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TREK-1, a K^+ channel involved in neuroprotection and general anesthesia

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TREK-1 is a two-pore-domain background potassium channel expressed throughout the central nervous system. It is opened by polyunsaturated fatty acids and lysophospholipids. It is inhibited by neurotransmitters that produce an increase in intracellular cAMP and by those that activate the Gq protein pathway. TREK-1 is also activated by volatile anesthetics and has been suggested to be an important target in the action of these drugs. Using mice with a disrupted TREK-1 gene, we now show that TREK-1 has an important role in neuroprotection against epilepsy and brain and spinal chord ischemia. Trek1$^{-/-}$ mice display an increased sensitivity to ischemia and epilepsy. Neuroprotection by polyunsaturated fatty acids, which is impressive in Trek1$^{+/+}$ mice, disappears in Trek1$^{-/-}$ mice indicating a central role of TREK-1 in this process. Trek1$^{-/-}$ mice are also resistant to anesthesia by volatile anesthetics. TREK-1 emerges as a potential innovative target for developing new therapeutic agents for neurology and anesthesiology.

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Introduction

Two-pore-domain potassium channels (K_{2P} channels) form a novel class of K^+ channels identified in various types of neurons (Kim *et al*, 1995; Wei *et al*, 1996; Lesage and Lazdunski, 2000; Talley *et al*, 2003). They are open at membrane potentials across the physiological range and are therefore likely to contribute to the background or leak currents that help set the resting membrane potential and oppose depolarizing influences. They are key components in shaping the characteristics of neuronal excitability. TREK-1 (Fink *et al*, 1996) is expressed throughout the central nervous system (Fink *et al*, 1996; Lauritzen *et al*, 2000; Maingret *et al*, 2000b; Hervieu *et al*, 2001; Talley *et al*, 2001) and is an important member of this family. It is the probable mammalian homolog of the Aplysia S-type K^+ channel (Siegelbaum *et al*, 1982; Patel *et al*, 1998), a channel involved in simple forms of learning and memory. TREK-1 is activated by membrane stretch and intracellular acidification (Patel *et al*, 1998; Maingret *et al*, 1999b). TREK-1 is opened by arachidonic acid and other polyunsaturated fatty acids (PUFAs) as well as lysophospholipids (LPLs) (Patel *et al*, 1998; Maingret *et al*, 2000b). On the other hand, PUFAs and LPLs are potent protective agents against forebrain ischemia and seizures, and it has been proposed that this effect results, at least in part, from their action on TREK channels (Lauritzen *et al*, 2000; Blondeau *et al*, 2001, 2002). TREK-1 probably has a central role in the control of excitability by a variety of neurotransmitters. TREK-1 is potently inhibited by neurotransmitters that produce an increase in intracellular cAMP (Patel *et al*, 1998) and also by those that activate the Gq protein pathway (Lesage *et al*, 2000; Chemin *et al*, 2003). The inhibition of TREK channels by glutamate via the activation of group I Gq-coupled metabotropic glutamate receptors requires PTX-insensitive G proteins coupled to phospholipase C (Chemin *et al*, 2003). TREK-1 is also activated by volatile anesthetics and suggested to be a target in the action of these drugs (Patel *et al*, 1999). This paper definitively shows that TREK-1 plays a major role in the PUFAs/LPLs-induced neuroprotection against epilepsy and ischemia and that TREK-1-deficient mice display resistance to anesthesia.

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Results

Generation and characterization of TREK-1 null mice

The TREK-1 gene of mice was disrupted through homologous recombination using a Cre/loxp-based strategy (Figure 1A). The CRE-mediated excision of exon 3 led to the deletion of the first transmembrane domain of the TREK-1 channel. Heterozygous matings produced offspring with normal Mendelian ratios (Figure 1B and C). Homozygous (Trek1$^{-/-}$) mutant mice were healthy, fertile and did not display any visible morphological differences. PCR amplification of testicular cDNA (a tissue where TREK-1 is abundant; Hervieu *et al*, 2001; Talley *et al*, 2001) showed that the null mutant only expressed a truncated transcript (Figure 1D). Sequencing of this transcript confirmed that it results from the deletion of the 311 nucleotides of the targeted exon (Figure 1D). The brain morphology of Trek1$^{-/-}$ mice appeared normal. In brain regions known to express the KCNK2 gene, no TREK-1 messenger RNA was detected by *in situ* hybridization using a probe recognizing the 3′-end of the mRNA (Figure 1E). The absence of the TREK-1 protein in null mutants was confirmed by the lack of immunoreactivity to specific anti-TREK-1 antibody (Maingret *et al*, 2000a) in brain areas such as the cortex or the hippocampus where it is highly expressed (Figure 1F).

