SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Immunostaining of K_{2P} channels in the EC. **A**, Immunoractivities detected by the second set of K_{2P} ABs (Santa Cruz Biotechnology Inc.). *Upper panels*, low magnification; *Low panels*, high magnification of the regions denoted in the upper panels. The catalog numbers of the ABs were labeled on the top. **B**, Preabsorption of the first set of K_{2P} ABs used in Figure 4A with their corresponding blocking peptides failed to detect the immunoreactivities of K_{2P} channels corroborating the specificity of these ABs. *Upper panels*, low magnification; *Low panels*, high magnification of the regions denoted in the upper panels.

Figure S2. Intracellular application of the second set of ABs to TASK-1, TASK-3, TWIK-1 and TREK-1 (40 μg/ml) did not alter baclofen-induced increases in outward holding currents in the stellate neurons of the EC. **A**, Anti-TASK-1 (89.4±4.9 pA, n=6, p=0.43 vs. control IgG). **B**, anti-TASK-3 (104.4±12.5 pA, n=6, p=0.20 vs. control IgG). **C**, anti-TWIK-1 (77.7±14.7 pA, n=6, p=0.52 vs. control IgG). **D**, anti-TREK-1 (95.4±8.5 pA, n=7, p=0.30 vs. control IgG).

Figure S3. The specificities of the K_{2P} ABs were further confirmed by immunocytochemistry and electrophysiology in HEK293 cells transfected with individual K_{2P} channels. **A**, Immunoreactivity to individual ABs was detected only in HEK293 cells transfected with the corresponding K_{2P} channels and there were no cross reactions among the ABs. The transfected cells on sterile glass cover slips were washed briefly with PBS, fixed in methanol at -10^{o}C for 5 minutes and then washed 3 times with PBS. Cells were incubated with normal donkey

blocking serum (5%) containing 1% Triton-X-100 followed by incubation with goat ABs to TASK-1, TASK-3, TWIK-1, TREK-1 or TREK-2 at a dilution of 1:200 at 4^{o}C overnight. After washing 3 times in PBS, cells were incubated with donkey anti-goat IgG-rhodamine (1:200; Santa Cruz Inc., sc-2094) at room temperature. Finally, the cover slips with the stained cells were mounted on slides, viewed and photographed under an Olympus Fluoview 300 laser-scanning confocal microscope. Each staining was repeated 3 times in 3 different transfections. Note that each AB only stained the cells transfected with the corresponding K_{2P} channels. Scale bare: 20 μm. **B-E**, Intracellular application of ABs (40 μg/ml) to K_{2P} channels inhibited the functions of the channels. HEK293 cells were transfected with TASK-1 (**B**), TASK-3 (**C**), TWIK-1 (**D**) and TREK-1 (**E**). Whole-cell recordings with pipettes containing corresponding ABs were performed on the transfected cells. Holding currents at -60 mV were recorded immediately after the formation of whole-cell configuration and actual holding currents were used to plot the figures. Intracellular dialysis of individual ABs to the HEK293 cells expressing the corresponding channels induced the development of an inward holding current (n=5-10 cells for each group, $p<0.001$, two-way ANOVA) compared with anti-TREK-2. Results for application of TREK-2 AB in HEK293 cells transfected with TREK-2 channels were shown in Figure 5D and 5F.

Figure S4. Baclofen-induced increases in outward HCs were sensitive to intracellular acidification, heat and arachidonic acid (AA). Intracellular acidification induced an outward HC (226.6±27.3 pA, n=6, ##$p<0.001$ vs. baseline=0) and significantly reduced the effect of baclofen (27.1±4.8 pA, n=6,

