

KCC2-Deficient Mice Show Reduced Sensitivity to Diazepam, but Normal Alcohol-Induced Motor Impairment, Gaboxadol-Induced Sedation, and Neurosteroid-Induced Hypnosis

Janne Tornberg¹, Mikael Segerstråle^{1,2}, Natalia Kuleskaya¹, Vootele Voikar¹, Tomi Taira^{1,2} and Matti S Airaksinen^{*1}

¹Neuroscience Center, University of Helsinki, Helsinki, Finland; ²Department of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland

GABA_A receptors mediate both fast phasic inhibitory postsynaptic potentials and slower tonic extrasynaptic inhibition. Hyperpolarizing phasic GABAergic inhibition requires the activity of neuron-specific chloride-extruding potassium–chloride cotransporter KCC2 in adult CNS. However, the possible role of KCC2 in tonic GABAergic inhibition and the associated behaviors is unknown. Here, we have examined the role of KCC2 in phasic vs tonic GABA inhibition by measuring the behavioral effects of pharmacological agents that presumably enhance phasic vs tonic inhibition in mice that retain 15–20% of normal KCC2 protein levels. These KCC2-deficient mice show decreased sensitivity to diazepam-induced sedation and motor impairment consistent with the reported role for KCC2 in fast hyperpolarizing inhibition. In contrast, the mice exhibit normal responses to low-dose alcohol-induced motor impairment, gaboxadol-induced sedation, and neurosteroid-induced hypnosis; behaviors thought to be associated with tonic GABAergic inhibition. Electrophysiological recordings show that the tonic conductance is not affected. The results suggest that KCC2 activity is more critical for behaviors dependent on phasic than tonic GABAergic inhibition.

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INTRODUCTION

In adult mammalian CNS, most of the fast neurotransmitter-mediated inhibitory activity is mediated through the activation of ionotropic GABA_A receptors. Neuronal inhibition mediated by GABA_A receptors is essential for information processing in the CNS, and GABA_A receptor dysfunction is implicated in many pathological processes such as epilepsy, pain, and anxiety (Rudolph and Mohler, 2004). Ionotropic GABAergic inhibition can be divided into two components; transient and fast, synaptic ‘phasic’ inhibition, and persistent, slower ‘tonic’ inhibition, which mainly derives from extrasynaptic GABA_A receptors (Farrant and Nusser, 2005; Mody and Pearce, 2004; Semyanov *et al*, 2004). Importantly, synaptic and extra-

synaptic GABA_A receptors differ in their subunit compositions (Nusser *et al*, 1998) that determine whether the receptor function is enhanced by allosteric modulators, such as benzodiazepines and neurosteroids (Hevers and Luddens, 1998; Korpi and Sinkkonen, 2006; Sieghart and Speck, 2002). A classical benzodiazepine, diazepam, binds to predominantly synaptic GABA_A receptor subtypes; whereas extrasynaptic receptors that mediate tonic currents in the thalamus, cerebellum and dentate granule cells are unable to bind the drug (Rudolph and Mohler, 2004). On the other hand, the extrasynaptic GABA_A receptors can be selectively enhanced by neurosteroids and alcohol both *in vitro* (Hamann *et al*, 2002; Stell *et al*, 2003; Wallner *et al*, 2003; Wei *et al*, 2004; Wohlfarth *et al*, 2002; but see Borghese *et al*, 2006) and *in vivo* (Hancher *et al*, 2005, 2006; Maguire *et al*, 2005; Mihalek *et al*, 1999; Wallner *et al*, 2006).

Activation of GABA_A receptors at synapses opens an anion conductance, which typically results in both post-synaptic hyperpolarization (mediated by Cl⁻ influx) and shunting of excitatory currents (because of increased conductance), which leads to a transient decrease in the probability of action potential initiation. The hyperpolarizing response requires an efficient chloride extrusion

*Correspondence: Dr MS Airaksinen, Neuroscience Center, University of Helsinki, Viikinkaari 4, PO Box 56, FIN-00014 Helsinki, Finland, Tel: +3589 191 57650, Fax: +3589 191 57620,