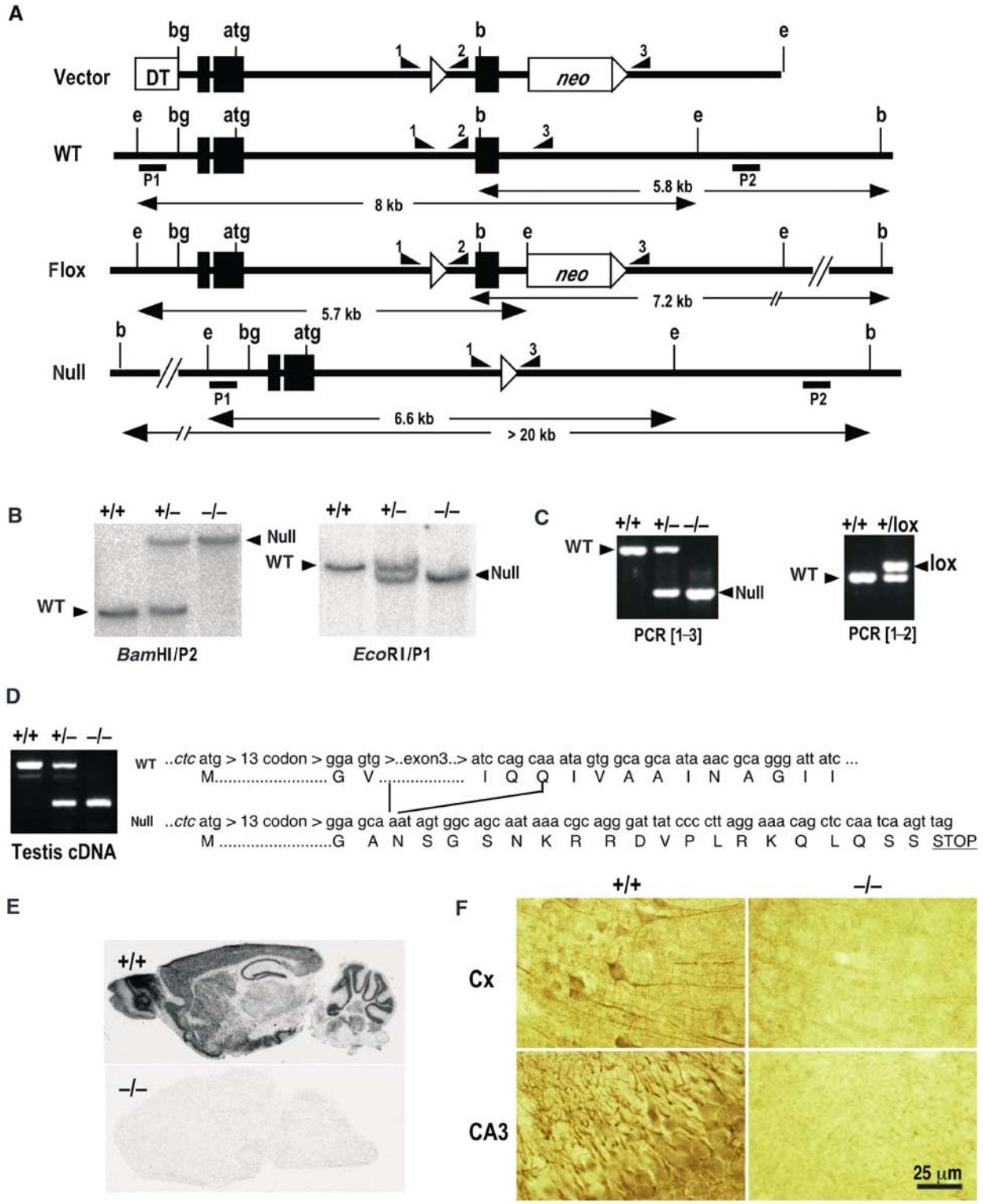

Figure 1 Disruption of the KCNK2 gene. (**A**) Targeting vector (▶ = loxp), native (WT) and recombined floxed (Flox) alleles. External probes used to characterize homologous recombination are designated as P1 and P2. Arrowheads (1–3) display locations of the primers used for PCR analysis of the different products. Double-headed arrows indicate the expected size of restriction fragments for Southern analysis (bg = *Bgl*II; b = *Bam*HI; e = *Eco*RI). (**B**) Southern blot analysis of *Eco*RI- and *Bam*HI-digested tail DNA from wild-type (+/+), heterozygous (+/−) or homozygous (−/−) KCNK2 mice probed with P1 and P2, respectively. (**C**) PCR amplification from tail genomic DNA. (**D**) PCR amplification from +/+, +/− and −/− mouse testis cDNA with primers surrounding the deletion. (**E**) *In situ* hybridization analysis shows the lack of mRNA expression in $Trek^{-/-}$ mouse brain on X-ray films. (**F**) Immunocytochemical TREK-1 staining in neocortex (Cx) and hippocampal CA3 subfield sections using a specific α-TREK-1 antibody (Lauritzen *et al*, 2000).

The TREK-1 mutation did not interfere with the mRNA expression in brain and cerebellum of other K_{2P} channels and of the GABAα6 subunit whose deletion causes an increased expression of TASK-1, another K_{2P} channel (Brickley *et al*, 2001) (Figure 2A). There was no compensatory upregulation of genes for other neuronal K_{2P} channels such as TWIK-1, TREK-2, TRAAK, TASK-1, TASK-3 or the GABAα6 subunit in Trek1$^{-/-}$ mice ($P<0.01$).

Primary behavioral testings (see Supplementary Materials and methods) showed that the TREK-1-deficient mice did not display any abnormal phenotype in appearance (Figure 2B). There was no difference in skin color, body tone or body weight. Trek1$^{-/-}$ mice did not display any abnormalities in body position, respiration or spontaneous activity. Stereotypies or tremor were not observed. There was no difference in frequency and volume of defecation or urination. Locomotor activity of the Trek1$^{-/-}$ mutant was not different from Trek1$^{+/+}$ control in the open field test as well as in the rotarod. No difference was seen in the touch escape response or in the positional passivity test. Recordings of reflexes and autonomic functions did not show any significant differences. Scorings were comparable in the visual placing test, grip strength, corneal and pinna reflex and in the righting reflex. No significant difference was seen between Trek1$^{+/+}$ and Trek1$^{-/-}$ mice in the object recognition test.

For comparative purpose, we have also deleted the TRAAK gene (see Supplementary Materials and methods and Supplementary Figure 1) to be able to evaluate the respective properties of Trek1$^{-/-}$ and Traak$^{-/-}$ mice. The TRAAK channel is closely related to the TREK-1 channel. Like TREK-1, it is a background outward rectifier K^+ channel, opened by membrane stretch, cell swelling and activated by PUFAs and LPLs. However, unlike TREK-1, the TRAAK channel is not activated by intracellular acidification (Maingret *et al*, 1999b) nor volatile anesthetics (Patel *et al*, 1999) and not inhibited by neurotransmitters that increase cAMP via a protein kinase A-dependent phosphorylation process (Fink