**p<0.01 vs. baclfoen alone). Elevation of the temperature in the recording chamber to 33^{o}C also induced an outward HC (85.2±15.8 pA, n=7, ##p<0.001 vs. baseline=0) and significantly inhibited the effect of baclofen (30.1±5.8 pA, n=7, **p<0.01 vs. baclfoen alone). Whereas TREK-2 channels are activated by AA, bath application of AA (10 μM) induced an inward HC (-25.1±6.7 pA, n=6, #p<0.05 vs. baseline=0) in the normal extracellular solution. Bath application of AA (10 μM) induced an outward HC (48.6±15.5 pA, n=9, #p<0.05 vs. baseline=0) when NaCl in the extracellular solution was replaced by the same concentration of NMDG-Cl and Ca^{2+} in the extracellular solution was omitted. Under this condition (AA+NMDG+0 Ca^{2+}), application of baclofen induced a smaller outward HC (58.0±9.4 pA, n=9, p=0.04) compared with baclofen-induced outward HC (108.8±22.7 pA, n=6) when the extracellular solution contained only NMDG and 0 Ca^{2+} without AA.

Figure S5. $GABA_BR$-mediated depression of spatial learning is not due to non-spatial learning factors. **A**, Swimming speed of the rats was not significantly different among the groups of rats microinjected with normal saline (control), baclofen, Rp-cAMPS, CGP55845 or CGP55845 followed by injection of baclofen ($F_{(4,33)}$=0.154, p=0.96) suggesting that these treatments did not alter the motor functions of the rats. **B**, Microinjection of normal saline, baclofen or Rp-cAMPS did not influence the latencies to find the platform in a visible-platform water maze test ($F_{(2,18)}$=1.725, p=0.206, among Control, Baclofen and Rp-cAMPS groups). **C_1**, Rats were randomly divided into 3 groups and conducted acquisition trials in the hidden-platform test for two days without microinjection. The rats

demonstrated rapid spatial learning as shown by significant decreases in escape latency for each group (Group 1: $F_{(11,66)}$=6.21, p<0.001; Group 2: $F_{(11,66)}$=8.47, p<0.001; Group 3: $F_{(11,66)}$=5.69, p<0.001). There were no significant differences for the acquisition trials among these 3 groups ($F_{(2,18)}$=0.82, p=0.46). **C_2**, On day 3, the 3 groups of rats in **C_1** were microinjected with normal saline (0.9% NaCl), baclofen and Rp-cAMPS, respectively and a probe trial with the platform unavailable was conducted for each group of rats 15 min after microinjection. There were no significant differences for the time spent in the target quadrant ($F_{(2,18)}$=0.27, p=0.77) among the three groups (saline: 39.2±3.7%, n=7, p=0.008; baclofen: 38.6±1.7%, n=7, p=0.0002; Rp-cAMPS: 36.8±1.7%, n=7, p=0.0005, vs. 25% chance level). **D**, siRNA treatment did not change the swimming speed of the rats ($F_{(3,27)}$=0.069, p=0.98) suggesting that siRNA treatment did not influence the motor function of the rats.

Figure S6. Injection of siRNA inhibited the expression of TREK-2 channels in a region of ~400-500 μm around the injection site. *Left panels*, low magnification; *Right panels*, high magnification of the region selected in the left panels showing the immunoreactivity of TREK-2 channels in Scr-siRNA-treated (*upper*) and siRNA-treated (*lower*) rats.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Slice preparation

Horizontal brain slices (400 μm) including the EC, subiculum and hippocampus were cut using a vibrating blade microtome (VT1000S; Leica, Wetzlar, Germany) from 15- to 20-day-old Sprague Dawley rats. After being deeply anesthetized

with isoflurane, rats were decapitated and their brains were dissected out in ice-cold saline solution that contained (in mM) 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 5.0 $MgCl_2$, and 10 glucose, saturated with 95% O_2 and 5% CO_2, pH 7.4. Slices were initially incubated in the above solution at 35^oC for 40 min for recovery and then kept at room temperature (~23^oC) until use. All animal procedures conformed to the guidelines approved by the University of North Dakota Animal Care and Use Committee.

