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Discrete Change in Volatile Anesthetic Sensitivity in Mice with Inactivated Tandem Pore Potassium Ion Channel TRESK

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ABSTRACT

Background: We investigated the role of tandem pore potassium ion channel (K_{2P}) TRESK in neurobehavioral function and volatile anesthetic sensitivity in genetically modified mice.

Methods: Exon III of the mouse TRESK gene locus was deleted by homologous recombination using a targeting vector. The genotype of bred mice (wild type, knockout, or heterozygote) was determined using polymerase chain reaction. Morphologic and behavioral evaluations of TRESK knockout mice were compared with wild-type littermates. Sensitivity of bred mice to isoflurane, halothane, sevoflurane, and desflurane were studied by determining the minimum alveolar concentration preventing movement to tail clamping in 50% of each genotype.

Results: With the exception of decreased number of inactive periods and increased thermal pain sensitivity (20% decrease in latency with hot plate test), TRESK knockout mice had healthy development and behavior. TRESK knockout mice showed a statistically significant 8% increase in isoflurane minimum alveolar concentration compared with wild-type littermates. Sensitivity to other volatile anesthetics was not significantly different. Spontaneous mortality of TRESK knockout mice after initial anesthesia testing was nearly threefold higher than that of wild-type littermates.

Conclusions: TRESK alone is not critical for baseline central nervous system function but may contribute to the action of volatile anesthetics. The inhomogeneous change in anesthetic sensitivity corroborates findings in other K_{2P} knockout mice and supports the theory that the mechanism of volatile anesthetic action involves multiple targets. Although it was not shown in this study, a compensatory effect by other K_{2P} channels may also contribute to these observations.

What We Already Know about This Topic

- Two pore potassium channels regulate neuronal excitability and may be targets of anesthetic action.
- One of these channels, TRESK, is affected by anesthetics *in vitro*.

What This Article Tells Us That Is New

- Mice generated lacking the TRESK channel were slightly less sensitive to isoflurane but similarly sensitive to other volatile anesthetics.

TANDEM pore potassium ion channels (K_{2P}) are important contributors to background potassium (K^+) currents in excitable cells. These channels are composed of two subunits, each with four transmembrane domains and two pore-forming domains arranged in tandem, a unique structure that distinguishes them from other K^+ channel subunits.[1] Background currents conducted by K_{2P} channels help maintain the resting membrane potential of cells and regulate the action potential of excitable cells.[2–4] K_{2P} channels are modulated by biophysical and biochemical factors such as pH, temperature, stretch, molecular oxygen, phospholipids, cyclic nucleotides, neuroprotective agents, protein kinases, G-protein-coupled receptors, and neurotransmitters.[5–14] K_{2P} channels participate in regulating adrenal aldosterone secretion,[15,16] renal proximal tubule cell volume,[17,18] cardiac action potential and rhythms,[19,20] and neuronal apoptosis and neuroprotection.[21,22] K_{2P} channels may also be involved

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with significant pathologic states such as tumorigenesis and depression.[23,24]

Many *in vitro* studies have suggested a role of K_{2P} channels in the mechanism of action of volatile anesthetics. Currents passed by TWIK (tandem pore weak inward rectifying K^+ channel)-related acid-sensitive K^+ channel (TASK)-2 expressed in *Xenopus laevis* oocytes are potentiated by halothane, isoflurane, enflurane, desflurane, and chloroform.[25] Human TWIK-related K^+ channel (TREK)-1 and TREK-2 currents expressed heterologously in COS-7 cells are strongly activated by chloroform, halothane, and isoflurane.[26,27] TASK-1 currents found in rat motor neurons and locus ceruleus cells are also strongly potentiated by halothane and sevoflurane.[28]

These cellular findings have been corroborated in whole animal studies. TREK-1 knockout mice are resistant to the anesthetizing action of several volatile anesthetics (chloroform, halothane, isoflurane, sevoflurane, desflurane) and require higher concentrations to achieve inhibition of righting reflex and nociception.[29] However, inactivation of other K_{2P} channel genes produce knockout mice with less definitive changes in anesthetic sensitivity. Both TASK-1 and TASK-3 knockout mice show small changes in sensitivity to some volatile anesthetics[30,31] Furthermore, TASK-2 and KCNK7 knockout mice do not have altered sensitivity to volatile anesthetics.[32,33] Thus, the involvement of K_{2P} channels in the mechanism of volatile anesthetics seems variable and must be determined individually for each K_{2P} family member.

The tandem pore potassium ion channel TRESK was the last K_{2P} family member isolated and was initially found expressed only in human spinal cord.[34] Subsequently, TRESK expression was also detected in human brain[35] and nonneuronal (liver, testis, spleen, heart, and lung) tissues.[36,37] Like other K_{2P} channels, TRESK channels conduct background K^+ currents to help set the membrane potential near the K^+ equilibrium potential.[34] In dorsal root ganglion (DRG) neurons, TRESK's primary role is to help shape the action potential.[38]

As with other K_{2P} channels, biochemical and biophysical factors regulate TRESK activity, including unsaturated free fatty acids (arachidonic, docosahexaenoic, and linoleic acids), changes in intracellular and extracellular pH,[34,37] intracellular Ca^{2+} concentrations,[39] and volatile anesthetics.[35] Human TRESK K^+ currents heterologously expressed in mammalian and amphibian cells are strongly potentiated (up to threefold) by halothane, isoflurane, sevoflurane, and desflurane at clinically relevant concentrations.[35]

We generated a mouse with inactivated TRESK gene to study its sensitivity to various volatile anesthetics. We also studied the development, growth, and behavior of these mice and compared them with wild-type littermates. The recovery and survival of these mice during and immediately after anesthetic treatments were also monitored and recorded. This report describes the first characterization of a TRESK knockout mouse.