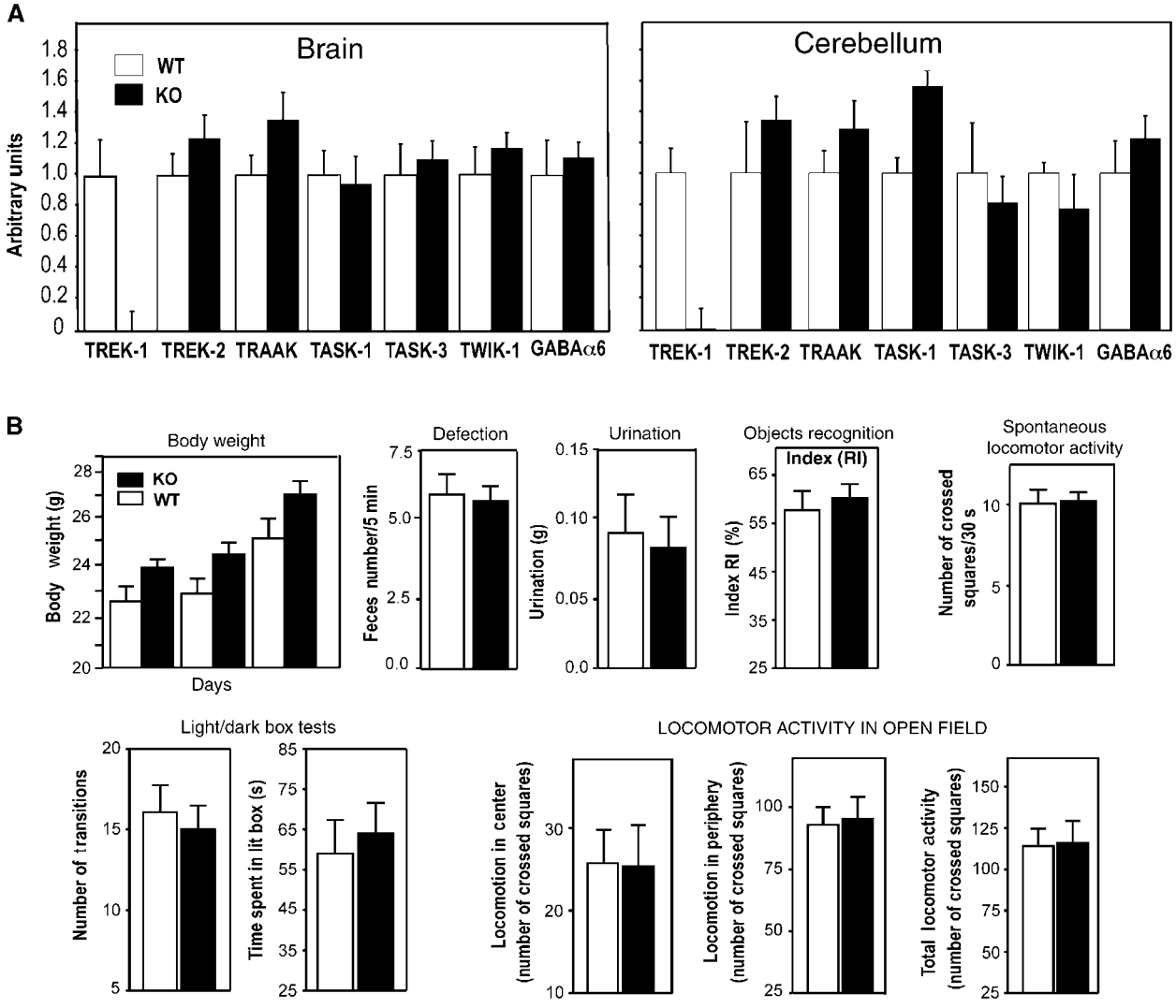

Figure 2 Characterization of TREK-1 null mice. (**A**) Relative expression of TREK-1, TREK-2, TRAAK, TASK-1, TASK-3 and GABAα6 mRNA levels in brain and cerebellum from Trek1$^{-/-}$ and Trek1$^{+/+}$ mice. Mean levels of gene expression, normalized to cyclophilin D, are displayed in arbitrary units on the vertical axis ($n=3$ mice, $P<0.01$, Student's *t*-test). (**B**) Primary behavioral test battery showing the lack of abnormal phenotype in TREK-1-deficient mice. Results are expressed as mean±s.e.m. Statistical significance was set at $P<0.05$ (Student's *t*-test or a Mann–Whitney test).

et al, 1998; Maingret *et al*, 1999a) or by those that activate the Gq protein pathway (Chemin *et al*, 2003).

Electrophysiological recordings

To test whether TREK-1 currents could be recorded in neurons from wild-type mice and were absent in neurons from TREK-1 null mice, we performed patch clamp recordings in striatal neurons in culture. These neurons were chosen because they strongly express TREK-1 but not TREK-2 or TRAAK channels (Hervieu *et al*, 2001; Talley *et al*, 2001), two K_{2P} channels that are also activated by membrane stretch, PUFAs and LPLs (Lesage and Lazdunski, 2000; Lesage *et al*, 2000; Patel and Honoré, 2001). In the striatum, the primary type accounting for 85% of the neurons is the GABAergic medium-size spiny neuron (Kita and Kitai, 1988). Using an antibody against GABA, we have checked that most neurons in our culture were indeed GABAergic (data not shown). The resting membrane potential of the striatal neurons from Trek1$^{+/+}$ and Trek1$^{-/-}$ mice was not significantly different (Student's *t*-test, $P=0.0586$) with -47.2 ± 1.6 mV ($n=30$) and -51.5 ± 7.5 mV ($n=26$), respectively. Neurons with resting membrane potential less negative than -30 mV were discarded. Using the inside-out configuration and in the presence of K^+ channels blockers (TEA, 4-AP and glibenclamide), a native TREK-1-like current was regularly recorded in cultures from wild-type mice. This current was reversibly activated by 10 μM arachidonate (AA) (Figure 3A) and by internal acidification (Figure 3B), as previously described (Maingret *et al*, 1999b, 2000b). The conductance was 55.8 ± 0.9 pS at $+50$ mV ($n=6$), which is close to the conductance of the cloned TREK-1 (Patel *et al*, 1998). The outwardly rectifying current reversed around the potassium equilibrium potential (Figure 3C). Like TREK-1 (Patel *et al*, 1998), the native current was also activated by membrane stretch (Figure 3D). The effect of volatile anesthetics was also studied on the TREK-like current recorded in striatal cultures from wild-type mice (Figure 3E, inset) and in TREK-1-transfected COS cells (Supplementary Figure 2A and B). Halothane in striatal neurons (Figure 3E, inset) as well as halothane and sevoflurane in COS cells (Supplementary Figure 2A and B) highly stimulated a TREK-1 channel activity. The loss of functional TREK-1 channels in TREK-1 null mutants was demonstrated by outside-out patch clamp recordings in striatal neurons. Figure 3E and F shows that in the presence of TEA and 4-AP to block voltage-dependent K^+ channels, there was no expression of basal current in wild-type neurons and in null mutants. Upon perfusion with the TREK-1 activator AA (20 μM), a robust TREK-1-like current was recorded in Trek1$^{+/+}$ neurons, whereas no significant variation was observed in Trek1$^{-/-}$ neurons. This electrophysiological analysis confirmed (i) that the TREK-1 deletion had taken place and (ii) that there was no compensatory upregulation of genes for other neuronal K_{2P} channels.