Whole-cell recordings from stellate neurons in layer II of the EC

Whole-cell patch-clamp recordings using an Axopatch 200B or two Multiclamp 700B amplifiers (Molecular Devices, Sunnyvale, CA) in current- or voltage-clamp mode were made from stellate neurons in layer II of the EC visually identified with infrared video microscopy (Olympus BX51WI) and differential interference contrast optics. Unless stated otherwise, all the recordings were conducted at room temperature (~23^oC). The recording electrodes were filled with (in mM) 130 K^+-gluconate, 0.5 EGTA, 2 $MgCl_2$, 5 NaCl, 2 ATP_2Na, 0.4 GTPNa and 10 HEPES, pH 7.4. The extracellular solution comprised (in mM) 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgCl_2$ and 10 glucose, saturated with 95% O_2 and 5% CO_2, pH 7.4. Data were filtered at 2 kHz, digitized at 10 kHz, acquired on-line and analyzed after-line using pCLAMP 9 software (Molecular Devices).

AP firing was recorded from stellate neurons in layer II of the EC. Usually, for most of the cells a positive current injection was needed to bring the RMP to ~ -50 mV to induce AP firing. Baclofen was applied after the AP firing had been stable for 5-10 min. Frequency of APs was calculated by Mini Analysis 6.0.1

(Synaptosoft Inc., Decatur, GA). HCs at -60 mV were recorded in the extracellular solution containing TTX (0.5 μM) and then averaged per minute. We subtracted the average of the HCs recorded for the last minute prior to the application of baclofen from those recorded at different time points to zero the basal level of HCs for better comparison unless stated otherwise. Voltage-current curves were constructed by using the external solution supplemented with (in μM) 0.5 TTX, 100 $CdCl_2$, 10 DNQX, 100 *dl*-APV and 10 bicuculline. Voltage-current relationship was obtained by using a ramp protocol from -140 mV to 0 mV at a speed of 0.1 mV/ms. We compared the voltage-current curves recorded before and during the application of baclofen for ~5 min.

Expression of $GABA_B$Rs and K_{2P} channels in HEK293 cells and electrophysiological recordings from the transfected cells

The N-terminus c-myc- and HA-tagged GB1a and GB2 $GABA_B$ receptor subunit coding sequences described previously (Pagano et al., 2001) subcloned into the pRK5 expression vector were obtained from Laurent Prézeu (CNRS, Montpellier, France). cDNA constructs coding for TREK-2 (NM_021161, subcloned into the pCMV6-XL4 vector), TREK-1 (NM_014217, subcloned into the pCMV6-entry vector) or TWIK-1 (NM_002245, subcloned into pCMV6-XL5 vector) were purchased from Origene (Rockville, MD). cDNA constructs coding for TASK-1 (AF_031384) and TASK-3 (AF_391084) subcloned into pcDNA3.1 were generous gifts from Dr. Douglas A. Bayliss (University of Virginia, Charlottesville, VA). In addition, a previously characterized PKA-insensitive TREK-2 mutant (S359A) subcloned into pcDNA3.1 was a generous gift from Dr. Donghee Kim

(Rosalind Franklin University of Medicine and Science, Chicago, IL). An empty pEGFP N-3 green fluorescent protein (GFP) fusion protein vector (GenBank accession number U57609) was purchased from Clontech (Palo Alto, CA). Detailed methods for transfection of HEK293 cells and electrophysiological recordings from the transfected cells were described previously (Deng et al., 2007).

Immunocytochemistry

The methods for immunocytochemistry were described previously (Deng and Lei, 2008). Briefly, horizontal sections (20 μm) from rat brain were washed in 0.1 M PBS and then treated with 0.3% hydrogen peroxide (H_2O_2) for 10 min to quench endogenous peroxidase activity. After being rinsed in 0.1 M PBS containing 1% Triton X-100 and 5% normal donkey serum for 30 min, sections were incubated with the primary goat ABs to TASK-1, TASK-3, TWIK-1, TREK-1 and TREK-2 (Santa Cruz Biotechnology Inc.) at a dilution of 1:200 at 4°C for 12 h. Sections were incubated at room temperature initially with biotinylated donkey anti-goat IgG (Immunocruz^{TM} Staining System, Santa Cruz Biotechnology Inc.) for 1 h, and then with HRP-streptavidin complex (Immunocruz^{TM} Staining System) for 1 h. After each incubation, sections were washed three times for a total of 30 min. Diaminobenzidine (Immunocruz^{TM} Staining System) was used for a color reaction to detect the positive signals. We strictly controlled the incubation time to obtain comparable results. Finally, sections were mounted on slides, dehydrated through an alcohol range, cleared in xylene and covered with cover-slips. We stained ~15 nonadjacent sections for each rat and each staining was repeated by