E-mail: mairaksi@operon.helsinki.fi

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mechanism, which is achieved through the activity of the chloride-extruding potassium–chloride cotransporter KCC2 (Mercado *et al*, 2004; Payne *et al*, 2003) (also known as Slc12a5). This role of KCC2 is directly evidenced in slice cultures of hippocampal neurons in which antisense knockdown of KCC2 results in a decrease of hyperpolarizing GABA responses (Rivera *et al*, 1999), and in KCC2-knockout mice in which the normally hyperpolarizing motoneuron GABA responses are absent (Hubner *et al*, 2001). Whereas synaptic inhibition may operate through both hyperpolarization and shunting, tonic inhibition probably mediates its effect predominantly through shunting mechanism (Brickley *et al*, 1996; Mitchell and Silver, 2003). Thus, drugs acting on phasic inhibition would be less effective when GABA is less hyperpolarizing, but that this should have no effect on the efficacy of drugs acting on tonic inhibition. To date, no experimental evidence exists concerning the role of KCC2 in the tonic form of GABAergic inhibition. We hypothesized that the shunting inhibition, which operates even if GABA is depolarizing (Staley and Mody, 1992), would remain effective in hypomorphic KCC2-deficient mice (Tornberg *et al*, 2005). These KCC2^{hy/null} mice retain 15–20% of normal KCC2 protein levels in the CNS and show various behavioral deficits (Tornberg *et al*, 2005).

In the present study, we have investigated the requirements for KCC2 in the tonic *vs* phasic GABA_A receptor-mediated inhibition in the adult CNS *in vivo*. For this aim, we tested the effect of drugs that are selective for the synaptic or extrasynaptic GABA_A receptors on KCC2-deficient mice. The experiments are based on four important previous observations. (1) Sedative and motor-impairing effects of diazepam are due to its action on $\alpha 1$ subunit containing receptors that are low-affinity, synaptic receptors (Crestani *et al*, 2000). (2) The sedative effect of gaboxadol (THIP) at low concentrations is presumably mediated through extrasynaptic δ -subunit containing GABA_A receptors (Brown *et al*, 2002; Belelli *et al*, 2005; Cope *et al*, 2005; Maguire *et al*, 2005). (3) The effects of low-dose alcohol on motor impairment are mediated at least in part by $\alpha 4/\alpha 6$ - and δ -subunit containing GABA_A receptors that are expressed extrasynaptically and mediate tonic inhibition (Hancher *et al*, 2005, 2006). (4) The effect of neurosteroids is predominantly through an action on extrasynaptic δ -subunit containing GABA_A receptors that mediate tonic inhibition (Mihalek *et al*, 1999). To simplify the interpretation, we selected behavioral tasks, in which the basal drug-free performance of KCC2-deficient mice does not differ from their wild-type (WT) littermates (Tornberg *et al*, 2005).

MATERIALS AND METHODS

Animals

Generation and genotyping of hypomorphic KCC2-deficient (KCC2^{hy/null}) mice has been described previously (Tornberg *et al*, 2005). The KCC2^{hy/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 hypomorphic allele, a *neo*-cassette lies within intron 3 in the opposite orientation relative to the KCC2 gene. KCC2^{hy/null}

and WT littermates in a (C57BL/6 \times 129S2)F₁ hybrid background were used in all the experiments described in this study. The mice were housed in standard polycarbonate cages covered with wire lid (Macrolon, Scanbur A/S, Karlsrunde, Denmark), 2–4 adult mice per cage (27 \times 21 \times 14 cm³), in specific pathogen-free conditions under a regular dark/light cycle (lights on 0700, off 1900 hours) with free access to standard rodent food and water.

A group of 3- to 5-month-old littermate mice (20 WT male, 14 KCC2^{hy/null} male, 16 WT female, 23 KCC2^{hy/null} female mice) was subjected to behavioral testing. As behavioral testing revealed no differences between the sexes, the results were pooled. An additional cohort of 2-month-old littermate (21 WT and 19 KCC2^{hy/null}) mice was used for testing the effect of gaboxadol. All animal experiments were approved by the ethics committee for animal research at the University of Helsinki.

Open Field

The effect of diazepam (Stesolid Novum, Dumex-Alpha A/S, Copenhagen, Denmark) and gaboxadol hydrochloride (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol; THIP, Sigma-Aldrich) on spontaneous locomotor activity was measured in the automated open field (30 \times 30 cm²; MedAssociates, VT) for 30 min. The mice, unfamiliar with the testing environment, were administered intraperitoneally (i.p.) with diazepam (2 mg/kg), gaboxadol (10 mg/kg) or vehicle (0.9% NaCl) 30 min before the testing.

Accelerating Rotarod

The mice were trained on an accelerating rotarod (Ugo Basile, Comerio, Italy) equipped with automatic fall detector in six trials on 3 succeeding days (two trials a day). The speed of rotation increased at a constant rate (from 3 to 30 r.p.m.) over 5 min and cutoff time was 6 min. The first three trials showed no difference between the genotypes, consistent with our previous study reporting normal motor coordination in the KCC2 mutants (Tornberg *et al*, 2005). However, the KCC2^{hy/null} mice displayed a slight deficit during the subsequent learning trials (data not shown), suggesting that the KCC2^{hy/null} mice may exhibit a mild deficit in motor learning (but not in motor coordination *per se*). Mice that failed to stay on the rod for 120 s on the pretrial test were excluded from the experiment (three WT and six KCC2^{hy/null} mice).