Materials and Methods

Production of TRESK Knockout Mice

All animal experiments were done at University of California San Francisco and were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Mice were housed under standard conditions with a 12-h light-dark cycle in a pathogen-barrier facility at University of California, San Francisco, and given *ad libitum* access to food and water. TRESK knockout mice were produced under contract with Ozgene Pty Ltd (Perth, Australia). A targeting vector was constructed to delete the entire sequence of exon III of mouse TRESK gene by homologous recombination. Exon III is the largest of three TRESK exons and encodes the majority of the TRESK channel subunit. The 5′ and 3′ regions of the C57/BL6 strain chromosomal locus (~5 kilobases [kb] each) flanking TRESK exon III were cloned from mouse genomic DNA and used as homologous arms to construct the targeting vector with a neomycin cassette (fig. 1A). The targeting construct was transfected into C57/BL6 embryonic stem cells and the recombinant embryonic stem cell lines were selected. The isolated TRESK knockout embryonic stem cells were microinjected into C57/BL6 mouse blastocysts to generate chimeric mice. The neo cassette was removed by interbreeding with mice constitutively expressing Cre recombinase. Heterozygous offspring with neo cassette deleted were used to breed TRESK knockout mice. To increase the number of animals with the desired TRESK genotypes, we also used some homozygous knockout offspring for breeding.

Genotyping

At 3 weeks of age, newborn mice were weaned, separated, and identified by ear tags. Genomic DNA was isolated from tail snip samples (less than 1 cm) with DNeasy Blood & Tissue kit (QIAGEN, Valencia, CA). Polymerase chain reaction (PCR) was performed with primers flanking TRESK gene exon III as follows: forward primer (P1) 5′-ACCAACACCAAGCTGTCTTGTTTCTC-3′ and reverse primer (P2) 5′-AGACAGATGGACGGACAGACATAGATG-3′ (fig. 1A). The PCR mixture contained 1 μl DNA, 2 μl PCR buffer (10× concentration), 0.4 μl dNTP (10 mM), 0.4 μl 10 mM forward and reverse primers, 0.2 μl Platinum Taq DNA polymerase (5 U/ml), and 16 μl water (PCR Core Kit Plus; Roche Applied Science, Mannheim, Germany). The PCR was carried out with a programmable thermal cycler (PTC-200 DNA engine; MJ Research Inc., Waltham, MA) programmed with initial heating to 95°C for 2 min, then 30 cycles of 92°C for 1 min, 58°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis on 1% agarose gels. The expected sizes of bands were as follows: wild type, 2.6 kb; knockout/+neo, 2.0 kb; knockout/−neo, 0.15 kb (representative gel shown in fig. 1B).

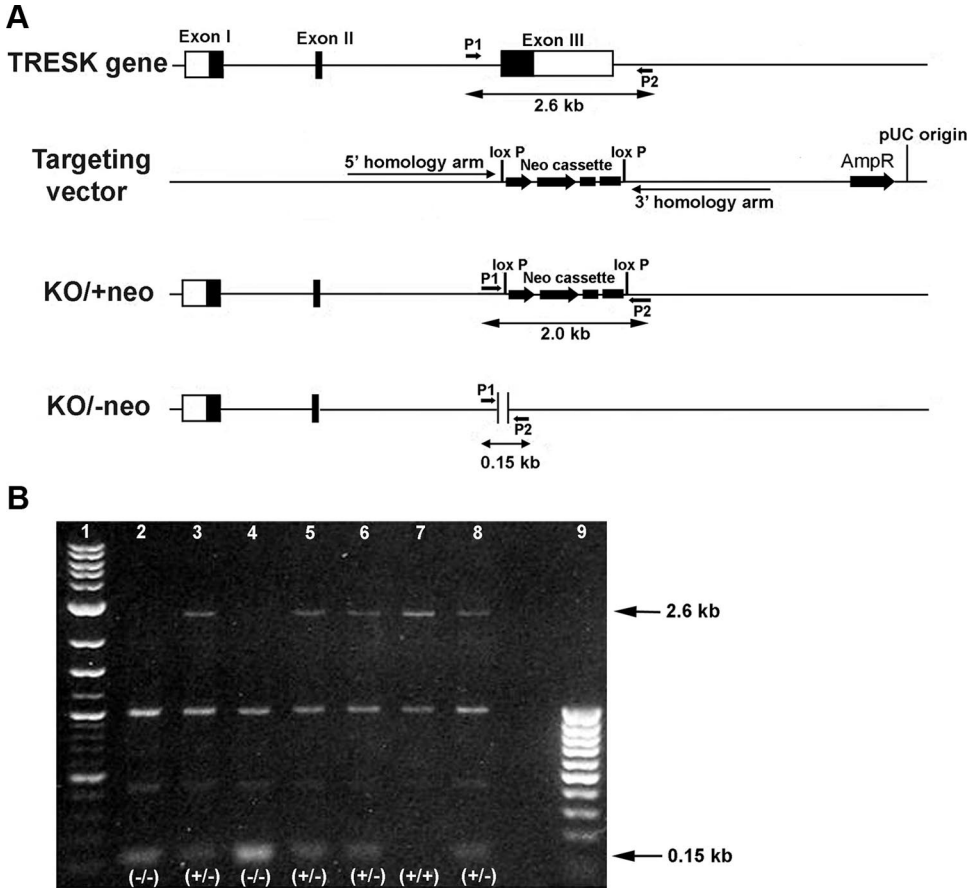

Fig. 1. Genomic structure of the TRESK genetic locus and genotyping results confirming TRESK gene knockout. (*A*) Basic structure of TRESK gene and a schematic of the targeting vector used to transfect mouse strain C57BL6 embryonic stem cells. For each exon, an open box represents an untranslated region and a closed box represents a protein-coding region. P1 and P2 indicate the location of primers used for genotyping. Knockout (KO)/+neo indicates the structure of the TRESK gene locus after successful homologous recombination with the targeting vector and KO/−neo shows the structure after deletion of the neo cassette *via* the action of Cre recombinase. AmpR = ampicillin-resistant gene (β-lactamase), neo = neomycin resistance gene, lox P = flanking sequences targeting the deletion of intervening sequence by Cre. (*B*) Representative agarose gel of genotyping results (ethidium bromide fluorescence). lane 1 = 1 kilobase (kb) DNA ladder, lane 9 = 0.1 kb DNA ladder. The symbol +/+, +/−, or −/− below each lane identifies a banding pattern indicating wild-type, heterozygous, or homozygous genotype, respectively.

