Supplement

Vascular responses in mice deficient in the potassium channel, TREK-1

Khodadad Namiranian, MD

Eric E. Lloyd, PhD

Randy F. Crossland, M.S.

Sean P. Marrelli, PhD

George E. Taffet, MD

Anilkumar K. Reddy, PhD

Craig J. Hartley, PhD

Robert M. Bryan, Jr., PhD

**Methods Supplement**

*PCR genotyping:* Tail DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA). The *KCNK2*$^{+}$ allele was detected by PCR using primer pairs: 5'-GCTGGGTGAAGTTCTTCAGC (WT-forward) and 5'-CATTACCTGGATGAGTTCGTC (WT-reverse in Figure 1A). The *KCNK2*$^{-}$ allele was detected using primer pairs: 5'-GCAGCGCATCGCCTTCTATC (mutant-forward) and 5'-AGGAGATGAAGACCTCTGCAAAGG (mutant-reverse in Figure 1A). The reaction consisted of denaturation for 4 minutes at 94°C, followed by 35 cycles of denaturation (15 seconds at 94°C), annealing (30 seconds), and elongation (40 seconds at 72°C). The annealing temperature used was 65°C for the first cycle, decreasing by 1°C every cycle from cycle 1 to 10, and maintained

at 55°C for cycles 11 to 35. The resulting amplified PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide.

*Reverse transcriptase-PCR:* After flash freezing in liquid nitrogen, total RNA was isolated from brain, aorta, and cerebral arteries using Trizol® reagent (Invitrogen), RNeasy Mini Kit (Qiagen) and RNeasy Micro Kit (Qiagen) respectively according to the manufacturer's instruction. DNase-treated RNA (1ug total RNA) was primed with random hexamers and reverse transcribed (SuperScript® III, Invitrogen). The cDNA product was treated with RNase H (Invitrogen).

PCR was performed using Platinum® Taq DNA Polymerase (Invitrogen). The reaction started with denaturation for 5 minutes at 94°C, followed by 35 cycles of denaturation (15 seconds at 94°C), annealing (30 seconds at 56°C), and elongation (40 seconds at 72°C). The PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide. Selected bands were cut and purified with MinElute Gel Extraction Kit (Qiagen).

The following primers were used for reverse-transcriptase-PCR (Figure 1B):

| | |
|---|---|
| m*KCNK*2-433F | 5'-GAAAATGCCTACCCGTGCA |
| m*KCNK*2-529R | 5'-TCCAAACCGAGGCTCTCATTC |
| m*KCNK*2-729F | 5'-CCAGAAGCAGACCTTCATAGCC |

m*KCNK*2-829R 5’-GGATAATCCCTGCGTTTATTGC

Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a reference/control gene, were:

mGAPDH-F 5’-AGCCTCGTCCCGTAGACAAAA

mGAPDH-R 5’-TGGCAACAATCTCCACTTTGC

All primer pairs spanned at lest two exons in order to avoid amplification of genomic DNA. Additional methods and analysis of TREK-1 mRNA is presented in the accompanying Online Supplement.

Additional expression of *KCNK2* mRNA was determined using whole brains from WT and TREK-1 KO mice (n=5 each). The schematic location and direction of the primers and the results of the PCR are shown in Supplemental Figure I. The following primers were used for the results described in the Supplement:

m*KCNK*2-100F GGGCGCCTCACAAAGACA

m*KCNK*2-1256F GCAGGTGGATCAGACATTGA

m*KCNK*2-1356R CATGCTCAGAACAGCTGCAA

m*KCNK*2-1878R CAACACCCTCCCTGGATGTAA

Neomycin-658F GTTGGCTACCCGTGATATTG

*Real-time RT-PCR:* RNA from brain, aorta, and cerebral arteries (middle cerebral arteries and basilar artery) of three adult male TREK-1 KO mice and three adult male WT mice was isolated as described above. After reverse transcription,

