Neuronal Chloride Regulation via KCC2 Is Modulated through a GABA_B Receptor Protein Complex

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GABA_B receptors are G-protein-coupled receptors that mediate inhibitory synaptic actions through a series of downstream target proteins. It is increasingly appreciated that the GABA_B receptor forms part of larger signaling complexes, which enable the receptor to mediate multiple different effects within neurons. Here we report that GABA_B receptors can physically associate with the potassium-chloride cotransporter protein, KCC2, which sets the driving force for the chloride-permeable ionotropic GABA_A receptor in mature neurons. Using biochemical, molecular, and functional studies in rodent hippocampus, we show that activation of GABA_B receptors results in a decrease in KCC2 function, which is associated with a reduction in the protein at the cell surface. These findings reveal a novel "crosstalk" between the GABA receptor systems, which can be recruited under conditions of high GABA release and which could be important for the regulation of inhibitory synaptic transmission.

Key words: chloride; GABA-B receptor; KCC2; protein complex; synaptic inhibition

Significance Statement

Synaptic inhibition in the brain is mediated by ionotropic GABA_A receptors (GABA_ARs) and metabotropic GABA_B receptors (GABA_BRs). To fully appreciate the function and regulation of these neurotransmitter receptors, we must understand their interactions with other proteins. We describe a novel association between the GABA_BR and the potassium-chloride cotransporter protein, KCC2. This association is significant because KCC2 sets the intracellular chloride concentration found in mature neurons and thereby establishes the driving force for the chloride-permeable GABA_AR. We demonstrate that GABA_BR activation can regulate KCC2 at the cell surface in a manner that alters intracellular chloride and the reversal potential for the GABA_AR. Our data therefore support an additional mechanism by which GABA_ARs are able to modulate fast synaptic inhibition.

Introduction

GABAergic synaptic inhibition is mediated by two major receptor systems: ionotropic GABA_A receptors (GABA_ARs) and metabotropic GABA_B receptors (GABA_BRs). GABA_ARs rely on transmembrane chloride gradients to generate fast inhibitory synaptic currents (Kaila, 1994; Payne et al., 2003). GABA_BRs, in contrast, generate slower inhibitory actions via the activation of guanine nucleotide-binding protein (G-protein) signaling pathways (Bettler et al., 2004).

It is becoming increasingly clear that to understand the function and regulation of GABA_BRs requires a more complete understanding of the molecular associations that underlie GABA_B complexes in the brain. For instance, recent proteomic approaches have identified auxiliary subunit proteins that modulate the receptor's agonist response and kinetics of G-protein signaling (Schwenk et al., 2010). GABA_B complexes can also include proteins that are the downstream targets following agonist activation of the
receptor (Ciruela et al., 2010b; Park et al., 2010) and proteins that are well placed to control the receptor’s dimerization or desensitization (Couve et al., 2001; Pontier et al., 2006). The identification of molecular partners for the GABAB R has also revealed a wider range of functions. These include associations that enable GABAB R subunits to regulate gene transcription (Nehring et al., 2000; White et al., 2000; Vernon et al., 2001) or the intracellular trafficking of other membrane proteins (Boyer et al., 2009). Further diversity in GABAB R function is also likely to relate to the temporal and spatial regulation of the receptor. Recent reports have indicated that the recycling of GABAB R s at the cell surface is dynamic and can be modulated through receptor activation, composition, phosphorylation, or degradation (González-Maeso et al., 2003; Fairfax et al., 2004; Gramp et al., 2007, 2008; Laffray et al., 2007; Vargas et al., 2008; Wilkins et al., 2008; Hannan et al., 2011).

Here we identify and investigate a novel association between postsynaptic GABAB R s and the potassium-chloride cotransporter protein, KCC2. KCC2 contributes to the low intracellular chloride concentrations found in mature neurons and thus establishes the conditions for the hyperpolarizing effect of GABAB R s (Rivera et al., 1999). Furthermore, KCC2 is a locus for modulating the strength of fast synaptic inhibition. Rapid changes in KCC2 function have been shown to be elicited in an activity-dependent fashion and involve different post-translational regulation of the transporter protein, including its phosphorylation state and regulation at the cell surface (Woodin et al., 2003; Rivera et al., 2004; Fiumelli et al., 2005; Lee et al., 2007; Wake et al., 2007; Watanabe et al., 2009; Lee et al., 2010; Chamma et al., 2012; Puskarjov et al., 2012; Medina et al., 2014; Mahadevan and Woodin, 2016).

Using a combination of proteomic, biochemical, and molecular studies, we demonstrate that GABAB R s and KCC2 can functionally associate with one another at the membrane of neurons. Activation of the GABAB R s results in reduced levels of KCC2 at the cell surface, which parallels an increase in intracellular chloride and depolarizing shift in the reversal potential for the GABAB R. Our data support a novel mechanism by which GABAB R s can modulate KCC2 and thereby fast synaptic inhibition mediated by the ionotropic GABA R.

**Materials and Methods**

**Mass spectrometry.** All experiments using animal tissue were in accordance with regulations from the United Kingdom Home Office Animals (Scientific Procedures) Act. Cortical membranes were prepared by dissecting the cortex from 5 male adult (2 months old) Sprague Dawley rats (Harlan) and homogenizing in 0.32 m sucrose, 50 mM Tris-HCl, pH 7.4 (10 ml/g tissue). The homogenate was centrifuged for 10 min at 690 × g, 4°C, and the supernatant was divided equally and rotated with either 5 ml of 0.85 M to 1.0 M to 1.2 M sucrose in 50 mM Tris-HCl, pH 7.4. Gradients were centrifuged for 2 hr at 111,000 × g, 4°C. Each pellet was resuspended in 0.32 m sucrose and layered at the top of a sucrose gradient (0.85 m to 1.0 m to 1.2 m sucrose in 50 mM Tris-HCl, pH 7.4). Gradients were centrifuged for 2 hr at 111,000 × g, 4°C. The membranes were removed, resuspended in 0.32 m sucrose, and centrifuged for 20 min at 19,500 × g, 4°C. Each pellet was resuspended in 50 ml of cold dH2O with protease inhibitors and placed on ice for 30 min. The samples were centrifuged for 20 min at 34,700 × g, 4°C, and the pellets resuspended in 50 ml Tris-HCl, pH 7.4, before determining the protein concentration and freezing at −80°C. For each affinity purification (3 in total), 10 mg of the prepared membranes was solubilized in 50 mM Tris-HCl, pH 7.4, containing 1% sodium deoxycholate, 10 mM iodoacetamide, 1% w/v β-mercaptoethanol, 0.64% w/v β-mercaptoethanol, 1% w/v B27 (all from Invitrogen; 350–360 mOsm) at 36°C in a 5% CO2 humidified incubator. The organotypic hippocampal brain slice enabled us to conduct electrophysiological imaging, and biochemical experiments in the same preparation. A potential source of variance when investigating chloride homeostasis mechanisms in acutely prepared brain slices has been associated with neuronal damage caused during the slicing procedure. Indeed, previous work has shown that the pyramidal neurons in the organotypic hippocampal brain slice are lost during the culturing period. Indeed, previous work has shown that the pyramidal neurons in the organotypic hippocampal brain slice are lost during the culturing period. Indeed, previous work has shown that the pyramidal neurons in the organotypic hippocampal brain slice are lost during the culturing period. Indeed, previous work has shown that the pyramidal neurons in the organotypic hippocampal brain slice are lost during the culturing period. Indeed, previous work has shown that the pyramidal neurons in the organotypic hippocampal brain slice are lost during the culturing period. Indeed, previous work has shown that the pyramidal neurons in the organotypic hippocampal brain slice are lost during the culturing period. Indeed, previous work has shown that the pyramidal neurons in the organotypic hippocampal brain slice are lost during the culturing period.
being active in these neurons and contributing to a mature and hyper-polarizing $E_{\text{GABA}_A}$. Although many aspects of organotypic hippocampal slice cultures have been shown to resemble the in vivo state (De Simoni et al., 2003), excitatory neurons in this experimental system exhibit increased axonal sprouting, which is likely to underlie the higher levels of synchronous network activity (Dyrhøj-Johnsen et al., 2010).