Real-time Quantitative Reverse Transcription

Real-time quantitative reverse transcription was performed to analyze the K_{2P} gene expression in bred mice. Poly $(A)^+$ RNA from brain, spinal cord, and DRG were extracted using a Micro-FastTrack messenger RNA (mRNA) isolation kit (Invitrogen, Carlsbad, CA) with extraction volumes adjusting to 1 ml per 100 mg wet tissue weight. The final concentrations and purity of the mRNA preparation were determined by measuring the absorbance at 260 nm (A_{260}) and the ratio of A_{260}/A_{280}. The yields of mRNA from the same tissues of different mice were adjusted by dilution with RNase-free water to reach the same concentration per mg of tissue. Reverse transcription was performed with 0.01–0.1 μg mRNA using a QuantiTect RT kit (QIAGEN). Quantitative PCR was carried out on the Mx3000P real-time PCR system (Stratagene, Cedar Creek, TX) with the following thermal cycles: initial heating to 95°C for 10 min, then 30 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 30 s. Total 50 μl PCR mixture included 25 μl QuantiTect SYBR Green PCR Master Mix (2× concentration, from QIAGEN kit), 0.5 mM each primer, and 2 μl reverse-transcribed complementary DNA. Quantitative PCR primers for the four K_{2P} channels were as follows: TRESK: forward, 5′-GACAGTGAGGTGT-GGGTCTG-3′; reverse, 5′-CCAGAGCTGTTGCATAG-GAA-3′; TREK-1: forward, 5′-TGTGGTTATCACTCT-GACG-3′, reverse, 5′-CAGCCCAACGAGGATCCAG-3′; TASK-1: forward, 5′-CTGCTCATTCACTCGTCCAT-3′, reverse, 5′-AAGAACTGCCCAGGTGACTT-3′; and TASK-3: forward, 5′-GACGCTGGTTATGTTCCAGA-3′, reverse, 5′-CGGTCACCATGTTCTCCATA-3′. Each sample was set in triplicate and the results of three mice of each genotype group were pooled.

To establish standard curves, fragments of TRESK, TREK-1, TASK-1, and TASK-3 were amplified by PCR from C57BL6 mouse mRNA, gel purified and diluted serially before quantitative PCR experiments. Five serial dilu-

tions with a factor of 10 for each K_{2P} standard were included in parallel with the quantitation of the K_{2P}-channel expression in every PCR run. A linear standard curve of threshold concentration *versus* concentration of standard was established for each gene. The expression level of each gene was quantified as a relative expression against the standard.

Immunocytochemistry

Perfusion and fixation of mice were performed according to our recently described methods.[40] Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection) and injected with heparin sodium (1,000 unit/kg; American Pharmacological Partners, Schaumburg, IL) into the left ventricle to prevent blood clotting. Approximately 30 ml 0.1 M phosphate-buffered saline prewarmed to 37°C was perfused into the circulation system through the left ventricle and drained out of the right atrium, followed by a perfusion of approximately 40 ml fixative containing 4% formaldehyde (v/v) in 0.2 M phosphate buffer precooled to 4°C. After perfusion, the brain, spinal cord, and lumbar DRG were dissected, fixed for another 3–4 h at 4°C in the same fixative, followed by overnight incubation in 30% sucrose in phosphate-buffered saline.

Sectioning of fixed tissues of brain (section thickness, 30 μm), spinal cord (20 μm), and lumbar DRG (10 μm) was performed on a cryostat (CM1900; Leica, Solms, Germany), from three different TRESK wild-type and knockout mice. Immunostaining using specific antisera against TRESK (goat polyclonal IgG, TRESK/V-12; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was carried out according to the avidin-biotin-peroxidase method described by Hsu *et al.*[41] with minor modifications.[40] Selected representative sections were immunostained in pretreated 12-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ). Tissue sections in each well were washed three times for 5 min each with washing buffer containing 10 mM phosphate-buffered saline, 1% normal rabbit serum (Vector Laboratories, Burlingame, CA), and 0.3% Triton X-100. After wash, the sections were incubated for 1 h in blocking buffer (washing buffer containing 3% normal rabbit serum), and then incubated with 1:100 diluted TRESK antisera for 4 h with a gentle shaking at room temperature, followed by continuous overnight incubation at 4°C. After incubation with the TRESK antisera, sections were washed and incubated with a biotinylated rabbit–anti-goat antibody (the secondary antibody) that reacted to avidin/biotinylated peroxidase complex (Vector Laboratories) for 2 h with gentle shaking at room temperature. In parallel, the following three control incubations were done: without primary antisera, without secondary antibody, and with the primary antisera preneutralized with a blocking peptide that was used to raise the antisera. Diaminobenzidine substrate kit for peroxidase (Vector Laboratories) was used to localize peroxidase reaction sites. Immunostained sections were mounted on glass microscopic slides, air dried, and covered with a coverslip using Permount histologic mounting medium (Thermo Fisher Scientific, Waltham, MA).

Images taken at 10×, 30×, and 50× magnification were captured with a microscope (MZFL III; Leica) connected to a digital camera (DXM 1200; Nikon, Melville, NY). Images at 200× and 400× magnification were also captured (Eclipse E400; Nikon) connected to a digital camera (D300; Nikon). All images were converted to grayscale and digitalized. The stereotaxic coordinates of coronal sections of the mouse brain were assigned according to Paxinos and Watson.[42] The levels of the spinal cord sections were determined according to Paxinos.[43]

Behavioral Testing

Standardized tests were applied to TRESK knockout mice and wild-type littermates to elicit neurobehavioral differences.

General Neurologic Screening

Basal Behavior Observation. Mice were placed in an open field box free of distinct odor and objects. They were allowed to freely explore the open field box for 5 min. Behavior was observed and scored as follows: active moves = sum of running, rearing, or jumping events; inactive periods = sum of sniff and immobile events; grooming; total defecations and urinations.

Hanging Wire Test. Mice were placed on 1-inch square mesh grid and then shaken briefly to ensure full grip. The grid was then inverted to allow the mouse to maintain grasp for a maximum of 60 s. The timer was stopped when the mouse fell (all four paws no longer grasping mesh grid). If the mouse climbed on top of the grid, the grid was reinverted to ensure upside-down grasping. Each animal was tested once.

Tail Suspension Test. Using electrical tape and a wooden bar 60 cm above table, mice were suspended by their tails. The following parameters were scored: latency to immobility, cumulative duration of immobility over a 4-min period, clasping (curling into fetal position).

Motor Testing

Rotarod Tests. Mice were habituated to testing room 30 min before testing.

