Title: Seizures and reduced lifespan in mice lacking the potassium channel subunit Kv1.2, but hypoexcitability and enlarged Kv1 currents in auditory neurons

Short title: Enlarged I_Kv1 in Kcna2-null MNTB

Authors: Helen M. Brew1,2, Josh X. Gittelman1,3, Robert S. Silverstein1,3, Timothy Hanks3, Vas P. Demas1, Linda C. Robinson1, Carol A. Robbins1, Jennifer McKee-Johnson1, Shing Yan Chiu5, Albee Messing6 and Bruce L. Tempel1,2,3,4


Corresponding authors: Helen M. Brew, V. M. Bloedel Hearing Research Center, Box 357923, University of Washington, Seattle, WA 98195-7923, U.S.A. Phone: 206-616-4694. Fax: 206-616-1828. e-mail address: hbrew@u.washington.edu B. L. Tempel, same address, phone: 206-616-4693, bltempel@u.washington.edu

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Abstract

Genes Kcna1 and Kcna2 code for the voltage-dependent potassium channel subunits Kv1.1 and Kv1.2 which are co-expressed in large axons and commonly present within the same tetramers. Both contribute to the low-voltage-activated potassium current I_Kv1 which powerfully limits excitability and facilitates temporally precise transmission of information, e.g. in auditory neurons of the medial nucleus of the trapezoid body (MNTB).
Kcnal-null mice lacking Kv1.1 exhibited seizure-susceptibility, and hyperexcitability in axons and MNTB neurons, which also had reduced I_{Kv1}. To explore whether a lack of Kv1.2 would cause a similar phenotype, we created and characterized Kcna2-null mice (-/-). The -/- mice exhibited increased seizure-susceptibility compared to their +/+ and +/- littermates, as early as P14. The mRNA for Kv1.1 and Kv1.2 increased strongly in +/+ brainstems between P7 and P14, suggesting these subunits’ increasing importance for limiting excitability. Surprisingly, MNTB neurons in brainstem slices from -/- and +/- mice were hypoexcitable despite their Kcna2 deficit, and voltage-clamped -/- MNTB neurons had enlarged I_{Kv1}. This contrasts strikingly with the Kcnal-null MNTB phenotype. Toxin block experiments on MNTB neurons suggested Kv1.2 was present in every +/+ Kv1 channel, approximately 60% of +/- Kv1 channels and no -/- Kv1 channels. Kv1 channels lacking Kv1.2 activated at abnormally negative potentials, which may explain why MNTB neurons with larger proportions of such channels had larger I_{Kv1}. If channel voltage-dependence is determined by how many Kv1.2 subunits each contains, neurons might be able to fine-tune their excitability by adjusting the Kv1.1:Kv1.2 balance rather than altering Kv1 channel density.

Introduction

Neuronal information processing is shaped by voltage-dependent potassium (Kv) channel tetramers, whose properties depend partly on which four subunits they contain. The subfamily of mouse genes Kcnal-Kcna8 codes eight Kv1 subunit types, Kv1.1-Kv1.8, potentially leading to high functional diversity in Kv1 channels (Lock et al., 1994). Alternatively, if there is functional redundancy, it may occur between subunit types Kv1.1 and Kv1.2 because they are coded by the two most closely related Kcna genes, are present at many of the same CNS locations, and either type forms channels with rapid activation and slow inactivation when expressed in oocytes (Stuhmer et al., 1989; Wang et al., 1993; Hopkins et al., 1994; Lock et al., 1994; Wang et al., 1994). Kv1.1 and Kv1.2 are also the two most abundant Kv1 subunit types and are commonly in the same tetramers; Kv1.1:Kv1.2 and Kv1.1:Kv1.4 are common combinations detected by coimmunoprecipitation from mammalian brains along with several different combinations of Kv1.1, Kv1.2, Kv1.3, Kv1.4 and Kv1.6 (Wang et al., 1993;
Scott et al., 1994; Rhodes et al., 1997; Shamotienko et al., 1997; Coleman et al., 1999; Wang et al., 1999). This makes it hard to discern whether Kv1.1 and Kv1.2 possess distinct functional roles.

One approach to dissecting out separate functions for individual Kv1 subunit types is genetic deletion. For example, evidence for an axonal role of Kv1.1 was provided by Kcna1-null mice in which several axonal and axon terminal sites exhibited hyperexcitability, thought to underly their seizure-susceptibility, cold-swim-induced myoclonus and hyperalgesia (Clark and Tempel, 1998; Smart et al., 1998; Zhou et al., 1998; Zhang et al., 1999). Such evidence is difficult to obtain in direct recordings from the sites where Kv1.1 and Kv1.2 are likely most strongly expressed, at the juxtaparanodes of large diameter axons, because they are beneath myelin which also impedes toxin access and makes their physiological role controversial (Wang et al., 1993; David et al., 1995). In the present study we created Kcna2-null (-/-) mice lacking Kv1.2 and compared them with control +/- and +/+ littermates, as well as previous results from Kcna1-nulls. The -/- mice had reduced lifespans and exhibited spontaneous generalized seizures. To explore developmental processes pertaining to the P15 onset of -/- seizures, we performed quantitative PCR (qPCR) on +/+ brainstems which showed Kcna2 mRNA was strongly upregulated from P7 on.

Kv1 currents have been characterized at some neuronal somata using direct recordings and toxins which block channels containing at least one sensitive subunit type. For example, channels containing at least one Kv1.2 subunit are blocked by tityustoxin-Kα (TsTx) a component of Tityus serrulatus scorpion venom (Werkman et al., 1993; Hopkins, 1998) and dendrotoxin-I (DTX) from black mamba snake venom (Strydom, 1976) blocks channels containing at least one of the DTX-binding subunits Kv1.1, Kv1.2 and Kv1.6 (Robertson et al., 1996). This probably means DTX blocks all Kv1 channels native to neurons, especially in auditory neurons of the medial nucleus of the trapezoid body (MNTB) where Kv1.1, Kv1.2 and Kv1.6 are the predominant Kv1 subunit types (Fonseca et al., 1998; Dodson et al., 2002). Applying DTX to murine MNTB neurons and other auditory neurons strongly expressing Kv1.1 and Kv1.2 has shown that Kv1 channels underly a rapidly activating low-voltage-activated potassium current I_{kl} (Wang et al., 1994; Grigg et al., 2000; Bal and Oertel, 2001; Adamson et al., 2002; Brew et al., 2003). Proposed functions of auditory I_{kl} are to: powerfully repolarize large synchronized EPSPs; reduce membrane time constants; minimize temporal summation;
facilitate temporally precise transmission across synapses; preserve phase-locking to sound peaks (Oertel, 1983; Manis and Marx, 1991; Brew and Forsythe, 1995; Trussell, 1999).

The homogeneity of the MNTB (90% principal neurons) makes it a prime location for combining the above approaches: direct recordings, toxin applications, and genetic deletion. For example, MNTB neurons in brainstem slices from Kcna1-null mice had 30% reduced I_kl amplitudes and were hyperexcitable (they fired more action potentials and had smaller threshold currents than controls, Brew et al., 2003). In analogous recordings described below, Kcna2-null MNTB neurons were markedly hypoexcitable and possessed enlarged I_kl, exactly opposite to the changes in Kcna1-null MNTB. To distinguish subcomponents of I_kl due to Kv1 channels with or without Kv1.2 subunits, TsTx and DTX were applied. One unexpected finding was that all Kv1 channels in +/+ mouse MNTB neurons contained Kv1.2, in marked contrast to rat MNTB neurons in which only a subset contained Kv1.2 (Dodson et al., 2002). An analysis of voltage-dependence suggests Kv1.2-free channels produce larger currents because they activate at more negative potentials than Kv1.2-containing channels, whose voltage-dependence may also depend on the number of Kv1.2 subunits they contain. Hence, neurons may adjust their balance between Kv1.2 and Kv1.1 expression to fine-tune their excitability for specialized information processing tasks. Some MNTB results were described in abstracts (Brew et al., 2000, 2001).

Methods:

Generation of Kcna2-null mice

A genomic phage clone containing the Kcna2 gene was isolated from a 129/Sv mouse liver library (Stratagene, La Jolla, CA). For the targeting construct, the neomycin resistance cassette (provided by Dr. R. Behringer, M.D. Anderson Cancer Center, University of Texas) was inserted between a Kcna2 5’ XbaI-EagI and 3’ NcoI-XbaI genomic fragment (see Figure 1A below). The thymidine kinase cassette (provided by Dr. R. Behringer) was cloned into the 3’ portion of the targeting construct. The targeting vector was linearized and electroporated into AB-1 embryonic stem (ES) cells (gift of Dr. A. Bradley, Wellcome Trust Sanger Institute, Cambridge,
U.K.). The ES cells were then subjected to positive-negative selection for 8 days in 300 µM G418 and 200 nM fialuridine. Doubly resistant clones were expanded and analyzed by Southern blot analysis.

Southern blot analysis with 5’ (XbaI-XbaI) and 3’ (XbaI-SacI) probes flanking the targeted region were used to confirm that a homologous recombination event had occurred in the genomic DNA. The EcoRV restriction endonuclease site in the Kcna2 open reading frame (ORF) was removed following homologous recombination allowing the 5’ and 3’ probes to detect fragments of increased size in the targeted allele (Figure 1B below shows sizes detected by the 5’ probe). Positive clones were expanded and injected into C57BL/6J blastocysts (University of Cincinnati Core Facility), and the chimeric mice generated from these blastocysts were bred to test for germline transmission of the mutant allele.

Breeding and genotyping

The mutation was transferred into C3HeB/FeJ mice (backcross generations N5-20) to create Kcna2-null mice (referred to here as -/- mice) and their wildtype (+/+ ) and heterozygous (+/-) littermates. A chimeric founder was also crossed to C57BL/6J mice, establishing a hybrid line and providing mutant mice referred to as -/- (B6/129) in this text. Breeding of the +/- mice took place in AAALAC-approved specific-pathogen-free facilities. Offspring were genotyped using tail clips taken from mice aged P6 or older and ear-punched for later identification on the day of experiments. When necessary, mice were euthanized by CO₂ exposure, followed by decapitation. All animal protocols were reviewed and approved by the University of Washington IACUC or the University of Wisconsin IACUC.

DNA was isolated from the tail and then PCR amplified using two primer sets (Table S1): one to match the ORF of Kcna2, indicating the presence of an intact Kcna2 gene; the other to match the neomycin resistance cassette, indicating a Kcna2-null chromosome. The size of the DNA fragments generated told us which mice in a litter were wildtypes, heterozygotes and Kcna2-nulls. For further details, see [http://depts.washington.edu/tempelab/Protocols/KCNA2.html](http://depts.washington.edu/tempelab/Protocols/KCNA2.html).
Western blot analysis of Kv1.2 protein expression.

Total protein was isolated from whole brains and enriched for membrane-associated proteins. Brains were homogenized in 320 mM sucrose with protease inhibitors and cellular debris was pelleted by centrifugation at 1000 x g. The supernatant was centrifuged at 120k x g for 1 hour and the resulting pellet resuspended in 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100 with protease inhibitors. Unsolubilized membrane proteins were removed by centrifugation at 120k x g for 1 hour and the supernatant saved. All steps were done on ice or at 4°C. Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL).

Protein samples from each animal (20 µg) were denatured at 50°C for 10 minutes in loading buffer containing 100 mM dithiothreitol (DTT) and separated by 8% SDS-PAGE. Protein was then electroblotted to a nitrocellulose membrane (BioRad, Hercules, CA). Membranes were blocked in PBS-T with 10% non-fat milk and probed with an anti-Kv1.2 monoclonal antibody (1:5000; Upstate, Waltham, MA). Anti-mouse HRP-conjugated secondary antibodies (1:1000) were used for detection with enhanced chemiluminescent reagents (Amersham Biosciences, Piscataway, NJ). Blots were exposed to film for the amount of time necessary to obtain a clear image. Blots were then re-probed with anti-β-actin monoclonal antibody (1:1000; Abcam, Cambridge, MA) and re-processed for chemiluminescence detection.

Quantitative real time PCR

qPCR techniques used here were similar to recently published procedures (Duncan et al., 2006; Silverstein and Tempel, 2006) including the normalization of Kcna expression levels to the geometric mean of the three most stable internal reference genes from panel of ten candidate reference genes, as described in (Vandesompele et al., 2002). To measure Kcna gene expression versus genotype, +/-, +/- and -/- mice were sacrificed at age P14, and the whole brain was homogenized in 6 ml of Trizol (Invitrogen, Carlsbad, CA) and purified. To measure changes in brainstem Kcna expression during development C3HeB/FeJ control mice were sacrificed at various ages P1 through P29. The brain was placed in RNALater (Qiagen) then the brainstem was dissected away and
frozen at -80ºC, for subsequent purification. Total RNA yield was measured spectrophotometrically and RNA integrity was verified by gel electrophoresis.

cDNA was reverse transcribed from 1 µg of total RNA using 50 pM of Random Hexamers (Amersham Biosciences) and 1 µL Powerscript Reverse Transcriptase (Clontech, BD Biosciences, Franklin Lakes, NJ) as detailed in (Duncan et al., 2006). For each transcript of interest, qPCR primer pairs were designed using Primer3 software (Rozen and Skaletsky, 2000)and following design criteria of: amplicon size 50-150 bp, primer T_m of 60-64ºC, primer %GC of 35-65%, and complementarity between and within primers minimized (see Table S1 for sequences).

qPCR using ~5 ng starting total RNA was measured using SybR Green Supermix (BioRad). Cycling parameters were as follows: 95ºC x 3m (activate enzyme), 40 repeats of 95ºC x 30s, 60ºC x 30s (amplification), 95ºC x 1m, 55ºC x 1m (pre-melt curve), 90 repeats of 10s each starting at 55ºC and incrementing 0.5ºC per step (melt curve). Real-time fluorescence was measured using the iCycler I/Q Module (BioRad). In each experiment, all samples were divided into two replicate reactions (same biological sample and reaction mixture), and the average threshold cycle number for the two replicates was used as a single data point. For each primer set melt curves were inspected and efficiencies determined as described in (Duncan et al., 2006).

*Kcna* expression levels were normalized to the geometric mean of multiple internal reference genes, as described (Vandesompele et al., 2002). For all of the samples of a given data set, the qPCR threshold cycles were determined for a panel of ten candidate reference genes, the three most stable reference genes were used, as ranked by geNORM software (http://medgen31.ugent.be/jvdesomp/genorm/). These were β-actin, γ-actin, and succinate dehydrogenase complex, subunit A for the cross genotype study (Figure 1D) and β-actin, γ-actin and hydroxymethylbilane synthase for the developmental study (Figure 3). The primer sequences used for the three *Kcna* genes and these four reference genes are shown in supplementary Table S1.

The expression levels for potassium channel genes *Kcna1, Kcna2* or *Kcna6* were normalized as kcnai/r where kcnai is the copy number for the relevant *Kcna* gene (i=1, 2 or 6) and r is the geometric mean of the copy numbers for the three reference genes. The copy number for each gene was its “base of amplification” value B.
raised to the power of its threshold cycle number. Final reported values of kcna/r were based on multiple independent qPCR runs from multiple animals (details in Figure 1 and 3 legends).

**Motor tests and seizure testing**

We tested gross motor behavior and susceptibility to flurothyl-evoked seizures in 28 mice aged P14 (3 litters). Mice were weighed before the motor tests, which included: (1) lowering the mouse towards a cage top, to see if they could grasp the rungs with their forelimbs, and testing the grip strength by pulling gently upwards on their tail; (2) letting the mice climb vertically upwards on cage rungs; and (3) letting them balance on a 1 cm diameter aluminium rod held horizontally about 10 cm above the cage floor.

Later on the same day, these mice were tested for seizure susceptibility. After placing a mouse into a plexiglass chamber of volume 10.7 liters, we applied the volatile convulsant flurothyl (Sigma-Aldrich, St Louis, MO) by dripping it in liquid form onto a filter paper at 20 µl/min. The chamber was cleaned and aerated between subjects. Two human observers noted the latencies from the first drip of flurothyl to the onset of various seizure-related behaviors, which included “flagpole” tail dorsiflexion (Straub tail), unilateral myoclonic jerks, tremors and bilateral forelimb clonus. Also noted was the latency to a fully generalized seizure, which comprised two to five seconds of running-bouncing seizure followed by full tonic extension. At this point, the mouse was rapidly removed into fresh air and given abdominal massage to restore breathing, before being placed in a cage for observation.

Because Kcnal-null mice display a dramatic tremor when forced to swim in cold water (Zhou et al., 1998) we performed similar swim tests on Kcna2-null mice (in this case the -/- (B6/129) mice). A tank, 18 cm wide by 29 cm long, was filled with water to a depth of 7 cm. Mice were placed in the middle of the tank to swim. The water temperature was 17°C. The swim time was 2 min. After swimming, the mice were placed on a dry platform at room temperature for observation of abnormal motor behavior.
MNTB electrophysiology: brainstem slice preparation and recording techniques

The preparation of brainstem slices containing MNTB neurons was carried out using previously published techniques (e.g. Brew et al., 2003). In brief, mice aged P9-P16 were euthanized by brief CO₂ exposure, followed by decapitation and brain dissection. A tail clip was collected for confirmation of prior genotyping (see above). Dissection and slicing took place in ice-cold sucrose-based solution containing in mM: 250 sucrose; 2.5 KCl; 26 NaHCO₃; 1.25 NaH₂PO₄; 2 CaCl₂; 1 MgCl₂; 10 glucose; 2 Na pyruvate; 0.5 Na ascorbate; 3 myo-inositol and whose pH was maintained at 7.4 by gassing with 95% O₂/5% CO₂ mixture. After separation of the forebrain by a transverse cut through the colliculi, the cut rostral surface of the brainstem was glued to the chamber of a vibrating slicer (Vibratome Series 1000, Technical Products International, St. Louis, MO). The brainstem was re-covered with ice-cold sucrose-based solution while 150 µm slices were cut, up to five of which contained MNTB. Slices were kept for one hour at 37°C in an incubation chamber filled with artificial cerebrospinal fluid (ACSF) and gassed with 95% O₂/5% CO₂ mixture. The ACSF was identical to the sucrose-based solution described above except that it contained 125 mM sodium chloride instead of the 250 mM sucrose. The incubation chamber was allowed to cool to room temperature and slices were maintained there for up to eight hours before use. Slices were then placed in a recording chamber, also perfused with gassed ACSF at room temperature (22 to 25°C), sited on a stable recording platform built around a microscope (on an X-Y stage) fitted with differential interference contrast optics (Axioskop or Axioskop FS2, Zeiss, www.zeiss.com).