cDNA was quantified by real-time PCR using SYBR® Green PCR Master Mix on an Applied Biosystems 7000 Sequence Detection System. Each sample was run in triplicate. The following primer sets were used:

| | | |
|---|---|---|
| TREK-1 | m*KCNK*2-F | 5'-GAAAATGCCTACCCGTGCA |
| | m*KCNK*2-R | 5'-AATGAGAGCCTCGGTTTGGA |
| TREK-2 | m*KCNK*10-F | 5'-CACTGATTCAGCATGCACTCG |
| | m*KCNK*10-R | 5'-CGGAGCAATATTCCCATACCC |
| TRAAK | m*KCNK*4-F | 5'-GGCCACATCGAAGCAATCTT |
| | m*KCNK*4-R | 5'-TGGCTTCTAACTTGCTCCAGC |
| TWIK-1 | m*KCNK*1-R | 5'-TTCTGGTTGTAGCCTTCCCCT |
| | m*KCNK*1-F | 5'-TCCTGCTTCTTCTTCATCCCA |
| TWIK-2 | m*KCNK*6-F | 5'-CCCTCAACGACTTAACAGGCA |
| | m*KCNK*6-R | 5'-CATTGGCAAAATATGGCCCAG |
| TASK-1 | m*KCNK*3-F | 5'-TGCTTCCCCAATGCCTTATG |
| | m*KCNK*3-R | 5'-GCGCCATTCTCAGTGTGAAGA |
| $BK_{Ca}$ | m*KCMA*1-F | 5'-TACAGCACTCCGCAGACATTG |
| | m*KCMA*1-R | 5'-CACCATAACAACCACCATCCC |
| GAPDH | mGAPDH-F | 5'-AGCCTCGTCCCGTAGACAAAA |
| | mGAPDH-R | 5'-TGGCAACAATCTCCACTTTGC |

*Pressurized cerebral arteries:* Cerebral arteries were mounted on pipettes in a chamber containing physiological buffer consisting of (in mM) 119 NaCl, 4.7 KCl, 24 $NaHCO_3$, 1.18 $KH_2PO_4$, 1.17 $MgSO_4$, 0.026 EDTA, 5.5 Glucose, and 1.6

$CaCl_2$. The buffer was gassed with 5% $CO_2$ (5%), 20 % $O_2$, and 75% $N_2$ to achieve a pH of 7.4. The arteries were pressurized to 75 mm Hg by raising reservoirs connected to the pipettes above the vessel chamber. Physiological buffer was perfused through the lumen of each middle cerebral artery at a rate to achieve a shear stress of ~20 dyne/$cm^2$ on the endothelial surface (20-35 µl/min depending on the artery radius). Each artery was magnified by a microscope, the image was displayed on a video monitor (final magnification 650X), and recorded on video cassette. Diameters were continuously measured using Optimas edge-detection software (v5.0, Optimas Corporation, Bothell, WA).

Dilator responses in preconstricted (3 µM phenylephrine) middle cerebral arteries were measured after administering ATP ($10^{-5}$ and $10^{-4}$ M) to the vessel lumen (1; 8; 9). Dilator responses to the abluminal administration of acetylcholine and the polyunsaturated fatty acids, arachidonic acid and $\alpha$-linolenic acid, were measured in basilar arteries after preconstricting with 10 nM endothelin-1. L-NAME (10 µM) and indomethacin (10 µM) were used to block NO synthase and cyclooxygenase respectively. Penitrem A (200 nM) was used to selectively block large-conductance calcium-activated potassium channels ($BK_{Ca}$). At the end of the experiment, the maximum diameter of each artery was determined by replacing the physiological buffer with calcium-free/3mM EGTA buffer.