Fixed Rotarod. On the first day, mice were placed on the rotarod apparatus with the rod rotating at the constant speed of 16 rotations per minute (rpm) for three trials. The trial ended when the mouse fell or for a maximum of 5 min.

Accelerated Rotarod. On the second day, mice were placed on the rotarod apparatus with the rod rotating at an accelerating speed, from 4 to 40 rpm. Every 30 s, the rotation speed was increased by 4 rpm. The trial ended when the mouse fell or for a maximum of 5 min.

Challenge Beam. Mice were trained for two consecutive days to traverse down a tapered cue stick, with the tip leading to their home cage. On the first day, mice received two assisted trials. Training was resumed on the second day with three unassisted trials. On the third day, mice were tested with a mesh grid (1-cm squares) placed over the cue stick leaving 1 inch between the grid and the cue surface. Animals were then

videotaped for three trials. Latency to finish, the number of falls, and the number of missed footsteps were scored for each mouse. The average for all three trials was used for the analysis.

Open Field Test. Time spent in an open field was measured in an automated clear plastic chamber (41 × 41 × 30 cm) with two photobeam arrays (16 × 16) detecting horizontal and vertical movements. Mice were habituated to the testing room 30 min before testing. During testing, mice were placed in one of four identical clear plastic chambers, as mentioned above, for 15 min. Open field movements were automatically recorded by photobeams.

Nociception Testing

Tail Flick Test. Mice were habituated to the testing room 30 min before testing. The mouse was placed with its tail protruding within a restraining tube on the platform of the stimulus unit. The tail was positioned on a slot of adjustable width to guarantee stability of the tail underneath a thermal stimulus. The latency to respond was measured (maximum latency, 15 s).

Hot Plate Test. Mice were habituated to the testing room 30 min before testing. Surface of the hot plate was heated to a constant temperature of 52–55°C as measured by a built-in digital thermometer. During testing, mice were placed in a clear, open-ended cylindrical (11 × 15 cm) enclosure placed on top of the hot plate. The latency to respond with a hind paw lick, hind paw flick, or jump (whichever occurred first) was timed. The mouse was immediately removed from the hot plate and returned to its home cage. Each animal was tested once.

Minimum Alveolar Concentration Assay

Bred mice aged 2 months or older underwent measurement of minimum alveolar concentration (MAC) according to methods previously established.[32,44] Genotyping of the animals was done after MAC testing so investigators were completely unaware of the genotypes during testing.

First, individually tagged animals were randomly selected from their cages, weighed, and placed in their own gas-tight plastic cylinders connected to a system containing a fan and oxygen source. Up to 10 mice were tested in one experimental setting. Temperature was monitored by rectal probe and kept between 35.5 and 37.5°C throughout the experiment. Volatile anesthetics were administered by agent-specific precision vaporizers and the concentrations monitored by a Datex-Ohmeda 5250 RGM monitor (Louisville, CO). Individual mice going through multiple MAC assays had a rest period of at least 1 week between test sessions.

Four volatile anesthetics, isoflurane, halothane, sevoflurane, and desflurane were tested. Anesthetic agents were administered at an inspired oxygen concentration of 1.0. Administration began at low concentration and continued to the equilibrium appropriate for each agent (20 min for desflurane, 30 min for isoflurane and sevoflurane, 40 min for halothane). At equilibrium, the exact anesthetic concentration was determined by gas chromatography (Gow-Mac Instrument Co., Bethlehem, PA) equipped with a flame ionization detector. A mechanical clamp was applied on the middle of the tail to test responsiveness. If the animal responded by movement of an extremity or head jerking, the anesthetic concentration was increased by 0.1% for halothane, isoflurane, and sevoflurane, and 0.2% for desflurane. The location of tail clamping was changed for subsequent measurement at the next concentration. On reaching the concentration of anesthetic at which the mouse did not move in response to tail clamping, the mouse was removed from the chamber and allowed to recover at warm temperature. The MAC value of a volatile anesthetic for a given mouse was defined as the average of two sequential concentrations at which responsiveness to tail clamping changed from positive to negative.

Survival/recovery or death during or immediately after MAC assays were recorded. To characterize the death as a direct result of anesthetic treatment, we counted only deaths that occurred within 24 h of MAC determination. Deaths due to other causes, such as infection or diseases not related to anesthetic exposure, were excluded from this death rate analysis.

Statistical Analysis

The Prism graphing, curve fitting, and statistical package (version 4; GraphPad Software, Inc., La Jolla, CA) was used for the analysis. We assumed a Gaussian distribution was present in the MAC assays and behavioral datasets. The choice of a parametric test, one-way analysis of variance, was based on discussion with Edmond Eger II, M.D. (Professor, Department of Anesthesia and Perioperative Care, University of California, San Francisco, December 2009), the originator of MAC testing with more than 40 years of experience. He advised that population normality can be assumed with genetically pure groups (wild type, heterozygotes, and homozygotes derived from a small number of breeding pairs). Power analysis for sample size computation was also performed after discussion with Dr. Eger. We compared the following three groups: homozygous, heterozygous, and wild-type animals. To have a probability (power) of 0.9 to detect an effect at the 0.05 significance level with the SD in the MAC measurement being 6% and an expected effect size of 10% change in MAC requires 15 mice per group. Thus, we needed at least 60 bred mice to have enough mice representing each genotype given a normal Mendelian distribution of genotypes.

One-way analysis of variance was used to analyze the differences in MAC testing among the three genotypes of TRESK knockout mice. The criteria for determining statistical significance was a P value of less than 0.05. Data are presented as mean ± SEM with the number of animals undergoing each MAC test shown above the upper error bar.

