-Hz stimulation also, the KCC2hy/null mice showed fatigue of fEPSPs, yet the difference in fEPSP slope in the end of the stimulation persisted (Fig. 3B). Moreover, with 100-Hz stimulation, there was a more prominent slow negative shift in field potential in KCC2hy/null mice (Fig. 3C, right), reflecting the tetanus-induced GABA-AR-mediated depolarization (Kaila et al. 1997; Taira et al. 1997).

**Difference in synaptic dynamics between the WT and KCC2hy/null mice is independent from GABA-AR activation**

More prominent phasic depolarizing or excitatory GABA-AR-mediated responses in KCC2hy/null mice could at least partially account for the accentuated short-term facilitation of fEPSPs seen in the mice. To explore this possibility, we next performed a series of experiments under blockade of GABA-ARs to see whether the difference in short-term plasticity was indeed dependent on different efficacy of GABAergic inhibition. Because the blockade of the GABA-ARs in hippocampal slices easily results in epileptiform activity thus hampering the measurements of fEPSPs, especially when repetitious stimuli are applied, the experiments were done using the whole cell patch-clamp technique in a voltage-clamp mode in the continuous presence of AP-5 (50 μM) and PiTX (100 μM).

Paired-pulse experiments using IPIs of 50 and 150 ms revealed that the difference in PPF between the genotypes was also seen in the absence of GABA-AR-mediated inhibition. The PPF of excitatory postsynaptic currents (EPSCs) in WT and KCC2hy/null mice at 50 and 150 ms IPIs was 163 ± 12 and
Having established that the genotypes displayed different PPF even in the absence of fast GABA-AR-mediated inhibition, we then investigated the synaptic properties in WT and KCC2hy/null mice using longer trains of stimuli applied at 1 and 10 Hz under the same conditions. In line with the results obtained with field potential recordings, with 1-Hz stimuli, there was practically no synaptic fatigue toward the end of the stimulus train in the mutant KCC2hy/null mice, whereas the WT animals displayed the characteristic decay of the EPSCs (Fig. 4A). When the EPSC amplitudes were normalized against the pretetanus responses, the average EPSC amplitudes of the last five responses in the train of 100 pulses were 55 ± 2% and 108 ± 25% in WT and KCC2hy/null mice, respectively (n = 7/3 and 10/4; P < 0.01). When a 10-Hz/20-pulse stimulus train was used, an initial facilitation and a subsequent fatigue of the EPSCs was seen in both genotypes. However, whereas the maximal facilitation (usually seen during the 2nd or 3rd pulse) in the WT mice was 130 ± 12%, in KCC2hy/null mice the facilitation was significantly higher being 178 ± 18% (n = 7/3 and 10/4; P < 0.01; Fig. 4B). Moreover, the normalized EPSC amplitudes in the end of the stimulus train were 53 ± 11 and 101 ± 13% of the pretetanus values in WT and KCC2hy/null mice, respectively (n = 7/3 and 10/4; P < 0.01). Thus WT and KCC2hy/null mice display different synaptic dynamics also in the absence of the fast GABA-AR-mediated inhibition.

**Difference in synaptic dynamics between the WT and KCC2hy/null mice is dependent on the extracellular K⁺ concentration**