*Isometric tension from aortic rings:* Changes in isometric tension were measured in aortic ring segments from male TREK-1 KO mice and littermate WT mice (8-12

wk). Each mouse was anesthetized and decapitated. The thoracic aorta from the arch of aorta to the diaphragm was carefully dissected, flushed with buffer to remove blood, cleaned, and cut into four 2mm long segments. Each segment was mounted in a myograph (ChuelTech, Houston, TX) to measure isometric tension changes. The ring segments were bathed in Krebs buffer (119 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$, 11.1 mM Glucose, and 2.5 mM CaCl2) maintained at 37°C and gassed with $CO_2$ 5% / $O_2$ 20% (pH = 7.4) (4).

Changes in tension were measured using Model 2 Research Grade isometric force transducer (Harvard Apparatus, Holliston, MA). The outputs from the transducers were digitized by Powerlab/8sp (AD Instruments, Colorado Springs, CO), captured at 1 KHz by LabChart v4.2.4 (AD Instruments), and stored for later analysis. Aortic ring segments were equilibrated in the organ chamber for one hour during which time they were periodically constricted using 40 and 60 mM KCl (4). The optimum resting tension, defined as the tension showing the maximum contraction to 60 mM KCl, was determined to be 1.2 g. This optimal resting tension was used in subsequent studies of aortic rings. Constrictor responses in aortas from male TREK-1 KO mice and male WT littermates were evaluated using phenylephrine (0.01μM to 100μM).

Endothelial-mediated relaxations in aortas from male WT mice and male TREK-1 KO littermates were measured using carbachol (0.01μM to 100μM), a cholinergic

agonist that stimulates the production of NO in endothelium. Prior to administering the carbachol, each ring was constricted with 0.13 μM phenylephrine, a concentration that produced ~70% of the maximum response of phenylephrine. The concentration of phenylephrine that produced 70% of maximum constriction was not significantly different between WT mice (0.146 μM ± 0.05) and TREK-1 KO mice (0.103 μM ± 0.07). Relaxations were normalized to the constriction produced by phenylephrine.

*Isolation of vascular smooth muscle cells (VSMCs):* Single CVSMCs were enzymatically isolated from a basilar artery and two middle cerebral arteries, which had been pooled, using a modification of previously described protocols (2; 7). Basilar and middle cerebral arteries were harvested, cleaned of connective tissue, and placed in digestion buffer consisting of (in mM): NaCl 135, KCl 5, $MgCl_2$ 1.5, $Na_2HPO_4$ 0.42, $NaH_2PO_4$ 0.44, $NaHCO_3$ 4.17, HEPES 10. The pH was adjusted to 7.25 with NaOH. Arteries were digested in a solution containing 1mg/ml papain, 1mg/ml dithioerythritol, 1mM EDTA, and 1 mg/ml bovine serum albumin (BSA) in digestion buffer for 30 minutes. After two washes with 1 mg/ml BSA in digestion buffer, arteries were further digested with 1.2 mg/ml collagenase, 60 U/ml elastase, 0.8 mg/ml soybean trypsin inhibitor, and 1mg/ml BSA in digestion buffer for 8 minutes. Following two washes, the arteries were triturated and the dispersed cells were placed on ice and used within 8 hours for the electrophysiological studies.

*Electrophysiological measurements:* Aliquots of cerebrovascular smooth muscle cells (CVSMCs) were placed in a chamber on the stage of an inverted microscope (Olympus IX 71) and allowed to adhere to the glass bottom. The chamber was continuously perfused with buffer by a gravity-fed reservoir. An integrating patch-clamp amplifier (Axopatch 200B) and pCLAMP 9.2 software (Molecular Devices) were used to measure whole cell currents in individual CVSMCs. Data were filtered at 1 kHz with a four-pole Bessel filter, digitized at 5 kHz, and stored on a hard disk. There was no compensation for cellular capacitance, series resistance, or leak current. The liquid junction potential was calculated using pClamp and corrected. Currents are expressed as current density (pA/pF) to normalize for differences between cell sizes. Patch electrodes were pulled from glass tubing (OD: 1.65 mm, ID: 1.28 mm, Warner Instruments) in two stages by a pipette puller (model PP-830, Narishige) and polished with a microforge (model MF-830, Narishige). Pipette resistances were 5–6 MΩ.