**Heterologous cell culture and transfection of KCC2 constructs.** CHO cells stably expressing GABAB$_{R1a/R2}$ or GABAB$_{R1b/R2}$ were grown as described previously (Pontier et al., 2006) in DMEM/F12 Ham (Invitrogen) with 2 mM glutamine (Invitrogen), 10% v/v FBS (Invitrogen), 0.5 mg/ml genicin, 0.4 mg/ml hygromycin B, and 2.5 µg/ml puromycin (all from Invitrogen; pH 6.8–7.2). 290–330 mM Oscm). Full-length rat KCC2 cDNA sequence encoding amino acids 1–1116, as well as KCC2 deletion mutants transmembrane domain (TMD) + carboxy-terminal domain (CTD) amino acids 97–116), amino-terminal domain (NTD) + TMD (amino acids 1–640), TMD (amino acids 97–640), and CTD (amino acids 637–1116), were cloned into pEGFP-N3 (Clontech) to generate C-terminally fused proteins. KCC2 NTD (amino acids 1–100) was cloned into pEGFP-C3. Constructs were transfected into CHO cells using JetPEI (Polyplus) and expressed for 48 h before immunoprecipitation or biotinylation analysis (see below).

**Communoprecipitation.** Organotypic hippocampal slices or transfected CHO cells were homogenized in CHAPS buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% w/v CHAPS, and protease inhibitors; Roche). Precleared lysates were probed for GABAB$_{R1}$, KCC2, GFP, or IgG. Protein A+G+ agaroose was added for 2 h before washing in CHAPS buffer. Agarose beads were eluted in 2X sample buffer at 60°C for 10 min, before loading on to 4% or 8% SDS-PAGE gels. Gels were immunoblotted onto Protran nitrocellulose membranes (Sigma) and probed with indicated primary antibodies overnight at 4°C before addition of relevant secondary HRP-conjugated antibodies and development with Pierce ECL substrate (Thermo Fisher Scientific).

**Biotinylation of cell surface proteins.** Rat organotypic hippocampal slices were incubated for 20 min at 28°C–30°C in either control ACSF or ACSF containing 5 µM SKF97541 while continuously bubbling with 95% O$_2$-5% CO$_2$. For biotinylation of both slices and CHO cells, every subsequent step was performed on ice. Samples were incubated for 30–45 min with 100 µM cleavable biotin (EZ-Link Sulfo-NHS-SS-Biotin, Thermo Fisher Scientific), then washed with 100 µM lysine and lysed with lysis buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1% w/v SDS, 1% v/v Triton X-100 containing protease inhibitors; Roche). The lysate was centrifuged, and 50 µl of the resultant supernatant was removed as the “total” protein lysate sample. Biotinylated proteins were captured by incubation with washed NeutraAvidin Ultralink Resin (Thermo Fisher Scientific) on a rotator overnight at 4°C. The beads were washed 3× with lysis buffer and the “Surface” sample eluted at 37°C for 30 min in 2X sample buffer. Prepared protein samples were subjected to SDS-PAGE/immunoblotting, as described above. In the CHO cell experiments, fluorescent signals were analyzed using a LI-COR Odyssey scanner. For slice experiments, the ECL signal was captured digitally using a Zeiss LSM510 confocal scanning microscope, mounted on an Axiovert 100M inverted microscope (Carl Zeiss). Hippocampal neurons in the dissociated cultures were identified as having a large soma and dendritic spines.

**Electrophysiological recordings.** Organotypic hippocampal slices were transferred to a recording chamber and continuously superfused with 95% O$_2$-5% CO$_2$ ACSF, heated to 28°C–30°C. These conditions ensured thermal stability and permitted long-term patch-clamp recordings from CA3 pyramidal neurons. The ACSF was composed of the following (in mM): 120 NaCl, 3 KCl, 2 MgCl$_2$, 1 CaCl$_2$, 1.2 Na$_2$HPO$_4$, 23 NaHCO$_3$, 11 glucose, pH 7.3–7.4. With the exception of the synaptic stimulation experiments, the ACSF also contained 1 µM TTX (Tocris Bioscience) to eliminate any potential effects at the network level. For perforated patch recordings, the internal solution contained the following (in mM): 135 KCl, 4 NaATP, 0.3 NaGTP, 2 MgCl$_2$, and 10 HEPES, osmolality 290 mosM, pH 7.35. Gramicidin (Calbiochem) was added on the day of the experiment to achieve a final concentration of 0.01 µM. Coverslips were washed and mounted with Vectashield mounting medium (Vector Laboratories). Images were collected using a Zeiss Plan-Apochromat 63×, 1.4 NA oil objective, mounted on a Zeiss LSM510 confocal scanning microscope, mounted on an Axiovert 100M inverted microscope (Carl Zeiss). Hippocampal neurons in the dissociated cultures were identified as having a large soma and dendritic spines.

**Immunofluorescence.** Organotypic hippocampal slices (P7 + 7–14 DIV) were fixed either in ice-cold methanol (for KCC2 labeling) or in 4% PFA followed by cold methanol (for GABAB$_{R2}$ labeling). Slices were blocked in PBS containing 0.3% Triton X-100 and 3% normal goat serum. Incubation with primary antibodies (1/1000 dilution for both rabbit and mouse anti-KCC2) was performed at 4°C overnight. Slices were washed 4 times with PBS containing 0.3% Triton X-100 and incubated for 4 h at room temperature in the same buffer supplemented with 5% normal goat serum and containing either a 568- or 488-coupled anti-rabbit secondary antibody (Invitrogen). Slices were then washed a further 4 times before mounting in 50% glycerol/PBS.

Immunofluorescence was also examined in dissociated hippocampal neurons, where antibody and optical access is better, and where we were able to develop a protocol to quantify expression of both proteins within the same cell. Rat dissociated hippocampal cells were prepared at embryonic day 18 (E18) as described previously (Pooler et al., 2009). After 18–21 DIV cells were fixed and permeabilized in ice-cold methanol, blocked in donkey serum, and sequentially colabeled for GABAB$_{R2}$ and either KCC2 or β-tubulin. Cells were incubated with the rabbit anti-GABA$_{R2}$ primary antibody (1/200 dilution), followed by incubation with a donkey anti-β-tubulin Cy3-conjugated secondary antibody. Appropriate controls were performed to ensure that the Cy3 Fab fragment blocked all available GABA$_{R2}$ sites. Coverslips were washed and mounted with Vectashield mounting medium (Vector Laboratories). Images were collected using a Zeiss Plan-Apochromat 63×, 1.4 NA oil objective, mounted on a Zeiss LSM510 confocal scanning microscope, mounted on an Axiovert 100M inverted microscope (Carl Zeiss). Hippocampal neurons in the dissociated cultures were identified as having a large soma and dendritic spines.
our protocol produced reliable estimates of steady-state $E_{\text{GABA}_A}$. All drugs were added to the ACSF, with the exception of pertussis toxin (PTX, Sigma) and okadaic acid (Tocris Bioscience), which were added directly to the tissue culture media before experimentation. As required, the following antagonists and blockers were added to the extracellular bathing solution; SKF97541, CGP53845, SCH23390, K252a, t-A, kynurenate, arsphenamine, thapsigargin, and monodansylcadaverine (DC) (all from Sigma).

For synaptic stimulation experiments, glutamatergic transmission was blocked by adding 2 mM kynurenate to the ACSF, and GABA release at synaptic terminals was evoked by delivering electrical stimuli via a bipolar tungsten stimulating electrode (FHC), placed 50–100 μm from the recorded pyramidal cell, at the border of the stratum pyramidale and stratum radiatum (Scanziani, 2000). To establish the stimulation conditions under which synaptic $GABA_A$ and $GABA_B$ responses are evoked, a series of recordings were first performed in whole-cell mode using a low-chloride internal solution containing the following (in mM): 140 K-glucuronate, 2 Na2ATP, 3 Na3GTP, 2 MgCl2, 1 EGTA, and 5 HEPES. To improve detection of $GABA_B$ergic currents in the whole-cell recordings, the cells were clamped at −50 mV, and $GABA_A$ and $GABA_B$ conductions were calculated by dividing the isolated currents by their driving force (see Fig. 7C).

Synaptic $E_{\text{GABA}_A}$ was determined from glicentin perfused patch recordings using a step voltage-protocol from a holding potential of −60 mV. The holding potential of the cells was stepped at 5 mV increments between −60 and −90 mV, during which pure $GABA_A$ currents were elicited using single presynaptic stimuli (see Fig. 7). Again, 5 min were allowed between presynaptic stimuli to allow time for the intracellular chloride to reequilibrate (Ehrlich et al., 1999). In synaptic conditioning experiments, a stimulation protocol was used to strongly activate $GABA_B$Rs (bursts of 6 stimuli at a frequency of 20 Hz, repeated every 5 s for a period of 75 s; see Fig. 7) and the effects upon synaptic $E_{\text{GABA}_A}$ were measured. During the $GABA_B$ synaptic conditioning protocol, the postsynaptic neuron was held at its $E_{\text{GABA}_A}$ to avoid transient loading of the cells with chloride (see Fig. 7B). In addition, to allow time for any transient changes to intracellular chloride to fully reequilibrate (Raimondo et al., 2012; Ehrlich et al., 1999), the first measurement of synaptic $E_{\text{GABA}_A}$ following the $GABA_B$ stimulation protocol was made after 5 min. As before, synaptic $E_{\text{GABA}_A}$ was measured using single presynaptic stimuli to activate pure $GABA_A$ currents.