using 6 rats. For the siRNA experiments, we stained sections from 2 Scr-siRNA-treated (control) rats and 2 siRNA-treated rats each time and repeated three times. The images were analyzed with Image-Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD). Briefly, we set the range of optical densities and area (size of cells) to define the countable positive cells. Only neurons with optical densities five times more than background were considered positive. For the cell size, we set the range (area) from 70 to 130 μm^2 according to the size of stellate cells in our setting. Under this condition, the software counted the cell number automatically. Each detected cell was then inspected visually to exclude obvious artifacts.

Water maze task

All surgical procedures were performed under aseptic conditions. After being anesthetized with pentobarbital sodium (40 mg/kg), rats were placed on a stereotaxic frame, and implanted bilaterally with stainless steel guide cannulae (23 gauge, Plastics One, Roanoke, VA) in the entorhinal cortices. The cannulae were cemented into place with dental acrylic. The coordinates for the implantation were based on the brain atlas of Paxinos and Watson (Paxinos and Watson, 1986) and our preliminary experiments; anteroposterior (AP) -6.5 mm, mediolateral (ML) $\pm$4.5 mm and dorsoventral (DV) -5.6 mm. Cannulation sites were confirmed at the end of each experiment by slicing rat brain, Nissl staining with cresyl violet (0.1%) and viewing under a microscope (Figure 6D). Data from the rats with incorrect injection sites were excluded from analysis. For TREK-2 siRNA or scrambled siRNA treated rats, Osmotic Pumps Cannulae/Guide

Combination (23 gauge, Plastics One) were used to combine acute injections (from the vertical guide tubes) with administration of siRNA or the scrambled siRNA via osmotic pumps connected to the side tubes of the cannulae which feed into the vertical guide tubes. Osmotic pumps (Alzet model 2001, Durect, Cupertino, CA) were filled with TREK-2 siRNA or scrambled siRNA and implanted subcutaneously on the back of rats for infusion at a rate of 1 µl per hour for 1 week. The pRNAT-U6.1/Neo siRNA expression vector (GenScript, Piscataway, NJ) was used. The siRNA target sequences inserted into the BamHI/HindIII multi-cloning site of this vector included 5'-CGGAATTACTCTCTGGATGAA-3' corresponding to positions 1794-1814 of the rat TREK-2 mRNA sequence (NM_023096) and 5'-ACCGTAATGGAGCTGTACATC-3' as the scrambled siRNA sequence generated using proprietary algorithm (GenScript, Piscataway, NJ). We used the JetSI^{TM}/DOPE cationic lipid based technique (Guissouma et al., 2006; Akhtar and Benter, 2007; Hassani et al., 2007) for *in vivo* delivery of siRNA sequences. Briefly, 10 mM JetSI™ (a commercially available mix of cationic lipids from Polyplus Transfection, Illkirch, France) in alcoholic suspension was mixed with 20 mM DOPE (dioleoylphosphatidylethanolamine, neutral lipid) dissolved in 80% ethanol and 20% chloroform at a ratio of 1:1. Aliquots of this mixture were kept at −20 °C. Each time, 1 ml of the injection mixture was prepared: 90 µl JetSI^{TM}/DOPE lipids, 100 µl siRNA (20 µM), 200 µl glucose (25%) and 610 µl H_2O. Each osmotic pump was filled with 200 µl of this mixture solution.