Recording pipettes were borosilicate glass (Vertical Pipette Puller 700B, David Kopf Instruments, Tujunga, CA) and filled with a solution containing (in mM): K gluconate 97.5, KCl 32.5, EGTA 5, HEPES 10, MgCl₂ 1 (adjusted to pH 7.2 using approximately 14 mM KOH). For voltage-clamp recordings, pipettes were coated with Sylgard (Dow-Corning, Midland, MI) to reduce their capacitance. Pipettes were connected to the patch clamp amplifier (Axopatch 200, Axon Instruments, Foster City, CA) via the amplifier headstage mounted on a micromanipulator (Narishige, Japan or EXFO Burleigh, Victor, NY) bolted to the recording platform. Pipette resistance was 3-6 MΩ prior to gigaohm seal formation, measured from the responses to −5 mV voltage-clamp steps. Though recording locations were not measured, attempts were made to record from a variety of locations throughout the MNTB, to try to fully represent the properties of all MNTB neurons, and also minimize bias.
from any variations in Kv1-based potassium current amplitudes across the tonotopic axis (e.g. as reported in rat MNTB (Brew and Forsythe, 2005). The access resistance was monitored frequently throughout recordings, and typically rose to approximately 10 MΩ on achieving the whole cell recording configuration (range 5 to 18 MΩ). Recordings were discontinued if the pipette access resistance went above 20 MΩ. There was a -7 mV liquid junction potential that is included in all the membrane potential values given below.

Soon after the start of voltage-clamp recordings, the slice was perfused with ACSFV (a low-calcium version of the ACSF) containing 0.5 mM CaCl2, 2.5 mM MgCl2 and 0.5-1 µM tetrodotoxin to minimize sodium currents, calcium currents and synaptic activity. The pipette access resistance was compensated using the series resistance compensation circuitry of the patch clamp amplifier (“correction” dial at 85% and “prediction” dial at 85%).

Chemicals were from one source (Sigma-Aldrich) except sucrose and NaH2PO4 (J.T. Baker, Phillipsburg, NJ). Tetrodotoxin (TTX), dendrotoxin-I (DTX) and tityustoxin-Kα (TsTx) were from another source (Alomone Labs, Israel). 100 nM TsTx or 100 nM DTX plus 100nM TsTx were added to the ACSFV and applied by perfusion. Because DTX block is almost irreversible in slices, for recordings following previous toxin applications, we used a fresh slice, replaced the perfusion tubing and reservoirs, and either replaced the recording chamber or washed it with dilute HCl for 60 minutes.

MNTB electrophysiology: stimulus generation and data acquisition

The software for stimulus generation and data acquisition (either Synapse, Synergy, Bethesda, MD or Axograph, Axon Instruments, Foster City, CA) ran on a Macintosh computer (7100/80AV or G3) and sent command sequences to the patch clamp amplifier via an AD/DA interface (ITC-16, Instrutech Corporation, Inc., NY). This interface also digitized signals on two channels from the patch clamp amplifier so that they could be digitally recorded by the software. During current clamp experiments, the two channels recorded were the current clamp pulse amplitude and the resulting pipette potentials, each recorded at a digitization rate of 10 kHz (amplifier filtering 5 kHz). During voltage clamp experiments, the two channels recorded were the voltage clamp pulse
amplitude and resulting pipette currents, each recorded at a digitization rate of 5 kHz (amplifier filtering 2 kHz).

Current clamp pulses were applied at 1 s intervals, were of 180 ms duration, and increased in 10 pA increments from 0 to 200 pA, or from -100 pA to 200 pA. This is the same protocol as used previously for the study of MNTB neurons in \textit{Kcnal}-null mice, to allow straightforward comparisons between the datasets (e.g. Table S2 below and Table 1 of Brew et al., 2003).

To measure current-voltage (I-V) relations in voltage-clamped MNTB neurons, a standard I-V protocol was used (also used previously for the \textit{Kcnal}-null MNTB study (Brew et al., 2003). Voltage-clamp command test pulses were applied at approximately 1.3 s intervals, were of 180 ms duration, and increased in 10 mV increments from -40 mV to +90 mV. These test pulses were applied over a continual background potential of -60 mV set using the amplifier, and the -7 mV liquid junction potential. This -67 mV holding potential was necessary to inactivate the large A-currents present in murine MNTB neurons (see Figure 7A and supplementary Methods). The resulting patch pipette potentials were -107 mV to +23 mV. Each pulse sequence included an initial 150 ms pulse to -107 mV (intended to remove any potassium channel inactivation that had occurred during previous test pulses), an 870 ms prepotential of -67 mV (to inactivate A-type potassium currents), the 180 ms test pulse, a 50 ms post-potential at -37 mV and a subsequent step back to -67 mV for 50 ms. The 245 ms section recorded included the final 15 ms of the prepotential, the 180 ms test pulse, and the 50 ms post-potential.

Two alternative protocols differed from the standard protocol described above only during the 180 ms test pulses section. One was a “high-voltage-resolution” I-V protocol, designed to increase the accuracy of the measurements of voltage-dependence, by using smaller test pulse increments of 5 mV within a smaller range of test potentials, -107 mV to -22 mV (this protocol and the standard I-V protocol were both used for the analyses of voltage-dependence in Figure 12). The other was a “toxin-monitoring” protocol which did not contribute to any data analyses. It consisted of repeated test pulses to -47 mV to facilitate visualization of the rapid decreases in current amplitudes caused by toxin perfusion (as shown in Figure 9A). The frequent monitoring allowed experimenters to judge when current amplitudes had become stable (typically 1-3 minutes after the start of recordings or a switch of perfusion solutions) which prompted the collection of I-V data (by performing several runs of the I-V protocols).
**MNTB electrophysiology: data analysis**

Current clamp data and voltage clamp data from MNTB neurons were analyzed, fitted and graphed using macros within the acquisition software and additional software (Kaleidagraph, Synergy Software, PA).

Current clamp data for each neuron were measured from a single run of the current pulse protocol before any averaging across runs or between neurons was performed. All membrane potentials presented include the addition of the -7 mV junction potential and a voltage correction for current flow across the pipette tip access resistance, both carried out offline. The sustained potential response to a particular current pulse amplitude was the average potential during a 10 ms time window towards the end of the pulse (100 datapoints, 160 ms to 170 ms after current pulse initiation). The resting potential was taken to be the sustained potential response to the 0 pA current pulse. Sustained potential responses were also used for calculations of input resistance (in Table S2).

In voltage clamped neurons, the sustained outward current amplitudes, I, were measured from each test potential of the standard I-V protocol or the “high-voltage-resolution” I-V protocol as follows. First the absolute current amplitude was measured at the end of each 180 ms test pulse, as the average of the 50 datapoints between 160 ms and 170 ms after test pulse initiation. The current amplitudes at membrane potentials between -97 mV and -77 mV represented background “leak current” and were used to calculate the linearly extrapolated current at other test potentials, which was then subtracted from the absolute currents to yield the sustained outward current amplitudes, I. For each neuron and in each perfusion solution, these values of I were also averaged across 2-3 runs of the respective I-V protocol. To obtain a measure of that neuron’s current through Kv1 channels, the sustained $I_{Kv1}$, these average I values in the presence of both TsTx and DTX were subtracted from the I values measured in ACSFV alone.

The membrane potentials referred to in the voltage-clamp results and figures below are the pipette potentials with a -7 mV correction for the liquid junction potential, but do not include any voltage correction for current flow across the uncompensated 15% of the pipette tip access resistance (range 1-3 MΩ). This omission facilitated the averaging of data collected at the same pipette test potentials, and is justified because
the estimated corrections were all small in the potential range relevant to Kv1 channels (e.g. at –37 mV, the mean amplitude of the sustained current was +760 pA prior to leak subtraction, resulting in an average correction of -1.5 mV).

To fit the apparent activation timecourse of an MNTB neuron’s I_{Kv1}, the current records were averaged from 2-3 runs of the standard I-V protocol in ACSFV and 2-3 runs in the presence of both TsTx and DTX and the latter were subtracted from the former (which removed capacitative current transients). The resulting currents were well fitted by curves increasing with a single exponential timecourse at test potentials –47 mV and –57 mV. Fits were performed on a time period of 20 ms (i.e. 100 datapoints) beginning soon after the start of the test pulse (1.2 ms through 21.1 ms). Because data were available from a larger number of neurons in ACSFV alone, the apparent activation timecourse was also fitted from each neuron’s averaged I-V responses in ACSFV, which required a non-standard method of subtracting the leak and capacitative transient currents, as follows. First, the record containing the –77 mV pulse was subtracted from that containing the –87 mV pulse, which yielded the estimated passive “leak plus capacitance” current response to a single –10 mV pulse (the –67 mV pulse could not be used because some sustained outward current including I_{Kv1} was usually present). Next, this passive “leak plus capacitance” estimate was multiplied by the relevant n before adding it to the current responses to test pulses differing by n increments of 10 mV from the –67 mV holding potential. Finally the current fits were performed (as described above for I_{Kv1}).

**MNTB electrophysiology: data sets and data exclusion criteria**

The current clamp data are from a total of 68 MNTB neurons and 27 mice (26 from 15 -/- mice, 23 from 12 +/- mice, and 19 from 10 +/- mice). The voltage clamp data are from another 29 MNTB neurons and 21 mice (11 from 8 +/- mice, 8 from 7 +/- mice, and 10 from 6 -/- mice). The recordings from neurons of each genotype had similar mean pipette access resistances and capacitances, as read off the amplifier dials, and were from mice of similar ages (see Table S2).

Current clamped neurons were excluded if the resting potential was smaller than –57 mV (including the –7 mV liquid junction potential). Voltage clamped neurons were excluded if a holding current larger than –100
pA was required to clamp at the background holding potential of –67 mV. We made exceptions to this criterion for two voltage-clamped neurons to which we subsequently successfully applied both toxins (one +/+ neuron and one –/- neuron requiring –160 pA and –190 pA, respectively). On average, the holding current was near-zero for the remaining +/+ and –/- voltage-clamped neurons (+/+, –2 ± 24 pA, n=10; –/-, –5 ± 18 pA, n=9) and slightly larger for +/- neurons (–50 ± 11 pA, n=8).

**MNTB electrophysiology: fitting of conductance data**

The conductance, \( G \), and its toxin-sensitive component \( G_{Kv1} \) were calculated at each test potential, \( V \), by dividing sustained current amplitudes (\( I \) or \( I_{Kv1} \)) by the driving force (\( V \) minus -80 mV, the approximate reversal potential for DTX-sensitive channels, Stansfeld and Feltz, 1988; Brew and Forsythe, 1995). The calculated \( G \) values were plotted versus \( V \) (-107 mV through -37 mV) and fitted with a single Boltzmann function

\[
G = G_{\text{max}}/(1+\exp((V-V_{\text{half}})/-k))
\]

with variable parameters \( G_{\text{max}} \), the maximum conductance, \( V_{\text{half}} \), the half-activation voltage and \( k \), the slope factor. The fitting used a least-squares curve-fitting method (the Pearson’s R general curve fit within Kaleidagraphe software, which finds the minimum value of \( \chi^2 \), the sum of the squared residuals, using partial derivatives according to the Levenburg-Marquardt algorithm, described in Press et al., 1992). In Figure 12, 56 fits and data are shown normalized to their fitted \( G_{\text{max}} \) values, to facilitate visual comparisons, but the description of goodness-of-fit below is based on the fits before normalization.

The 56 fits shown in Figure 12 appeared good to the naked eye, had small mean errors returned with each fitted parameter (\( G_{\text{max}}, 0.9 \) nS; \( V_{\text{half}}, 1.6 \) mV; and \( k, 0.8 \) mV) and values of \( R^2 \) larger than 0.93 (53 fits had \( R^2 \) larger than 0.98). Of these, 46 fits were judged “very good” because the returned errors were smaller than 2 units for all three parameters (shown by solid lines in Figure 12). Fits were judged too poor to be included if one or more parameters were returned with estimated errors larger than 10 units, but in most cases the same neuron’s current responses from an alternative protocol returned a good fit. Hence, only one neuron was excluded from a plot because of a poor fit (a –/- neuron excluded from Figure 12A). If both protocols returned very good fits, those from the high-voltage-resolution protocol were preferred for inclusion in Figure 12 and statistical comparisons. Also shown are 10 “less-good” fits, for which at least one parameter was returned with
an error estimate larger than 2 units but smaller than 10 units (dashed lines in Figure 12). The majority of these “less-good” fits came from +/+ neurons (7 out of 10). This was probably partly because the conductance was not near its maximum value at -37 mV in +/+ neurons, whereas at that potential the +/- and -/- conductances had reached or closely approached their maxima (Figure 12A, also see Results). The results and discussion therefore focus on the +/- and -/- results, and only tentative conclusions are made from the +/+ data.

Statistical evaluation

Tests were unpaired two-tailed Student’s t-tests assuming equal variance in each sample, except as noted for paired comparisons or comparisons involving markedly non-normal distributions, for which we used Mann-Whitney U-tests. All tests were performed using StatView (SAS Institute Inc., Cary, NC) or Kaleidagraph. All averaged values are expressed in text and figures as mean ± the standard error of the mean.

Results:

Verification of Kcna2 knockout and Kcna expression in +/+, +/- and -/- brains

Our targeting strategy successfully knocked out the entire open reading frame of the Kcna2 gene (Figure 1A,B). The lack of Kv1.2 protein in the brains of Kcna2-null (-/-) mice was confirmed by Western blot (Figure 1C). By qPCR there was also no Kcna2 mRNA present in brains from -/- mice aged P14, and Kcna2 mRNA expression was approximately halved in +/- compared to +/+ littermates’ brains (Figure 1D). The Western blot is suggestive of a reduced amount of Kv1.2 protein in +/- brain relative to +/+ brain (Figure 1C). These results verify that both Kcna2 mRNA and Kv1.2 protein are absent in the -/- mouse brain.

Figure 1 near here
To test whether other *Kcna* genes had altered expression as a result of the absence of *Kcna2* in -/- mice, the qPCR experiments also measured *Kcna1* and *Kcna6* mRNA expression, which were found to be very similar between +/+, +/- and -/- brains (Figure 1D). These two genes code for Kv1.1 and Kv1.6 subunits, which are known to be expressed along with Kv1.2 in both rat and mouse MNTB neurons (Fonseca et al., 1998; Dodson et al., 2002; Brew et al., 2003). Because the expression of each *Kcna* gene was normalized to the same three reference genes, these data suggest that *Kcna1* mRNA may be expressed at a higher level than either *Kcna2* or *Kcna6* in +/- brain. The brains of P14 -/- mice did not exhibit any signs of compensatory changes or greater variability in mRNA expression for *Kcna1* or *Kcna6* because both their mean expression and variability were similar to those of +/- and +/+ brains (Figure 1D).

**Lifespan and gross motor behavior in *Kcna2*-null mice**

The -/- mutation did not cause embryonic lethality, judging by the Mendelian proportions of each genotype in 14 litters comprising 88 mice, none of which were subjected to any experimental testing (+/+, n=21, 23.8%; +/-, n=45, 51%; and -/-, n=22, 25%). However, the -/- mice had a severely reduced lifespan, averaging 17 ± 0.2 postnatal days (Figure 2A, range P16-P19). Their littermate +/+ and +/- mice had normal lifespans. In litters removed from the specific pathogen free facility for experimental testing, some -/- mice had even shorter lifespans (range P14-P19). The mean lifespan was also 17 ± 2 postnatal days in 8 -/- (B6/129) mice, though the range was much larger (one -/- (B6/129) mouse died at P6, the rest at P14-P25) perhaps because the mutation was in a genetic background with seizure resistant (B6) and relatively seizure sensitive (129) alleles sorting in the mixed background (Frankel et al., 2001). Nonetheless, the data suggest the null mutation was penetrant in more than one genetic background. Adult +/- mice were good breeders, suggesting that they flourish despite the substantially reduced expression of *Kcna2* mRNA and Kv1.2 protein demonstrated at P14 (Figure 1C,D).

Figure 2 near here
The -/- mice appeared physically normal during their first two weeks of life. At P14 the majority of -/- mice had their eyes open and exhibited age-appropriate motor behavior (walking, running, rearing, grooming, exploration). We tested gross motor behavior in three litters of 28 mice aged P14 (see Methods). All the mice passed motor tests 1-3, i.e. could grasp and climb vertically on cage rungs, and balance on the horizontal rod. The -/- mice weighed approximately 1 g less (7.5 ± 0.17 g, n=7) than their +/- littermates (8.9 ± 0.20 g, n=6, p<0.05) and their +/+ littermates (8.3 ± 0.19 g, n=15, p<0.05).