Lowered activity of KCC2 can result in decreased buffering of extracellular K⁺ (K⁺o) and thus during repetitious activity
FIG. 3. Analysis of fEPSPs during repetitious stimulations in WT and KCC2hypo/null mice. At each frequency, significant ($P < 0.05$) augmentation of fEPSPs during repeated stimulation. A: analysis of fEPSP slope during the train of 100 pulses at 1 Hz (WT, $n = 6/5$; KCC2hypo/null, $n = 7/6$). Example traces represent average of the 3 1st and 3 last fEPSPs in the train. There was a significant difference between the genotypes in the size of the 3 last pulses in the train ($P < 0.05$). B: 100 pulses at 10 Hz (WT, $n = 6/6$; KCC2hypo/null, $n = 6/6$; $P < 0.05$). C: 100 pulses at 100 Hz (WT, $n = 4/3$; KCC2hypo/null, $n = 7/6$; $P < 0.05$). One and 10 Hz: plot represents analysis of fEPSP slope during the train; 100 Hz: plot represents analysis of the fEPSP amplitude from the baseline before tetanization, showing a significant DC shift during the high-frequency stimulation (HFS).
FIG. 4. Whole cell patch-clamp recordings show different synaptic dynamics WT and KCC2hypo/null mice. A: paired-pulse experiments using interpulse intervals (IPIs) of 50 and 150 ms reveal that the difference in paired pulse facilitation (PPF) between the genotypes is also seen in the absence of GABA-AR-mediated inhibition. The PPF of excitatory postsynaptic currents (EPSCs) in WT and KCC2hypo/null mice at 50 ms IPIs is 163 ± 12% (WT) and 201 ± 13% (KCC2hypo/null), and 145 ± 10% (WT) and 177 ± 11% (KCC2hypo/null), respectively (n = 7/3 and 10/4; P < 0.05. B, left: sample averaged EPSC traces from WT and KCC2hypo/null mice taken during the last 5 pulses of the 1-Hz/100 pulses stimulation train (light gray) and superimposed on pretetanus EPSCs (dark gray). Right: averaged EPSC amplitudes plotted against the number of pulse. C: at 10-Hz/20 pulses stimulation the WT and KCC2hypo/null mice show enhanced initial facilitation of EPSCs and less fatigue toward the end of the stimulus train. Left: sample traces from WT and WT and KCC2hypo/null mice. Right: Statistics as in B. Left bottom: summary statistics showing the averaged and normalized change in the EPSC amplitudes during 1- and 10-Hz stimulation trains in WT and KCC2hypo/null mice.
lead to accentuated accumulation of $K^+_o$ (Payne 1997). This, in turn, can result in prolonged depolarization of synaptic terminals and increased transmitter release (Poolos et al. 1987). To test the effect of changes in $K^+_o$ on EPSC dynamics in WT and KCC2<sub>hy/null</sub> mice, we applied trains of stimuli (10 Hz/20 pulses) in the presence of low (1 mM) and high (6 mM) $K^+_o$. Whole cell recordings from CA1 pyramidal neurons revealed that lowering the $K^+_o$ to 1 mM abolished the difference in short-term plasticity between the genotypes (Fig. 5A). However, in the presence of high (6 mM) $K^+_o$, the difference in EPSC facilitation was still seen (EPSC amplitudes in the end of the stimulus train were $36 \pm 7$ and $97 \pm 2\%$ of the pretetanus values in WT and KCC2<sub>hy/null</sub> mice, respectively ($n = 7/3$ and $7/3$; $P < 0.05$) (Fig. 5C).

Amplitude and the frequency of miniature EPSCs do not differ between the WT and KCC2<sub>hy/null</sub> mice

Change in synaptic dynamics might reflect altered release probability, which can be assessed by measuring mEPSCs (see Lauri et al. 2006). Thus we recorded miniature AMPAR-mediated EPSCs in WT and KCC2<sub>hy/null</sub> mice in the pres-

![Figure 5](http://jn.physiology.org/)

**FIG. 5.** The difference in synaptic dynamics between the WT and KCC2<sub>hy/null</sub> mice is dependent on the extracellular $K^+_o$ concentration. **A**: sample traces from whole cell recordings in CA1 pyramidal cells demonstrating the effect of changes in $K^+_o$ on EPSC facilitation in WT (left) and KCC2<sub>hy/null</sub> mice (right). **B**: averaged EPSC amplitudes plotted against the number of pulse. **C**: summary statistics showing the averaged and normalized change in the 20th EPSC amplitudes during 10-Hz stimulation trains in WT ($n = 7/3$) and KCC2<sub>hy/null</sub> mice ($n = 7/3$) in different $K^+_o$ ($P < 0.05$).
ence of 100 μM PiTX, 50 μM d-AP-5, and 1 μM TTX. Whole cell recordings from CA1 pyramidal neurons showed that in terms of the frequency (WT: 10.3 ± 2; KCC2hyo/null: 10 ± 2 event/min, n = 8/3 and 12/4, respectively; Fig. 6A) or the amplitude (WT: 25.4 ± 1 pA; KCC2hyo/null: 24.4 ± 1 pA; Fig. 6B) of the mEPSCs, the genotypes were indistinguishable. This finding is consistent with the data showing that the basic transmission in the KCC2hyo/null mice was unaltered (cf. Fig. 2).