Morris Water Maze test were conducted after 1 week of postsurgical recovery. For the hidden-platform test, rats were trained to locate a hidden platform (10 cm in diameter, submerged 1 cm) in a water maze (1.2 m in diameter) as described previously (Xu et al., 2004). The water maze was filled with opaque water at a temperature of 20±0.5°C. Video tracking was performed using a computerized water maze system (2020 Plus Tracking System), allowing measurement of a number of parameters, including latency to find the platform, swimming distance, swimming speed, thigmotaxic behavior and quadrant analyses. Rats received 2 days of acquisition trials, each day comprising 6 consecutive trials, during which the platform was in a fixed position of a quadrant. On each trial, the rat was gently put in the water maze facing the wall with semi-randomized starting positions, released and allowed to search for the platform for 90 s. If the rat failed to find the platform within this time, it was guided to the platform by the experimenter. The rat was allowed to stay on the platform for 30 s before next trial. The inter-trial time was approximately 45 s. After completion of all 6 trials, the rats were dried off with towels. A probe trial with the platform unavailable for 90 s was conducted on day 3. The rats were released from the pool side opposite to the target (platform) quadrant. Time spent in the target quadrant was compared with time spent in other quadrants. The chance level spent in each quadrant is 25%. Rats received drug infusion 15 min prior to the acquisition trials on days 1 and 2 but no drug treatment on day 3 (probe trial) unless stated otherwise. All animals received 1 μl of one or two specific drugs via the 27 gauge

internal cannulae that protruded approximately 0.5 mm from the guide cannulae. Infusions (0.5 μl/min) were made with a microinfusion pump (WPI, SP230IW).

For the visible-platform test, the escape platform was protruded to 1.5 cm above water surface and the edge of the platform above water surface was covered with a black water-proof tape. The visible platform was placed in distinct positions for each trial of the 6 trials of each day for 2 days. The animal was placed into the tank facing the wall at a start point opposite to the platform from trial to trail. The same amount of saline, baclofen and Rp-cAMPS was microinjected each day before the visible-platform test.

Western blot

Tissues for Western blot were taken from 3 Scr-siRNA- and 3 siRNA-treated rats. Horizontal brain slices were cut initially and the medial EC region around the injection site was punched out from the slices under a microscope. The isolated brain region was lysed in tissue protein extraction buffer containing protease inhibitors (Pierce, Rockford, IL). The lysates were centrifuged at 10,000 g for 10 min to remove insoluble materials and protein concentrations in the supernatant were determined (Bradford, 1976). An equivalent of 20 μg total protein was loaded to each lane. Proteins were separated by 12 % SDS–PAGE and transferred to the polyvinylidene difluoride (PVDF, Immobilon-P, Millipore, Billerica, MA) membranes using an electrophoretic transfer system (BioRad, Hercules, CA). Blots were blocked with 5% powdered milk, and then incubated with TREK-2 (1:500, sc-11559, Santa Cruz Biotechnology Inc.) primary antibody overnight at 4 °C followed by incubation with the secondary antibody (donkey

anti-goat IgG-HRP, 1:2000) for 1 h at room temperature. Tris-buffered saline with 1% Tween-20 was used to wash the blots 3 times (10 min each) after incubation with both primary and secondary antibodies. Immunoreactive bands were visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and detected by a Biospectrum Imagining System (UVP, Upland, CA). Optical density of protein bands were quantified using ImageJ 1.41o (National Institutes of Health, USA).

Chemicals

RpcAMPs, tertiapin, CGP 55845, pertussis toxin and guanosine-5'-O-(2-thiodiphosphate) (GDP-β-S) were from BIOMOL (Plymouth Meeting, PA). Baclofen and MDL-12,330A were purchased from TOCRIS (Ellisville, MO). St-Ht31 inhibitory peptide and St-Ht31P control peptide were purchased from Promega (Madison, WI). Two sets of K_{2P} ABs purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) were used for immunohistological and electrophysiological experiments; set 1: TWIK-1 (sc-11483), TASK-1 (sc-32067), TASK-3 (sc-11322), TREK-1 (sc-11554) and TREK-2 (sc-11560); set 2: TWIK-1 (sc-11481), TASK-1 (sc-11309), TASK-3 (sc-11317), TREK-1 (sc-11556) and TREK-2 (sc-11559). A TREK-2 AB (APC-055) and its corresponding blocking peptide were purchased from Alomone labs (Jerusalem, Israel) and used for electrophysiological experiments. Other chemicals were products of Sigma-Aldrich (St. Louis, MO).

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