Wherever possible, electrophysiological recordings were conducted under a “within cell” experimental design. This means that each neuron had its own $E_{\text{GABA}_A}$ measurements before (“baseline”) and after drug treatment, so that each neuron served as its own control. This “within-cell” experimental design reduces the impact of cross cell variability, means that drug effects can be examined using paired statistical tests. For clarity, we report the mean absolute values of $E_{\text{GABA}_A}$ and the mean change in $E_{\text{GABA}_A}$. To minimize the potential effect of changes in recording conditions that may have taken place over the course of the study, we took the additional step of restricting comparisons to recordings that were performed during similar time periods. For example, over the course of the study, the effect of SKF97541 upon $E_{\text{GABA}_A}$ was measured in a total of 20 neurons. However, when compared with another experimental group, the SKF97541 data were restricted to recordings performed during a similar time period as the experimental group. Finally, to avoid potential contamination effects across experiments, only one electrophysiological recording was performed per organotypic hippocampal slice culture by bioelectronic transfection (Bio-Rad). At 2–3 d after transfection, CI-Sensor protein expressing neurons were imaged using an FV300 confocal microscope (Olympus), custom-converted for multiphoton imaging, and equipped with a MaiTai-HP Ti:sapphire femtosecond pulsed laser (Newport Spectra-Physics). Images were acquired using Fluoview software (version 5.0, Olympus). Cells were excited at 850 nm and a 510 nm dichroic mirror was used to separate emitted light into CFP and YFP channels, which were filtered at 460–500 nm and 520–550 nm, respectively, and detected simultaneously using two externally mounted PMTs (Hamamatsu). Image stacks were flat-field corrected, collapsed along the z-plane, background subtracted, and the YFP/CFP ratio was calculated by dividing the respective images on a pixel-by-pixel basis. The ratio was calibrated to absolute intracellular chloride values using the K+ / H+ exchanger nigerinic and the Cl− / OH− exchanger tributyltin chloride (both at 20 μM) in a high K+ , HEPES-buffered solution at pH 7.35, as described previously (Boyarsky et al., 1988; Kuner and Augustine, 2000).

Data analysis and statistics. Data and statistical analyses were performed using MATLAB R2008b (The MathWorks) and SPSS (IBM). All data are reported as mean ± SE. Statistical comparisons were made using either paired or unpaired Student’s $t$ tests, and one-way ANOVAs with post hoc Dunnett (two-sided) corrections. All statistical tests were two-tailed, and a $p$ value of <0.05 was deemed statistically significant.

Results

$GABA_B$Rs form a protein complex with the potassium-chloride transporter KCC2 at the neuronal membrane

We used a combination of coimmunoprecipitation and mass spectrometry to identify functionally important components of $GABA_B$ protein complexes in the brain. An anti-$GABA_B$R1 antibody was used to isolate antibody-protein complexes from membrane preparations generated from freshly dissected adult rat cortex (see Materials and Methods). Analysis of the resulting peptides revealed a series of proteins that have previously been shown to associate with the $GABA_B$R, including G-protein subunits (Bettler et al., 2004), potassium channel tetramerization proteins (Schwenk et al., 2010), NEM-sensitive fusion protein (Ponter et al., 2006), and 14-3-3 signaling proteins (Couve et al., 2001) (Fig. 1A). In addition, the mass spectrometry revealed a novel protein association between the $GABA_B$R and the solute carrier family 12, member 5 protein (SLC12A5), also known as the potassium chloride cotransporter KCC2 (Fig. 1A). As with all of the associated proteins, KCC2 was present in three independent neuronal membrane isolates, where it was identified from multiple peptides on each occasion and did not appear in IgG control precipitates (Fig. 1B).

KCC2 coimmunoprecipitated robustly with $GABA_B$R1 when protein complexes were isolated from rat organotypic hippocampal brain slices using either a $GABA_B$R1 (Fig. 1B) or a $GABA_B$2 antibody (Fig. 1C). KCC2 coimmunoprecipitated with the two splice isoforms of the $GABA_B$R subunit ($GABA_B$R1a and $GABA_B$R1b) (Fig. 1C). KCC2 appeared as two bands (130 and 270 kDa) on immunoblots (Figs. 1B, C), consistent with previous reports that KCC2 can exist as both a monomer and a dimer (Blaesse et al., 2006; Uvarov et al., 2009). This confirmed that the association between $GABA_B$R and KCC2 is present in organotypic hippocampal rat brain slices as well as acutely dissected rat cortex, and revealed that the $GABA_B$R associates with both monomeric and dimeric forms of KCC2, although it is not clear whether this is a direct interaction or whether other proteins are involved. Consistent with the biochemical evidence, immunofluorescence staining in rat organotypic hippocampal slices (P7 + 7–14 DIV) and rat dissociated hippocampal cultures (E18 + 18–21 DIV) confirmed that the $GABA_B$R and KCC2 are both found at somatic and dendritic membranes and exhibit overlapping labeling (Fig. 1D, E). The immunofluorescence protocol in dissociated cultures enabled us to examine coexpression of both proteins within the same cell (see Materials and Methods; Fig.
We therefore quantified the GABABR and KCC2 staining pattern in the dissociated hippocampal neurons and found that the vast majority exhibited overlapping labeling at the membrane, consistent with colocalization of the proteins (91%; 52 of 57 GABABR-positive cells).

To further characterize the association between GABABRs and KCC2, we used a heterologous CHO cell line that constitutively expresses rat GABABR1b and GABABR2 (CHO GABABR1b/R2) (Pontier et al., 2006). GABABR1b from this cell line was detected as multiple bands on immunoblots, consistent with differential glycosylation of the GABABR1b protein in this system (Fig. 2A).

CHO GABABR1b/R2 was transfected with recombinant versions of rat KCC2 fused to GFP. Coimmunoprecipitation experiments using antibodies against GABABR1, KCC2 (Fig. 2B), or GFP (Fig. 2E) confirmed that the association between GABA Rs and full-length KCC2 (FL-KCC2) could be reconstituted in this system. KCC2 is predicted to consist of a cytoplasmic amino-terminal domain and a cytoplasmic carboxy-terminal domain, either side of a transmembrane domain that contains 12 transmembrane helices (Fig. 2C) (Payne et al., 1996). We generated GFP fusions of KCC2 that were comprised of only the NTD, only the CTD, or only the TMD. In addition, we generated GFP fusions of KCC2 that lacked either the amino-terminal domain (TMD/H11001) or the carboxy-terminal domain (NTD/H11001). Biotinylation experiments confirmed that each of the fusion proteins containing the transmembrane domain was trafficked, at least in part, to the cell surface in CHO cells (Fig. 2D). This is consistent with previous studies that have shown that KCC cytoplasmic domains (N and C terminal) are not essential for membrane delivery in heterologous cell systems (Casula et al., 2001; Li et al., 2007). We then performed coimmunoprecipitation experiments to establish the KCC2 region responsible for the association with GABAB R. These revealed that GABAB R can form a complex with versions of KCC2 that lack both intracellular terminal domains. However, GABAB R do not associate with versions of KCC2 that lack the transmembrane domain (Fig. 2E). The association appears spe-
Figure 2.  The GABA\textsubscript{R} associates with the transmembrane domain of KCC2.  

A. The GABA\textsubscript{R}1 monoclonal antibody 2D7 used for immunoblotting is specific for GABA\textsubscript{R}1. In Western blots of total rat brain and rat hippocampus homogenate, two bands corresponding to GABA\textsubscript{R}1a and GABA\textsubscript{R}1b were detected. Blots of lysates from CHO cells stably expressing either GABA\textsubscript{R}1a/R2 or GABA\textsubscript{R}1b/R2 revealed multiple bands, consistent with differential glycosylation of GABA\textsubscript{R}1 proteins in this system.  

B. The KCC2-GABA\textsubscript{R} association can be reconstituted in a heterologous cell system. CHO cells stably expressing GABA\textsubscript{R}1b/R2 were transiently transfected with FL-KCC2-GFP and used for coimmunoprecipitation experiments. Western blots of resulting complexes showed that KCC2 can be coimmunoprecipitated with GABA\textsubscript{R}1b (left), and the reciprocal coimmunoprecipitation confirms the association (right). IgG controls were included in each experiment. Sh, Sheep; Rab, rabbit.  