After the learning trials, the animals were used in ethanol and diazepam experiments with at least 5 days and one injection-free trial on rotarod in-between the drug injections. The tested dose of ethanol (Primalco, Rajamäki, Finland; 12% weight/volume, diluted in 0.9% NaCl) was 1.25 g/kg (i.p.). The performance was measured as the latency to fall from the rotating rod before (pretrial) and 10 and 40 min after the ethanol injection. Similarly, the effect of diazepam (3 or 10 mg/kg, i.p.) on rotarod performance was tested before and 30 and 60 min after the drug injection.

Loss of Righting Reflex (LORR)

Mice were tested for the duration of the loss of the righting reflex ('sleep time') in response to the neuroactive steroid,

pregnanolone (5 β -pregnan-3 α -ol-20-one; Sigma-Aldrich, 8 mg/kg) as described previously (Mihalek *et al*, 1999). The drug was dissolved in a 22.5% (wt/vol) solution of 2-hydroxypropyl- β -cyclodextrin (Sigma-Aldrich). Upon the intravenous injection of the drug, the mice became immobile in 5 s and were placed on their backs in a v-shaped trough. Mice were monitored until they could right themselves three times in 30 s, and the time to pass the criteria was recorded. A heat lamp was used to ensure normothermia.

Electrophysiology

Acute hippocampal slices were prepared from anesthetized WT and KCC2^{hy/null} mice (16–20-day-old), using standard methods. Briefly, the brain was quickly dissected into ice-cold solution containing the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, 26 NaHCO₃, 10–15 D-glucose, and 1 CaCl₂ (bubbled with 5% CO₂/95% O₂). A tissue block containing the hippocampi was dissected and glued into the stage of a vibratome (Vibratome, St Louis, MO). Slices (350 μ m thick) were cut sagittally in the above solution and stored at room temperature in a solution containing the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 4 MgSO₄, 26 NaHCO₃, 10–15 D-glucose, and 2 CaCl₂ (5% CO₂/95% O₂). The slices were used 1–4 h after cutting.

For electrophysiological recordings, the slices were placed in a submerged recording chamber and perfused with extracellular solution containing the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 26 NaHCO₃, 10–15 D-glucose, and 2 CaCl₂ (bubbled with 5% CO₂/95% O₂ at 32°C). Whole-cell recordings were made from visually identified CA3 interneurons, with patch electrodes (3–5 M Ω) filled with a solution containing the following (in mM): 120 CsCl, 10 HEPES, 0.5 EGTA, 4 Mg-ATP, 0.3 Na-GTP, and 8 NaCl (285 mosM), pH 7.2. Perforated patch clamp recordings were made with high resistance electrodes (12–14 M Ω) to prevent spontaneous rupture of the gigaseal. The tip of the electrodes were filled with a solution containing the following (in mM): 135 K-gluconate, 10 HEPES, 5 EGTA, 4 Mg-ATP, 0.5 Na-GTP, 2 KCl, and 2 Ca(OH)₂, 290 mosM, pH 7.2; and then backfilled with the same solution containing gramicidin (100 μ g/ml, Sigma). Recordings were started when access resistance lower than 150 M Ω was reached.

The experiments were made in voltage-clamp mode, where the membrane was clamped to –80 mV. In the break-in whole-cell experiments, the pipette solution contained a high concentration of cesium chloride, so that GABA_A receptor-mediated currents at –80 mV were inward. In the perforated patch experiments, gramicidin was used to get a stable access to the cell. Access and input resistances were monitored throughout the experiments using a 5 mV voltage step. AMPA/kainate, NMDA, and GABA_B receptors were routinely blocked with NBQX (50 μ M), AP5 (50 μ M), and CGP52432 (5 μ M), respectively. The tonic GABA_A receptor-mediated current was calculated as the change in baseline holding current produced by blocking GABA_A receptors with picrotoxin (PiTX, 100 μ M). Currents were acquired with a multiclamp 700A amplifier (Axon Instruments, Foster City, CA). Recordings were filtered at 2 kHz

and sampled at 4 kHz. Access and input resistances were monitored throughout the experiments using a 5 mV voltage step.