The pipette buffer consisted of (in mM) 100 gluconate ($K^+$ salt), 43 KCl, 0.1 EGTA, 1 $MgCl_2$, 10 HEPES (5). The pH was adjusted to 7.1 with KOH. The bath buffer contained (in mM) 137 NaCl, 5.6 KCl, 0.1 $CaCl_2$, 1 $MgCl_2$, 5 Glucose, 10 HEPES. The pH was adjusted to pH 7.4 using NaOH. Cells were ruptured using negative pressure in the patch pipette and whole cell currents were measured using a ramp protocol.

*Drugs and reagents:* Arachidonic acid (Sigma) and α-linoleic (NuChek Prep, Inc., Elysian, MN) were dissolved in ethanol. Penitrem A (Biomol) was dissolved in DMSO and iberiotoxin (Sigma) was dissolved in buffer. Indomethacin (Sigma) was dissolved in 10mM $Na_2CO_3$. ATP, phenylephrine, endothelin, and acetylcholine (all from Sigma) were dissolved in water. MAHMA NONOate (Methylamine hexamethylene methylamine NONOate, Cayman Chemical), an NO donor, was dissolved in water.

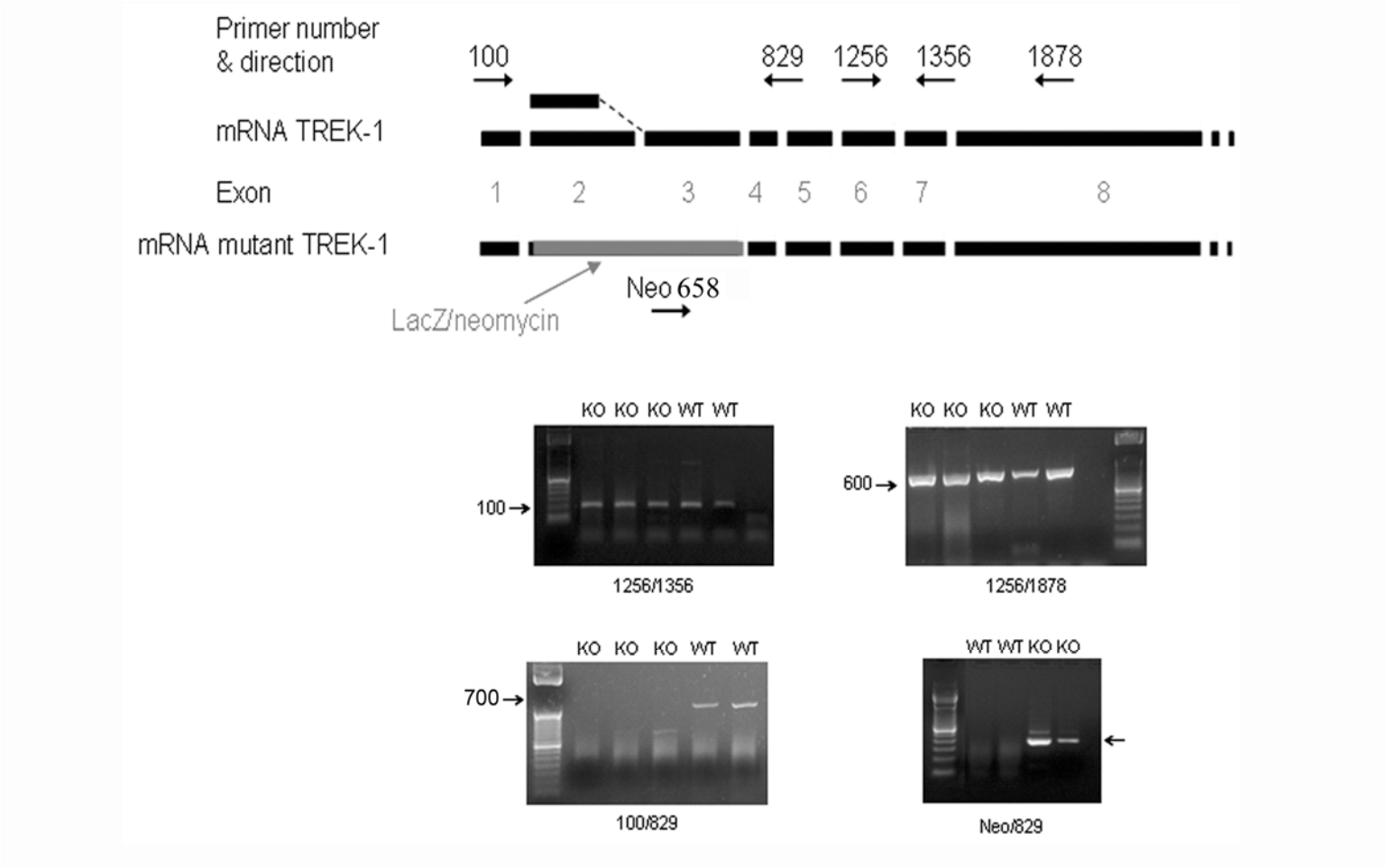