C. Schematic diagram of KCC2 showing the intracellular NTD, CTD, and TMD with its 12 predicted transmembrane helices.  

D. GFP fusion proteins of FL-KCC2 and different KCC2 deletion constructs containing the TMD are expressed and transported to the plasma membrane. Biotinylation experiment comparing total (T) and cell surface (S) protein levels in CHO GABA\textsubscript{R}1b/R2 cells, transiently transfected with different GFP fusion constructs (left). FL-KCC2 (predicted molecular mass 150 kDa, although the TM region is glycosylated), TMD\textsubscript{H11001}/NTD\textsubscript{H11001} (predicted 140 kDa), TMD (predicted 96 kDa), and TMD (predicted 86 kDa) are all detected on the cell surface, whereas GFP (27 kDa) alone is not. Additional bands detected likely represent alternatively glycosylated, degraded, or aggregated proteins. The blot was reprobed with GABA\textsubscript{R}1 antibody to confirm surface expression of the receptor (bottom).  

E. The transmembrane domain of KCC2 is required for the association with the GABA\textsubscript{R}. Coimmunoprecipitation experiments on CHO GABA\textsubscript{R}1b/R2 cells transiently expressing KCC2-GFP deletion constructs were performed using anti-GFP as the precipitating antibody. When the resulting complexes were probed for GABA\textsubscript{R}1 (top), all KCC2-GFP fusion proteins containing the TMD successfully coimmunoprecipitated GABA\textsubscript{R}1b. However, GFP fusion proteins containing only the NTD (predicted 38 kDa) or the CTD (predicted 82 kDa) did not capture GABA\textsubscript{R}1b. Under these conditions, and compared with FL-KCC2 (100%), (Figure legend continues.)
pecific as another transmembrane protein, the transferrin receptor, was not found in the KCC2 isolates (Fig. 2F). Thus, KCC2 associates with the GABA<sub>R</sub> via its transmembrane domain, which is consistent with the idea that KCC2 and GABA<sub>R</sub> can form a protein complex at the neuronal membrane.

**GABA<sub>R</sub> activation affects transmembrane chloride gradients**

Signaling interactions across GABA<sub>R</sub> protein complexes have been shown to be capable of modulating the activity of both the receptor and its associated proteins (Balasubramanian et al., 2004; Pontier et al., 2006; Ciruela et al., 2010b; Park et al., 2010). Given the evidence that the GABA<sub>R</sub> and KCC2 can associate at the membrane, we investigated whether activation of the GABA<sub>R</sub> can influence how KCC2 contributes to transmembrane chloride gradients. To assess KCC2 function, intracellular chloride concentration ([Cl<sup>-</sup>]) was monitored by calculating the reversal potential of the ionotropic GABA<sub>A</sub>-induced current (E<sub>GABA</sub>-<sub>A</sub>). To avoid disrupting [Cl<sup>-</sup>] gradient, we measured baseline chloride concentration (35.5 ± 11.3 pA in SCH23390-treated neurons compared with 86.0 ± 11.4 pA in control neurons, n = 8 and 13, respectively, p = 0.01, ANOVA followed by post hoc Dunnett’s correction; Fig. 3E). In contrast, using SCH23390 (10 μM) (Kuzhikandathil and Oxford, 2002) to block the downstream GIRK channels directly, did not prevent the SKF97541-induced change in E<sub>GABA</sub>-<sub>A</sub>. The efficacy of the GIRK channel block by SCH23390 was evident from the significant reduction in the SKF97541-evoked current (35.5 ± 11.3 pA in SCH23390-treated neurons compared with 86.0 ± 11.4 pA in control neurons, n = 8 and 13, respectively, p = 0.01, ANOVA followed by post hoc Dunnett’s correction; Fig. 3E).

Further experiments established that the GABA<sub>R</sub>-mediated effect requires associated G-proteins, but is independent of downstream, G-protein-coupled inwardly rectifying potassium (GIRK) channels. First, G-protein signaling via the GABA<sub>R</sub> was disrupted by pretreating the organotypic hippocampal slices with the G<sub>αi</sub>-selective antagonist PTX (5 μM) onto the cell soma. The mean resting membrane potential was −71.5 ± 0.9 mV, compared with an E<sub>GABA</sub>-<sub>A</sub> value of −82.5 ± 1.4 mV (n = 13). Thus, the neurons displayed a mature hyperpolarizing E<sub>GABA</sub>-<sub>A</sub> profile at the time of recording, consistent with KCC2 expression and function.

To investigate whether agonist activation of GABA<sub>A</sub>Rs mediates functional changes in [Cl<sup>-</sup>], E<sub>GABA</sub>-<sub>A</sub> was measured before and after the application of the specific GABA<sub>A</sub> agonist, SKF97541 (1 μM). GABA<sub>R</sub> activation was found to result in a depolarizing shift in E<sub>GABA</sub>-<sub>A</sub> which was evident 5–10 min following GABA<sub>R</sub> activation and persisted for the remainder of the recording (Fig. 3A–D). Across a population of CA3 pyramidal neurons, the mean E<sub>GABA</sub>-<sub>A</sub> shifted from a baseline value of −82.5 ± 1.4 mV to −78.2 ± 1.3 mV following GABA<sub>R</sub> activation. This represented a mean change in E<sub>GABA</sub>-<sub>A</sub> of 4.2 ± 0.7 mV (p = 0.0009, n = 13, paired t test; Fig. 3D). In control experiments, blocking GABA<sub>A</sub>Rs with a selective, competitive antagonist (5 μM CGP55845) prevented the change in E<sub>GABA</sub>-<sub>A</sub> in response to SKF97541 (p = 0.2, n = 6, paired t test). This confirmed that the effects were specific to the GABA<sub>R</sub>, and not alternative receptors such as the GABA<sub>B</sub>-<sub>R</sub>.

Further experiments established that the GABA<sub>R</sub>-mediated effect requires associated G-proteins, but is independent of downstream, G-protein-coupled inwardly rectifying potassium (GIRK) channels. First, G-protein signaling via the GABA<sub>R</sub> was disrupted by pretreating the organotypic hippocampal slices with the G<sub>αi</sub>-selective antagonist PTX (5 μg/ml for 24 h before recordings). We could confirm that PTX treatment did disrupt GABA<sub>R</sub>-mediated chloride currents that is consistent with a decrease in KCC2 function.

**GABA<sub>R</sub> activation can regulate KCC2 at the membrane**

To directly test the hypothesis that GABA<sub>R</sub> activation modulates KCC2 function at the membrane, we performed a combination of electrophysiological recordings and biotinylation experiments. First, to establish that the GABA<sub>R</sub>-mediated shift in E<sub>GABA</sub>-<sub>A</sub> occurs through a reduction in KCC2 function, cells were exposed to furosemide, which blocks KCC2 activity. CA3 pyramidal neurons exposed to furosemide (1 mM) exhibited a significantly more depolarized resting E<sub>GABA</sub>-<sub>A</sub> (−70.2 ± 2.9 mV, n = 12) than untreated control cells (−83.0 ± 1.9 mV, n = 9, p = 0.002, ANOVA followed by post hoc Dunnett’s correction; Fig. 4C). The furosemide-induced shift in E<sub>GABA</sub>-<sub>A</sub> was evident within 5 min, highlighting that KCC2 functions to continuously maintain the hyperpolarized E<sub>GABA</sub>-<sub>A</sub> under these conditions. Importantly, ap-
Fig. 3. GABABR activation causes a depolarizing shift in $E_{\text{GABA}}$ and increase in intracellular chloride. A, Example GABABR i–v plot from a gramicidin perforated patch recording of a CA3 pyramidal neuron in a rat organotypic hippocampal slice. Inset, Raw current traces recorded at different holding potentials. GABABR currents were evoked by local application of muscimol (10 μM, arrowhead) to the cell soma. $E_{\text{GABA}}$ was defined as the holding potential at which the GABABR current had an amplitude of zero. Calibration: 100 pA, 1 s. B, Example i–v plot from the same cell in A recorded after 10 min of bath application of the GABABR agonist, SKF97541 (1 μM). C, GABABR activation led to a significant depolarizing shift in $E_{\text{GABA}}$ across a population of neurons ($n = 13$). **$p = 0.0009$ (paired $t$-test). Each connected pair of dots corresponds to an individual neuron. Gray horizontal bars represent population means. D, Change in $E_{\text{GABA}}$ plotted as a function of time before and after the onset of SKF97541 application (gray bar; $t = 0.98$, ANOVA followed by post hoc Dunnett’s correction; Fig. 4B,C). Furthermore, bumetanide treatment did not prevent the depolarizing shift in $E_{\text{GABA}}$ following GABABR activation. Upon SKF97541 application, the mean $E_{\text{GABA}}$ shifted to a new value of $-77.8 \pm 2.7$ mV, a change that was indistinguishable to that seen in control cells ($4.5 \pm 1.1$ mV, $p = 0.98$, ANOVA followed by post hoc Dunnett’s correction; Fig. 4E,F). GABABR activation led to an equivalent shift in $E_{\text{GABA}}$ in cells treated with both 10 μM bumetanide (baseline $E_{\text{GABA}} = -84.3 \pm 3.2$ mV, SKF97541-treated $E_{\text{GABA}} = -80.2 \pm 4.2$ mV, change = $4.1 \pm 1.5$ mV; $p = 0.99$, $n = 6$) or 100 μM bumetanide (baseline $E_{\text{GABA}} = -80.2 \pm 3.5$ mV, SKF97541-treated $E_{\text{GABA}} = -75.4 \pm 3.5$ mV, change = $4.8 \pm 1.7$ mV; $p = 0.92$, $n = 6$, ANOVA followed by post hoc Dunnett’s correction). These data demonstrate that KCC2 is the mediator of the GABABR-dependent effect upon $E_{\text{GABA}}$. If this is the case, one prediction is that other manipulations that downregulate KCC2 protein levels should attenuate the expression of the GABABR agonist SKF97541 in the presence of furosemide failed to produce any further change in $E_{\text{GABA}}$ ($-70.0 \pm 2.3$ mV) (Fig. 4A,D). Compared with control cells, the effect of SKF97541 upon $E_{\text{GABA}}$ was significantly attenuated in furosemide-treated cells ($0.2 \pm 1.3$ mV, $p = 0.039$, ANOVA followed by post hoc Dunnett’s correction; Fig. 4F).