For behavioral testing, although fewer animals were available for testing, the same assumption of genetic homogeneity—and therefore normal distribution of behaviors—was appropriate. For analysis of behavioral tests, a two-tailed Stu-

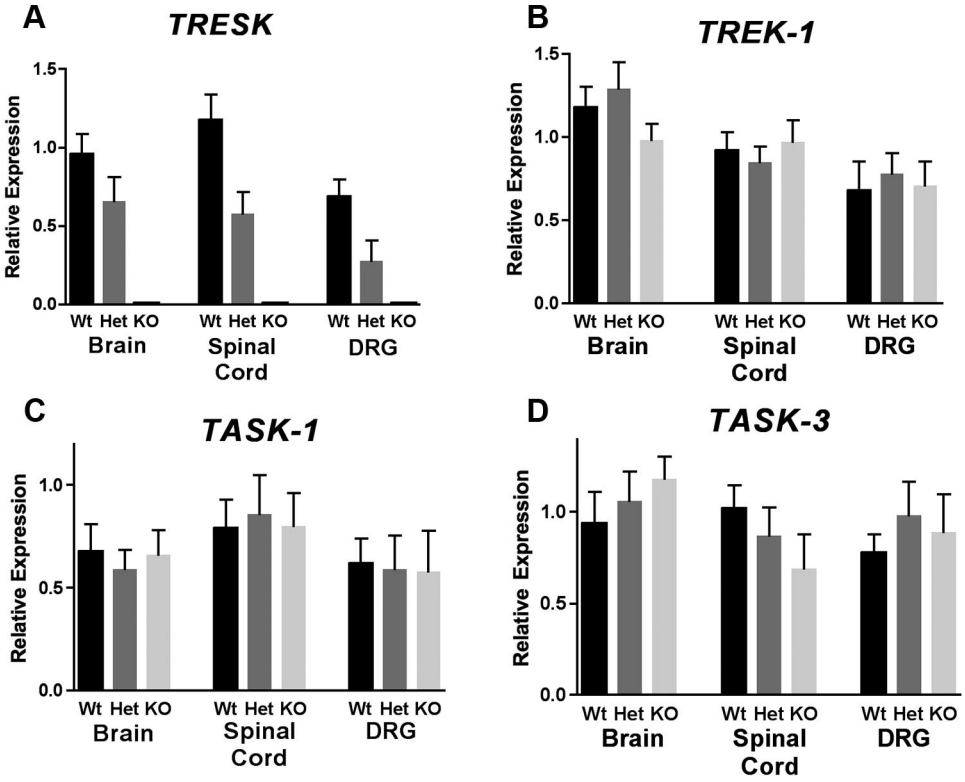

Fig. 2. Results of real-time quantitative polymerase chain reaction from whole brain, spinal cord, and dorsal root ganglion (DRG) tissues using K_{2P} channel–specific primers. Each column represents a reaction primed with total RNA isolated from wild-type (Wt), heterozygous (Het), or knockout (KO) mice. The expression levels are represented as relative to an internal calibration standard that was included in all experiments: TRESK primers (*A*), TREK-1 primers (*B*), TASK-1 primers (*C*), and TASK-3 primers (*D*).

dent *t* test was used. To compare differences in death rate after MAC assays between different genotypes, Fisher exact test was used.

Results

Figure 1 shows details of the procedure used to generate TRESK knockout mice. Exon III of mouse TRESK gene was targeted for deletion by homologous recombination because it is the largest of three TRESK exons and encodes the majority of the TRESK channel subunit. Figure 1A shows the genomic structure of the mouse TRESK gene locus and the elements of the targeting vector that was constructed to delete exon III. To eliminate potential off-targeting effects, mice transgenic for the homologously recombined deletion vector were bred with mice constitutively expressing Cre to produce the final knockout strain in which the neomycin selection cassette had been deleted.

Genotyping and Expression of TRESK and Other K_{2P} of Bred Mice

A total of 186 mice were bred for this study (97 male, 89 female). The genotypes of mouse pups were determined at 8–10 days of age using PCR on DNA samples isolated from tail snips. Typical results from PCR genotyping are shown in figure 1B. The presence of a 2.6-kb band indicated a wild-type allele whereas the presence of a 0.15-kb band indicated a knockout allele. The distribution of genotypes of the offspring from crossings of heterozygous parents closely followed a normal Mendelian ratio (1:2:1): wild-type (+/+) 36 (25%), wild-type/TRESK knockout heterozygous (+/−) 65 (46%), homozygous TRESK-knockout (−/−) 41 (29%).

To confirm the elimination of TRESK expression in homozygous knockout animals and to assess the effect of knockout on the expression of other K_{2P} channels, mRNA concentrations of TRESK, TREK-1, TASK-1, and TASK-3 in brain, spinal cord, and DRG were measured by real-time quantitative reverse transcription. In TRESK (−/−) mice, expression of TRESK mRNA was undetectable in the whole brain, spinal cord, and DRG; mice heterozygous for TRESK knockout (+/−) expressed approximately half the amount of TRESK mRNA compared with wild-type (fig. 2A). Real-time quantitative reverse transcription results also showed that the expression of TREK-1, TASK-1, and TASK-3 mRNA in the whole brain, spinal cord, and DRG of TRESK −/− and +/− mice remained at levels similar to those of their wild-type (+/+) littermates (fig. 2B–D).

As final proof of the absence of TRESK expression immunocytochemical staining of spinal cord and brain sections showed complete elimination of staining in brain, spinal cord, and DRG sections from knockout animals (fig. 3).