“Spontaneous” seizures in Kcna2-null mice

Many -/- mice were observed undergoing apparently spontaneous episodes which began with a sudden explosive onset of wild running and jumping, followed after 5-10 seconds by full tonic extension (TE). This sequence is highly reminiscent of a running-bouncing seizure (RBS) leading to TE which is a typical feature of a fully generalized seizure in rats and mice (Gale, 1992). When -/- mice were observed recovering from TE, they often exhibited myoclonic jerks and tremors, followed by 5-20 minutes of relative immobility before recovery of normal motor behavior, reminiscent of a post-ictal phase. The fatality rate of observed RBS/TE events was high, approximately 50%, and was probably due to the cessation of movement and breathing that accompanied TE. It is likely that all the -/- mice eventually experienced fatal RBS/TE, because all those that died unobserved were found with their limbs and bodies fully extended. Hence, the RBS/TE episodes in -/- mice are probably seizures with generalized onset, and the proximal cause of the reduced -/- lifespans was probably fatal apnea occurring during TE.

The exact frequencies, fatality rates and ages of occurrence of all RBS/TE events could not be recorded, but some conclusions can be drawn from the following quantitative estimates, as remembered by two human observers L.R. and H.B. who were with litters including -/- mice during up to 60 minutes per day of routine mouse care or experimental preparations. At least 50 RBS/TE episodes were observed in approximately 100 -/- mice aged P15-P19. This is more than would be expected if observed and unobserved RBS/TE events occurred at the same rate and had the same fatality rate (50%) in which case a typical -/- mouse would undergo only one or two RBS/TE events in its lifetime, and the expected number of events in 100 -/- mice would be 200, only 8 of
which should coincide with the daily hour of observation. Both observers reported that many RBS/TE events began when the observer moved the cage slightly, perhaps suggesting they were evoked by sounds or accelerations or stress. The fatality rate did not appear to increase with age because RBS/TE was fatal in the two youngest -/- mice ever observed undergoing RBS/TE, aged P14, and non-fatal RBS/TE events were observed in several -/- mice at ages P17 and P18, and in a -/- (B6/129) mouse aged P17 which survived but died later the same night. RBS/TE events were very rare and approximately 50% fatal during several days of quiet extended observation of 5 -/- mice in a cage with a foster mother, and were initiated from sleep or normal awake behavior without any provoking stimuli apparent to the human observer. Hence, RBS/TE events in -/- mice were probably infrequent (less than one per day between P15 and P19), 50% fatal, and some observed RBS/TE events may have been evoked by unknown stimuli.

Susceptibility to seizure induction of Kcna2-null mice

To test whether the -/- CNS was abnormally susceptible to evoked seizures, mice aged P14 were tested for their latencies to flurothyl-induced seizures (see Methods, same 7 -/- mice, 15 +/- mice and 6 +/+ mice as in the gross motor testing described above). At this age, it was likely that none of the -/- mice had yet experienced an RBS/TE generalized seizure event. All mice behaved normally for a few minutes after the first drip of flurothyl into the exposure chamber, then exhibited seizure-related behaviors (see Methods) eventually culminating in RBS followed by TE. The mean latency to occurrence of the first seizure-related behavior was shortest in -/- mice, intermediate in +/- mice and longest in +/+ mice (Figure 2B, left). The mean RBS latency for -/- mice was 40% shorter than in +/+ or +/- mice (Figure 2B, middle). The -/- mice progressed very rapidly from their first seizure-related behavior to RBS, taking an average of 23 seconds, whereas their +/+ and +/- littermates displayed ongoing seizure-related behaviors for about 2 minutes before progressing to RBS (Figure 2B, right). There was no overlap in the range of latencies to flurothyl-induced seizures aged P14 in the 7 -/- mice (182-251 s) and their +/- and +/+ littermates (311-465 s and 292-455 s, respectively). Abdominal massage restored breathing for 18 of these 28 mice, including 3 -/- mice. The shorter latencies to RBS in -/- mice suggest there was network hyperexcitability present in the -/- CNS at P14.
Though the seizure profile of -/- mice (described above) is distinct from that of Kcnal-null mice (partial seizures from age P21 onwards) the Kcnal-null mice did also have reduced seizure latencies in response to flurothyl at ages as young as P10 (Rho et al., 1999). Because Kcnal-null mice showed abnormal tremor following a cold swim, we performed similar cold swim tests on 5 -/- (B6/129) mice (see Methods). However, unlike the Kcnal-null mice, the Kcna2-null mice did not exhibit any signs of hyperexcitability (body tremors) after a cold swim.

Could sound be one of the stimuli able to induce RBS/TE in -/- mice? This question arises because RBS/TE can be induced audiogenically in certain strains of rats and mice which are either genetically susceptible or have been made susceptible by early partial deafening (see Discussion). Typically, loud sounds of 100-130 dB lead after 2-20 s to RBS, closely followed by clonic seizures and/or TE (Ross and Coleman, 2000). In preliminary tests, 70 out of 71 applications of an octave band stimulus 8 kHz to 16 kHz (112 dB SPL, 20 s duration) failed to induce RBS or TE in 6 -/- mice tested at ages P14-P18 (data not shown).

**Developmental expression of Kcna mRNA in +/+ brainstem**

To explore whether the second and third postnatal weeks might be an especially important period in the development of CNS Kv1 channels, the qPCR technique was used to measure the developmental timecourse of Kcna2 gene mRNA expression in the brainstems of 14 +/+ mice, two mice at each of seven ages tested (Figure 3A, error bars indicate the variability of repeated qPCR measures from one mouse). The expression level increased ten-fold between P1 and P29 for Kcna2 mRNA (Figure 3A). This increase in Kcna2 expression occurs at approximately the same age as the onset of seizures in -/- mice (see Discussion).

Figure 3 near here

The developmental timecourse of Kcnal and Kcnab gene mRNA expression was also measured using the same brainstem tissue samples (Figure 3B, Kcna2 expression replotted from A). The mRNA expression increased approximately thirty-fold for Kcnal but only two-fold for Kcnab (Figure 3B). Kcnal mRNA and Kcna2
mRNA reached their half-maximal expression at P12 and P11, respectively (values from fits to Boltzmann functions, Figure 3B). A comparison of the relative expression of these three Kcna genes is also of interest. The data suggest that neonate brainstems had higher expression of Kcna6 mRNA than Kcna1 and Kcna2 mRNA, whereas the opposite was true for juvenile brainstems (Figure 3B). Also, Kcna1 and Kcna2 mRNA were present at similar copy numbers in neonatal mice, whereas in juvenile mice Kcna1 mRNA contributed more than twice as many copies as Kcna2 mRNA (Figure 3B). If this reflects the ratios of Kv1 subunit proteins produced, it suggests that the order of expression strength in neonatal brainstem may be Kv1.6>>Kv1.2>Kv1.1, whereas this order is reversed during maturation, becoming Kv1.1>Kv1.2>>Kv1.6.

Kcna2-null MNTB neurons were hypoexcitable

One obvious way in which a lack of Kv1.2 might cause network hyperexcitability in the -/- CNS would be via reduced potassium currents causing hyperexcitability in individual neurons. Though it is probably unlikely that MNTB neurons within the brainstem auditory system play a role in seizure susceptibility in either Kcna1-null or Kcna2-null mice, MNTB neurons are a useful model for study because even small reductions in their I_{Kv1} have large effects on excitability (Brew and Forsythe, 1995; Brew et al., 2003).

To find out whether -/- MNTB neurons had abnormal excitability, we recorded and analyzed the responses to current pulses (180 ms duration, -100 pA to 200 pA or 0 pA to 200 pA, in 10 pA increments) of 63 MNTB neurons in brainstem slices from mice aged P9-P16 (9 +/+ mice, 14 +/- mice and 15 -/- mice). The responses of three example MNTB neurons, each typical of their genotype, are shown in Figure 4A. The -/- neuron fired the smallest numbers of APs, e.g. only a single initial AP at the start of a 200 pA pulse contrasting with 3 APs generated by the +/+ neuron (Figure 4A, top traces). The -/- neuron also had the highest threshold current amplitude (defined as the smallest current pulse amplitude that generated at least one AP) of 160 pA, contrasting with 80 pA for the +/+ neuron, and 130 pA in the +/- neuron. Overall, the threshold current amplitudes were significantly smaller in the 21 +/+ MNTB neurons than in the 25 -/- neurons (p<0.0001) or the 19 +/- neurons (p<0.005, Figure 4B). The +/- neurons threshold current amplitudes were distinctly intermediate between +/+ and +/- neurons because they were also significantly smaller than those of the -/- neurons (p<0.05).
Both the -/- and the +/- neurons fired fewer APs than +/+ neurons, for every current pulse amplitude 80 pA and larger (Figure 4C, small and large asterisks show significant differences). There were only three current pulse amplitudes at which +/- neurons fired significantly larger numbers of APs than -/- neurons (Figure 4C, circumflexes). The genotypic differences in AP numbers were also present when data were lumped across all the current amplitudes because the +/+ AP numbers differed significantly from -/- AP numbers or +/- AP numbers (repeated measures ANOVA, each at p<0.001). The small overall difference between -/- and +/- MNTB neurons’ AP numbers was also significant (repeated measures ANOVA, p<0.05).

To more accurately represent the excitability of each MNTB neuron, and compare the distribution of excitability within each genotype, the AP numbers were averaged across the five largest current pulses tested, 160 pA through 200 pA, and this average was termed the APN(160to200). The APN(160to200) values were significantly larger in +/+ neurons than in -/- neurons or +/- neurons (Figure 4D).

In Figure 4 and the related statistical comparisons above, 5 out of an original total of 68 recordings were excluded because they fired so many APs that they were statistical outliers (Figure S1A, arrows) and their tonic firing also disallowed their inclusion in Figure 5 and Table S2 (see below). These recordings were probably not from MNTB principal neurons and their exclusion made very little difference to any of the statistically significant differences between the genotypes (see legend to Figure S1).

There was only slight overlap between the +/+ and -/- APN(160to200) values (Figure 4D) which suggests almost all of the -/- MNTB neurons had abnormally low excitability, rather than some -/- MNTB neurons having similar excitability to +/+ neurons, having somehow compensated for their Kcna2 deficit. In principle, variable levels of compensation for the Kcna2 deficit in +/- and -/- neurons could lead to greater within-genotype variability than for +/+ neurons, but in fact they exhibited similar variability in threshold currents and smaller variability in AP numbers (e.g Figure 4B,C,D). There was also no sign that +/- neurons and -/- neurons underwent compensation in the age range studied of P9-P16, because they were not more
excitable in slices from mice aged P12-P16 than from mice aged P9-P11 (see Table S2). The significant differences between +/+ neurons and the hypoexcitable -/- and +/- neurons remained after this subdivision into two age-groups (Table S2).

These data show that -/- MNTB neurons and +/- MNTB neurons were hypoexcitable compared to their +/+ counterparts. This is especially surprising because it was previously shown that *Kcnal*-null MNTB neurons were hyperexcitable and had the reduced I_{kl} amplitudes expected because of their Kv1.1 deficit (Brew et al., 2003). In marked contrast, the deficits of Kv1.2 subunits in -/- and +/- MNTB neurons were associated with hypoexcitability, suggesting their I_{kl} amplitudes were enlarged, not reduced.

### Altered resting membrane properties and I-V relations in -/- MNTB neurons

An enlarged I_{kl} would be expected to cause slightly less depolarized steady-state I-V relations in the range –70 mV to –40 mV, as well as enlarged conductance (reduced membrane resistance). Analysis showed that -/- MNTB neurons did have less depolarized sustained membrane potentials than +/+ MNTB neurons, for all depolarizing current pulse amplitudes tested, as well as shallower I-V relations around rest (Figure 5A, same neurons and pulse protocol as in Figure 4, linked arrows in Figure 4A show the time-window of sustained potential measurement). The +/- neurons’ sustained potentials were similar to those of +/+ neurons, though there were small significant differences in potential at a few current amplitudes (Figure 5B). The abnormal sustained I-V relations in -/- MNTB neurons support the idea that their sustained I_{kl} amplitude is enlarged compared to +/- neurons.

Though the mean resting potentials were slightly larger in the -/- and +/- neurons (~66 mV and ~67 mV) than in the +/+ neurons (~65 mV) the differences were not significant (Table S2, resting potential was defined as the sustained membrane potential during the 0 pA current pulse). Table S2 compares the MNTB neurons of each genotype across a range of excitability-related membrane parameters. The mean resting input resistance was approximately 90 MΩ in -/- neurons, significantly lower than the 130-140 MΩ in +/+ neurons (Table S2, see legend for values and details of both methods used to quantify input resistance). The +/- neurons mean
resting input resistance was similar to that of +/+ neurons (Table S2). These data do not support the idea that there had been variable levels of compensation for the Kcna2 deficit in +/- and -/- neurons because the standard deviations for each parameter were generally similar in the MNTB neurons of each genotype (Table S2). As expected for mouse strains repeatedly backcrossed into the same inbred C3HeB/FeJ background, the distributions of AP numbers, threshold currents and other membrane properties for the +/+ MNTB neurons reported here were very similar to those for MNTB neurons from the control littermates of Kcna1-nulls (compare the present study’s Figures 4, 5, S1 and Table S2 with Figures 1, 5 and Table 1 of Brew et al., 2003).

**Action potentials and other properties were similar in +/+ and -/- MNTB neurons**

Aside from the above-described differences which could be ascribed to potassium currents, MNTB neurons had similar properties irrespective of genotype. For example, the -/- and +/- sodium channels were probably functioning normally because MNTB neurons of all three genotypes had a similar initial AP during a 200 pA pulse, with an approximate mean latency of 3 ms, mean half-width of 0.9 ms, and a rapid rising phase beginning at –45 mV (Table S2). MNTB neurons of each genotype were probably of similar size because they had similar capacitances (Table S2). Also, the size of MNTB and its principal neuron somata appeared similar in brainstem slices of each genotype viewed in our recording chamber (data not shown). The pipette access resistances and ages were similar for MNTB neurons of each genotype (Table S2). Hence the genotypic differences in threshold currents, AP numbers and IV relations described above could be produced solely by differences in potassium currents.

**Dendrotoxin-I had larger effects on -/- MNTB neurons and abolished genotypic differences**

Next, we measured the effects of dendrotoxin (DTX, see Introduction) on threshold current amplitudes, AP numbers and sustained potentials in 5 +/+ and 4 -/- MNTB neurons, in order to test whether -/- hypoexcitability could be caused solely by an enlarged I_{Kv1} (the subcomponent of I_{kl} clearly attributable to Kv1-type channels because of its DTX-sensitivity). If so, DTX should have larger effects on the excitability of -/- neurons than +/+
neurons and there should be no differences in excitability between +/+ and -/- neurons when DTX is present.
The typical effect of DTX on MNTB neuron firing is a reduction in the threshold current for an AP and a
conversion from phasic to tonic firing (e.g. in rat MNTB, Brew and Forsythe, 1995). This phasic firing during a
prolonged current pulse was shown above for two typical example +/+ and -/- MNTB neurons in control ACSF
(Figure 4A) and both converted to tonic firing throughout current pulses after 100 nM DTX was applied (Figure
6A,B). In terms of AP numbers, these +/+ and -/- MNTB neurons fired 3 APs and 1 AP respectively in ACSF at
the example pulse amplitude of 200 pA, and in DTX this increased to 20 APs and 16 APs. The DTX application
caused a much larger reduction in threshold current amplitude for the -/- neuron than the +/+ neuron (140 pA
versus 40 pA). Similar results were obtained in all 5 +/+ neurons and 4 -/- neurons which had threshold current
amplitudes in control ACSF averaging 100 pA and 170 pA, respectively, each typical for their genotype, and
differing significantly from each other (Figure 6D, left, compare with Figure 4B). After DTX application, the
+/+ neurons and -/- neurons had statistically indistinguishable threshold current amplitudes of about 40 pA
(Figure 6D, center) and fired similarly large numbers of APs (Figure 6C, triangles). The mean reduction in
threshold current amplitudes caused by DTX was approximately twice the size in -/- neurons as in +/+ neurons
(Figure 6D, right).

Along similar lines, if different amplitudes of $I_{Kv1}$ accounted for the different sustained I-V relations of +/+ and
-/- MNTB neurons, then DTX should cause larger shifts in sustained potentials in -/- neurons, and the I-V
relations of +/+ and -/- neurons should become similar in DTX. Consistent with this, the mean sustained I-V
relations were initially quite different for the 5 +/+ and 4 -/- neurons in ACSF (Figure 6E, filled and open circles)
but became very similar after DTX was applied (Figure 6E, triangles, and extrapolated lines). In DTX, sustained
potentials were not attained for pulse amplitudes larger than the threshold current (around 30 pA) because they
typically caused tonic firing (e.g. Figure 6A,B). However, the measurable sustained I-V relations were highly
linear in every neuron (data not shown) hence the fitted lines represent the data slightly beyond the average data
shown by the triangles, whose range was restricted by neurons with very low threshold currents of 0 pA and 10 pA (Figure 6E). Up to about the 30 pA current pulse amplitude, the depolarizations of the sustained potentials caused by DTX were approximately twice as large in -/- neurons as in +/+ neurons (Figure 6E, compare vertical distances between the circle symbols and the lines fitted through triangles). The mean depolarization caused by DTX relative to the resting potential (the sustained potential during the 0 pA pulse) was more than twice as large in the 4 -/- neurons (4.8 ± 0.90 mV) as in the 5 +/+ neurons (1.5 ± 0.47 mV, p<0.05, this included only one potential that was an extrapolated estimate, for one +/+ that neuron fired tonically at 0 pA in DTX).

These data suggest that $I_{\text{Kv1}}$ is larger in -/- than +/+ MNTB neurons, and that this could underly all their differences in excitability and membrane properties. The DTX-sensitivity of the -/- Kv1 channels shows they must each contain at least one DTX-sensitive subunit, i.e. Kv1.1 or Kv1.6, given that Kv1.2 subunits are absent (Figure 1C).