RESULTS

KCC2^{hy/null} Mice are Insensitive to Diazepam-Induced Sedation

The diazepam-induced depression of motor activity, a measure of sedation, was tested in the open field. The spontaneous locomotor activity of the vehicle-treated animals did not differ between the genotypes (Figure 1a), consistent with our previous results of similar open field performance of noninjected KCC2^{hy/null} and WT littermate mice (Tomberg *et al*, 2005). Treating the mice with diazepam (2 mg/kg, i.p.), however, induced a significant difference in the motor activity between the genotypes (Figure 1a; effect of genotype: $F_{1,69} = 4.77$, $p < 0.05$; effect of treatment: $F_{1,69} = 8.18$, $p < 0.01$). The KCC2^{hy/null} mice were

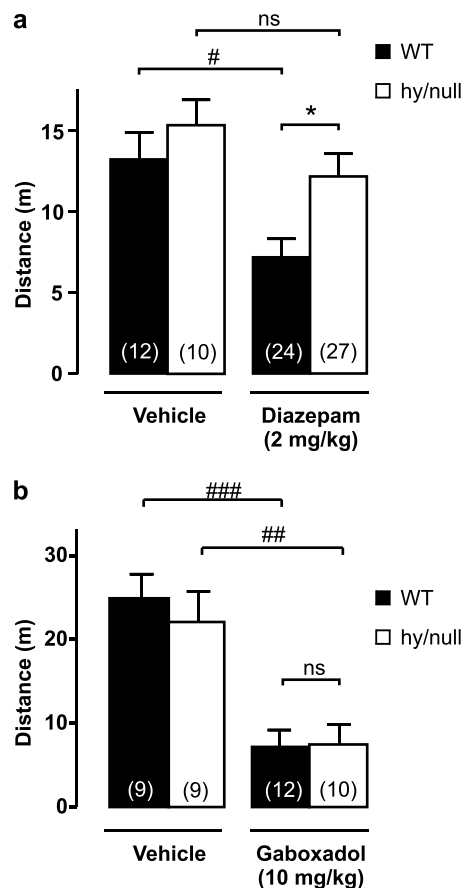


Figure 1 Effect of diazepam and gaboxadol in WT and KCC2^{hy/null} mice on total distance traveled during a 30-min trial in open field. (a) Diazepam (2 mg/kg, i.p.) significantly depressed the spontaneous locomotor activity in WT mice compared to vehicle-treated controls. The KCC2^{hy/null} mice showed insensitivity to diazepam-induced sedation and were less sedated compared to the WT littermate controls. (b) In contrast, gaboxadol (THIP, 10 mg/kg, i.p.) impaired the locomotor activity similarly in both WT and KCC2^{hy/null} mice. Data represent the mean \pm SEM. Number of mice in each group is shown in parenthesis. * $p < 0.05$ between the genotypes and # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ within the genotype by two-way ANOVA followed by Newman–Keuls *post hoc* test.

insensitive to diazepam-induced sedative effects, whereas WT mice became clearly less active by the treatment (Figure 1a; WT-vehicle vs WT-diazepam, $p < 0.05$; WT-diazepam vs hy/null-diazepam, $p < 0.05$).

KCC2^{hy/null} Mice Show Normal Gaboxadol-Induced Sedation

The sedative effect of low-dose gaboxadol (THIP) was tested in the open field using a new cohort of mice. In contrast to diazepam, gaboxadol (10 mg/kg, i.p.) impaired the locomotor activity similarly between the genotypes (Figure 1b; effect of genotype: $F_{1,20} = 0.01$, $p = 0.92$; effect of treatment for WT: $F_{1,19} = 25.18$, $p < 0.001$, for KCC2^{hy/null}: $F_{1,17} = 11.66$, $p < 0.003$).

KCC2^{hy/null} Mice Show Normal Alcohol-Induced Motor Coordination Impairment

The motor impairing effect of low dose of ethanol was assessed in the accelerating rotarod. The mice were first trained to stay on the rod on 3 consecutive days (two trials per day until no significant improvement took place). Next day following the last learning trial, the mice were tested on the rotarod before (pretrial) and 10 and 40 min after the ethanol (1.25 g/kg body weight, i.p.) injection. The KCC2^{hy/null} mice showed no motor impairment in the pretrial test compared to WT control mice (Figure 2a). *Post hoc* analysis detected a significant reduction in motor abilities measured 10 min after the ethanol injection in both genotypes (Figure 2a; WT $p < 0.001$; hy/null $p < 0.001$). Although the KCC2^{hy/null} mice showed slightly decreased alcohol sensitivity, the difference was not significant between the genotypes (Figure 2a; effect of genotype: $F_{1,45} = 0.104$, $p = 0.75$; interaction of genotype and trial: $F_{2,90} = 1.67$, $p = 0.19$).