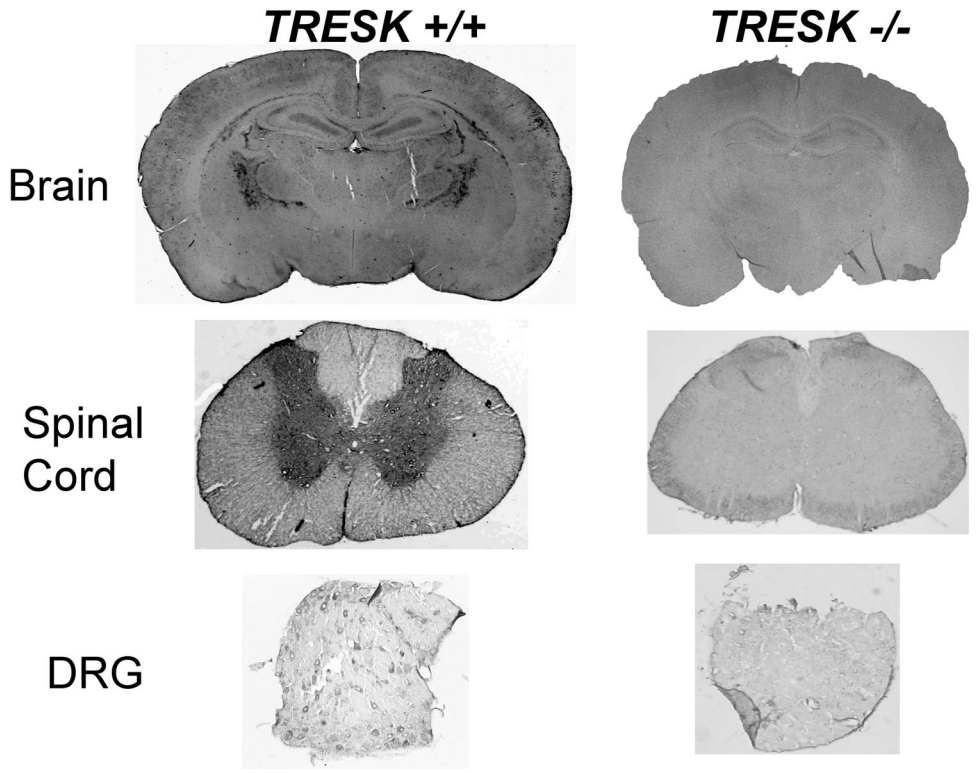

Fig. 3. Representative fixed sections of brain, spinal cord, and dorsal root ganglion (DRG) from wild-type (TRESK +/+) and knockout (TRESK −/−) mice showing immunolocalization of TRESK protein expression using anti-TRESK antisera.

Overall Development and Behaviors of TRESK Knockout Mice

TRESK knockout (−/−) and heterozygous (+/−) mice had healthy growth and showed no structural abnormalities. Mice bred from homozygous TRESK knockout (−/−) mice parents also developed and grew normally. Body size, body weight, and the appearance of fur and whiskers were normal compared with wild-type littermates. Knockout and wild-type littermates were evaluated in more detail for neurobehavioral differences (10 knockout, 9 wild-type) (fig. 4). Basic behavioral screening including analysis of active and inactive events, grooming, defecation, urination, movement in response to tail suspension, and ability to suspend from wire grid were not significantly different from wild-type littermates (fig. 4, A, C, and D). Knockout mice showed a small but significant decrease in inactive events compared with wild-type littermates (fig. 4B). Motor function was evaluated in two ways: ability to traverse a challenge beam and ability to maintain position on a rotarod (fixed and accelerating). In these tests, there were no significant differences in performance between wild-type and knockout mice (fig. 4, E and F). However, TRESK knockout mice had increased thermal nociceptive sensitivity, showing 20% decreased latency in hot plate testing (fig. 4G).

Sensitivity of TRESK Knockout Mice to Volatile Anesthetics

Four volatile anesthetics, isoflurane, halothane, sevoflurane, and desflurane were tested. Table 1 shows the number and gender of animals tested with each agent. The sensitivity of TRESK knockout and heterozygous mice to isoflurane was significantly reduced compared with that of wild-type mice as follows: MAC values (mean ± SEM) of isoflurane for knockout, 1.54 ± 0.016; heterozygote, 1.52 ± 0.017; and wild-type, 1.42 ± 0.016 ($P = 0.001$ between knockout and wild-type) (fig. 5A). However, the sensitivities of TRESK knockout or heterozygous mice to desflurane, halothane, and sevoflurane were not different from wild-type. MAC values of desflurane were knockout, 7.59 ± 0.09; heterozygote, 7.58 ± 0.07; and wild-type, 7.47 ± 0.10 (fig. 5B). MAC values of halothane were knockout, 1.16 ± 0.02; heterozygote, 1.15 ± 0.03; and wild-type, 1.16 ± 0.02 (fig. 5C). MAC values of sevoflurane were knockout, 2.86 ± 0.03; heterozygote, 2.89 ± 0.03; and wild-type, 2.87 ± 0.05 (fig. 5D).

Viability of TRESK Knockout Mice after MAC Assays

The recovery and survival of wild-type mice after MAC assays were consistent with previous studies using this mouse strain (death rate for C57BL6 ~12–15% during MAC testing, verbal communication, James Sonner M.D., Ph.D., Professor, Department of Anesthesia and Perioperative Care, University of California, San Francisco, December 2009). However, the recovery and survival of TRESK knockout mice after MAC assays were significantly impaired; knockout mice recovered slowly and incompletely after the MAC assay compared with wild-type littermates. Some knockout mice appeared to have recovered but were then found dead in their