**Larger $I_{\text{Kv1}}$ in voltage-clamped -/- MNTB neurons than +/+ neurons**

To measure and compare $I_{\text{Kv1}}$ amplitudes and properties, we carried out voltage clamp recordings from MNTB neurons in brainstem slices from +/+, +/- or -/- mice. After obtaining the whole-cell recording configuration for an MNTB neuron, the slice was perfused with ACSFV containing tetrodotoxin, low calcium and high magnesium to block sodium and calcium channels, and the currents were recorded in response to test pulses –107 mV to +23 mV, following a –67 mV prepotential (see Methods). Typical example +/+ and -/- MNTB neurons had current responses which appeared qualitatively similar in the -107 mV to –37 mV potential range (Figure 7A, note that following test pulses more negative than –67 mV, the A-currents can be very large during the –37 mV postpotential). The amplitudes of sustained outward current were slightly larger in the -/- neuron than the +/+ neurons within the -67 mV to –37 mV range within which Kv1 channels are expected to dominate the outward current (Figure 7A, bars show time window for measurements of sustained current). In 11 +/+ and 10 -/- MNTB neurons the mean amplitudes of sustained outward currents were similar and statistically indistinguishable at all test potentials except -67 mV and -57 mV (Figure 7B, asterisks denote p<0.05, Mann-Whitney U-tests, see Methods for details of leak current subtraction). The datapoints within the $I_{\text{Kv1}}$-relevant
range of potentials were replotted to show the larger mean amplitudes of sustained current in -/- MNTB neurons than +/+ neurons (Figure 7C, also including data from 8 +/- MNTB neurons). At the –67 mV test potential, the mean amplitudes of sustained current were almost twice as large in -/- neurons (45 pA) as in +/+ MNTB neurons (26 pA). This is highly consistent with the current clamp data above showing that -/- neuron hypoexcitability could be due to enlarged I_{Kv1} (Figures 4, 5, 6).

Figure 7 near here

The 8 +/- MNTB neurons had sustained current amplitudes which were significantly smaller than those of the -/- neurons at the test potentials –67 mV and –57 mV but statistically indistinguishable from the +/+ neurons (Figure 7C, p<0.05, Mann-Whitney U-tests). The similar current amplitudes in +/+ and +/- neurons were somewhat surprising given that +/- MNTB neurons had low excitability much more like that of -/- MNTB neurons than that of +/+ MNTB neurons (see Figure 4). However, the +/- current amplitudes appear closer to those of -/- neurons after the exclusion of a few neurons with current amplitudes which were outliers for their genotypes (see Figures 8, 11 below, note that the presence of outliers was why non-parametric statistical tests were used above).

**No other genotypic differences apparent**

As described above for the current-clamp MNTB data, apart from any differences in potassium currents the 29 voltage-clamped MNTB neurons of Figure 7 had similar properties irrespective of genotype. The background “leak” resistances were statistically indistinguishable between genotypes in the potential range used for leak subtraction (–97 mV to –77 mV, see Methods: +/+, 186 ± 14 MΩ, n=11; +/-, 228 ± 20 MΩ, n=8; -/-, 244 ± 32 MΩ, n=10). The pipette access resistances and neuronal capacitances noted from the compensation dials on our amplifier were also similar (+/+, 10.7 ± 1.1 MΩ; +/-, 11.8 ± 1.1 MΩ; -/-, 12.7 ± 1.1 MΩ: +/+, 13.2 ± 0.9 pF; +/-, 14.4 ± 0.7 pF; -/-, 13.0 ± 0.4 pF). The postnatal ages of the mice used were similar across genotypes (+/+, 12.4
days, n=11; +/-, 12.3 days, n=8; +/-, 12.6 days, n=10). There were no statistically significant correlations between any of the electrophysiological parameters we measured and the age of the mouse, at least within the small age range tested.

**Activation timecourse of I_{kl} after the –67 mV prepotential**

Different activation kinetics could in principle contribute to excitability differences between +/-, +/- and -/- MNTB neurons. However, the outward currents developed with very similar timecourses in all MNTB neurons, attaining their maximal amplitudes within 10 ms of step to -57 mV (Figure 8A, same neurons as in Figure 7B,C, arrows show outlier current amplitudes further addressed in Figure 11 below). The current traces from all these neurons were fitted well by single exponentials and gave almost identical mean time constants of approximately 3 ms for the neurons of each genotype (Figure 8B, left bars). Fits to the currents at –47 mV showed slightly more rapid current increase than at –57 mV, as would be expected, with time constants of approximately 2 ms in each genotype (Figure 8B, right bars). Very similar time constants were obtained from fits to the I_{Kv1} current component obtained by subtraction (e.g. 3 ms at –57 mV, data not shown, currents in the presence of Kv1-selective toxins were subtracted from those recorded a few minutes previously in ACSFV, see Methods). This suggests that when depolarized from –67 mV (similar to their resting potentials, Table S2) the potassium currents in MNTB neurons of each genotype increased with a similar timecourse, implying that amplitude differences alone may underly genotypic differences in excitability. However, subtle kinetic differences cannot be ruled out, and might be more easily measurable under conditions where I_{Kv1} activation was slow and sigmoidal, as occurred following holding potentials of –80 mV or more negative in (Rothman and Manis, 2003a). Also, inactivation properties may contribute to the apparent activation timecourses in Figure 8, though inactivation at –67 mV is probably less than 10% (Brew et al., 2003).

Figure 8 near here
The differential sensitivities to subunit-specific toxins of \( I_{\text{Kd}} \) in +/-, +/- and -/- neurons

To characterise the subunit contents of Kv1 channels in the MNTB neurons of each genotype, their currents were recorded while applying tityustoxin (TsTx), which blocks Kv1 channels containing at least one Kv1.2 subunit, and DTX which probably blocks all MNTB neuron Kv1 channels (see Introduction). Currents were recorded from MNTB neurons, initially during perfusion with control ACSFV alone, then during perfusion of ACSFV plus 100 nM TsTx, and finally during perfusion of ACSFV containing both 100 nM TsTx and 100 nM DTX. The effects of the toxins on sustained current amplitudes occurred within 1-3 minutes of the start of perfusion, as shown at –47 mV for typical example neurons of each genotype (Figure 9A, top row). The application of TsTx to +/- or +/- neurons caused decreased sustained current amplitudes within 1 minute, reaching a new equilibrium level within 3 minutes (e.g. Figure 9A, left, middle). TsTx had no effect on the currents in -/- neurons, as expected because of their lack of Kv1.2 subunits (e.g. Figure 9A, right). The current amplitudes in +/- neurons did not decrease further when the solution containing both TsTx and DTX was applied, presumably because the TsTx solution had already blocked all the DTX-sensitive channels (e.g. Figure 9A, left). The additional application of DTX to -/- and +/- neurons when TsTx was already present decreased their sustained currents substantially (e.g. Figure 9A, middle, right).

The analyses of toxin block presented below are all based on the sustained current amplitudes measured at the ends of test pulses, but very similar results would have been obtained from currents measured early during the test pulse, because the toxin effects were approximately uniform throughout 180 ms test pulses (Figure 9B, same neurons as directly above in A). When both toxins were present, the toxin-insensitive currents were revealed and were similar for neurons of each of the three genotypes, usually including a small initial A-current (mostly inactivated by the –67 mV prepotential) and a slowly activating outward current (Figure 9B, traces marked d). Note that the only substantial effect of any of the toxins on the baseline outward current amplitudes
at -67 mV prior to the test pulse was in the +/- neuron, in which the approximately 50 pA reduction probably reflected block of Kv1 channels open at -67 mV (Figure 9B, right).

The voltage-dependence of the sustained current amplitudes in the presence of the control, TsTx or TsTx+DTX solutions were calculated for 21 MNTB neurons and averaged within each genotype (Figure 9C, leak subtraction and averaging across 2-3 runs of the standard I-V protocol were as in Figure 7B,C and see Methods). At the most positive potentials, neither TsTx nor the combined toxins affected mean current amplitudes significantly (Figure 9C, insets) probably because Kv3-type high-voltage-activated currents are very large (Brew and Forsythe, 1995; Wang et al., 1998). In the potential range relevant to Kv1 channels, at test potentials -67 mV through -37 mV, TsTx produced substantial current block in the 7 +/- and 8 +/- MNTB neurons, but had no effect in 6 +/- MNTB neurons (Figure 9C, dashed lines). The addition of DTX had no further blocking effect in the +/- MNTB neurons but produced substantial current block in the -/- neurons (Figure 9C, dotted lines). Throughout this potential range, TsTx block appeared to account for 100% of the total toxin block in +/- neurons, in contrast with 0% in -/- neurons and approximately 50% in +/- neurons (Figure 9C, and see below for further analyses).

Some of these MNTB neurons exhibited toxin-induced current increases at positive potentials (e.g. see +/- inset panel in Figure 9C). The probable reason for this and details of how this also limits the reliability of the calculated $I_{Kv1}$ values at more negative potentials are described further in Supplementary Methods. This is relevant when considering the fits to the calculated $G_{Kv1}$ and the parameters for voltage-dependence in Figures 12 and 13 below because the same mechanism could slightly distort some of the calculated $G_{Kv1}$ values (see Supplementary Methods).

Next, the amplitudes of current blocked at -47 mV by TsTx and by subsequent addition of DTX are presented for individual MNTB neurons (Figure 10A, dark gray bars and light gray bars, respectively, same 21 neurons as Figure 9C). The mean amplitude of the current reduction caused by TsTx was 185 ± 29 pA in the 7 +/- neurons and only a little smaller at 153 ± 19 pA in the 8 +/- neurons, but only 9 pA in the 6 -/- neurons. The consecutive addition of DTX reduced current amplitudes by only 4 pA in the +/- neurons, 129 pA in the +/- neurons and 287 pA in the -/- neurons. The total current blocked by the combined TsTx+DTX solution
compared to the control ACSFV solution is the $I_{Kv1}$ component (represented by the total heights of the bars in Figure 10A, unless one subcomponent was “negative”, i.e. when a toxin application was associated with an increased current amplitude). The mean amplitude of $I_{Kv1}$ at -47 mV was 188 ± 29 pA in +/- neurons, significantly smaller than the 282 ± 30 pA in +/- neurons (p<0.05). Though the $I_{Kv1}$ was even larger in -/- neurons, at 297 ± 61 pA, this was not significantly larger than either +/- or +/- neurons, probably because one -/- neuron had extremely small $I_{Kv1}$ (shown at far right in Figures 10, 11, S2). The relatively tiny current shifts that occurred when TsTx was applied to -/- neurons were likely due to experimental noise; the shifts were not statistically significant, averaged 9 pA and were not correlated with the $I_{Kv1}$ amplitude for that neuron (Figure 10A, also see Figure 9A above for sample noise/drift). The apparent effects of the addition of DTX to TsTx-treated +/- neurons were also small and not significant (averaging 4 pA, and the largest value was 33 pA) and probably attributable to noise.

Figure 10 near here

To estimate the percentage of Kv1 channels that contain Kv1.2 in each MNTB neuron, we calculated the percentages of current blocked by TsTx relative to the total toxin block when DTX was also present. At -47 mV, the average percentage block accounted for by TsTx was almost 100% for +/- neurons, 56% for +/- neurons and approximately 0% for -/- neurons (Figure 10B, from data of panel A). The percentages of TsTx block were similar when averaged across the four test potentials -67 mV through -37 mV (+/+, 98%: +/-, 49%: -/-, -1%). The absence of Kv1.2 protein in the -/- brain obviously makes it reasonable to assume that the true percentage of Kv1 channels containing Kv1.2 is 0% in the -/- neurons. However, though the +/- data suggest that 100% of the +/- Kv1 channels contain Kv1.2, this conclusion implies that all of the percentage variability is due to noise, but we cannot rule out the alternative idea that the four +/- neurons with TsTx block percentages clustering in the 80-90% range (Figure 10B) actually reflect reality and have 10-20% of their Kv1 channels lacking Kv1.2. Similarly, although the 35% to 93% range for +/- neurons TsTx block percentages at –
47 mV may be due to noise, these values might reflect actual differences in each +/- neuron’s proportion of Kv1 channels containing Kv1.2 (Figure 10B, also see Figure 13 below).

**Typical current amplitudes and proportions of I\_Kv1 for MNTB neurons of each genotype**

MNTB neurons within each genotype exhibited some variability in their sustained current amplitudes in the control ACSFV solution (e.g. at -47 mV, Figure 11A). This variability may reflect real differences between recordings because the current amplitudes at -47 mV, -57 mV and -67 mV were highly correlated (data not shown). Of the 29 neurons shown, 4 had current amplitudes which were outliers for their genotypes at two or three of these test potentials (asterisks in Figure 11A, see legend for statistical definition). Without these 4 outliers, the MNTB neurons within each genotype had approximately normally distributed current amplitudes, and there was very little overlap in current amplitudes between +/- and -/- neurons (Figure 11A). This is consistent with the current clamp excitability data above, which showed little overlap between +/- and -/- neurons in the numbers of APs fired (Figure 4D). Hence both voltage-clamp and current-clamp data suggest that the majority of -/- MNTB neurons were abnormal, rather than having compensated for their lack of Kv1.2 subunits by up- or down-regulation of other genes or proteins.

In order to compare current amplitudes for more representative populations of MNTB neurons of each genotype, the I-V data from Figure 7C were replotted after excluding the outliers (Figure 11B, excludes the 4 neurons asterisked in A). After the exclusion, the -/- current amplitudes remained significantly larger than in +/- neurons at all three test potentials, and larger than in +/- neurons at -57 mV and -67 mV (Figure 11B, p<0.005). The +/- currents had intermediate amplitudes similar to those of +/- neurons at -67 mV, but closer to -/- values at -47 mV (Figure 11B) which might relate to the findings above that +/- MNTB neurons had properties similar to +/- neurons when at their resting potentials, but more like their -/- counterparts at the slightly depolarized membrane potentials crucial to determining excitability (Table S2, e.g. resting input
resistances, AP numbers). While the exclusion of outliers makes statistical comparisons speculative, the current amplitudes were significantly larger in +/- neurons than in +/+ neurons at –67 mV, -57 mV and –47 mV (Figure 11B, small asterisks, p<0.005) and remained significantly different when all 8 +/- neurons were included (p<0.05). Abnormally large +/- currents also seem a probable explanation for the significantly lower excitability in +/- MNTB neurons than in +/+ neurons, shown above (Figure 4).

In the majority of MNTB neurons of each genotype, most of the sustained Ikl amplitude at –47 mV was accounted for by the toxin-sensitive component IKv1 (70-80% for +/- and -/- MNTB neurons, and 60-70% for +/+ neurons, Figure S2). This was after excluding 3 neurons with atypically large proportions of non-Kv1 current, all of which were also outliers in terms of their Ikl amplitudes (Figure 11A, asterisks). This helps to justify the inclusion in the analyses of voltage-dependence below of 4 +/+ and 4 -/- MNTB neurons to which toxins were not applied, because we can assume that most of their total Ikl was IKv1. Except for the 3 excluded neurons, the toxin-insensitive non-Kv1 currents had similar amplitudes irrespective of genotype, averaging approximately 100 pA at –47 mV (Figure S2). This is consistent with the idea that the genotypic differences in sustained current amplitudes are attributable to different amplitudes of IKv1.

The finding of increasing current amplitudes between +/+, +/- and -/- MNTB neurons suggests an inverse dependence on their respective gene dosages of Kcna2 (2, 1 and 0) and possibly on the proportions of their Kv1 channels that include Kv1.2 (roughly 100%, 50% and 0%). In principle, individual +/- neurons with differential expression of Kv1.2 subunits might also exhibit an inverse dependence between current amplitudes and their proportion of Kv1.2-based current, but no such correlation is evident (compare middle panels of Figures 10 and 11A). Possible differences between +/- neurons are explored further in the analyses of Figures 12 and 13 below, which address whether the enlarged potassium current amplitudes in +/- and -/- neurons might be due to abnormally negatively activating Kv1 channels.

-/- GKv1 activated at more negative potentials than +/- GKv1

To explore whether the genotypic differences in current amplitudes were partly based on differences in voltage-dependence, fits were made to the total conductance, G (Figure 12A) and, where available, its explicitly toxin-
sensitive component, $G_{Kv1}$ (Figure 12B, calculated from sustained $I_{kl}$ or $I_{Kv1}$ respectively, as described in Methods). For each neuron, the $G$ and $G_{Kv1}$ values were first plotted against test potential and fitted by a Boltzmann function $G_{Kv1} = G_{max}/(1+\exp((-V-V_{half})/k))$ with fitted parameters as follows: $G_{max}$, the maximum conductance; $V_{half}$, the half-activation voltage; and $k$, the slope factor. Note that the necessary use of the $-67$ mV prepotential (see Supplementary Methods) means that these curves could represent a mix of activation and inactivation for each channel in the population, but previous results suggest inactivation is probably less than 10% for the +/- neurons (Brew et al., 2003). The data and fit for each neuron are shown normalized to the $G_{max}$ value for the fit (Figure 12). Of the 56 fits shown, 46 were very good, using the fitting criteria described in the Methods (Figure 12, solid lines). Of the 10 “less-good” fits, 7 came from +/- neurons (Figure 12, dashed lines). The focus of the analysis below is therefore on the results from -/- and +/- neurons, though +/- data are also presented. In each panel, the gray shaded region covers the same 9 mV range between $-48$ mV and $-57$ mV in order to facilitate visualization of the differences in conductance curves between neurons and between genotypes (Figure 12). For example, in all 8 +/- neurons the fits to $G$ yielded $V_{half}$ values between $-48$ mV and $-55$ mV, within the range shown by the gray bar (Figure 12A, middle, 0.5 on the Y-axes corresponds to half-activation at $V_{half}$ on the X-axes). In contrast, 6 of the 8 -/- neurons had $V_{half}$ more negative than $-57$ mV, to the left of the gray bar (Figure 12A, bottom). The mean $V_{half}$ value from +/- neurons was 5 mV less negative than in -/- neurons ($p<0.001$). The $V_{half}$ values returned from 8 out of 9 fits to +/- $G$ data were less negative than $-48$ mV, i.e. more negative than any of the fits to +/- or -/- $G$, and reaching $V_{half}$ at potentials beyond the right edge of the gray bar; only one +/- $V_{half}$ value of $-49$ mV fell within the gray bar (Figure 12A, top). The mean +/- $V_{half}$ value was 7 mV less negative than the mean for +/- neurons, and 12 mV less negative than for -/- neurons ($p<0.0001$).