As furosemide can block multiple cotransporter proteins, this experiment could not exclude a contribution by the sodium-potassium-chloride cotransporter protein, NKCC1, which has also been shown to contribute to [Cl$^-$], regulation in hippocampal pyramidal neurons, particularly during development (Dzhala et al., 2005). We therefore examined the effect of GABABR activation in the presence of bumetanide, a more selective blocker of NKCC1. The baseline $E_{\text{GABA}}$ after incubation with bumetanide was similar to untreated control cells, indicating that NKCC1 does not make a major contribution to the [Cl$^-$], measured in these neurons ($E_{\text{GABA}} = -82.3 \pm 2.3$ mV, $n = 12$, $p = 0.97$, ANOVA followed by post hoc Dunnett’s correction; Fig. 4B,C). Furthermore, bumetanide treatment did not prevent the depolarizing shift in $E_{\text{GABA}}$ following GABABR activation. Upon SKF97541 application, the mean $E_{\text{GABA}}$ shifted to a new value of $-77.8 \pm 2.7$ mV, a change that was indistinguishable to that seen in control cells ($4.5 \pm 1.1$ mV, $p = 0.98$, ANOVA followed by post hoc Dunnett’s correction; Fig. 4E,F). GABABR activation led to an equivalent shift in $E_{\text{GABA}}$ in cells treated with both 10 μM bumetanide (baseline $E_{\text{GABA}} = -84.3 \pm 3.2$ mV, SKF97541-treated $E_{\text{GABA}} = -80.2 \pm 4.2$ mV, change = $4.1 \pm 1.5$ mV; $p = 0.99$, $n = 6$) or 100 μM bumetanide (baseline $E_{\text{GABA}} = -80.2 \pm 3.5$ mV, SKF97541-treated $E_{\text{GABA}} = -75.4 \pm 3.5$ mV, change = $4.8 \pm 1.7$ mV; $p = 0.92$, $n = 6$, ANOVA followed by post hoc Dunnett’s correction). These data demonstrate that KCC2 is the mediator of the GABABR-dependent effect upon $E_{\text{GABA}}$. If this is the case, one prediction is that other manipulations that downregulate KCC2 protein levels should attenuate the
Figure 4. \(\text{GABA}_B\)-R activation downregulates the chloride transporter KCC2. A, The \(\text{GABA}_B\)-R-induced shift in \(E_{\text{GABA}}\) is occluded by blocking the activity of chloride transporter proteins. \(E_{\text{GABA}}\) recorded in a CA3 pyramidal neuron in a rat organotypic hippocampal slice under baseline conditions (white symbols), following application of furosemide (1 mM, black symbols), and then after addition of SKF97541 (1 \(\mu\)M, gray symbols). Insets, Raw current traces recorded at different holding potentials. Calibration: 100 pA, 1 s.

B, The \(\text{GABA}_B\)-R-induced shift in \(E_{\text{GABA}}\) is not prevented by blocking the chloride transporter NKCC1. \(E_{\text{GABA}}\) recorded in a CA3 pyramidal neuron in a rat organotypic hippocampal slice under baseline conditions (white symbols), after bumetanide treatment (10 \(\mu\)M, black symbols), and then after the subsequent addition of SKF97541 (1 \(\mu\)M, gray symbols). Calibration: 100 pA, 1 s.

C, Compared with control cells \((n = 9), E_{\text{GABA}}\) was significantly more depolarized in neurons treated with furosemide \((n = 12), ***p < 0.002\) (ANOVA, with post hoc Dunnett’s correction). Neurons treated with bumetanide \((10-100 \mu M)\) had values comparable with control \((p = 0.97\) (ANOVA, with post hoc Dunnett’s correction)). D, The SKF97541-induced change in \(E_{\text{GABA}}\) in a population of CA3 pyramidal neurons treated with furosemide (black bar) and plotted as a function of the time of SKF97541 application (gray bar; \(n = 12\)). E, The SKF97541-induced change in \(E_{\text{GABA}}\) observed in control cells \((n = 9)\) was blocked in furosemide-treated neurons \((p = 0.039\) (ANOVA, with post hoc Dunnett’s correction)). Bumetanide treatment did not affect the depolarizing shift in \(E_{\text{GABA}}\) caused by \(\text{GABA}_B\)-R activation \((p = 0.98\) (ANOVA, with post hoc Dunnett’s correction)).

The effects upon surface KCC2 did not appear to involve degradation as the total amount of KCC2 protein was not different between control and SKF97541-treated slices (Fig. 5C,D).

The effects upon surface KCC2 did not appear to involve degradation as the total amount of KCC2 protein was different between control and SKF97541-treated slices. When examined after the same period of SKF97541 application (5 \(\mu\)M for 20 min), the normalized levels of total KCC2 monomer were 104.0 \(\pm\) 7.1\% of control and total KCC2 dimer was 103.7 \(\pm\) 10.2\% of control \((p = 0.002, n = 14, t\) test), whereas dimeric KCC2 was reduced to 83.3 \(\pm\) 7.7\% of control \((p = 0.048, n = 14, t\) test; Fig. 5A, D). The effect was specific to KCC2 as surface levels of the transferrin receptor \((94.2 \pm 21\%); p = 0.79, n = 8, t\) test) and NKCC1 \((27.2 \pm 8.9\%); p = 0.4, n = 13, t\) test) were not statistically different between control and SKF97541-treated slices (Fig. 5C, D).

Interestingly, at this later time point, an effect upon surface KCC2
was also not detectable (surface/total KCC2 monomer was 104.1 ± 8.3% of control and surface/total KCC2 dimer was 106.3 ± 9.9% of control, p = 0.63 and p = 0.54, respectively; n = 12, t test). These data suggest that GABA\(_B\)R activation does not lead to KCC2 degradation but can rather affect the surface trafficking (endocytosis and recycling) of KCC2.