**Supplemental Results**

In total, 965 mice from the *KCNK2* colony were genotyped. *KCNK2*$^{-/-}$ mice were viable, fertile, and did not show any obvious anatomical abnormalities. Mating between *KCNK2*$^{-/+}$ mice led to the birth of 583 mice. The genotype of the offspring were 151 *KCNK2*$^{+/+}$ (26%), 269 *KCNK2*$^{-/+}$ (46%), 163 *KCNK2* $^{-/-}$ (28%). This distribution of genotype was not significantly different from the predicted Mendelian ratio (p=0.14 chi-square test).

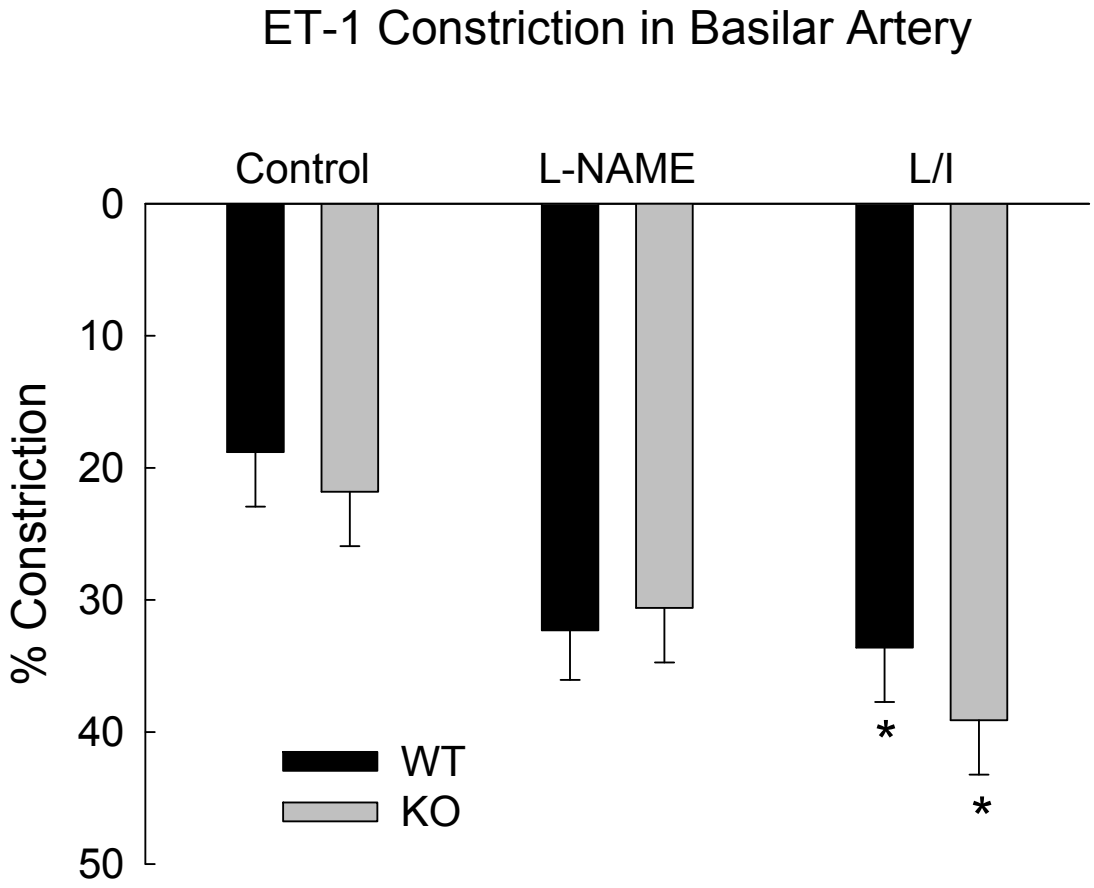
Since the *LacZ/neomycin* cassette had two polyadelylation sites to terminate transcription, there should not be mRNA corresponding to exons 4 through 8 for the mutant *KCNK2* gene. Unexpectedly, we found mRNA corresponding to exons 6, 7, and 8 in *TREK-1 KO* mice (Supplemental Figure I). The identity of the amplicons in both genotypes was confirmed by sequencing. Two hypotheses were tested in an attempt to understand the transcription of this distal message in the *KCNK2*$^{-}$ allele. The first hypothesis was that the first exon was spliced to