mEPSC frequency but not amplitude is altered in KCC2hyo/null mice

To assess whether the compromised hyperpolarizing GABA-AR-mediated inhibition was accompanied by homeostatic changes in GABAergic synaptic transmission (see Mody 2005), we recorded mIPSCs from CA1 pyramidal cells in WT and KCC2hyo/null mice. We found no difference in the mIPSC amplitude between the genotypes (WT: 12.9 ± 2.5 pA; KCC2hyo/null: 10.2 ± 0.6 pA, n = 6/5 and 7/4, respectively; Fig. 7A), the result thus suggesting that the postsynaptic GABA-A conductance was unaltered in the mutant mice. In contrast, the frequency of the mIPSCs was lower in the KCC2hyo/null mice than in WT mice (6.3 ± 0.6 and 14.5 ± 1.9 Hz, respectively, P < 0.01), suggesting a reduction in the GABA release or in the density of GABAergic synapses in these animals (Fig. 7B).

KCC2hyo/null mice display accentuated synchronous discharges in the area CA1

Attenuated GABAergic inhibition is known to easily result in large-scale synchronization and epileptiform activity in the hippocampal neuronal network. We therefore next examined whether the threshold for the induction of synchronous hip-
Poecampal bursting activity was altered in KCC2<sup>hy/null</sup> mice. Field recordings were done in the CA1 somatic layer (s. pyramidale) to get a better estimate of the synchronous action potential firing: the population spike. In line with the results obtained in recordings from s. radiatum, there were no differences in the input/output curves between the genotypes with respect to the initial slope of the fEPSP (Fig. 8<sup>B</sup>). However, the threshold for evoking population spike for a given stimulus strength was found to be lower in the KCC2<sup>hy/null</sup> mice than in the WT mice (Fig. 8<sup>A</sup>). Moreover, the occurrence of synchronous network bursting 20–80 ms after single stimulation pulse at high-intensity appeared to be much higher in the KCC2<sup>hy/null</sup> mice as indicated by CBI (Fig. 8<sup>C</sup>).

The altered network behavior in the KCC2<sup>hy/null</sup> mice was evidently attributed to the less efficient GABAergic inhibition in the mutant mice because in the presence of the GABA-AR antagonist PiTX, the difference in the CBI between the genotypes was evened out (Fig. 9, A and B, left). Further, the increase in the CBI after PiTX application was more prominent in the WT mice, thus further indicating that the efficacy of

**FIG. 7.** Miniature inhibitory postsynaptic current (mIPSC) frequency but not amplitude is altered in KCC2<sup>hy/null</sup> mice. Sample traces of mIPSC recordings from WT (left) and KCC2<sup>hy/null</sup> (right) mice. A: cumulative plot and histogram of mIPSC frequencies in WT (14.5 ± 1.9 Hz) (n = 6/5) and KCC2<sup>hy/null</sup> mice (6.3 ± 0.4 Hz; P < 0.01, n = 7/4). B: cumulative plot and histogram of mIPSC amplitudes in WT (12.9 ± 2.5 pA) and KCC2<sup>hy/null</sup> mice (10.2 ± 0.6 pA).
GABAergic inhibition at the pyramidal layer was higher in the WT animals (Fig. 9B, right).

Induction of neither LTP nor LTD is affected by the KCC2 deficiency

GABA-AR-mediated inhibition is one of the critical factors in setting the threshold for the N-methyl-D-aspartate receptor (NMDAR)-dependent forms of long-term synaptic plasticity, namely LTP and LTD (Wikström and Gustafsson 1986). Because activation of the NMDAR-linked cation channel is accentuated at positive membrane voltages, it is to be expected that nonhyperpolarizing or depolarizing GABA-AR-mediated responses would promote activation of the NMDARs, thus enhancing the induction of changes in synaptic efficacy (see Pavlov et al. 2004). Thus in the last series of experiments, we...
investigated whether the deficiency in the KCC2 expression would also predispose the CA1 synapses to undergo activity-dependent forms of plasticity.