GABA\(_B\)R dynamics at the cell surface can be affected upon receptor activation (Laffray et al., 2007; Grampp et al., 2008; Wilkins et al., 2008), suggesting that changes to surface trafficking of KCC2 could be associated with changes to surface GABA\(_B\)R. Indeed, our electrophysiological recordings revealed that SKF97541-evoked currents tended to decrease in the continued presence of the agonist (a decrease of 10.1 ± 3.7%, from 88.5 ± 12.8 pA to 79.0 ± 11.1 pA, with 10 min of SKF97541 exposure; n = 13, p = 0.012, paired t test), indicating that there may be an agonist-dependent change in GABA\(_B\)R signaling at the cell surface. To investigate this biochemically, we used our quantitative Western blot methods to examine GABA\(_B\)R behavior after exposure to SKF97541 and found that, at the same time point that surface KCC2 is reduced (Fig. 5A, D), levels of surface GABA\(_B\)R were also reduced. SKF97541 treatment (5 \(\mu M\) for 20 min) reduced GABA\(_B\)R1 surface expression in the organotypic hippocampal slices to 79.4 ± 8.7% of control (p = 0.046, n = 9, t test; Fig. 5B, D). Meanwhile, communoprecipitation experiments revealed that the amount of KCC2 pulled down in GABA\(_B\)R1 complexes was not significantly different between SKF97541-treated and matched control slices (p = 0.44, n = 3, t test; Fig. 5E). To investigate whether the internalization of KCC2 via GABA\(_B\)R could be reconstituted in the heterologous CHO cell system, we applied SKF97541 to CHO GABA\(_B\)R1b/R2 transiently expressing FL-KCC2. In this system, there was no significant effect upon surface levels of either GABA\(_B\)R1 (105.7 ± 3.4% of control; p = 0.12, n = 13, t test) or KCC2 (98.8 ± 6.1% of control; p = 0.85, n = 13, t test), suggesting that the mechanism may be sensitive to expression levels, post-translational modifications, or that intermediary proteins are involved in regulating surface expression in neurons.
Figure 6. The GABA_B R effect upon KCC2 requires clathrin-dependent endocytosis. A, Blocking clathrin-dependent endocytosis with DC prevents the depolarizing shift in $E_{\text{GABA}_{\text{A}}}$ following GABA_B R activation. Example $I$–$V$ plots are shown for a CA3 pyramidal cell in a rat organotypic hippocampal slice treated with DC (50 μM). $E_{\text{GABA}_{\text{A}}}$ at baseline (black data) is similar to that recorded after SKF97541 treatment (gray data). B, The SKF97541-induced change in $E_{\text{GABA}_{\text{A}}}$ in a population of CA3 pyramidal neurons treated with DC (black bar) and plotted as a function of the time of SKF97541 application (gray bar; $n = 8$). C, Blocking Ca^{2+} signaling via a combination of nimodipine (20 μM), thapsigargin (2 μM), and d-APV (100 μM) did not prevent the GABA_B R-mediated shift in $E_{\text{GABA}_{\text{A}}}$. Same conventions as in A, D. The SKF97541-induced change in $E_{\text{GABA}_{\text{A}}}$ in a population of CA3 pyramidal neurons treated with Ca^{2+} blockers (black bar), plotted as a function of the time of SKF97541 application (gray bar; $n = 9$). E, Summary plot of the effect of GABA_B R activation in the presence of different inhibitors. Blocking clathrin-dependent endocytosis significantly reduced the SKF97541-dependent shift in $E_{\text{GABA}_{\text{A}}}$ ($n = 8$) observed in control cells ($n = 11$), $p = 0.03$ (ANOVA followed by post hoc Dunnett’s correction). In contrast, treating cells with a combination of Ca^{2+} blockers ($n = 9$) had no effect on the SKF97541-dependent shift in $E_{\text{GABA}_{\text{A}}}$, $p = 0.99$ (ANOVA followed by post hoc Dunnett’s correction). Preincubation with the selective protein kinase C inhibitor, Go6976 (1 μM, $n = 9, p = 0.99$); the general kinase blocker, K252a (100 μM, $n = 6, p = 0.94$); the tyrosine phosphatase inhibitor, Na_3VO_4 (1 μM, $n = 6, p = 0.99$); or the protein phosphatase 1 and 2 inhibitor, okadaic acid (1 μM, $n = 6, p = 0.99$) did not prevent a significant shift in $E_{\text{GABA}_{\text{A}}}$ following GABA_B R activation (all ANOVA followed by post hoc Dunnett’s correction). F, Surface KCC2 levels are not altered when GABA_B Rs are activated in the presence of an inhibitor of clathrin-dependent endocytosis. Rat organotypic hippocampal slices were pretreated with DC (50 μM) and exposed to SKF97541 (DC + SKF). Slices pretreated with DC but not exposed to SKF97541 (DC) were used as controls and run in parallel (see Materials and Methods). G, Slices treated with a combination of Ca^{2+} blockers still showed reduced surface KCC2 levels following GABA_B R activation. H, Summary plot of the ratio of surface-to-total KCC2 protein, normalized to control values. Blocking clathrin-dependent endocytosis prevented the reduction in surface KCC2 following GABA_B R activation ($n = 7, p = 0.13$; t test), whereas blocking Ca^{2+} signaling ($n = 8$) did not prevent the effect of GABA_B R activation upon surface KCC2. *$p = 0.011$ (t test).

GABA_B R regulation of KCC2 involves clathrin-mediated endocytosis

Both GABA_B Rs and KCC2 undergo endocytosis via the clathrin-mediated endocytic pathway (Grampp et al., 2007; Laffray et al., 2007; Vargas et al., 2008; Zhao et al., 2008). Indeed, clathrin-mediated endocytosis contributes to the constitutive membrane recycling of GABA_B Rs, which can be accelerated by receptor activation (Grampp et al., 2007, 2008; Laffray et al., 2007). One possibility therefore is that the clathrin-mediated endocytic pathway is important for the change in surface KCC2 that results from GABA_B R activation. To test this, we used a selective blocker of the clathrin-mediated endocytic pathway, dansylcadaverine (DC). Importantly, CA3 pyramidal neurons pretreated with DC (50 μM) failed to show a change in $E_{\text{GABA}_{\text{A}}}$ following GABA_B R activation (Fig. 6A, B). $E_{\text{GABA}_{\text{A}}}$ shifted from a mean baseline of $-82.6 \pm 3.4$ mV to $-82.1 \pm 3.3$ mV following SKF97541 treatment, a shift of just $0.46 \pm 0.6$ mV ($n = 8$), which was significantly smaller than the change observed in control cells exposed to SKF97541 ($n = 11, p = 0.03$, ANOVA followed by post hoc Dunnett’s correction; Fig. 6E). DC-treated neurons also failed to show a decrease in the amplitude of SKF97541-evoked currents in the continued presence of the agonist (a decrease of $-0.9 \pm 3.9\%$, from $36.3 \pm 8.3$ pA to $35.5 \pm 7.8$ pA, with 10 min of SKF97541 exposure; $n = 8, p = 0.36$, paired t test), suggesting no net change in GABA_B R signaling at the cell surface. Finally, consistent with these electrophysiological recordings, the reduction in levels of surface KCC2 following GABA_B R activation (5 μM SKF97541 for 20 min) was reduced when clathrin-mediated endocytosis was blocked with DC (50 μM; $n = 7, p = 0.13$; Fig. 6F, H). Disrupting the clathrin-mediated endocytic pathway therefore occludes the GABA_B R-mediated change in surface KCC2.

KCC2 function has also been reported to be modulated by Ca^{2+}-dependent kinases, phosphatases, and proteases (Fiumelli et al., 2005; Lee et al., 2007, 2010; Wake et al., 2007; Xu et al., 2008; Watanabe et al., 2009; Puskarjov et al., 2012). To test whether the GABA_B R-dependent modulation of KCC2 involves intracellular Ca^{2+} signaling, hippocampal slices were treated with a combination of Ca^{2+} channel blockers and intracellular Ca^{2+} store blockers (20 μM nimodipine, 100 μM D-APV and 2 μM thapsigargin). Under these conditions, GABA_B R activation still resulted in a positive shift in $E_{\text{GABA}_{\text{A}}}$ that...
Figure 6

B. Isolating GABA_R and GABAB_R responses. Representative traces show monosynaptic GABAergic postsynaptic currents in a CA3 pyramidal neuron before (black data) and after (gray data) delivering a conditioning protocol designed to strongly activate postsynaptic GABA_Rs (90 stimuli delivered as 15 bursts of 6 stimuli at 20 Hz, at 5 s intervals). Insets, Raw traces. Calibration: 50 pA, 1 s.

Figure 7

A. Diagram of the experimental setup for synaptically activating postsynaptic GABA_Rs and GABAB_Rs. Presynaptic GABAergic interneurons were stimulated in rat organotypic hippocampal slices via a bipolar tungsten electrode positioned at the stratum radiatum/pyramidale border, 50–100 μm from the recorded cell. B. Isolating GABA_R and GABAB_R responses. Representative traces show monosynaptic GABAergic postsynaptic currents in a CA3 pyramidal neuron recorded in response to single presynaptic stimuli (left) or trains of 6 stimuli applied at 20 Hz (middle). GABA_R and GABAB_R responses could be pharmacologically isolated by application of the selective GABA_R antagonist SR95531 (10 μM) and then the GABAB_R antagonist GPP5845 (5 μM). GABA_R responses were not evoked by single stimuli but were evident for the multiple-stimuli condition. In the absence of these receptor blockers (right), the flux of chloride through GABAARs could be to strongly activate postsynaptic GABABRs (90 stimuli delivered as 15 bursts of 6 stimuli at 20 Hz, at 5 s intervals). Insets, Raw traces. Calibration: 50 pA, 1 s.