The mean $V_{half}$ values for the $G$ from MNTB neurons of each genotype remained similar when we excluded the 5 “less-good” fits (Figure 12 A, dashed lines, described above and in the Methods) because the remaining 5 +/-,
8 +/- and 7 +/- neurons had mean $V_{\text{half}}$ of $-45.0 \text{ mV}$, $-51.1 \text{ mV}$ and $57.2 \text{ mV}$, respectively, and the differences between genotypes remained significant at $p<0.005$ or better. The values of $G_{\text{max}}$ associated with the fits to $G$ were very similar for the MNTB neurons of each genotype (+/+; 17.2 ± 1.2 nS; +/-; 18.4 ± 1.5 nS; -/-, 19.0 ± 1.4 nS). This suggests that MNTB neurons of each genotype may possess the same numbers of Kv1 channels, and that the genotypic differences in current amplitudes may be solely due to differences in the voltage-dependence of their Kv1 channels (possibly involving differences in both activation and inactivation).

Overall, the voltage-dependence of $G$ was steepest in -/- neurons, intermediate in +/- neurons and shallowest in +/+ neurons (Figure 12A). Though the genotypic differences in $k$ values were significant, the differences were small and almost all the fits had $k$ values between 5 and 10 mV (averaged data not shown). If the differences are real, it might mean that individual +/+ Kv1 channels activated less steeply than individual -/- Kv1 channels and/or might reflect a greater spread of $V_{\text{half}}$ values among +/+ Kv1 channels.

The same ordering of voltage-dependence between the genotypes was also found in the fits to $G_{\text{Kv1}}$, for which $V_{\text{half}}$ values were 7 mV more negative in -/- neurons than +/- neurons ($p<0.005$, Figure 12B). The $V_{\text{half}}$ values from the fits to -/- $G_{\text{Kv1}}$ ranged between $-58 \text{ mV}$ and $-62 \text{ mV}$, exhibiting hardly any overlap with the $-48 \text{ mV}$ to $-57 \text{ mV}$ range for +/- $G_{\text{Kv1}}$ (Figure 12B). The fact that the fits yielded broadly similar $V_{\text{half}}$ values whether calculated from $G$ or $G_{\text{Kv1}}$ suggests that $G$ did largely reflect the voltage dependence of Kv1 channels. 

The fits to +/+ $G_{\text{Kv1}}$ could be regarded as consistent with the fits to $G$ if we focus on the 4 fits with $V_{\text{half}}$ values less negative than $-48 \text{ mV}$ (Figure 12B, top) but the other 2 fits yielded very negative $V_{\text{half}}$ values, probably distorted in the negative direction by toxin-induced space clamp effects (see Supplementary Methods). If Kv1 channels do activate at less negative potentials in +/+ MNTB neurons than in their +/- and -/- counterparts, this would itself contribute to making the +/+ $G$ and $G_{\text{Kv1}}$ data harder to fit because they would not yet be approaching their maximum values at $-37 \text{ mV}$, whereas the +/- and -/- $G$ and $G_{\text{Kv1}}$ data show clear signs of approaching or reaching their maxima (see Figure 12A and B).

Both sets of fits, to $G$ and $G_{\text{Kv1}}$ (Figure 12A,B) suggested that $V_{\text{half}}$ is approximately 6 mV more negative for -/- than +/- Kv1 channels. The actual $V_{\text{half}}$ values may be close to the $-60 \text{ mV}$ and $-53 \text{ mV}$ returned from the $G_{\text{Kv1}}$ fits, because the $G$ fits were probably shifted slightly to the right by the toxin-insensitive
conductance component, which, when fitted separately, gave a less steep voltage-dependence and substantially more positive $V_{\text{half}}$ than any of the fits shown (data not shown). Consistent with this, simulations in which we subtracted a typical amount of toxin-insensitive conductance (calculated from the neurons of Figure S2B) from the G data (as in Figure 12A) yielded +/- and -/- fits with $V_{\text{half}}$ values 3-5 mV more negative than before the subtraction, and slightly steeper. The same subtraction from the +/+ G data yielded +/+ $V_{\text{half}}$ values 7 mV more negative than before the subtraction, of ~51 mV, presumably because of the larger proportional contribution to +/+ G of toxin-insensitive channels. From this, our overall best estimates of the mean $V_{\text{half}}$ for +/+ , +/- and -/- MNTB neuron Kv1 channel populations are ~51 mV, -55 mV and ~60 mV. Hence, the $V_{\text{half}}$ may be only 9 mV more negative for -/- than +/+ Kv1 channels, rather than the 12 mV suggested by the G fits. These results on voltage-dependence should ideally be confirmed in future experiments when selective blockers become available for the various types of DTX-insensitive channels also present, which would allow improved isolation of the Kv1 channel currents and would probably reduce the effects of any DTX-induced space-clamp alterations (see Supplementary Methods).

The results in Figure 12A and B suggest that Kv1 channel populations activate at more negative potentials in neurons with smaller gene dosages of $Kcna2$ genes, shown above to have smaller proportions of Kv1.2 subunits (Figures 10,11). One simple explanation for this would be that Kv1 channels without Kv1.2 subunits activate at more negative potentials than those containing Kv1.2. If so, this might be detectable between the two distinct $G_{Kv1}$ components in +/- neurons, based on either Kv1.2-free or Kv1.2-containing channels. For the same 7 +/- neurons as in Figure 12B, the fits to the $G_{Kv1}$ component based on TsTx-sensitive currents activated at 4 mV less negative potentials (Figure 12C, top) than for the $G_{Kv1}$ component based on TsTx-insensitive but DTX-sensitive currents (Figure 12C, bottom, $p<0.05$). Note that the latter values for presumed Kv1.2-free +/- Kv1 channels are not as negative as for -/- $G_{Kv1}$, possibly because the TsTx block was not fully completed when DTX was added. Nonetheless, this provides additional support for the idea that Kv1 channels without Kv1.2 subunits activated at more negative potentials than those containing Kv1.2.
**G and G\textsubscript{Kv1} activate more negatively in +/- neurons with smaller percentages of TsTx block**

The \( V_{\text{half}} \) values for G were highly correlated with the number of \( Kcna2 \) genes present (0, 1 or 2) in the 25 neurons of all three genotypes (Figure 13A, \( p<0.01 \)). The relative gene dosage is probably a rough indicator of the relative proportions of Kv1.2 subunits present, but in +/- neurons a better indicator may be the proportion of \( I_{Kv1} \) that was blocked by TsTx at \(-47 \text{ mV} \) (Figure 10 above). For the 7 +/- neurons for which we obtained good fits to \( G_{Kv1} \) (in Figure 12B) the \( V_{\text{half}} \) values were significantly correlated with this TsTx block proportion (Figure 13B, \( p<0.05 \)). Also plotted for comparison are the \( V_{\text{half}} \) values from +/+ and -/- neuron \( G_{Kv1} \), assuming that their TsTx block proportions were 0% and 100%. These data show that the variations in \( V_{\text{half}} \) between +/- MNTB neurons are correlated with the proportion of their \( I_{Kv1} \) that is due to Kv1.2-containing channels, which is likely to be larger when Kv1.2 subunits make up a larger proportion of the total pool of Kv1 subunits.

So far, these data could be explained by assuming only two possible \( V_{\text{half}} \) values for Kv1 channels; either Kv1.2-free channels with \( V_{\text{half}} \) of \(-60 \text{ mV} \) or Kv1.2-containing channels with \( V_{\text{half}} \) of, say, \(-50 \text{ mV} \). If so, then the intermediate \( V_{\text{half}} \) of the Kv1 channel population in +/- MNTB neurons would be produced by a mix of these two channel types, but the \( V_{\text{half}} \) value should be the same for all Kv1.2-containing channels. However, the \( V_{\text{half}} \) values from the TsTx-sensitive component of +/- \( G_{Kv1} \) (from Figure 12C) varied and were themselves significantly correlated with the TsTx block proportion (Figure 13C, \( p<0.05 \)). This suggests that different \( V_{\text{half}} \) values may be possible among Kv1.2-containing channels, perhaps correlating with their possession of different numbers of Kv1.2 subunits. For example, most +/- channels containing Kv1.2 may include only a single Kv1.2 subunit and have a more negative \( V_{\text{half}} \) than channels containing two or more Kv1.2 subunits, which predominate in +/+ neurons.

Figure 13 near here
Discussion

We generated \(-/-\) mice lacking the \textit{Kcna2} open reading frame and Kv1.2 subunit proteins. Their primary overt phenotype comprised increased seizure-susceptibility (at P14), seizures (from P15) and reduced lifespan. The \(+/\) mice were overtly normal, despite the halved expression of \textit{Kcna2} mRNA in \(+/\) brain. At the individual neuron level, \(-/-\) auditory MNTB neurons were hypoexcitable and had abnormally large Kv1-based K\(^+\) currents (\(I_{Kv1}\)) probably because their Kv1.2-free channels activated at substantially more negative potentials than the \(+/+\) channels, all of which contained Kv1.2. Corresponding variations in Kv1.1:Kv1.2 stoichiometry between different neuronal types may represent one mechanism of fine-tuning potassium currents for particular information processing tasks.

Severe brainstem seizures probably cause the \(-/-\) reduced lifespan

Wild running/bouncing and tonic extension (TE) involve the whole body and indicate the \(-/-\) mice had generalized, brainstem seizures, rather than partial, forebrain seizures. Studies utilizing genetically epilepsy-prone rats (GEPRs) showed wild running and TE depend on brainstem structures and occur even when forebrain connections are severed, whereas partial seizures involve only parts of the forebrain and movements of parts of the body (Gale, 1992; Browning et al., 1999; Faingold, 1999). Though TE is usually not fatal in GEPRs, TE is commonly a fatal endpoint of mouse seizures (e.g. Rho et al., 1999). TE and apnea during seizures probably caused the reduced lifespan in \(-/-\) mice because their fatalities always coincided with TE. If so, this suggests a possible way to prolong the \(-/-\) lifespan, because TE with apnea became non-fatal for mice placed in 100\% oxygen just prior to audiogenic seizure induction (Venit et al., 2004).

If there were brainstem sites where Kv1.2 subunits dominated Kv1 channels, with no Kv1.1 expression, such sites would probably be hyperexcitable in \(-/-\) mice. However, Kv1.1 and Kv1.2 are co-expressed in all the same neurons of the mouse brain and brainstem, except in one neuron type in the olfactory bulb which expresses Kv1.2 without Kv1.1 and in the hippocampus which expresses Kv1.1 much more widely than Kv1.2 (Wang et al., 1993; Wang et al., 1994; Veh et al., 1995; Grigg et al., 2000). Though partial seizures exhibited
by mice lacking Kv1.1 probably initiate in the hippocampus, where CA3 neurons are hyperexcitable (Smart et al., 1998; Lopantsev et al., 2003) it seems unlikely that -/- seizures would initiate in the olfactory bulb. Instead the -/- brainstem may be hyperexcitable at sites where Kv1.2 subunits dominate channels in normal mouse brain, even with some Kv1.1 present (see below). Possibly, respiratory or cardiovascular abnormalities could also contribute to -/- seizures, because arterial smooth muscle expresses Kv1.2 with Kv1.5 but not Kv1.1 (Xu et al., 2000; Thorneloe et al., 2001; Albarwani et al., 2003). However, intrinsic neural network hyperexcitability was apparently present in all -/- mice tested at P14, which had reduced latencies to flurothyl-induced seizures, and rapid progression to generalized seizures (Figure 2B).

**Kv1 subunit expression and the phenotypes of mice with Kv1 deficits**

Kv1.2 may be more important than other Kv1 subunit types because -/- mice were more severely affected than mice with deficits of other Kv1 subunits. *Kcnal*-null mice lacking Kv1.1 exhibited milder partial seizures and lived approximately twice as long as -/- mice (Smart et al., 1998) whereas *Kcnal*-null mice had normal lifespans and only occasional seizures (London et al., 1998). Mice with deficits in Kv1.3 or Kv1.5 had normal lifespans and no reported seizures (Archer et al., 2001; Xu et al., 2003). The relative severity of each phenotype may reflect the relative abundance of each subunit type in normal mouse brain. Using antibody binding to DTX-sensitive channels from bovine brain, the order of subunit prevalence was Kv1.2 > 1.1 >> 1.6 > 1.4 (Scott et al., 1994). Kv1.3, Kv1.5 and Kv1.7 are weakly expressed or absent in neurons (Kalman et al., 1998; Trimmer and Rhodes, 2004).

These relative abundances may alter during development. Our qPCR data suggest that the dominance of Kv1.1 and Kv1.2 develops in the brainstem between P1 and P29, when their mRNA expression increased tenfold and fourfold, respectively, while mRNA for Kv1.6 subunits merely doubled (Figure 3B). Hence Kv1.6 subunits may play a relatively important role in early postnatal development, but be less important in adults. During the second and third postnatal weeks, the upregulation of Kv1.1 and Kv1.2 is probably important for myelination of axons, because both subunits are strongly co-expressed in large myelinated axons in adult mice (Wang et al., 1993) but the role of a transient strong embryonic expression of Kv1.1 is unknown (Hallows and
The qPCR data also showed an increasing ratio of *Kcnal* to *Kcn2* mRNA during development (Figure 3B). This ratio also trended upward with age in the rat cochlear nucleus (Bortone et al., 2006). The MNTB neuron results showing Kv1 channel voltage-dependence varied with their Kv1.2 content (Figures 10, 12 and see below) suggest this changing ratio might result in Kv1 channels that activated at more negative potentials, as the nervous system develops.

The usual timecourse of Kv1.1 or Kv1.2 subunit upregulation may have relevance to the age of -/- seizure onset. For example, large axons may be able to function with or without Kv1.2 during the first two postnatal weeks, after which Kv1 subunit production in -/- mice would fall behind requirements, causing reduced amplitudes of axonal *I*\textsubscript{Kv1} and seizures. Alternatively, the timing of -/- seizure onset may be determined by when *I*\textsubscript{Kv1} becomes enlarged due to Kv1.1-dominated channels with abnormally negative activation. However, an early juvenile onset is common for seizures of many different etiologies, perhaps related to increases in neuronal maximum firing rates (Swann and Hablitz, 2000). For example, mice with sodium channel abnormalities developed spontaneous seizures and died aged P13-P26 (Chen et al., 2004) and there was a P16-P18 onset of seizure-susceptibility in GEPRs (Reigel et al., 1986; Reigel et al., 1989).

**Hyperexcitability due to abnormal *I*\textsubscript{Kv1} in axons, synapses or inhibitory neurons?**

Possibly, Kv1.2-dominated channels populate axons via preferential targeting, though all three subunit types Kv1.1, Kv1.2 and Kv1.6 are targeted to axons via an interaction with Kvbeta2 (Gu et al., 2003). If so, -/- axons or synapses may have strongly reduced Kv1 channel density, leading to network hyperexcitability and seizures. In *Kcnal*-null axons, it is thought that reduced *I*\textsubscript{Kv1} contributes to the hyperexcitability induced by cold temperatures at the final heminode prior to neuromuscular synaptic terminals, thought to account for the cold-swim induced tremor in *Kcnal*-null mice, and in Kvbeta2-null mice (Zhou et al., 1998; McCormack et al., 2002). Kv1 conductance may be especially susceptible to cold temperatures, as shown in auditory octopus neurons (Cao and Oertel, 2005). However, there was no cold swim-induced tremor in -/(B6/129) mice (described above) arguing against abnormally small *I*\textsubscript{Kv1} in -/- axons, unless their Kv1.2-free channels are relatively cold-insensitive.
Preferential targeting of Kv1.2 to synaptic terminals was first suggested by localization discrepancies between Kv1.2 protein and mRNA within the hippocampus (Veh et al., 1995). A hippocampal lesioning study suggested Kv1.2 was in the synaptic terminals of entorhinal afferents, whereas Kv1.1 and Kv1.4 were found together along axons (Monaghan et al., 2001). Kv1.2 subunits dominated the Kv1 currents at the giant synaptic terminals of cochlear nucleus bushy cells, whereas Kv1.1 subunits dominated their somatic current (Dodson et al., 2003). Future direct recordings from these synapses in -/- mice could reveal reduced I_{Kv1}.

Alternatively, an enlarged -/- I_{Kv1} (as found in MNTB) could lead to seizures if it weakened inhibition more than excitation. A weakened glycinergic inhibitory output of -/- MNTB neurons seems unlikely to lead to seizures, but might interfere with sound localization using the cue of interaural level difference, calculated by lateral superior olive (LSO) neurons which integrate ipsilateral excitatory signals with contralateral inhibitory signals, arriving via the MNTB inverting relay (also see -/- auditory system section below). Neocortical pyramidal neurons had increased IPSC frequencies in Kcna1-null mice, while EPSC frequencies were normal (van Brederode et al., 2001). The powerful effects of Kv1 channels on excitability at GABA-ergic cerebellar basket cell synapses were shown when DTX led to an increased IPSC frequency onto Purkinje neurons, as was also found in mice with Kv1.1 deficits, though the basket cell somata fired at normal rates (Southan and Robertson, 1998; Zhang et al., 1999; Herson et al., 2003). Future recordings showing abnormal IPSC frequencies at these synapses in -/- CNS would point to whether the synapses have reduced or increased I_{Kv1}.