The protocols to induce synaptic plasticity included 100 Hz/100 pulses or 10 Hz/100 pulses (LTP) and 1 Hz/900 pulses (LTD). We found that there were no significant differences in stimulus-induced long-term changes in synaptic efficacy between the genotypes. One hundred and ten-hertz tetanic stimulation resulted in similar potentiation of the fEPSP slope both in the WT and KCC2hypo/null mice. The normalized values of fEPSP slopes at 60 min after 100-Hz stimulation were 149.7 ± 11% (10/8) and 149.6 ± 8% (8/8; Fig. 10, A and D) and after 10-Hz stimulation 129 ± 11% (8/8) and 133.4 ± 7% (6/6; Fig. 10, B and D) for WT and KCC2hypo/null mice, respectively. Further, 1-Hz stimulation induced no changes in the synaptic efficacy in either mouse strain. Normalized fEPSP slopes for WT and KCC2hypo/null mice after 60 min of the 1-Hz stimulation LTD were 101.5 ± 7% (6/5) and 106.7 ± 13% (8/8), respectively (Fig. 10, C and D).

Thus the lack of hyperpolarizing inhibition seemed to have no effect on these NMDAR-dependent forms of synaptic plasticity.

**DISCUSSION**

We have here characterized the properties of synaptic transmission in hippocampal CA1 pyramidal cells in KCC2hypo/null mice lacking hyperpolarizing GABA-AR-mediated responses. The maintenance of 15–20% of the WT KCC2 protein levels in these animals provided us with a model in which the hyperpolarizing GABA-AR-mediated inhibition was compromised but the shunting inhibition was still effective. As previously reported, the KCC2hypo/null mice are viable and show no gross changes in brain histology nor spontaneous seizures (Tornberg et al. 2005). Further, we have recently shown that the tonic GABA-AR-mediated conductance in KCC2hypo/null mice is not different from that seen in the WT mice (Tornberg et al. 2006). Thus unlike the previously reported KCC2 deficient mice, which had serious developmental defects and died either at birth or before the third postnatal week (Hubner et al. 2001; Woo et al. 2002), these mice allowed us to study the consequences of the absence of hyperpolarizing inhibition on hippocampal function in viable, adult animals. Furthermore, because no KCC2-specific pharmacological tools are currently available, the present results provide interesting insight into the physiological role of the transporter in the adult brain.
The prominent positive shift in $E_{\text{GABA-A}}$ in CA1 pyramidal cells in KCC2<sup>hy/null</sup> mice found in the present study corroborates the role of KCC2 as a major neuronal Cl<sup>-</sup> transport mechanism in CA1 (Hubner et al. 2001; Rivera et al. 1999; Zhu et al. 2005). In line with the previous reports on KCC2-deficient neurons displaying different levels of KCC2 expression, there were no differences in the resting membrane potential between the WT and KCC2<sup>hy/null</sup> mice (see Hubner et al. 2001; Zhu et al. 2005). As to the basic glutamatergic synaptic transmission, we found no differences in single pulse-evoked dendritic fEPSPs in the area CA1 between the WT and KCC2<sup>hy/null</sup> mice. However, action potential firing and consequently, the population spike are easily accentuated on erosion of somatic hyperpolarizing GABA<sub>A</sub> responses (Kaila et al. 1997; Perreault and Avoli 1992; Staff and Spruston 2003; Taira et al. 1997); this is consistent with our present data.