KCC2^{hy/null} Mice are Less Sensitive to Diazepam-Induced Motor Coordination Impairment

Five days after the alcohol test, the mice were assessed for the motor coordination impairing effects of diazepam. The mice were tested in the accelerating rotarod before (pretrial) and after (30 and 60 min) the drug injection. The pretrial test showed no difference in the motor abilities between the genotypes (Figure 2b), consistent with the alcohol pretrial test. Diazepam (3 mg/kg, i.p.) treatment revealed a significant difference between the genotypes (Figure 2b; effect of genotype: $F_{1,45} = 17.71$, $p < 0.001$; interaction of genotype and trial: $F_{2,90} = 12.49$, $p < 0.001$). *Post hoc* test revealed a reduction in motor abilities in both genotypes measured 30 min after the drug injection (Figure 2b; WT $p < 0.001$; hy/null $p < 0.01$). However, the KCC2^{hy/null} mice performed the task significantly better than WT controls ($p < 0.001$ measured at 30 and 60 min after the injection). Higher, clearly sedative dose of diazepam (10 mg/kg) severely impaired motor coordination in both genotypes (Figure 2c).

KCC2^{hy/null} Mice Show Normal Neurosteroid-Induced Hypnotic Effects

The LORR assay was conducted to determine whether the hypnotic effects of neuroactive steroid pregnanolone were

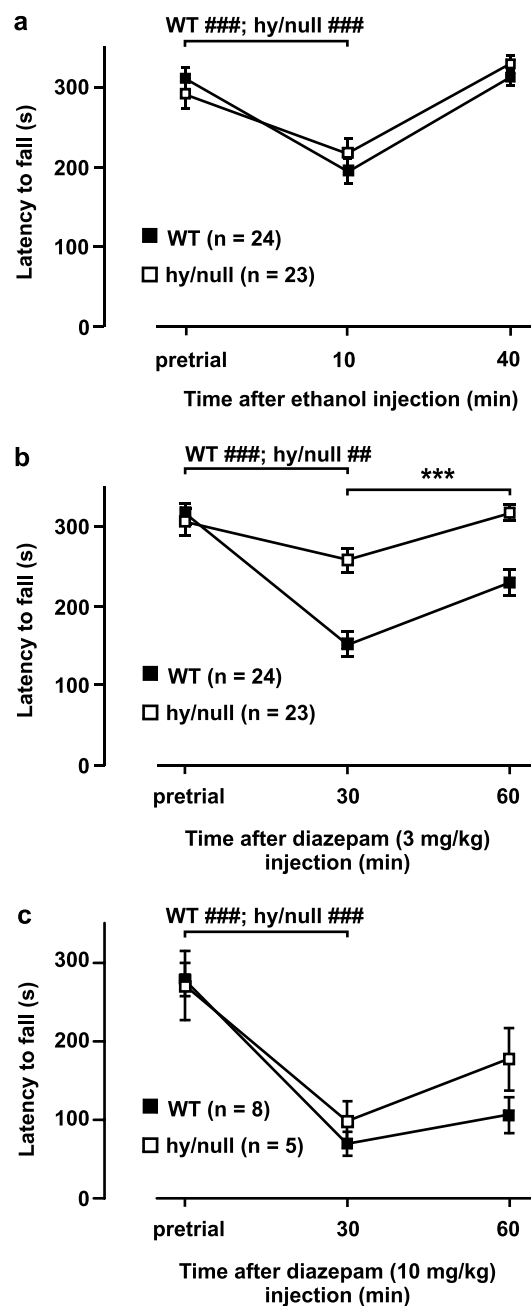


Figure 2 Effect of alcohol (1.25 g/kg, i.p.) and diazepam (3 or 10 mg/kg, i.p.) on rotarod performance in WT and KCC2^{hy/null} mice. (a) Low dose of ethanol reduced the motor abilities of both genotypes measured 10 min after the injection. The ethanol-induced motor impairment showed no difference between the WT and KCC2^{hy/null} mice measured at two postinjection data points (10 and 40 min after the ethanol injection). (b) Low dose (3 mg/kg) diazepam-treatment impaired the motor coordination in both genotypes measured 30 min after the injection. The effect of diazepam was, however, significantly weaker in KCC2^{hy/null} mice compared to WT controls measured at the two postinjection data points. (c) Higher, clearly sedative dose of diazepam (10 mg/kg) severely impaired motor coordination in both genotypes. Data represent mean \pm SEM. *** $p < 0.001$ between the genotypes; ## $p < 0.01$ and ### $p < 0.001$ within the genotype by repeated measures ANOVA followed by Newman-Keuls *post hoc* test.

altered in the KCC2 mutant mice. The pregnanolone-induced (8 mg/kg, intravenous (i.v.)) duration of LORR did not differ between the WT and KCC2^{hy/null} mice (Figure 3; $p = 0.9$ using the *t*-test).