C. gGABA_R and gGABA_B plots for a CA3 pyramidal neuron before (black data) and after (gray data) delivering a conditioning protocol designed to strongly activate postsynaptic GABA_Rs (90 stimuli delivered as 15 bursts of 6 stimuli at 20 Hz, at 5 s intervals). Insets, Raw traces. Calibration: 50 pA, 1 s.

D. Change in E_GABA_R (mV) with GABA_B stimulation. E. Diagram of the experimental setup for synaptically activating postsynaptic GABA_Rs and GABAB_Rs. Presynaptic GABAergic interneurons were stimulated in rat organotypic hippocampal slices via a bipolar tungsten electrode positioned at the stratum radiatum/pyramidale border, 50–100 μm from the recorded cell.
manner that is independent of calcium-dependent kinase and phosphatase activity, but is dependent upon clathrin-mediated endocytosis.

**Synaptically driven GABA\(_B\)R activity affects intracellular chloride regulation**

To investigate whether this mechanism could be recruited under physiological conditions, we examined whether the GABA\(_B\)R-mediated effect upon KCC2 occurs at inhibitory synaptic connections. Presynaptic GABAergic interneurons in organotypic hippocampal slices were stimulated via a bipolar electrode placed at the border of the stratum pyramidale and stratum radiatum, 50–100 μm from the recorded pyramidal cell (Fig. 7A). This enabled us to evoke monosynaptic GABA\(_B\)R responses and to measure synaptic E\(_{\text{GABA}}\). Baseline synaptic E\(_{\text{GABA}}\) was similar to muscimol-evoked E\(_{\text{GABA}}\), with a mean value of −76.7 ± 2.1 mV (n = 22; Fig. 7D). We next examined whether the GABA\(_B\)R-mediated effect upon E\(_{\text{GABA}}\) could be elicited via synaptic activation of GABA\(_B\)Rs. GABA\(_B\)Rs are located predominantly extrasynaptically in hippocampal pyramidal cells and are thought to be activated under conditions of strong GABA release, such as occur during periods of high-frequency presynaptic firing (Scanziani, 2000). Consistent with this, a single presynaptic stimulus generated a pure GABA\(_B\)R response in CA3 pyramidal neurons, which was entirely blocked by SR95531 (10 μM; Fig. 7B). In contrast, high-frequency trains of stimuli (e.g., 6 stimuli at 20 Hz) produced a postsynaptic response that was comprised of a large GABA\(_A\)R conductance and a smaller GABA\(_B\)R-mediated conductance that could be blocked by CGP55845 (5 μM; Fig. 7B). By varying presynaptic stimulation conditions, it was observed that the optimal presynaptic frequency for activating a GABA\(_B\)R response was close to 20 Hz (Fig. 7C).

Having established the stimulation parameters for isolating the GABA\(_B\)R response and for evoking robust GABA\(_B\)R responses, we asked whether synaptically driven GABA\(_B\)R activation could induce an activity-dependent shift in E\(_{\text{GABA}}\). Using gammadicin perforated patch recordings, baseline E\(_{\text{GABA}}\) was first determined by using single presynaptic stimuli to elicit a postsynaptic GABA\(_B\)R response at different holding potentials (Fig. 7E). A synaptic stimulation protocol was then administered, which had been shown to elicit strong GABA\(_B\)R activation and consisted of bursts of 6 stimuli at a frequency of 20 Hz, repeated every 5 s for a period of 75 s (GABA\(_B\)R synaptic conditioning protocol; see Materials and Methods). To avoid loading the cells with chloride during these stimulation trains, the holding potential of the recorded cell was clamped at E\(_{\text{GABA}}\), so that there was minimum flux of chloride through the GABA\(_B\)R (Fig. 7B). After the GABA\(_B\)R conditioning protocol, synaptic E\(_{\text{GABA}}\) was then reassessed before using single presynaptic stimuli. These experiments revealed that the GABA\(_B\)R synaptic conditioning protocol caused a robust depolarizing shift in E\(_{\text{GABA}}\) (Fig. 7E,F). Across a population of cells, the mean E\(_{\text{GABA}}\) shifted from a baseline value of −73.3 ± 3.4 mV to −67.2 ± 4.6 mV when recorded 15 min after synaptic GABA\(_B\)R stimulation, which represented a change in E\(_{\text{GABA}}\) of 6.1 ± 1.7 mV (p = 0.014, n = 6, paired t test; Fig. 7G). A temporal analysis of the data showed that the shift in E\(_{\text{GABA}}\) was evident 10 min following synaptic stimulation of the GABA\(_B\)R (E\(_{\text{GABA}}\) = −69.4 ± 4.1 mV, change = 4.0 ± 1.3 mV, p = 0.027, n = 6, paired t test) and was still detected at 30 min after stimulation, the longest population data point that we were able to record (E\(_{\text{GABA}}\) = −64.5 ± 4.2 mV, change = 8.9 ± 1.4 mV, p = 0.002, n = 6, paired t test; Fig. 7G).

To establish that this effect was dependent upon GABA\(_B\)R activation, we first confirmed that a control stimulation protocol that generated minimal GABA\(_B\)R activation (90 stimuli delivered at 1 Hz) did not elicit a change in E\(_{\text{GABA}}\). The baseline E\(_{\text{GABA}}\) was −77.1 ± 4.1 mV; and after delivering the control stimulation protocol, E\(_{\text{GABA}}\) was −75.9 ± 3.0 mV, which was a significantly smaller change in E\(_{\text{GABA}}\) (1.2 ± 1.4 mV) than observed after the GABA\(_B\)R conditioning protocol (p = 0.017, n = 6, ANOVA followed by post hoc Dunnett’s correction; Fig. 7G). Then we confirmed that blocking GABA\(_B\)Rs with a competitive antagonist (5 μM CGP55845) was able to significantly attenuate the shift in E\(_{\text{GABA}}\) caused by the GABA\(_B\)R synaptic conditioning protocol. Indeed, in these experiments, the baseline E\(_{\text{GABA}}\) was −79.3 ± 1.2 mV; and after delivering the GABA\(_B\)R synaptic conditioning protocol, the E\(_{\text{GABA}}\) was −78.2 ± 1.7 mV. This was a change of 1.1 ± 0.5 mV, which did not represent a significant depolarizing shift in E\(_{\text{GABA}}\) (1.1 ± 0.5 mV, n = 5; t test; p = 0.11) and was significantly smaller than the E\(_{\text{GABA}}\) change observed without the GABA\(_B\)R antagonist (p = 0.022, n = 5, ANOVA followed by post hoc Dunnett’s correction; Fig. 7G).

Finally, blocking KCC2 with the selective antagonist VU0240551 (25 μM) (Delpeire et al., 2009; Ivakine et al., 2013) also reduced any change in E\(_{\text{GABA}}\) following GABA\(_B\)R stimulation with the GABA\(_B\)R conditioning protocol (baseline E\(_{\text{GABA}}\) = −72.1 ± 3.6 mV, poststimulation E\(_{\text{GABA}}\) = −72.6 ± 2.6 mV, change = −0.5 ± 1.1 mV, p = 0.001, n = 7, ANOVA followed by post hoc Dunnett’s correction; Fig. 7G). These experiments demonstrate that the GABA\(_B\)R-mediated effect upon E\(_{\text{GABA}}\) via KCC2 is not only elicited by exogenous agonist activation of the GABA\(_B\)R but can also be elicited by synaptically evoked GABA release.

**Discussion**

By forming signaling complexes through specific interactions with other proteins, G-protein–coupled receptors convert extracellular signals into diverse neuronal responses. In the case of GABA\(_B\)Rs, this includes G-proteins that are required for their “classic” signaling, but also interactions with auxiliary proteins that modulate the kinetics of receptor signaling (Schwenk et al., 2010), desensitization (Pontier et al., 2006), subunit dimerization (Couve et al., 2001), and regulate the localization of the receptor or other proteins within cells (White et al., 2000; Boyer et al., 2009). Here we have identified a novel association between the GABA\(_B\)R and the potassium-chloride cotransporter KCC2. This association was discovered in an unbiased screen for proteins present within GABA\(_B\)R complexes at the neuronal membrane, was confirmed by biochemical experiments in hippocampal brain slices and heterologous cells, and was shown to be mediated via the transmembrane region of KCC2. Agonist activation of the GABA\(_B\)R elicits signaling events at the neuronal membrane via G-protein–coupled complexes. We observed that GABA\(_B\)R activation led to a rapid and sustained change in the ionic driving force for the chloride-permeable GABA\(_B\)R, consistent with a decrease in KCC2 function. Electrophysiological recordings and biotinylation assays confirmed that the effects were mediated via KCC2 and were associated with a change in the trafficking of KCC2 protein at the cell surface. A similar downregulation in KCC2 function could also be elicited by a synaptic conditioning protocol designed to strongly activate GABA\(_B\)Rs. While other signaling mechanisms may have been activated under our experimental conditions, the principle change in E\(_{\text{GABA}}\) was unlikely to be mediated by alternative GABA receptors, such as the GABA\(_A\), or by other signaling systems (Mahadevan and Woodin, 2016), because the effect of the GABA\(_B\) agonist and synaptically released
GABA were both blocked by a selective GABA\textsubscript{B}R antagonist. These results are consistent with evidence that GABA\textsubscript{B}R activation modulates proteins with whom the receptor is physically associated (Ciruela et al., 2010b; Park et al., 2010).