Neuronal communication normally involves brief bursts of activity with variable frequencies rather than single action potential firing (Dobrunz and Stevens 1999; Lisman 1997). Further, it is well known that prolonged activation of GABA-A Rs easily results in accumulation of intracellular Cl<sup>-</sup> thus shifting the $E_{\text{GABA-A}}$ toward positive values, sometimes resulting in postsynaptic excitation (Kaila et al. 1997; Lamasa and Taira 2003; Taira et al. 1997). The effect is expectedly even more prominent in neurons having deficient Cl<sup>-</sup> extrusion mechanism (see Zhu et al. 2005). We found here that the short-term facilitation of fEPSPs during repetitive stimulis was much more persistent in the KCC2<sup>hy/null</sup> mice than in the WTs regardless of the stimulation frequency used. Although the initial facilitation of the fEPSPs which was typically seen during the first 2–10 stimulation pulses was of similar magnitude in WT and KCC2<sup>hy/null</sup> mice, there was much less fatigue of the responses toward the end of the stimulus train in the latter. The accentuated slow negative shift in the extracellular potential in KCC2<sup>hy/null</sup> mice reflects the tetanus-induced GABA-A-AR-mediated slow depolarization in CA1 (Kaila et al. 1997; Staley et al. 1995; Taira et al. 1997), indicative of the decreased ability of neurons to recover from activity-induced transmembrane anion shifts (see Zhu et al. 2005).

GABAergic inhibition has a central role in gating the flow of information in synaptic circuitries. During afferent activity both feedforward and -back inhibitory circuits can be activated, thus resulting in an attenuation of subsequent postsynaptic excitatory responses, e.g., on paired-pulse stimulation. On the other hand, on repetitive stimulation at 0.5- to 50-Hz autoinhibition of GABA-release results in relieved postsynaptic inhibition, thus ensuing accentuated postsynaptic excitation and long-term plastic changes (Davies and Collingridge 1991; Davies et al. 1993). Curiously, the differences in the short-term facilitation between the WT and KCC2<sup>hy/null</sup> mice we observed in fEPSP recordings were retained in the absence of GABA-AR-mediated inhibition as shown in whole cell recordings. Moreover we found no differences either in the mEPSC amplitudes or frequencies between the genotypes, thus supporting our view that the basic excitatory synaptic function was intact in the KCC2<sup>hy/null</sup> mice. It is interesting to note that cultured cortical KCC2-deficient neurons (completely lacking KCC2) have a delayed spine development and a decreased number of functional glutamatergic synapses as indicated by reduction in mEPSC frequency; an effect independent of the transporter’s Cl- extrusion function (Li et al. 2007). Thus our result demonstrates that already 15–20% expression level of KCC2 may be sufficient to rescue its effects on glutamatergic function in vivo.

Thus the observed difference in synaptic dynamics between the WT and KCC2<sup>hy/null</sup> mice was not dependent on the changes in basic synaptic transmission properties or the compromised hyperpolarizing GABA-AR-mediated inhibition in the KCC2<sup>hy/null</sup> mouse. One plausible explanation for the enhanced EPSPs/EPSCs on repetitive stimulation in the hypomorphic mice is decreased buffering of extracellular K<sup>+</sup> accumulation leading to a prolonged depolarization of synaptic terminals and a consequent increase in the transmitter release (Payne 1997; Thompson and Gahwiler 1989; Voipio and Kaila 2000; Zhu et al. 2005). This mechanism would also explain the use dependence of the changes in EPSPs/EPSCs as well as the change in the EPSP/EPSC PPF with long IPIs. The fact that in the presence of 1 mM K<sub>+</sub>, there were no differences in the short term facilitation between the genotypes suggests that the KCC2 expression level in our KCC2<sup>hy/null</sup> mice (15–20% of WT level) was sufficient to maintain the normal transmembrane K<sup>+</sup>/Cl<sup>-</sup> gradient under the lowered K<sup>+</sup> load. The attenuation of EPSC amplitudes seen both in WT and KCC2<sup>hy/null</sup> mice in 6 mM K<sub>+</sub> is most likely due to high K<sup>+</sup>-induced sustained terminal depolarization and ensuing inactivation of voltage-dependent Na<sup>+</sup> channels, thus leading to decreased transmitter release (Poolos et al. 1987). The preceding findings thus reveal a novel physiological role for KCC2, namely the modulation of synaptic dynamics during repetitive synaptic activation.