Tonic Current in KCC2^{hy/null} Neurons is Normal Using Whole-Cell but Changed Using Gramicidin-Perforated Patch Recordings

When tonic GABAergic current was measured in hippocampal interneurons by conventional whole-cell-clamp technique (Figure 4a), PiTX caused a similar shift in the holding current between the genotypes (WT 41 ± 4 pA, $n = 7$; hy/null 45 ± 6 pA, $n = 4$), indicating that the tonic conductance was the same. However, in the perforated patch-clamp experiments (Figure 4b), the tonic current (PiTX-sensitive holding current) corrected for cell capacitance (Scimemi *et al*, 2005), was lower in WT than in KCC2^{hy/null} neurons when clamped at -80 mV (WT 320 ± 70 pA/nF, $n = 4$; hy/null 560 ± 30 pA/nF, $n = 3$; $p = 0.03$).

DISCUSSION

In this study, we show that the KCC2^{hy/null} mice display impaired responses to diazepam-induced sedation and motor impairment, but normal responses to alcohol-induced motor impairment, gaboxadol-induced sedation, and neurosteroid-induced hypnosis. These results are consistent with the reported role for KCC2 in the hyperpolarizing synaptic inhibition and indicate that a

profound (80–85%) deficiency of normal KCC2 levels (and thus Cl⁻ extrusion capacity) in adult CNS does not affect the correct functioning of tonic GABAergic inhibition at the behavioral level. This suggests that KCC2 is predominantly involved in the phasic but not in the tonic component of GABAergic neurotransmission.

The synaptic GABA_A receptor activation results typically in hyperpolarizing inhibitory postsynaptic potentials, which are dependent on the chloride extruding activity of KCC2 (Hubner *et al*, 2001; Rivera *et al*, 1999; Woo *et al*, 2002; Zhu *et al*, 2005). Although the synaptic (phasic) inhibition also includes a shunting component, extrasynaptic (tonic and presynaptic) inhibition is thought to operate mainly via the shunting mechanism (through the opening of the GABA_A receptor channel causing a decrease in membrane resistivity), which is effective even if GABA is depolarizing (Staley and Mody, 1992). We have previously reported several behavioral phenotypes of the hypomorphic KCC2-deficient mice (Tomberg *et al*, 2005), including increased anxiety and seizure susceptibility, poor spatial learning and memory, hyposensitivity to thermal and mechanical stimuli, and reduced weight but apparently normal locomotor activity and motor coordination. Interestingly, the mice showed normal anticonvulsant responses to an anesthetic drug, propofol, which preferentially enhances the tonic GABA_A receptor-mediated conductances in hippocampal neurons (Bai *et al*, 2001; Bieda and MacIver, 2004), suggesting that the tonic inhibition would remain effective in KCC2 mutant mice.

Mice with a point mutation in the GABA_A receptor $\alpha 1$ subunit are insensitive to low-dose diazepam-induced sedation and motor impairment (Crestani *et al*, 2000; McKernan *et al*, 2000; Rudolph *et al*, 1999), implying that these diazepam effects are mediated through the activation of receptors that contain $\alpha 1$ subunit, which are predominantly expressed postsynaptically (Farrant and Nusser, 2005). Thus, to specifically examine the role of KCC2 in the phasic (synaptic) form of GABAergic inhibition, we tested the locomotor-impairing/sedative effect of diazepam in the KCC2^{hy/null} mice in open field. A dose of diazepam (2 mg/kg, i.p.) that markedly depressed the motor activity (distance traveled) in the WT mice resulted in only a slight

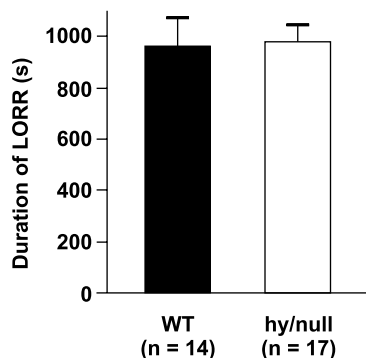


Figure 3 Duration of LORR induced by pregnanolone (8 mg/kg, i.v.) in WT and KCC2^{hy/null} mice. The duration of LORR was similar between the genotypes. Data represent mean \pm SEM.