In our recordings from CA3 pyramidal neurons, activation of the GABA\textsubscript{B}R by agonist or by synaptic activity released GABA resulted in an \(~5\) mV positive shift in \(E_{\text{GABA}}\), which is similar in amplitude to the shifts in \(E_{GABA}\) following other activity-dependent changes to chloride transporter proteins (Woodin et al., 2003; Wang et al., 2006; Xu et al., 2008; Ormond and Woodin, 2009). Shifts in \(E_{\text{GABA}}\) were evident within \(~10\) min following GABA\textsubscript{B}R activation, which is also consistent with previous evidence that \(E_{\text{GABA}}\) can be rapidly modulated within minutes (Woodin et al., 2003; Fiumelli et al., 2005; Wang et al., 2006; Balena and Woodin, 2008; Xu et al., 2008). Our longest recordings were unable to capture the reversal of the effects on \(E_{\text{GABA}}\) and showed that they were evident for at least 30 min, which is again similar to previous studies that have examined the activity-dependent regulation of KCC2 function (Woodin et al., 2003; Fiumelli et al., 2005; Kitamura et al., 2008; Lee et al., 2011; Puskarov et al., 2012; Zhou et al., 2012). Assuming \(E_{\text{GABA}}\) reflects \(E_{Cl}\) and that extracellular chloride remains constant, a 5 mV shift would equate to an increase in intracellular chloride of \(~1.2\) mM (from 5.4 to 6.6 mM, according to the Nernst equation). Changes in \(E_{\text{GABA}}\) over a narrow range (<5 mV) can have dramatic effects upon whether GABAergic inputs have an inhibitory or facilitating effect (Morita et al., 2006; Jedlicka et al., 2011) and \(E_{\text{GABA}}\) changes of the same magnitude can cause significant changes in the degree of NMDA receptor activation and action potential firing frequency (Akerman and Cline, 2006; Saraga et al., 2008), which can be further influenced by the frequency and location of GABAergic inputs (Prescott et al., 2006; Jean-Xavier et al., 2007).

The GABA\textsubscript{B}R-mediated effect upon KCC2 appears to be distinct from previously described, activity-dependent mechanisms that regulate KCC2. Post-translational regulation of the transporter has been linked to calcium signaling events and associated enzymatic modifications. KCC2 function is associated with its phosphorylation state (Woodin et al., 2003; Fiumelli et al., 2005; Lee et al., 2007; Wake et al., 2007), and the transporter has been reported to turnover rapidly and as a function of the phosphorylation of specific sites within the C-terminal (Rivera et al., 2004; Lee et al., 2010). Recent work has revealed that the total pool of KCC2 is much more stable, but that degradation can be triggered by intracellular calcium, which activates calcium-dependent proteases that cleave the C-terminal of KCC2 (Puskarov et al., 2012). In contrast to these mechanisms, the GABA\textsubscript{B}R-mediated effect upon KCC2 was not prevented by blocking calcium signaling processes, it was not affected by blockers of kinases and phosphatases implicated in the regulation of KCC2, and the total levels of KCC2 were not altered, suggesting that degradation pathways are not involved.

The stable physical association we observed between the GABA\textsubscript{B}Rs and KCC2, plus evidence that GABA\textsubscript{B}Rs can exhibit dynamic behavior at the membrane, offers a potential mechanism by which GABA\textsubscript{B}R activation could influence the surface stability and/or trafficking of the transporter protein. Previous work has provided differing results on the membrane dynamics of GABA\textsubscript{B}Rs. Some studies have reported that the receptor is stable at the cell surface, regardless of whether it is activated or not (Fairfax et al., 2004; Grampp et al., 2007). Other studies have provided evidence that GABA\textsubscript{B}Rs are mobile, being rapidly and constitutively internalized on a timescale of minutes via clathrin-dependent pathways, and in a manner that can be modulated by activation of the receptor (Laffray et al., 2007; Grampp et al., 2008; Wilkins et al., 2008). Our experiments in rat organotypic hippocampal slices revealed that GABA\textsubscript{B}R activation can result in a decrease in the surface expression of both GABA\textsubscript{B}Rs and KCC2 proteins. Such a reduction in surface GABA\textsubscript{B}R following receptor activation is consistent with previous observations in slice cultures (Laffray et al., 2007) but contrasts with studies in dissociated neuronal cultures (Fairfax et al., 2004; Vargas et al., 2008), suggesting that the experimental system (Vargas et al., 2008), or factors such as the dimerization state of the GABA\textsubscript{B}R (Laffray et al., 2007; Hannan et al., 2011), may be important. The effects we observed appeared to affect only a subset of the proteins (<25% decrease in both surface proteins) and were evident over a similar, but not identical, timescale to the downregulation in KCC2 function that we measured electrophysiologically. These differences in timescales of effect may reflect the sensitivities of the methods but could also indicate functional changes to KCC2 that result from being recycled to the membrane, perhaps due to changes in membrane domain, cellular location, and/or molecular interactions (Hartmann et al., 2009; Watanabe et al., 2009).

Internalized GABA\textsubscript{B}Rs are associated with the clathrin-binding adaptor protein-2 complex (Grampp et al., 2007), and disrupting clathrin-mediated endocytosis prevents internalization and recycling of GABA\textsubscript{B}R (Grampp et al., 2007; Laffray et al., 2007; Vargas et al., 2008). Similarly, KCC2 has been shown to bind to adaptor protein-2 in the brain and to undergo fast clathrin-mediated endocytosis (Zhao et al., 2008). Importantly, we found that blocking clathrin-mediated endocytosis prevented GABA\textsubscript{B}R from down-regulating KCC2 function and expression at the neuronal membrane. Together, these data support a model in which active GABA\textsubscript{B}Rs can modulate the surface stability of KCC2 via a mechanism that involves clathrin-mediated endocytosis and which impacts the transporter’s contribution to transmembrane chloride levels. It is worth noting that our data do not demonstrate a direct interaction between KCC2 and GABA\textsubscript{B}R; therefore, the potential for additional proteins to mediate the functional association in neurons should also be considered.

The fact that we observed a 20% reduction in surface KCC2 and a smaller GABA\textsubscript{B}R-mediated shift in \(E_{\text{GABA}}\) than was produced by furosemide is consistent with the idea that different pools of KCC2 exist, which differ in terms of their localization, protein associations, and/or stability in the membrane. For instance, recent work has revealed that a pool of KCC2 is not localized at GABAergic synapses but rather at glutamatergic postsynaptic structures, where it functionally associates with kainate receptors and has been implicated in regulating glutamatergic transmission (Gauvain et al., 2011; Mahadevan et al., 2014; Chevy et al., 2015). Our experiments did not distinguish between protein complexes located in different subcellular compartments, such as the soma or dendrites. Future experiments could therefore explore whether the KCC2-GABA\textsubscript{B}R association varies as a function of cellular location or the membrane lipid environment (Hartmann et al., 2009; Watanabe et al., 2009). It would also be interesting to examine the longer-term consequences of manipulating the KCC2-GABA\textsubscript{B}R association, where the use of transgenic mouse lines is likely to be informative (Schuler et al., 2001; Vigot et al., 2006).

In conclusion, GABA\textsubscript{B}Rs are able to associate in a protein complex with the potassium–chloride cotransporter KCC2. Activation of the GABA\textsubscript{B}R can result in a decrease in KCC2 function, which requires the clathrin-mediated endocytosis pathway, regulates the transporter protein at the cell surface, and alters the driving force for chloride-permeable GABA\textsubscript{B}Rs. These findings reveal a novel “crosstalk” between the GABA receptor systems,
which has important implications for the regulation of inhibitory synaptic transmission.

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