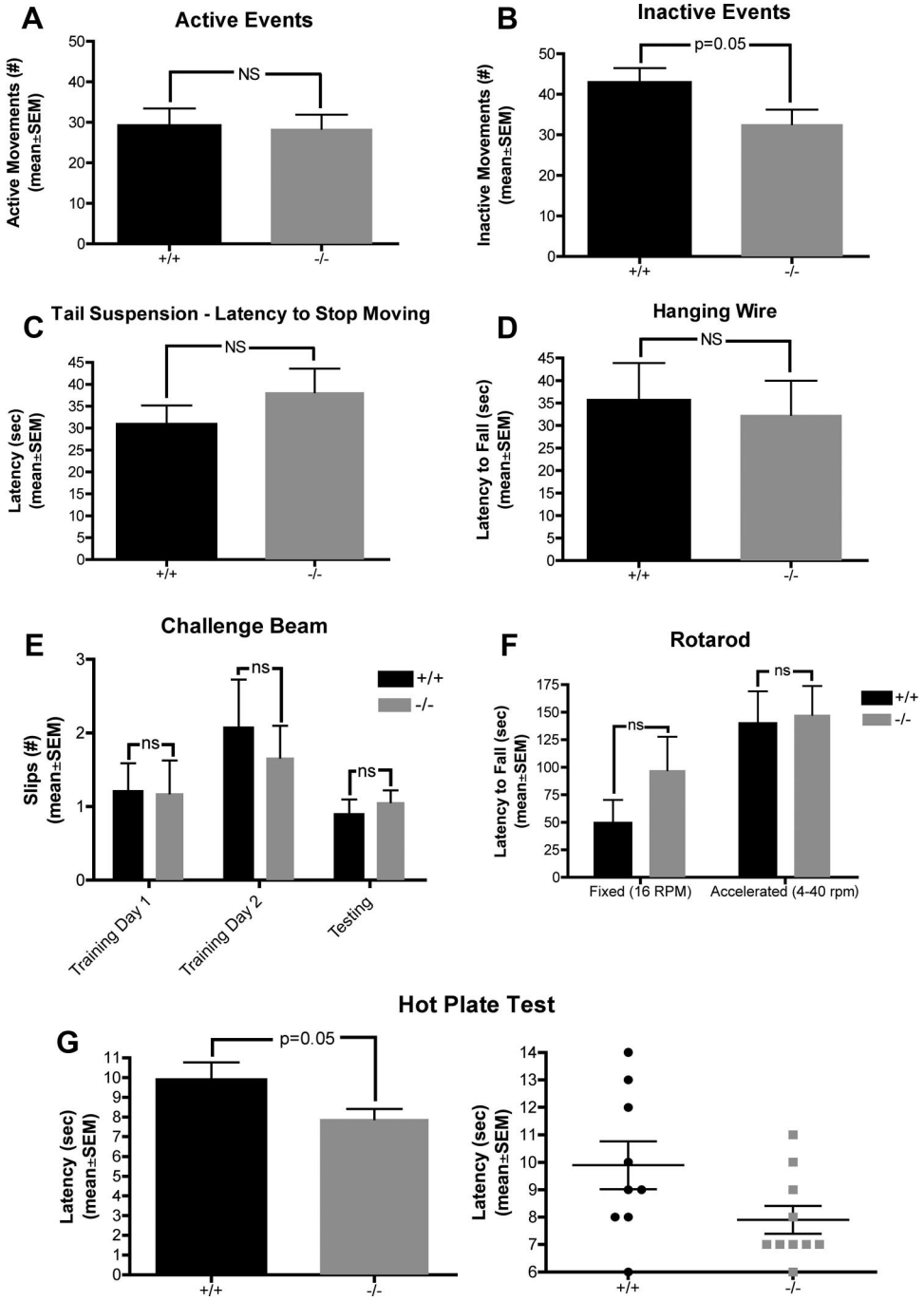

Fig. 4. Results of trials comparing wild-type (+/+) and TRESK knockout (−/−) mice in the indicated behavioral test. Each bar in *A–F* represents mean ± SEM. (*G*) Individual responses for each mouse tested are shown on right. NS = not significant; rpm = rotations per minute.

cages the next day. Mortality of knockout mice during and within the first 24 h after MAC assay was significantly higher than that of wild-type littermates (table 2). The difference in mortality between genotypes was significantly different for animals after a first MAC study (31.6% for knockout *vs.* 11.5% for wild-type, $P < 0.05$). Overall death rates after multiple (*i.e.*, two or more) MAC assays were also increased (73.7% for knockout *vs.* 53.8% for wild-type), but the difference was not statistically significant.

Discussion

In the current study, we evaluated the phenotype of mice with deletion of the K_{2P} channel TRESK gene. TRESK homozygous knockout mice appeared no different from wild-type littermates in growth, overall appearance, and reproduction, suggesting that TRESK is not an essential gene for these aspects of mouse development. Knockout of the TRESK gene produced no noticeable impairment in gross behaviors

Table 1. Genotypes and Genders of Mice Tested in MAC Assays

	Wild Type	Heterozygous	Knockout
Total Bred Mice, no. (m/f)	67 (33/34)	65 (34/31)	54 (30/24)
Isoflurane, no. (m/f)	23 (10/13)	29 (15/14)	24 (13/11)
Desflurane, no. (m/f)	17 (7/10)	33 (19/14)	20 (11/9)
Halothane, no. (m/f)	16 (7/9)	23 (11/12)	17 (6/11)
Sevoflurane, no. (m/f)	19 (9/10)	30 (13/17)	16 (7/9)

f = female; m = male; MAC = minimum alveolar concentration preventing movement in 50% of animals to tail clamp stimulus.

Table 2. Mortality by Genotype after MAC Assays

Deaths	Wild Type	Heterozygous	Knockout
After first MAC assay, no. (%)	3/26 (11.5)	8/40 (20.0)	12/38 (31.6)
Total, no. (%)	14/26 (53.8)	24/40 (60.0)	28/38 (73.7)

MAC = minimum alveolar concentration preventing movement in 50% of animals to tail clamp stimulus.

or motor ability. However, on closer testing, knockout mice were found to have mildly increased thermal sensitivity and decreased inactive behavior compared with wild-type mice. Although we found no compensatory changes in expression of other major K_{2P} channels when TRESK expression is lost, the lack of behavioral phenotype in these mice implies there is redundancy behind the functional contribution that TRESK makes to the function of the central nervous system.

We also investigated the role of TRESK in sensitivity to volatile anesthetics and found that mice with inactivated TRESK were less sensitive to isoflurane, but had unchanged sensitivity to desflurane, halothane, and sevoflurane. The difference in MAC for isoflurane was small but statistically significant. This isolated finding is similar to previously reported changes in anesthetic sensitivity in other K_{2P} knockout mice. TASK-1 knockout mice have partially reduced sensitivity to isoflurane or halothane depending on the assay[30] whereas TASK-3 knockout mice show decreased sensitivity to halothane but not isoflurane in standard MAC assay.[31] Furthermore, knockout mice with deletion of other proteins implicated in anesthesia mechanisms, such as the $\beta 3$ subunit of the γ-aminobutyric acid type A receptor[45] or the stomatin gene, show variable changes in volatile anesthetic sensitivity.[46] Only TREK-1 knockout mice manifest a consistent reduction in volatile anesthetic sensitivity. Yet, even in those mice, the changes in MAC varied widely, from as low as 7% for desflurane to as high as 48% for halothane.[29] We believe that our finding of decreased anesthetic sensitivity for only one drug in TRESK knockout mice adds to a growing