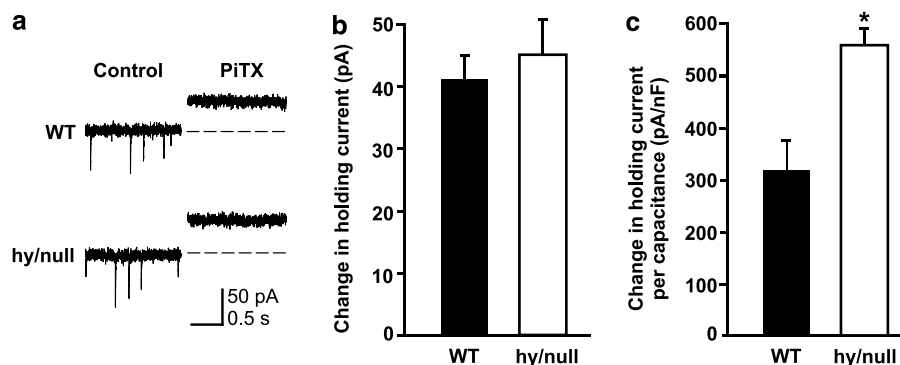


Figure 4 Effect of PiTX on holding current in CA3 stratum lucidum interneurons in WT and KCC2^{hy/null} mice. (a) Representative traces obtained in whole-cell mode from one WT and one KCC2^{hy/null} interneuron. PiTX abolished spontaneous IPSCs and decreased the holding current. (b) In whole-cell experiments, PiTX shifted the holding current similarly in WT and KCC2^{hy/null} neurons. (c) In gramicidin-perforated patch experiments, tonic current density, measured as the ratio of PiTX-sensitive holding current to cell capacitance, was lower in WT than in KCC2^{hy/null} neurons. The membrane potential was clamped to -80 mV in both experiments. Experiments were performed in the presence of glutamate receptor and GABA_B receptor antagonists. Data represent mean \pm SEM. * $p = 0.03$ using the *t*-test.

decrease in spontaneous motor activity in the KCC2^{hy/null} mice. This resistance to diazepam-induced sedation is consistent with compromised synaptic inhibition in the KCC2-deficient mice. In contrast to diazepam, low dose of gaboxadol impaired locomotor activity in the open field test similarly between the genotypes. As gaboxadol is thought to specifically target extrasynaptic GABA receptors (eg Belelli *et al* 2005), this result suggests that tonic GABA inhibition may be relatively intact in the KCC2-deficient mice.

A naturally occurring point mutation in GABA_A receptor subunit $\alpha 6$ is reported to render rats more sensitive to low-dose (1.25 g/kg) alcohol-induced motor impairment by selectively increasing the alcohol sensitivity of a specific extrasynaptic GABA_A receptor subtype, $\alpha 6\beta 3\delta$, which is exclusively expressed in cerebellar granule cells, suggesting that alcohol impairs motor coordination by enhancing granule cell tonic inhibition (Hancher *et al*, 2005). Although the mechanism by which ethanol modulates tonic GABAergic inhibition in cerebellar granule neurons is still a matter of debate (Borghese *et al*, 2006), recent studies provide further evidence that behavioral low-dose ethanol effects are to a large extent mediated by extrasynaptic GABA_A receptors (Hancher *et al*, 2006; Wallner *et al*, 2006). To investigate the effects of KCC2 alterations in this model of tonic inhibition, we tested the KCC2^{hy/null} mice in accelerating rotarod before and after the low dose of alcohol-treatment. Importantly, motor abilities were similar between the genotypes in the pretrial test and were significantly impaired after alcohol administration similarly in both genotypes. This result is consistent with a recent report showing that almost all GABAergic inhibition in cerebellar slices from adult rats is mediated by extrasynaptic currents, which are not affected by blocking ($\sim 80\%$) of KCl cotransport (Hamann *et al*, 2002). As the cerebellar information processing is profoundly modulated by GABAergic shunting inhibition (Mitchell and Silver, 2003; Chadderton *et al*, 2004), these results imply the integrity of tonic GABAergic inhibition in the cerebellum of KCC2-deficient mice.

To further compare the effect of phasic vs tonic enhancement of GABAergic inhibition in the KCC2^{hy/null} mice in the same behavioral task, we repeated the accelerating rotarod test by replacing ethanol with diazepam. Again, the pretrial test showed no differences in the motor abilities between the genotypes. The enhancement of phasic GABAergic inhibition by diazepam, however, revealed a significant difference between the WT and KCC2^{hy/null} mice rotarod performance. Diazepam at 3 mg/kg (i.p.) significantly impaired the motor abilities in both genotypes, but the effect was considerably stronger in the WT mice. This is consistent with the open field results and shows that the phasic GABAergic inhibition mediated by the $\alpha 1$ subunit containing GABA_A receptors is impaired in the KCC2^{hy/null} mice. The difference between genotypes was smaller using higher dose of diazepam (10 mg/kg, i.p.) that causes more severe sedation and impairment of rotarod performance. This is consistent with previous results that sedative/motor impairing effects of higher dose of diazepam are mediated also via other than synaptic $\alpha 1$ subunit containing GABA_A receptors (eg Crestani *et al*, 2000).