At the level of CA1 hippocampal network, the KCC2 deficiency and the lack of hyperpolarizing GABA-AR-mediated inhibition was reflected as a lowered threshold for stimulation-induced synchronous neuronal activity. This finding is in line with the data reported by Woo et al. (2002). However, we were surprised to note that the difference between the WT and KCC2<sup>hy/null</sup> mice became evident only when high stimulation intensities were used. Thus shunting type of inhibition was evidently powerful enough to curtail neuronal excitation and hypersynchronous activity unless high stimulation intensities of the afferents were employed. Our finding that the mIPSC amplitudes were unaltered in the KCC<sup>hy/null</sup> mice supports the idea that the postsynaptic GABA-A conductance and thus the efficacy of shunting was still effective in these animals. It was somewhat curious to find that there was a reduction in the mIPSC frequency in the mutant animals. In the light of the compensatory homeostatic mechanisms, one could have expected to see the opposite. However, the finding is consistent with the previous study by Chudotvorova et al. (2005), who showed that KCC2 overexpression increases the number of functional GABAergic synapses in cultured neurons. Also if one considers that the depolarizing shift in the GABA-AR-mediated responses in KCC2<sup>hy/null</sup> mice may easily result in GABAergic excitation (e.g., Taira et al. 1997), it is plausible to assume that the decreased mIPSC frequency in fact is a homeostatic mechanism reducing the overall network excitability.

Application of the GABA-AR blocker PiTX evened out the difference in the CBI. Thus the accentuation of network bursting in the KCC2<sup>hy/null</sup> mice was evidently linked to the lack of hyperpolarizing inhibition in these animals. Moreover, the relative change in the CBI after PiTX application was bigger in
FIG. 10. Synaptic plasticity in WT and KCC2<sup>hypo/null</sup> mice. Extracellular recordings from s. radiatum at control conditions; conditioning stimulation 100 pulses at different frequencies. A–C: pooled data showing the change in fEPSP slope after tetanic stimulation. There was no significant differences between the genotypes in long-term plasticity at any induction protocol used (100 pulses, 1–100 Hz). D: pooled data from the preceding experiments showing the level of potentiation at individual experiments + averaged data 10 min after HFS (1–100 Hz).
WT mice when compared with KCC2^hy/null mice, thus indicating that under control conditions the efficacy of GABA-AR-mediated inhibition is weaker in KCC2^hy/null mice.

Downregulation of KCC2 has previously been reported after LTP induction (Wang et al. 2006; see also Rivera et al. 2004). However, whether the transporter plays a role in the LTP induction has not been known. Surprisingly, there were no differences in the level of LTP or LTD in CA1 between the WT and KCC2^hy/null mice, although hyperpolarizing inhibition has been thought to be one of the key factors controlling the efficacy of the induction of NMDAR-dependent LTD and LTD in the area CA1 (Wikström and Gustafsson 1986). Thus our data again emphasize that shunting is the physiologically relevant mode of inhibition, particularly in the dendrites (e.g., Staley and Moldy 1992).

What might then explain the lack of difference in the levels of LTP and LTD between the genotypes? During 10- to 100-Hz stimulation, the LTP induction ensues rapidly already during the first pulses, and thus the EPSP/EPSC facilitation as well as the slow depolarizing shift seen during the 100 Hz tetanic stimulation may no longer affect the induction mechanisms. This idea is in line with the finding that attenuation of GABA-AR-mediated depolarization has no effect on the LTP induction by tetanic stimulation (Debray et al. 1997). Finally, it is plausible that the GabAergic excitation between interneurons is accentuated in the KCC2^hy/null mice (see Lamsa and Taira 2003). Thus during elevated neuronal activity (e.g., on high-frequency stimulation), there may be stronger inhibitory shunt to pyramidal neurons in the KCC2^hy/null mice, thus compensating for the possible effect of depolarizing GABA-AR-mediated responses on the LTP induction.

As a conclusion, our study provides new information on the significance of the hyperpolarizing inhibition in the hippocampal CA1 circuitry. Surprisingly, it appears that the lack of hyperpolarizing GABA-AR-mediated inhibition in the local neuronal circuitry in the KCC2^hy/null mice can retain its function showing only relatively minor changes when compared with the WT mice. The results underscore the central role of shunting type of inhibition in controlling the neuronal excitation/inhibition balance. Moreover, our data demonstrate a novel, unexpected role for the KCC2, namely the modulation of synaptic dynamics during physiological-type, repetitive afferent activity.

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