**Hypoexcitability of -/- MNTB neurons is due to enlarged I_{Kv1}**

MNTB neurons in brainstem slices from -/- mice were intrinsically less excitable than +/- MNTB neurons. Consistent with this, I_{Kv1} was larger in voltage-clamped +/- MNTB neurons than in the +/- neurons. This contrasted markedly with results from Kcna1-null MNTB neurons, lacking the closely related subunit Kv1.1, which had reduced potassium currents and were hyperexcitable (Brew et al., 2003). Interestingly, the excitability of Kcna1-heterozygote MNTB neurons was identical to that of control counterparts, whereas here we showed Kcna2-heterozygote MNTB neurons had excitability and I_{Kv1} properties intermediate between their -/- and +/- littermates (compare Table S2 with Table 1 in Brew et al., 2003).
The excitability differences between -/- and +/+ MNTB neurons were attributable solely to differences in their Kv1 channels, because genotypic differences were abolished by DTX and other electrophysiological properties such as capacitance and action potential shapes were similar across genotypes. The enlarged -/- IKv1 could also explain why DTX caused larger depolarizations from rest in -/- neurons, and larger reductions in their threshold currents (Figure 6 and related text).

The implications of toxin-sensitivities for Kv1 channel stoichiometries in MNTB neurons

Both Kv1.1 and Kv1.2 are probably present in almost all Kv1 channels in murine MNTB neurons, as follows. Both are strongly expressed in rat and mouse MNTB, with some Kv1.6 (Wang et al., 1994; Fonseca et al., 1998; Dodson et al., 2002; Brew et al., 2003). The Kv1.1-selective blocker dendrotoxin-K (DTX-K, Robertson et al., 1996) blocked more than 70% of the total sustained Ikl at –47 mV in MNTB neurons of the same mouse strain used here, C3HeB/FeJ (Gittelman and Tempel, 2006). This is consistent with the 50-70% block of Ikl, expected if IKv1 was fully blocked, because Ik could be blocked by 50-60% by DTX in Brew et al. (2003) and by 66% by TsTx+DTX above (Figure S2B). This suggests Kv1.1 is present in all the Kv1 channels. Kv1.2 subunits were in 80-100% of +/+ MNTB neuron Kv1 channels because when TsTx was already present, no additional current block was caused by DTX (Figures 9,10).

The 80-100% of Kv1 channels that contain Kv1.2 in murine MNTB neurons is a substantially larger percentage than the 50-60% in rat MNTB neurons. All the rat MNTB neuron Kv1 channels included Kv1.1 because either DTX or DTX-K blocked 90% of the total sustained current at –47 mV, but only 50% was TsTx-sensitive and therefore due to Kv1.2-containing channels (Brew and Forsythe, 1995; Dodson et al., 2002). Rat MNTB neurons also lack the large DTX-insensitive A-currents found in murine MNTB neurons (Figure 8 and equivalents in Brew and Forsythe, 1995; Brew et al., 2003). These different potassium currents may reflect species differences in information processing by MNTB. For example, the rat medial superior olive (MSO) receives substantial inhibitory input from MNTB (Smith et al., 2000) whose phase-locking is thought to improve MSO neurons’ encoding of interaural time difference, a useful cue for localizing low frequency sound sources, but mice hear only at higher frequencies (more suited to interaural level comparisons by LSO) and their MSO is
correspondingly vestigial. Perhaps very negatively activating Kv1 channels facilitate the preservation of phase-locked information across the giant synapse onto rat MNTB neurons, by ensuring consistent resetting of the membrane between every synaptic event, whereas potassium channels with less extreme properties may be suited to the accurate temporal representation of the sound envelope to be used in the murine LSO level comparison (possibly by increasing membrane resistance at rest and maximum firing rates). Note that when Kcna2 expression was reduced in the +/- MNTB neurons with only one Kcna2 gene, they became very similar to rat MNTB neurons in terms of their percentage of Kv1 channels containing Kv1.2, because in both cases TsTx block was 50-60% of the DTX current block amplitude.

Next, we explore the possible stoichiometries of Kv1 channels in +/- and +/- MNTB neurons, in terms of their Kv1.2 subunits. Because the +/- neurons each possess a single Kcna2 gene, they probably have half the usual +/- copy numbers of Kcna2 mRNA (as in whole brain, Figure 1D) and of Kv1.2 subunit production, accounting for a smaller proportion of their Kv1 current being TsTx-sensitive. This probably also means that the +/- Kv1.2-containing channels would have fewer Kv1.2 subunits per channel, on average, than the Kv1.2-containing channels in +/- MNTB neurons. The channel stoichiometries can be estimated if we assume that subunits assemble randomly into tetramers (binomial assembly of Kv1.2 and non-Kv1.2 subunits) and that stoichiometry does not affect the surface expression of channels. The mean proportional TsTx-sensitivity in +/- MNTB neurons can be approximately simulated using binomial assembly if the Kv1 subunit population consists of 20% Kv1.2 subunits, resulting in 59% of Kv1 channels containing at least one Kv1.2 subunit. This 59% comprises 41% containing a single Kv1.2 subunit and 18% containing two or more Kv1.2 subunits. If +/- MNTB neurons produced the same numbers of non-Kv1.2 subunits as +/- neurons, and twice the numbers of Kv1.2 subunits, their overall proportion of Kv1.2 subunits would be 33%. Using binomial assembly, this would result in 80% of the Kv1 channels containing Kv1.2, not dissimilar to the values found for +/- Kv1 channel TsTx-sensitivity (80% to 90%, excluding 3 values greater than 100%, Figure 10). Of these simulated +/- channels, 40% contain a single Kv1.2 subunit and another 40% contain two or more Kv1.2 subunits. Hence, we estimate that more than two thirds of the Kv1.2-containing channels in +/- neurons contain only one Kv1.2
subunit (xxx2) whereas at least half of the Kv1.2-containing channels in +/- neurons possess two or more Kv1.2 subunits (xx22 or x222).

Stoichiometry and differences in $V_{\text{half}}$ between MNTB neuron Kv1 channel populations

The results suggest that there are at least three different $V_{\text{half}}$ values for MNTB neuron Kv1 channels containing zero, one or two Kv1.2 subunits, as follows. Our best estimates of $V_{\text{half}}$ were $-60$ mV for +/- Kv1 channels (Kv1.2-free) and $-51$ mV for +/- Kv1 channels (at least 80% Kv1.2-containing channels). In principle, different proportions of Kv1 channels having one or other of these two $V_{\text{half}}$ values could produce the mean $V_{\text{half}}$ of $-55$ mV for +/- Kv1 channels, and explain the significant correlation between $V_{\text{half}}$ and TsTx-percentage block in individual +/- MNTB neurons (Figure 13B). However, if there were also distinct $V_{\text{half}}$ values for Kv1 channels containing one or two Kv1.2 subunits (xxx2 versus xx22) this could explain why the $V_{\text{half}}$ values from the subpopulation of +/- channels that contained Kv1.2 were also significantly correlated with the TsTx-percentage block (Figure 13C). In oocytes, four distinct $V_{\text{half}}$ values have been demonstrated for channels formed from Kv1.1 and Kv1.2 subunits (stoichiometries 1111, 1122, 1222 and 2222 had $V_{\text{half}}$ of $-31$ mV, $-27$ mV, $-23$ mV and $-16$ mV respectively (Akhtar et al., 2002). An unknown modulating factor may cause Kv1 channel $V_{\text{half}}$ values to be approximately 20 mV more negative in auditory neurons than in oocytes (Trussell, 1999). Our MNTB neuron results suggest that any such modulating factor does not overwhelm $V_{\text{half}}$ differences between channels containing different numbers of Kv1.2 subunits.

The +/- Kv1 channels’ 10 mV negative shift in $V_{\text{half}}$ relative to +/- channels suggests they may be dominated by Kv1.1 subunits, or even be Kv1.1 homomers, because the published literature suggests Kv1.1 subunits would be the only Kv1 subunit type likely to exert a strong negative effect on $V_{\text{half}}$ when substituting for Kv1.2 subunits. In oocyte expression systems, the average $V_{\text{half}}$ values for each of Kv1.1 homomers, Kv1.2 homomers and Kv1.6 homomers were $-33$ mV, $-26$ mV and $-17$ mV and other Kv1 homomer types had $V_{\text{half}}$ of $-26$ mV to $+4$ mV (Stuhmer et al., 1989; Grupe et al., 1990; Swanson et al., 1990; Grissmer et al., 1994; Hopkins et al., 1994; Kalman et al., 1998; Lang et al., 2000; Akhtar et al., 2002). One caveat to this is that in some mammalian cell lines, Kv1.2 homomers had much more positive $V_{\text{half}}$ values (Werkman et al., 1992;
Grissmer et al., 1994). Preliminary experiments showed $-/-$ \( I_{\text{kl}} \) had high sensitivity to block by external tetraethylammonium (TEA, \( K_d < 1 \) mM) in contrast to $+/+$ \( I_{\text{kl}} \) (> 5 mM, unpublished data from J.X.G. and H.M.B.). The \( K_d \) of expressed Kv1.1 homomers was also below 1 mM (0.3-0.6 mM) contrasting with homomers of Kv1.6 (4 mM, 7 mM) or homomers of Kv1.2, Kv1.3, Kv1.4 or Kv1.5 (> 10 mM (Stuhmer et al., 1989; Grupe et al., 1990; Swanson et al., 1990; Grissmer et al., 1994). Hence $-/-$ Kv1 channels are probably Kv1.1 homomers or have 111x stoichiometry, given that TEA block is additive (Heginbotham and MacKinnon, 1992).

**Kv1.1-dominated channels, Kvbeta2 and surface expression**

A strong surface expression of Kv1.1 homomers in $-/-$ CNS would be particularly interesting both because Kv1.1 homomers have not been detected in mammalian brains (Shamotienko et al., 1997; Coleman et al., 1999; Wang et al., 1999) and because Kv1.1 subunits possess a strong ER retention motif (Shi et al., 1996). Though this retention motif is also present in Kv1.2 and Kv1.6 subunits, Kv1.1 subunits exhibit the greatest retention in expression systems (95%) and can apparently dominate retention in heteromers with one or two subunits of Kv1.2, or Kv1.4 which lacks the motif (Manganas and Trimmer, 2000). Possibly MNTB neurons produce so much Kv1.1 and Kv1.2 that surface \( I_{\text{Kv1}} \) is substantial despite very strong ER retention, accounting for Kv1.1 and Kv1.2 antibody staining being so strongly intracellular (Brew et al., 2003). Surface Kv1 channel density might instead be limited by the availability of the accessory protein Kvbeta2, present in MNTB neurons, which facilitates surface expression (Fonseca et al., 1998; Manganas and Trimmer, 2000). However, mice lacking Kvbeta2 had only occasional seizures, lived for many months and exhibited cold-swim induced tremor, all consistent with a mild version of the \( Kcna1 \)-null phenotype (Smart et al., 1998; Zhou et al., 1998; McCormack et al., 2002).
Kv1 channel voltage-dependence in MNTB neurons and elsewhere

Some of the variation in reported $V_{\text{half}}$ values for $G_{Kv1}$ between neuronal types may arise from differences in the Kv1.1:Kv1.2 balance of their Kv1 channel populations. In auditory neurons in rodent brain slices, $G_{Kv1}$ had $V_{\text{half}}$ between $–50 \text{ mV}$ and $–45 \text{ mV}$ and toxin experiments suggested all the underlying channels contained Kv1.1, whereas a little over 50% contained Kv1.2: the percentages of TsTx-sensitive $I_{Kv1}$ were 65% in murine octopus neurons, 50% in rat MNTB and 70% in rat bushy neurons (Bal and Oertel, 2001; Dodson et al., 2002; Dodson et al., 2003). If there were a gradient of Kv1.1:Kv1.2 expression across the tonotopic axis of rat MNTB, it could underly the gradients of current amplitudes and $V_{\text{half}}$, which increased significantly from $–46 \text{ mV}$ laterally to $–50 \text{ mV}$ medially (Brew and Forsythe, 2005). Tonotopic gradients of the Kv1.1:Kv1.2 expression ratio have been detected along the mouse spiral ganglion and within chick nucleus magnocellularis (NM) the avian equivalent of bushy neurons, along with gradients of firing properties indicating larger $I_{Kv1}$ where the ratio was largest, in regions with high best frequency (Adamson et al., 2002; Fukui and Ohmori, 2004). Acutely isolated murine spiral ganglion neurons and chick NM neurons yielded the most negative reported $V_{\text{half}}$ values for auditory $G_{Kv1}$, of $–62 \text{ mV}$ and $–58 \text{ mV}$, (Rathouz and Trussell, 1998; Mo et al., 2002) perhaps suggestive of strongly Kv1.1-dominated channels.

DTX-sensitive conductances with less negative $V_{\text{half}}$ values of $–27 \text{ mV}$, $–22 \text{ mV}$ and $–5 \text{ mV}$ occur in non-auditory neurons (striatal neurons, Shen et al., 2004) and pyramidal cells in neocortex and amygdala (Bekkers and Delaney, 2001; Faber and Sah, 2004). This may be because Kv1.1 is scarce or may mean these Kv1 channels lack whatever modulating factor causes the very negative $V_{\text{half}}$ values for $G_{Kv1}$ in auditory neuron somata. At the giant synapse onto rat MNTB neurons, all the Kv1 channels contained Kv1.2 but only 37% of $I_{Kv1}$ was through Kv1.1-containing channels (Dodson et al., 2003). With binomial assembly this balance could be produced if the Kv1.1:Kv1.2 subunit ratio was 10:90, with most channels Kv1.2 homomers and 1222 channels. Based on our MNTB neuron results above, we predict that this Kv1.2-dominated channel population would have $V_{\text{half}}$ less negative than any of the values from auditory neuron somata.

Although the populations of Kv1 channels in auditory neurons probably include channels with distinct $V_{\text{half}}$ values, the voltage-dependences of these channel populations were quite steep, with slope factors between
5 and 10 mV in the present study, and 6-10 mV in previous studies (Brew and Forsythe, 1995; Bal and Oertel, 2001; Dodson et al., 2002; Mo et al., 2002; Rothman and Manis, 2003a). Similar values were found for homogenous populations of expressed homomers of Kv1.1, Kv1.2 or Kv1.6, and some Kv1.1:Kv1.2 heteromers (Stuhmer et al., 1989; Grupe et al., 1990; Swanson et al., 1990; Bosma et al., 1993; Grissmer et al., 1994; Hopkins et al., 1994; Akhtar et al., 2002). The steep voltage dependence means quite small differences in \( V_{\text{half}} \) between \(+/+\) and \(-/-\) MNTB neuron G and G\(_{Kv1}\) are associated with large changes in channel open probabilities, sufficient to account for the genotypic differences in I\(_{Kv1}\) amplitudes at particular membrane potentials (calculations not shown, but see Figure 10). By analogy, a positive shift in \( V_{\text{half}} \) of a few mV could explain why I\(_{Kv1}\) was reduced by 41% in MNTB neurons from \( Kcna1 \)-null mice (Brew et al., 2003).

If differences in I\(_{Kv1}\) are produced solely by changes in channel open probability, MNTB neuron somata may possess the same numbers and densities of Kv1 channels, irrespective of genotype (also with identical single channel conductance). In support of this, the mean G\(_{\text{max}}\) was similar in \(+/+\), \(+/-\) and \(-/-\) MNTB neurons (see Results). It is reasonable to assume that \(+/+\) MNTB neurons produce a larger total number of Kv1 subunit proteins than \(+/-\) neurons, which in turn produce larger numbers than \(-/-\) neurons, because of the different numbers of Kv1.2 subunits produced, but our data argue against the idea that \(+/+\) MNTB neurons have the highest Kv1 channel densities, at least at their somata. Hence, the factors determining Kv1 channel density may be relatively independent of Kv1 subunit production.

**Does the absence of Kv1.2 affect information processing by the -/- auditory system?**

Enlarged I\(_{Kv1}\) is likely in -/- spiral ganglion (SG) neurons whose axons are the auditory nerve fibers, and in their synaptic targets the -/- cochlear nucleus bushy cells (which send synapses to MNTB neurons) because in normal mice they exhibit MNTB-like firing, I\(_{Kv1}\) and strong Kv1.1 and Kv1.2 expression (Manis and Marx, 1991; Wang et al., 1994; Adamson et al., 2002; Mo et al., 2002; Brew et al., 2003). This enlarged I\(_{Kv1}\) would predict reduced activity in both excitatory (SG and bushy cells) and inhibitory (MNTB projections) -/- auditory pathways, but -/- auditory responses *in vivo* are harder to predict because they would depend on the balance between the two (e.g. at LSO) and there could also be compensatory synaptic changes. Results from \( Kcna1\)-
null mice also suggest auditory system abnormalities went beyond reduced $I_{Kv1}$ in SG cells, bushy cells and MNTB neurons (Brew et al., 2003) because, in vivo, spontaneous firing rates and auditory thresholds were normal in bushy and MNTB neurons, though they exhibited increased jitter to auditory stimulation, but their maximum firing rates were actually reduced, perhaps suggesting compensatory strengthening of inhibition (Kopp-Scheinpflug et al., 2003).

Preliminary behavioral data show that +/- mice do not share the auditory abnormalities found in Kcna1-null mice, which had impaired ability to detect rapid sound offsets and changes in the azimuthal location of sounds, using reflex modification audiometry (Ison et al., 2002; Allen et al., 2003) because +/- mice aged P12 to P18 performed at least as well as controls (Brew et al., 2005). This suggests that firing patterns are approximately normal in the +/- auditory nerve. One interesting possibility is that the +/- auditory nervous system might have better than normal temporal resolution on some auditory processing tasks, if enlarged $I_{Kv1}$ leads to decreased time constants for membrane potential changes.