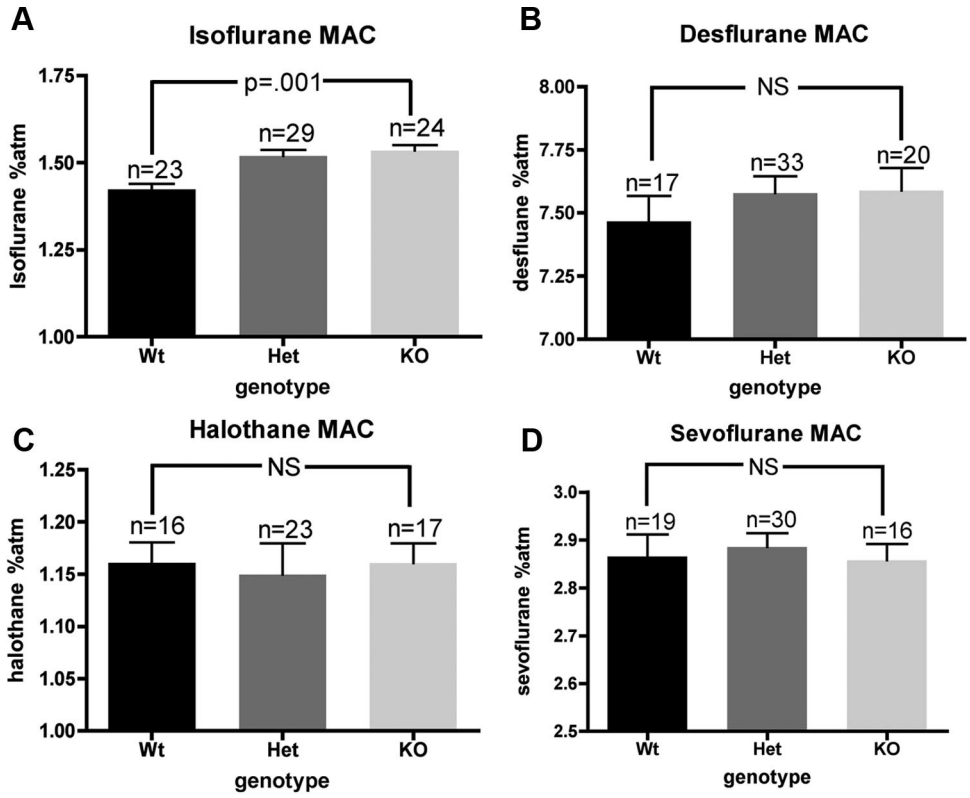

Fig. 5. Minimum alveolar concentration (MAC) suppressing movement to tail clamp in 50% of wild-type (Wt), TRESK heterozygous (Het), or complete knockout (KO) mice for isoflurane (*A*), desflurane (*B*), halothane (*C*), and sevoflurane (*D*). Each column shows mean ± SEM. The number of animals tested is shown above each error bar. NS = not significant.

consensus that volatile anesthetics are acting on multiple targets, possibly including K_{2P} channels, whose sum total effect achieves the pharmacodynamic action of volatile anesthetics.

The behavioral evaluation of mice with inactivated TRESK gene are also congruent with previous studies of other K_{2P} family knockout mice.[47] TREK-1, TWIK-1, TASK-2, and KCNK7 knockout mice all are healthy, fertile, and have normal morphology and behaviors.[29,32,33,48] TASK-1 knockout mice act normally in several behavioral tests, with only minor changes in paw withdrawal response and motor function.[30,49] TASK-3 knockout mice show no gross abnormality apart from a minor increased locomotor activity during the dark phase and slower swimming ability.[31] Even the TASK-1/TASK-3 double-knockout mouse shows no obvious neurologic abnormalities or health problems.[50] These observations reinforce the idea that the expression of individual members of the K_{2P} gene family is not essential for healthy growth, development, and reproduction.

We did document a greater mortality rate in TRESK knockout mice after undergoing MAC testing compared with their wild-type littermates. We speculate that this impaired survival ability may result from reduced tolerance to the stress of MAC testing. The MAC assay itself, consisting of a prolonged anesthetic with varying agent concentrations and exposure to noxious stimuli under, at times, a light plane of anesthesia represents a significantly stressful event. We do not believe that the higher mortality of knockout *versus* wild-type mice occurred as a result of the difference in isoflurane MAC because knockout and wild-type animals received similar concentrations of isoflurane during testing. In addition, greater mortality also occurred in animals exposed only to the other volatile anesthetics, for which the knockout mice did not display any change in MAC. The exact mechanism underlying reduced survival ability in these mice remains to be further investigated and could include alterations in respiratory dynamics, abnormal response to changes in Pa_{CO_2}, or interference with other cardiorespiratory functions.

As with all global knockout studies, it is possible that a compensatory effect by other K_{2P} channels or other genes responsible for anesthetic action may occur in response to TRESK gene deletion. Quantitative PCR showed that knockout of TRESK gene did not significantly alter mRNA expression of TREK-1, TASK-1, and TASK-3 in the whole brain, spinal cord, and DRG. However, these results do not rule out changes in expression of these K_{2P} family members at a regional or cellular level or of compensation by other ion-channel expression. Changes in gene expression of other anesthetic-sensitive ion channels could compensate for loss of TRESK, as has been described with μ opioid receptor knockout.[51]

Another physiologic effect that could be masking the role of TRESK and other K_{2P} channels in knockout mice is the fact that the anesthetics studied in the MAC assays were delivered in 100% oxygen. Given that several K_{2P} channels are activated by molecular oxygen, it is possible that the anesthetic response of knockout animals was blunted by this factor.

In conclusion, inactivation of TRESK gene expression in mice does not cause a significant effect on development, growth, reproduction, and gross behavior, but it does cause a slight decrease in the sensitivity of the mice to isoflurane along with increased thermal nociception. Decreased sensitivity to isoflurane—but not to halothane, sevoflurane, and desflurane—indicates that TRESK may partially mediate the action of this volatile anesthetic. A significantly higher death rate in TRESK knockout mice after the MAC assays could indicate a role for TRESK in an endogenous survival mechanism in response to stress.

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ANESTHESIOLOGY REFLECTIONS

The Irish Connections of Patrick Sim, M.L.S.

Patrick P. Sim, MLS (1939 - 2010)

Decades before he would be celebrated as the American Society of Anesthesiologists' Paul M. Wood Distinguished Librarian Emeritus, a young boy named Sim Pui-Kam matriculated at Hong Kong's famous St. Joseph's College, a Catholic boys' school where most classes were taught in English (*i.e.*, English with an Irish accent). The Lasallian Brothers who ran the school were mostly Irishmen, so, naturally, they christened the young man "Patrick" Pui-Kam Sim. In 2001 Sim was granted an Honorary Membership in the Anesthesia History Association, one of whose council members would donate a Murphy Chloroform Inhaler to the Wood Library-Museum (WLM) in honor of "Irish" Patrick Sim. The WLM salutes our fallen Librarian, Patrick Sim (1939–2010). (Copyright © the American Society of Anesthesiologists, Inc. This image appears in color in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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