exon 4, 5, or 6. This possibility was tested using a primer pair consisting of a forward primer (*m KCNK2*-100F) located in the exon 1 and a reverse primer (*m KCNK2*-829R) located in the exon 4 (Supplemental Figure I). While this primer set amplified a corresponding product in brain from WT mice, no product was found in brain from the TREK-1 KO mice (Supplemental Figure I).

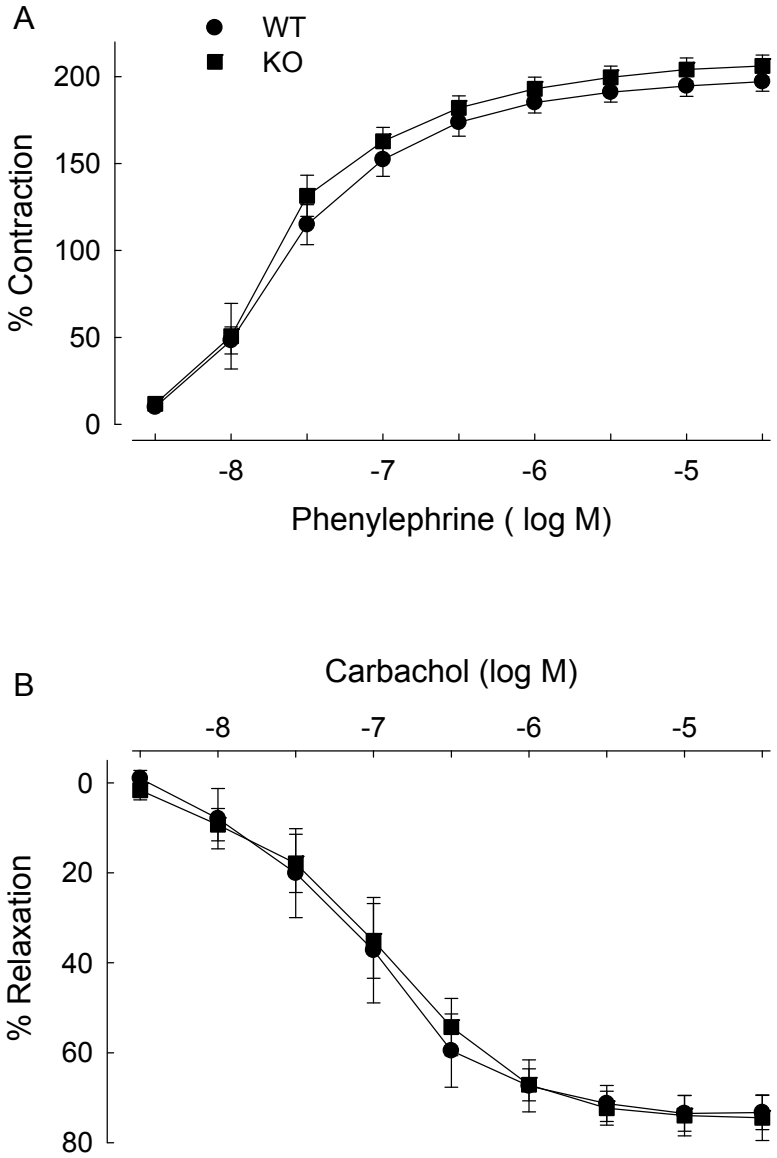
The second hypothesis stated that the Neomycin cassette of the mutated allele was spliced to exon 4 despite polyadelylation sites to terminate transcription. In order to test this hypothesis, we used a forward primer located in the *Neomycin* cassette and a reverse primer located in exon 4 (reverse 829). A PCR product of ~300 bp was amplified in the $KCNK2^{-/-}$ but not in $KCNK2^{+/+}$ mice (Supplemental Figure I). After sequencing, we determined that the LacZ/neomycin cassette was spliced to the beginning of the fourth exon of the mutated *KCNK2* allele. It is rare for splicing to occur after poly-adenylation sites (AAUAAA) that exists at the end of the Neomycin gene. However, the efficiency of these sites also depends on downstream GU-rich sequences (3; 6). For the $KCNK2^{-}$ allele, it appears that the downstream sequence did not allow for transcription termination. Although the message for exons 4-8 was detected in TREK-1 KO mice, the mutated *KCNK2* transcript was not translated into protein. This conclusion is based on IP-western blotting where protein corresponding to exons 7 & 8 was not expressed in TREK-1 KO mice (see Figure 1C in the primary publication).