The known link between reduced firing in auditory afferents and audiogenic seizure susceptibility (ASS) may have some relevance to the +/- phenotype. Studies showing that experimentally-induced early hearing impairments led to ASS in normal mice (e.g. Chen and Fuller, 1976) led to the idea that rodent ASS is caused by “reduction of neural activity in the auditory pathways from deafness during development” (Ross and Coleman, 2000). In GEPRs, audiogenic seizures initiate in the inferior colliculus, where there is weakened GABA circuitry, thought to occur as compensation for the reduced activity in excitatory auditory inputs caused by their hearing impairment (Faingold et al., 1986; Faingold, 2002). Although enlarged $I_{Kv1}$ is likely in +/- excitatory auditory afferents and could lead to reduced activity, we were not able to induce seizures audiogenically in young +/- mice. Note that GEPRs did also exhibit reduced latencies to flurothyl-induced seizures, as well as heightened susceptibility to seizures induced by other chemical, electrical or sensory stimuli (Reigel et al., 1986; Franck et al., 1989). However, none of the genes and chromosomes thus far linked to ASS in mice have been Kcna genes or the chromosomes containing them (Neumann and Collins, 1992; Lock et al., 1994; Skradski et al., 2001; Misawa et al., 2002).
Functionally distinct Kv1 channels and neuronal information processing

The range of $V_{\text{half}}$ values possible for different Kv1.1/Kv1.2 stoichiometries suggests different neurons could tune their channel properties for different roles. For example, Kv1 channels are found in auditory pathways which rapidly relay temporally precise signals from the cochlea to the brainstem, via large axons with large synapses, and their main role is to powerfully and rapidly reset the membrane potential between synaptic events (Oertel, 1983; Trussell, 1999). (This may be analogous to a role for Kv1 channels in axonal conduction, with large depolarizing sodium currents at nodes of Ranvier substituting for synaptic inward currents). Very negatively activating Kv1.1-dominated channels may be particularly well-suited to this role. In contrast, membrane resetting between synaptic events would be disastrous in most other neurons, where instead temporal summation is crucial and less negatively activating Kv1 channels can shape onset latencies and firing rates as they inactivate during prolonged responses (e.g. Storm, 1988; Bekkers and Delaney, 2001; Faber and Sah, 2004; Shen et al., 2004). Consistent with this, neurons within low frequency parts of chick NM had the highest Kv1.2:Kv1.1 expression ratio and the smallest threshold currents and were also most likely to perform temporal summation because they received several smaller synapses, rather than a single large synapse from one auditory nerve fiber (Fukui and Ohmori, 2004).

If the Kv1 channel density was the same in +/-, +/- and -/- MNTB neurons, even though the latters’ production of Kv1 subunits was probably reduced by deficits in Kv1.2 subunit numbers, this suggests neurons can adjust their potassium currents by fine-tuning channel properties rather than altering channel densities. This idea could be tested by generating mice with different $Kcna$ gene configurations and recording from their MNTB neurons. For example, $G_{Kv1}$ should have the same amplitude and properties in MNTB neurons with two $Kcna2$ genes and four $Kcna1$ genes as the +/- neurons described here.
Figure legends

Figure 1. Generation and verification of *Kcn2*-null mice

A) Diagram of the targeting strategy used to remove the *Kcn2* open reading frame (ORF). Homologous recombination of the *Kcn2* chromosomal locus (top) with the targeting vector construct (middle) generated a targeted locus (bottom) in which the neomycin resistance cassette (Neo) has replaced the *Kcn2* ORF.

B) Southern blot analysis of genomic DNA from offspring of a heterozygote intercross. An *EcoRV* digest probed with the 5’ (*XbaI*-*XbaI) fragment detected an 8.5 kb wildtype allele and a 11.3 kb targeted allele. In this litter there were two +/- mice, three +/- mice and one -/- mouse.

C) Western blot analysis of Kv1.2 in protein isolated from whole brains. Kv1.2 protein was detected in +/-, reduced in +/- and not detected in -/- brains. The broad band of Kv1.2 staining probably reflects variable levels of glycosylation. Blots were reprobed with anti-β-actin to control for loading.

D) Quantitative PCR (qPCR) expression of mRNA for *Kcn2*, *Kcn1* and *Kcn6* in whole brains from mice aged P14. For each qPCR experimental run, the expression of the three *Kcn* genes was measured and normalized relative to the geometric mean of the expression levels for three reference genes: β-actin; γ-actin; and succinate dehydrogenase complex, subunit A (Table S1 and Methods). The mean relative expression levels from 1-4 qPCR runs were calculated for each mouse and each gene (only two mice had only a single qPCR run per gene) before averaging across mice, and plotting the mean and s.e.m. *Kcn1* and *Kcn6* mRNA expression were similar across genotypes. *Kcn2* mRNA expression was approximately halved in +/- brain compared to +/- controls, and *Kcn2* mRNA was absent from -/- brain (the calculated -/- expression level and error bar were both smaller than the thickness of the X-axis).

Figure 2. Survival curves and seizure susceptibility in +/-, +/- and -/- mice

A) The percentage of mice surviving through each postnatal age (e.g. if they died at age P16, they survived through P15). All of the 22 -/- mice died at age P19 or younger, whereas none of the 21 +/- mice or the 45 +/+
mice died during the first 25 days. The total of 88 mice were from 14 litters, with roughly Mendelian proportions of each genotype and balanced gender proportions (45 males and 43 females). The line through the points was drawn by eye.

B) The mean latencies to the first seizure-related behavior (left bars) and running-bouncing-seizure (RBS, middle bars) for 6 +/+ mice, 15 +/- mice and 7 -/- mice, following the first drip of flurothyl onto the filter paper in the exposure chamber. The mean time between the first seizure-related behavior and the RBS is also shown (right bars). The significant differences between genotypes are denoted by single or double asterisks (p<0.05 or p<0.005, Mann-Whitney U-tests). The -/- mice differed significantly from +/+ and +/- mice on all three measures.

Figure 3. The developmental expression in brainstem of mRNA for *Kcna2*, *Kcna1* and *Kcna6*

A) The expression of *Kcna2* mRNA in +/+ brainstems from 14 mice sacrificed at ages P1 through P29. Expression is given relative to the geometric mean of the three control genes β-actin, γ-actin and hydroxymethylbilane synthase (Table S1 and Methods). At each age, the expression level is plotted for two mice, as the mean of 3-5 qPCR measurements (s.e.m. also plotted for each mouse using either narrow or wide error bars). The line is a Boltzmann fit to the 14 mean values plotted.

B) Relative mRNA expression as in B, but shown as the average of both mice at each age, for *Kcna2* (triangles) *Kcna1* (inverted triangles) and *Kcna6* (diamonds). At each age, *Kcna1* and *Kcna6* expression are shown as the mean and s.e.m. for 4 qPCR measurements, two from each mouse. *Kcna2* expression was the mean and s.e.m. from 6-9 qPCR measurements. Each line is a Boltzmann fit to the 7 mean values for that gene.

Figure 4. -/- MNTB neurons are less excitable by current pulses than +/+ neurons

The threshold currents and AP numbers were measured from +/+, +/- and -/- MNTB neurons subjected to 180 ms duration current pulses –100 to 200 pA, applied at 1 s intervals, incrementing by 10 pA.
A) Example responses of typical +/+, +/- and -/- MNTB neurons to the current pulse protocol, shown here at 50 pA intervals. The linked arrows on the upper traces mark the time during which sustained membrane potential was measured (used for Figure 5 and for calculating the input resistances and resting potentials as described in the Results text and Table S2).

B-D) Summary data on threshold currents and AP numbers from 21 +/+ neurons (filled circles) 17 +/- neurons (triangles) and 25 -/- neurons (open circles). For each neuron, data came from a single run of the current clamp protocol. Statistical tests were two-tailed unpaired t-tests assuming equal variance. Significant differences between the genotypes are shown by single, double or triple symbols (p<0.05, P<0.01 and p<0.005, respectively).

B) The threshold current amplitudes required to generate at least one AP in the MNTB neurons of each genotype. The threshold current amplitudes were larger in -/- neurons and +/- neurons than in +/+ neurons. Asterisks show significant differences between the genotypes.

C) The mean number of APs generated at each current pulse amplitude. Error bars show the s.e.m. The -/- and +/- neurons fired significantly fewer APs than the +/+ neurons. Significant differences between +/+ and -/- values are shown by small asterisks, between +/+ and +/- values by large asterisks and between +/- and -/- values by circumflexes.

D) The mean numbers of APs during the five largest current steps tested (160 pA through 200 pA). This value was termed the APN(160to200) and it was largest in +/+ neurons, smallest in -/- neurons and intermediate in +/- neurons. Asterisks show significant differences between the genotypes.

Figure 5. Sustained potentials were more depolarized in +/+ than in -/- or +/- MNTB neurons.

A) The mean sustained potential responses to current pulses, measured in ASCF for 21 +/+ MNTB neurons and 13 -/- neurons. Depolarizing current pulses led to larger depolarizations in the +/+ neurons than the -/- neurons. (Same current pulse protocol as in Figure 4, same +/+ and -/- neurons as in Figure 4C and Table S2, except excluding data from 12 -/- neurons to which only depolarizing pulses were applied. These 12 neurons’ mean responses were identical to within 1 mV of the 13 -/- neurons shown). Sustained potential was defined as the
mean of 600 data points towards the end of the current pulse, during the 30 ms period marked by linked arrows in Figure 4A. The sustained potentials were corrected for current flow across the pipette access resistance.

S.e.m. is shown by error bars, sometimes obscured by the size of the symbols. Significant differences between genotypes are denoted by single and double asterisks (p<0.05 and p<0.001).

B) As in A, but comparing 17 +/- neurons with the 21 +/+ MNTB neurons. Depolarizing current pulses led to larger depolarizations in the +/+ neurons than the +/- neurons.

**Figure 6. Dendrotoxin has larger effects on -/- MNTB neuron excitability than on +/+ MNTB neuron excitability.**

The responses of MNTB neurons to –100 pA through 200 pA pulses (protocol as in Figure 4) were measured in control ACSF, then in DTX (ACSF containing 100 nM DTX). For panels C-F the data from each neuron were averaged from 2-3 runs of the protocol, before averaging across 5 +/+ neurons or 4 -/- neurons.

A) The responses of an example +/+ MNTB neuron in DTX. (Same neuron as in Figure 4A, recorded several minutes previously in control ACSF).

B) Responses of an example -/- neuron in DTX (Control data from same neuron shown in Figure 4B).

C) The mean numbers of APs generated at each current pulse amplitude for +/+ and -/- neurons in ACSF and in DTX. In DTX the AP numbers were similar across genotypes.

D) The mean threshold current amplitudes in control ACSF and in DTX for +/+ neurons (black bars) and -/- neurons (white bars). Before DTX application, the -/- threshold currents were significantly larger than in +/+ (left bars) but DTX abolished the differences, reducing the threshold currents to approximately 40 pA for each genotype (middle bars). The magnitude of the DTX-induced decrease in threshold current was significantly larger for the -/- neurons (right bars). Single and double asterisks denote significant differences (at p<0.05 and p<0.001, respectively).

E) The mean sustained potentials (measured as in Figure 5) from 5 +/+ neurons and 4 -/- neurons in response to current pulses in ACSF (circles) and in DTX (triangles). The -/- neurons (open symbols) had stronger I-V rectification in ACSF than the +/+ neurons (filled symbols) and the -/- rectification arose at a more negative
potential. In DTX, the rectification was abolished and the responses became linear in each individual MNTB neuron (data not shown). The DTX responses are shown only up to the current amplitude where all neurons had measurable sustained potentials (rather than spiking tonically as in most examples of panels A,B). The extrapolated linear fits were very similar for the mean DTX responses of -/- neurons (open triangles, solid line) and +/- neurons (filled triangles, solid line, shown slightly lengthened to distinguish it from -/- fit) suggesting that in the presence of DTX, neurons of each genotype have similar background leak conductance.

Figure 7. Outward K+ currents in +/-, +/- and -/- MNTB neurons

A) The qualitatively similar currents recorded from two MNTB neurons, each typical of their genotypes (+/+, left; +/-, right), in response to voltage clamp test pulses –107 mV through –37 mV, from within the standard I-V protocol (test pulses were applied in 10 mV increments between –107 mV and +23 mV, at 1.3 s intervals, each preceded by a -67 mV prepotential and followed by a -37 mV postpotential, see Methods). Slices were perfused with ACSFV (see Methods). A large transient A-type potassium current was visible during the –37 mV postpotential if the test pulse was –67 mV or more negative, but its inactivation by the –67 mV prepotential meant it was absent or very small during test pulses. The bars show the times of measurement of sustained currents for panels B and C.

B) The sustained current amplitudes from 11 +/- neurons (8 mice) and 10 -/- neurons (6 mice) at test potentials –107 mV through +23 mV. For each run of the standard I-V protocol, the sustained current amplitudes were measured towards the end of each test pulse (during time marked by bars in A) and leak currents were subtracted (see Methods). Then, for each neuron, the sustained current amplitudes were averaged across 2-3 runs of the I-V protocol, before averaging across the neurons within each genotype. Asterisks indicate significant differences between +/- and -/- neurons (p<0.05, Mann-Whitney U-test).

C) The boxed region from panel B, plotted on an expanded scale to highlight the potential range where there were significant differences between genotypes (asterisks as in B). Also shown are the sustained currents from 8 +/- neurons (7 mice). There were no significant differences between the +/- and +/- currents. Circumflexes (hats) indicate significant differences between +/- and -/- current amplitudes (p<0.05, Mann-Whitney U-tests).
Figure 8. The timecourse of $I_{\text{leak}}$ in +/+, +/- and -/- MNTB neurons

A) The current responses of individual +/+, +/- and -/- neurons during the first 10 ms of -57 mV pulses from the standard I-V protocol (same neurons as in Figure 7C). To facilitate visual comparisons of amplitude and kinetics, we subtracted capacitative and leak currents (see Methods) and also offset the baseline current at -67 mV to zero. Each current trace increased in amplitude with an approximately single exponential timecourse. Arrows show neurons with current amplitudes that were outliers for their genotype (as defined in Figure 11A below).

B) The mean time constants of the current increases shown in A at -57 mV (left bars, same neurons as in A) or at -47 mV (right bars, at this potential one -/- neuron could not be included because it yielded a poor fit; it was the -/- neuron with tiny current arrowed in panel A). The time constants were determined by fitting a single exponential curve to the current from each neuron before averaging across neurons (see Methods for fitting details and criteria).

Figure 9. The effects of TsTx and DTX in +/+, +/- and -/- MNTB neurons

A) The sustained current amplitudes in response to -47 mV test pulses for an example MNTB neuron of each genotype (+/+, left; +/-, middle; -/-, right). The currents were measured first in control ACSFV, then while the perfusion solution was switched to ACSFV containing 100 nM TsTx and finally to ACSFV containing both 100 nM TsTx and 100 nM DTX-I (times of toxin applications shown by black bars). In the +/- neuron, TsTx led to substantial current block but the additional presence of DTX caused no further block (left). In the +/- neuron, TsTx led to substantial current block and the addition of DTX also led to substantial current block (middle). In the -/- neuron, TsTx had no effect but DTX led to substantial current block. Here leak current was not subtracted because most data points came from the toxin-monitoring protocol and only 9-12 datapoints came from the I-V protocols which allow leak current subtraction (see Methods). For each neuron in each solution, 3 runs of the standard I-V protocol described in Figure 7 were applied at times when the currents appeared approximately
stable (e.g. shown by the letters c, t and d in each panel which also denote when the data in panel B were
collected). For some neurons the “high resolution” I-V protocol was also applied for 3 runs per solution (see
Methods).

B) The current responses during –47 mV pulses of the same example neurons as in A (directly above) at the
approximate times shown by letters c, t or d in panel A, i.e. in ACSFV, TsTx or (TsTx+DTX). Each trace is the
average response from 2-3 runs of the standard I-V protocol. In the +/- neuron, the current block appeared
identical in the TsTx solution and the (TsTx+DTX) solution (left). In the +/- neuron, the current amplitude in
TsTx fell about halfway between the control amplitude and that in the solution containing both toxins (middle).
In the -/- neuron, the current appeared identical in the control ACSFV or in the TsTx solution, and only the
(TsTx+DTX) solution led to substantial current block (right). These characteristic effects of toxins on the
current amplitudes of neurons of each genotype were as evident at the beginning of test pulses as at the end,
when sustained current amplitudes were measured (see bars in Figure 7A).

C) The voltage-dependence of the mean sustained current amplitudes in +/-, +/- and -/- neurons, in control
ACSFV (c, solid lines) ACSFV plus TsTx (t, dashed lines) and ACSFV plus TsTx and DTX-I (d, dotted lines).
For each neuron, the currents from each run of the standard IV protocol were subjected to leak subtraction (see
Methods) before averaging the amplitudes from 2-3 runs per solution. The inserts show the mean current
amplitudes for all the test pulses –107 mV through +23 mV, and the larger panels show the same data plotted on
an expanded scale to highlight the toxins’ effects in the range of potentials relevant to Kv1 channels. In the +/-
neurons, the current amplitudes at each potential were similar in TsTx or (TsTx+DTX). In the +/- neurons, TsTx
led to substantial current block and (TsTx+DTX) caused even greater block. In the -/- neurons, TsTx had no
effect at any potential and DTX led to substantial current block.

**Figure 10. Proportional sensitivities to TsTx and DTX in MNTB neurons at –47 mV**

A) The amplitudes of sustained current blocked at –47 mV for each of 21 MNTB neurons by TsTx (dark gray
bars, control current minus current in TsTx) and by subsequent addition of DTX (light gray bars, TsTx current
minus current in TsTx+DTX solution). If both subcomponents are positive, then the heights of the combined
light and dark gray bars reflect the total current blocked by both toxins, or $I_{Kv1}$ (also shown by the vertical
extent of the black bars in Figure S2A). Only very small current shifts were ever associated with DTX addition
in +/+ neurons (light gray, left) or TsTx application in -/- neurons (dark gray, right).

B) The proportion of the total toxin block at $-47$ mV that is accounted for by TsTx in each of the neurons
shown in A. The TsTx-sensitive current (control current minus current in TsTx solution) was divided by $I_{Kv1}$
(control current minus current in TsTx+DTX solution). At $-47$ mV, the average TsTx percentage block relative
to TsTx+DTX block was 98% for +/+ neurons, 56% for +/- neurons and 1.2% for -/- neurons.