The tonic form of GABAergic inhibition is also highly sensitive and selectively enhanced by neuroactive steroids

acting through δ subunit containing GABA_A receptors (Stell *et al*, 2003; Wohlfarth *et al*, 2002). This has been assessed at the behavioral level by δ subunit-knockout mice that show a reduced sleep time induced by neuroactive steroid pregnanolone (Mihalek *et al*, 1999). Consistent with the gaboxadol-induced sedation and alcohol-induced motor impairment, pregnanolone-induced LORR showed no difference in duration between WT and KCC2^{hy/null} mice. This further supports our conclusion that a profound reduction of KCC2 levels in the adult CNS has little if any effect on tonic GABA signaling at the behavioral level.

Consistent with previous reports that KCC2 is the predominant Cl⁻ extrusion mechanism in adult neurons (Hubner *et al*, 2001; Rivera *et al*, 1999; Woo *et al*, 2002; Zhu *et al*, 2005), hippocampal pyramidal neurons from the KCC2^{hy/null} mice show a marked positive shift in the reversal potential for GABA (E_{GABA} , WT: -69 ± 4 mV, $n = 6$, hy/null: -48 ± 5 mV, $n = 5$) (R Riekk *et al*, Unpublished data). Consequently, the increase in intracellular Cl⁻ in KCC2^{hy/null} neurons moves the GABA reversal potential to more positive values, and in some neurons this may even lead to a switch in the polarity of the GABA_A receptor-mediated responses from hyperpolarizing to depolarizing; a finding consistent with the observed reduction in diazepam sensitivity in the KCC2 mutant mice. As diazepam remains inhibitory in the mice, particularly at the higher (10 mg/kg) dose, either most neurons do not switch the polarity of GABA_A receptor-mediated responses or that the shunting component of synaptic receptors plays an important role in the inhibitory actions of classical benzodiazepines.

When tonic GABAergic current was measured in hippocampal interneurons by conventional whole-cell-clamp technique, no change in holding current was observed between the genotypes. This indicates that tonic GABA_A conductance, receptor expression, and extracellular GABA levels are comparable between the genotypes and that no adaptive plasticity in the GABA_A receptors mediating the tonic current had taken place (Scimemi *et al*, 2005). However, when measured using the gramicidin-perforated patch-clamp configuration that maintains internal Cl⁻ homeostasis, tonic current density (change in holding current per cell capacitance) was higher in the KCC2^{hy/null} neurons when the cells were held at -80 mV. This indicates a more positive E_{GABA} of the tonic current in these animals that is consistent with the positive shift in E_{GABA} in synaptically induced GABA_A receptor-mediated responses. However, as the physiological role of the tonic GABAergic transmission is to maintain inhibitory background tone in the neuronal network via shunting (Semyanov *et al*, 2004), the positive shift in the E_{GABA} does not necessarily compromise this.

In contrast to hyperpolarizing inhibition, shunting inhibition is nonlinear phenomenon behaving in an on-off like manner (Koch *et al*, 1983) and is efficient even if GABA is depolarizing (Staley and Mody, 1992). The key element in shunting inhibition is the increase in membrane conductance, which limits depolarization above E_{GABA} and therefore inhibits the membrane potential from reaching an action potential threshold, provided that the E_{GABA} is below this value (Owens and Kriegstein, 2002). We propose that the functional outcome of tonic GABA inhibition assessed at the behavioral level remains intact, because E_{GABA} still

remains hyperpolarized relative to action potential threshold so that the tonic current can still effectively diminish excitatory inputs. However, it remains possible that under certain conditions (such as during prolonged hyperexcitability) that lead to accumulation of intracellular chloride levels, the altered tonic current (due to reduced chloride extrusion capacity) in KCC2^{hy/null} mice could manifest at the behavioral level, such as increased seizure susceptibility (Maguire et al, 2005).

In summary, the role of KCC2 in hyperpolarizing GABA responses (shifting the E_{GABA} toward more negative potentials) is emphasized in phasic GABA inhibition. We show here that weakened hyperpolarizing GABA responses due to KCC2 deficiency are directly correlated in the animal behavior, whereas changed tonic GABA current does not necessarily manifest in behavior. This suggests that, at least under most physiological conditions, 15–20% of KCC2 is sufficient to maintain the E_{GABA} in the shunting range of GABA_A receptor activation and thus preserve the correct functional outcome of tonic GABAergic neurotransmission.

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