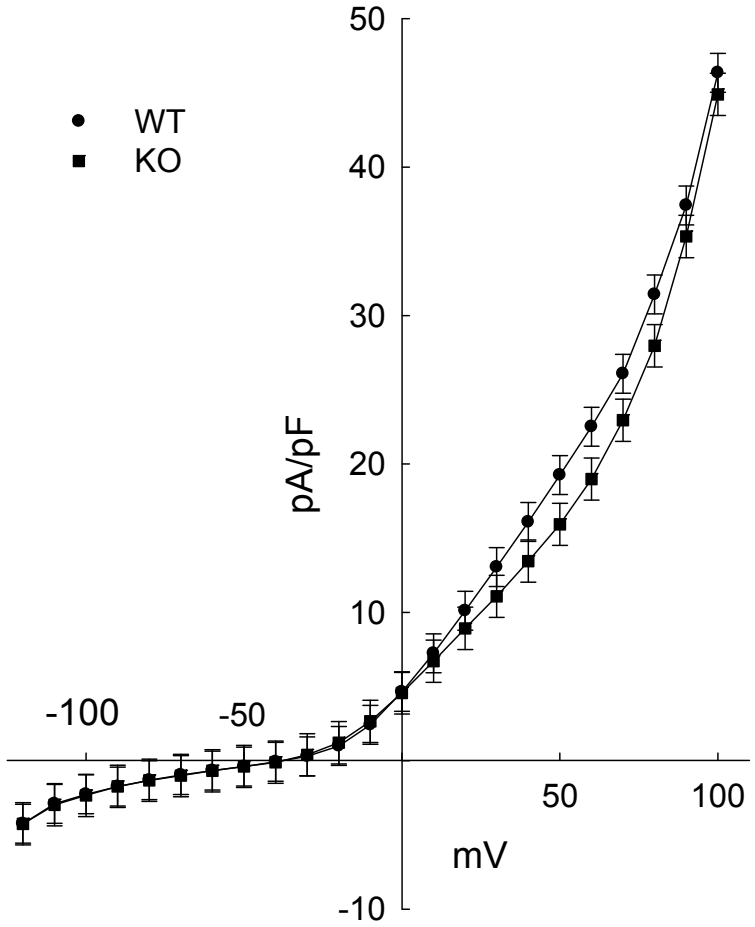

Supplemental Figure I. A diagram of TREK-1 mRNA with primer pairs used to detect strands of message (top). The number for the primers represents the base number of the mRNA with the arrow denoting the direction. The gel shows results from mRNA isolated from brain in wild type and TREK-1 KO mice (bottom).




Supplemental Figure II. Constrictions of basilar arteries from WT and TREK-1 KO mice to 10 nM ET-1 in the absence and presence of L-NAME, or L-NAME-indomethacin (L/I). * $p<0.05$ compared to Control (n=5 for each group).




Supplemental figure III. (A) isometric contractions of the aortas from WT and TREK-1 KO mice with the cumulative administration of phenylephrine (n=3 for each group). Dose dependent relaxations to carbachol, a cholinergic agonist, are shown in Supplemental Figure IIIB (n=3 for each group). There were no differences in the isometric constrictions or relaxations between aortas from littermate WT and TREK-1 KO mice.




Supplemental Figure IV. Whole cell currents (normalized to cell capacitance) in cerebrovascular smooth muscle cells from WT and TREK-1 KO mice as a function of membrane potential (n= 39 for WT and 33 for TREK-1 KO). There was no effect of genotype (p=0.33) or interaction between genotype and membrane potential (p=0.97).

Supplement Reference List


1. Andresen JJ, Shafi NI, Durante W and Bryan RM, Jr. Effects of carbon monoxide and heme oxygenase inhibitors in cerebral vessels of rats and mice. *AJP - Heart and Circulatory Physiology* 291: H223-H230, 2006.