**Figure 11. Outward $K^+$ current amplitudes in individual MNTB neurons of each genotype**

A) A bar plot of the sustained current amplitudes in ACSFV for 29 MNTB neurons at $-47$ mV. The amplitudes
were approximately normally distributed for each genotype, with the exception of 2 +/+ neurons, 1 +/- neuron
and 1 -/- neuron with amplitudes that were outliers for their genotype (asterisks, outliers defined as being more
than 1.5 times the interquartile range above the upper quartile or below the lower quartile, both at this test
potential and at one or both of the test potentials $-57$ mV or $-67$ mV).

B) I-V relations plotted as in Figure 7C, but excluding 4 outlier neurons (the neurons with outlier values of
sustained current, asterisked in A). Small asterisks indicate significant differences between +/+ and -/- neurons,
circumflexes indicate significant differences between +/- and -/- neurons and large asterisks indicate significant
differences between +/+ and +/- neurons. All symbols indicate significance levels of $p<0.005$ (two-tailed
unpaired t-tests assuming equal variance).

**Figure 12. Voltage-dependence curves for +/+, +/- and -/- $G_{kl}$ and $G_{Kv1}$**

A-C) Symbols show $G/G_{max}$ values from individual MNTB neurons plotted against test potentials $-87$ mV
through $-37$ mV (see Methods for conductance calculations and fitting methods). The conductances were
calculated from the current responses obtained using the standard IV protocol (or the high resolution IV protocol
if available) after leak subtraction and averaging across 2-3 runs. Fits of Boltzmann functions to these data are
shown as solid lines, or dashed lines for slightly “less good” fits (see Methods). The gray bars are visual aids showing the range –57 mV to –48 mV.

A) Symbols show $G_{\text{KL}}/G_{\text{max}}$ values plotted against test potential for 10 $+$/+$+$ neurons (top) 8 $+$/-$-$ neurons (middle) and 9 $-$/-$-$ neurons (bottom, same neurons as in Figure 7C but excluding one $+$/+$+$ neuron and one $-$/-$-$ neuron which yielded poor fits). The $V_{\text{half}}$ values for the $+$/+$+$ neurons and $-$/-$-$ neurons fell in distinct ranges whereas the $+$/-$-$ neurons had intermediate $V_{\text{half}}$ values (approximately within the gray bar).

B) $G_{\text{Kv1}}/G_{\text{max}}$ values from individual MNTB neurons plotted against test potentials for 17 MNTB neurons (same neurons as in Figures 9, 10 and S2, but excluding two $+$/+$+$ neurons and two $-$/-$-$ neuron yielding poor fits, and one $+$/-$-$ neuron for which the DTX-sensitive component was too small to be well-fitted). The mean $V_{\text{half}}$ values and their ranges and the genotypic differences between them were generally similar to those from panel A, though the mean $V_{\text{half}}$ values were a little more negative than those in panel A.

C) Fits as in A and B, but to the distinct components of $+$/-$G_{\text{Kv1}}$ either TsTx-sensitive or TsTx-insensitive but DTX-sensitive. The TsTx-sensitive components’ $G_{\text{Kv1}}/G_{\text{max}}$ became half-activated at less negative potentials than the TsTx-insensitive components’ $G_{\text{Kv1}}/G_{\text{max}}$, but the $V_{\text{half}}$ values for these two components did not fall neatly into the $+$/+$+$ and $-$/-$-$ ranges, respectively.

Figure 13. Relationship between $V_{\text{half}}$ and Kv1.2 subunit content

A) The $V_{\text{half}}$ values from the fits to total $G_{\text{KL}}$ shown in Figure 12A, plotted against the $Kcna2$ gene dosage for each neuron. The regression line through all 27 points shows a significant correlation (p<0.01).

B) The $V_{\text{half}}$ values from the fits to $G_{\text{Kv1}}$ shown in Figure 12B, plotted against the estimated proportion of channels containing at least one Kv1.2 subunit. In the case of $+$/+$+$ and $-$/-$-$ neurons, we estimated these proportions were 0% and 100%, whereas for $+$/-$-$ neurons the estimate was taken as the percentage of $I_{\text{Kv1}}$ blocked by TsTx at –47 mV. Here the regression line is through only the 7 $+$/-$-$ points, and shows a significant correlation (p<0.05). This supports the idea that $+$/-$-$ Kv1 channel $V_{\text{half}}$ values are determined by the proportion of Kv1.2-containing channels. The clusters of $-$/-$-$ and $+$/+$+$ $V_{\text{half}}$ values falling on the regression line suggest that
most +/- neurons have channel populations with $V_{\text{half}}$ values distinct from the other two genotypes, and that the two more negative +/+ $V_{\text{half}}$ values may be due to distorted data (see Results and Methods).

C) As in B, but including only TsTx-sensitive channels. The $V_{\text{half}}$ values from fits to +/+ $G_{Kv1}$ and TsTx-sensitive +/- $G_{Kv1}$ (shown in Figure 12C) are plotted against the estimated proportion of channels containing at least one Kv1.2 subunit. Again the regression line is through only the 7 +/- points, and shows a significant correlation ($p<0.05$). This suggests that +/- Kv1 channels including Kv1.2 can have different $V_{\text{half}}$ values, probably depending on the number of Kv1.2 subunits each channel contains.

**Supplementary Figure S1. Justification for exclusion of 5 MNTB current clamp recordings**

This supplementary figure shows data from the original total 68 current clamp MNTB recordings which met the criteria for inclusion of resting potentials larger than –57 mV and pipette access resistance below 20 MΩ. (see Methods). This includes 5 hyperexcitable recordings (arrowed in panels A,B) that were excluded from Figures 4,5 and Table S2. Similar high-spiking outliers among murine MNTB neurons were reported and graphed in (Brew et al., 2003) and neurons with small or no $I_{kl}$ were previously reported in rat MNTB and suggested to be non-principal neurons, synaptic terminals or neurons whose processes had been cut off (Brew and Forsythe, 1995). These did not seem to be due to unhealthy slices, because recordings with typical properties could be obtained later from the same slice, or another slice from the same animal. The 5 high-spiking recordings often included tonic firing throughout current pulses, which meant they did not attain the sustained steady potentials which were addressed by Figure 5 and used for many parameters of Table S2. For consistency, they were also excluded from Figure 4. Their exclusion from Figure 4D was also beneficial because it allowed the neurons with more typical APN(160to200) values to be plotted on a scale that allowed clear visualization of their distributions, essential for seeing whether some +/- and -/- neurons might have wholly or partly compensated for their $Kcna2$ deficit. Because only 5 of a total 68 recordings were hyperexcitable, and they were evenly distributed between the genotypes, there were only small effects on mean parameter values when they were excluded. The main statistical differences between the genotypes were present before the exclusion, as shown in panels A,B for APN(160to200) values and threshold currents. Also, the 68 MNTB neurons of all three
genotypes differed significantly in their AP numbers compared across all current amplitudes (p<0.01, Kolmogorov-Smirnoff test).

A) The APN(160to200) plotted as in Figure 4D, but including 5 extremely hyperexcitable recordings which were statistical outliers (arrowed) and were evenly distributed between the genotypes (2,2,1). The triple asterisks indicate significant differences between MNTB neurons of each genotype (p<0.005, Mann-Whitney U-tests). The +/- and -/- neurons did not differ significantly on this parameter. All 5 arrowed neurons were statistical outliers according to the definition of being more than 1.5 times the interquartile distance above the upper quartile or below the lower quartile. Technically, two additional -/- neurons were also outliers according to this definition, but this was mainly because the -/- neurons as a group fired so few APs it made their variability very small, hence these neurons were included in the data analyses of Figures 4, 5 and Table S2.

B) The threshold current amplitudes from all 68 neurons, including the 5 high-spiking neurons (arrowed). These 5 recordings were not statistical outliers in terms of this parameter. The single or triple asterisks indicate significant differences between the genotypes using two-tailed unpaired t-tests (p<0.05 and p<0.005, respectively).

Supplementary Figure S2. Relative proportions of Kv1 and non-Kv1 current in MNTB neurons

A) The sustained current amplitudes at –47 mV for the 21 MNTB neurons to which both toxins were successfully applied. The total height of each bar shows the total sustained current amplitude in ACSFV, broken down into two components: the light grey section shows the current amplitude that remained when both TsTx and DTX were present; and the black shows the amplitude of current block by TsTx and DTX combined. In all but three neurons, the black bars made up more than 50% of the total amplitude, showing that most of their current at –47 mV was toxin-sensitive. In the other three neurons the black bars comprised less than 50% of the current (asterisks). These were also 3 of the 4 outliers in terms of current amplitudes, as defined in Figure 11A.

B) The mean amplitudes of sustained current (solid lines) and its toxin sensitive component (dashed lines) calculated from the same neurons as in A, but excluding 3 neurons in which the toxin-sensitive component
comprised less than 50% of the total (asterisked in A). The toxin-sensitive component comprised approximately 70-80% of the total sustained current.

References


Zhang CL, Messing A, Chiu SY (1999) Specific alteration of spontaneous GABAergic inhibition in cerebellar
Table S1. Primers used for qPCR and genotyping

Primer sequences for three \textit{Kcna} genes and the four reference genes used in the final normalizations, but not the other six reference genes (albumin 1; glyceraldehyde-3-phosphate dehydrogenase; hypoxanthine guanine phosphoribosyl transferase; TATA binding protein; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; and ubiquitin C). Key to gene IDs for which primers are shown: Actb=\(^{\beta}\)-actin; Actg=\(^{\gamma}\)-actin, Hmbs=hydroxymethylbilane synthase, Sdha=succinate dehydrogenase complex, subunit A. \textit{Kcn1}, \textit{Kcn2} and \textit{Kcn6}=members 1, 2 and 6 of shaker-related subfamily of potassium voltage-gated channel subunits. Neo=the neomycin resistance cassette.

| Gene ID | Forward primer  | Reverse primer  | Product
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<td>Neo</td>
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### Table S2. Excitability parameters in *Kcna2* +/+ , +/- and +/- MNTB neurons

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<th>Parameter</th>
<th>+/+ , n=21</th>
<th>+/− , n=17</th>
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<th>+/−:-/+</th>
<th>−/− , n=25</th>
<th>−/−:+/+</th>
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<tr>
<td>APN(160to200)</td>
<td>2.9 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td>***</td>
<td>*</td>
<td>0.86 ± 0.09</td>
<td>***</td>
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<td>Threshold current pA</td>
<td>95 ± 5</td>
<td>128 ± 8</td>
<td>***</td>
<td>*</td>
<td>151 ± 8</td>
<td>***</td>
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<td>Sustained potential at 100 pA mV</td>
<td>-55.1 ± 0.73</td>
<td>-57.8 ± 1.2</td>
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<td></td>
<td>-59.2 ± 0.65</td>
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<td>Resting potential mV</td>
<td>-64.9 ± 0.9</td>
<td>-67.4 ± 1.3</td>
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<td></td>
<td>-66.4 ± 0.9</td>
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<td>Input resistance (0-20 pA) MΩ</td>
<td>134 ± 14</td>
<td>126 ± 8</td>
<td>***</td>
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<td>90.0 ± 8</td>
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<td>Input resistance (-67 mV) MΩ</td>
<td>148 ± 10</td>
<td>125 ± 9</td>
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<td>90.5 ± 6 (22)</td>
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**Features of 1st AP at 200 pA**

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<tr>
<th>Parameter</th>
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<th>+/−:-/+</th>
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<td>Latency ms</td>
<td>2.7 ± 0.2 (20)</td>
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<td>3.1 ± 0.2 (24)</td>
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<td>Inflexion potential mV</td>
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<td>AP duration ms</td>
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<td>Latency of threshold AP ms</td>
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<td>5.3 ± 0.38</td>
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<td>Latency of subthreshold peak ms</td>
<td>8.3 ± 0.53 (20)</td>
<td>7.3 ± 0.42</td>
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<td>5.7 ± 0.41 (22)</td>
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<td>Pipette series resistance MΩ</td>
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<td>Postnatal age of mouse days</td>
<td>11.9 ± 0.48 (16)</td>
<td>11.9 ± 0.42 (12)</td>
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<td>12.6 ± 0.31 (22)</td>
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<td>Capacitance pF</td>
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<td>15.1 ± 0.5 (16)</td>
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<td>16.5 ± 0.64</td>
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<tr>
<td>APN(160to200) (P9 to P11)</td>
<td>3.27 ± 0.50 (9)</td>
<td>1.43 ± 0.22 (7)</td>
<td>*</td>
<td>**</td>
<td>0.65 ± 0.13 (8)</td>
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<td>APN(160to200) (P12 to P16)</td>
<td>2.52 ± 0.58 (10)</td>
<td>1.08 ± 0.16 (10)</td>
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<td></td>
<td>0.96 ± 0.11 (17)</td>
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Values are mean ± s.e.m. for the same neurons as in Figure 4, except where numbers in parentheses indicate reduced datasets. Statistical tests were two-tailed unpaired Student's t-tests. Asterisks show significant differences for comparisons described at top of columns: *= p<0.05, **=p<0.01 and ***=p<0.005. Parameter measurements not already described in legends to Figures 4,5 or Methods were as follows. Input resistance was measured using the difference in the sustained responses to 0 pA and 20 pA pulses, or by fitting potentials in the 10 mV range centered on –67 mV (between –72 mV and –62 mV). (3 −/− neurons were missing from the latter measure because the sustained potential responses had not been recorded in the relevant range, because they had resting potentials smaller than –60 mV and were tested only with depolarizing current pulses). AP features: inflexion potential was the sampled potential after which the depolarization rate exceeded 20 mV/ms; latency was the latency of the inflexion potential, relative to the start of the current pulse; AP duration was measured using a cursor at –27 mV.
A

Kcna2 gene
probes:

Targeting Vector

Mutant Gene

B Diagnostic digest: EcoRV 5' XbaI probe:

mutant band 11.3 kb
8.5 kb genomic band

C

β-actin

Kv1.2 protein

D

Kcna mRNA expression (relative to control genes)

Kcna2 Kcna1 Kcna6
A

Postnatal age (days)

+/-, n=21

and

+/-, n=45

-/-, n=22

Percentage survival

B

Latency to RBS

Latency to first seizure-related behavior

Duration of seizure-related behavior

+/-, n=6

+/-, n=15

-/-, n=7

**

*

*
**A**

Postnatal age (days)

Kcna2

mRNA expression in +/+ brainstem, relative to three control genes

**B**

Postnatal age (days)

Kcna1, Kcna2, Kcna6

mRNA expression in +/+ brainstem, relative to three control genes
A) Wildtype MNTB neuron

B) Threshold current for AP

C) Mean numbers of APs at each current pulse amplitude

D) Mean numbers of APs (160 pA to 200 pA)

Kcna2-heterozygous MNTB neuron

Kcna2-null MNTB neuron

Mean numbers of APs during pulses of 160 pA to 200 pA

+/-  n=21

+/-  n=17

-/-  n=25

Mean no. of spikes in 180 ms pulse

Current step amplitude (pA)
B  
Ikl activation time constants

<table>
<thead>
<tr>
<th>Voltage</th>
<th>n (at -57 mV)</th>
<th>n (at -47 mV)</th>
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<td>11, 8, 9</td>
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<tr>
<td>-/-</td>
<td>10</td>
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A

B

C $+/+$ $+/-$ $-/-$

nA $+/+$ $n=7$ $+/-$ $n=8$ $-/-$ $n=6$

Membrane potential (mV)

I(sustained), at -47 mV, including I(leak)
A

Control - TsTx
TsTx - (DTX & TsTx)

B

TsTx-sensitive I(sustained)/
(DTX & TsTx)-sensitive I(sustained)

+/+ 98%
+/− 56%
−/− 1.2%
**Figure A**

Membrane potential (mV)

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<tr>
<th>Membrane Potential (mV)</th>
<th>I(sustained) at -47 mV (nA)</th>
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<tr>
<td>+/+</td>
<td><strong>0.6</strong></td>
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<tr>
<td>+/-</td>
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<td>-/-</td>
<td><strong>0.4</strong></td>
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**Figure B**

-/+, n=9
+/-, n=7
/-, n=9

Membrane potential (mV) vs. I(sustained) (nA)
Figure 8: Ion Channel Conductance and Half-Activation Potential

A: Total G in ACSF

- Half-activation potential: $V_{\text{half}} = 44.0 \text{ mV}$
- Standard deviation: +/-
- Sample size: $n=8$

B: Toxin-sensitive $G_{K_{V1}}$

- Half-activation potential: $V_{\text{half}} = 48.9 \text{ mV}$
- Standard deviation: +/-
- Sample size: $n=6$

C: TsTx-sensitive $G_{K_{V1}}$

- Half-activation potential: $V_{\text{half}} = 52.4 \text{ mV}$
- Standard deviation: +/-
- Sample size: $n=7$

D: TsTx-insensitive but DTX-sensitive $G_{K_{V1}}$

- Half-activation potential: $V_{\text{half}} = 55.9 \text{ mV}$
- Standard deviation: +/-
- Sample size: $n=4$

Graphs show the normalized conductance ($G/G_{\text{max}}$) as a function of membrane potential (mV).
A. $V_{\text{half}}$ values from fits to total $G$
- $-65$ to $-35$ mV
- $+/-$ neurons
- $+/+$ neurons
- $R = 0.91$
- $n = 25$
- $p < 0.01$

B. $V_{\text{half}}$ values from fits to toxin-sensitive $G_{Kv1}$
- $-65$ to $-35$ mV
- $0$ to $100$
- $R = 0.75$
- $n = 7$, $+/-$ only
- $p < 0.05$

C. $V_{\text{half}}$ values from fits to TsTx-sensitive $G_{Kv1}$
- $-65$ to $-35$ mV
- $0$ to $100$
- $R = 0.75$
- $n = 7$, $+/-$ only
- $p < 0.05$