2. Bryan RM, Jr., You J, Phillips SC, Andresen JJ, Lloyd EE, Rogers PA, Dryer SE and Marrelli SP. Evidence for two-pore domain potassium channels in rat cerebral arteries. *Am J Physiol Heart Circ Physiol* 291: H770-H780, 2006.
3. Buratowski S. Connections between mRNA 3' end processing and transcription termination. *Curr Opin Cell Biol* 17: 257-261, 2005.
4. Chen J, Crossland RF, Noorani MMZ and Marrelli SP. Inhibition of TRPC1/TRPC3 by PKG contributes to NO-mediated vasorelaxation. *AJP - Heart and Circulatory Physiology* 297: H417-H424, 2009.
5. Cohen KD and Jackson WF. Membrane hyperpolarization is not required for sustained muscarinic agonist-induced increases in intracellular Ca2+ in arteriolar endothelial cells. *Microcirculation* 12: 169-182, 2005.
6. Connelly S and Manley JL. A functional mRNA polyadenylation signal is required for transcription termination by RNA polymerase II. *Genes Dev* 2: 440-452, 1988.

7. Jackson WF, Huebner JM and Rusch NJ. Enzymatic isolation and characterization of single vascular smooth muscle cells from cremasteric arterioles. *Microcirculation* 4: 35-50, 1997.
8. You J, Johnson TD, Marrelli SP and Bryan RM, Jr. Functional heterogeneity of endothelial P2 purinoceptors in the cerebrovascular tree of the rat. *Am J Physiol* 277: H893-H900, 1999.
9. You JP, Johnson TD, Marrelli SP, Mombouli JV and Bryan RM, Jr. P2u-receptor mediated release of endothelium-derived relaxing factor/nitric oxide and endothelium-derived hyperpolarizing factor from cerebrovascular endothelium in rats. *Stroke* 30: 1125-1133, 1999.