Role for the TREK-1 channel in the control of epileptogenesis

The high level of TREK-1 channel expression in the cortex and thalamic nuclei and its colocalization on GABAergic cortical and hippocampal interneurons, which are inhibitory to pyramidal cell activity (Hervieu *et al*, 2001; Talley *et al*, 2001), suggest a possible involvement of the TREK-1 channel in the control of epileptic seizures. To analyze the seizure

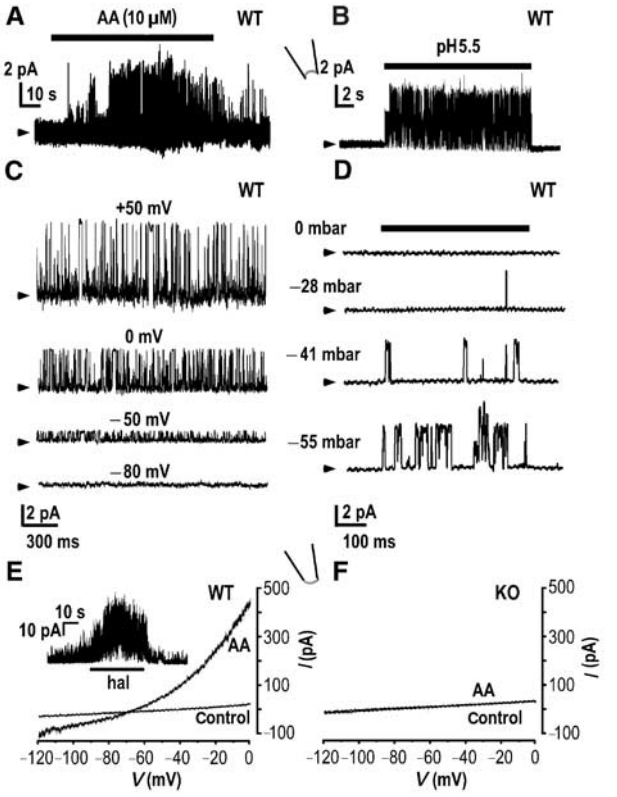

Figure 3 Patch clamp recordings in striatal neurons from Trek1$^{+/+}$ and Trek1$^{-/-}$ mice. (**A**) Activation of the TREK-like current by 10 μM AA. (**B**) Activation of the TREK-like current by internal acidification to pH 5.5. Currents in (A, B) were recorded in inside-out configuration at 0 mV. (**C**) Single channel currents recorded as in (A) at various potentials as indicated. (**D**) Activation by membrane stretch recorded as in (A) at various negative pressures as indicated. (**E**) Typical TREK-like current recorded in outside-out configuration before (control) and after activation by 10 μM AA in striatal neurons from wild-type mice (WT). Values are average of two consecutive current traces elicited with voltage ramps starting from 0 mV down to −120 mV, from a holding potential of 0 mV. Inset: Effect of 2 mM halothane (hal) on TREK-like activity recorded at 0 mV in outside-out configuration. (**F**) Same recordings in neurons from TREK-1 knockout mice (KO).

susceptibility of Trek-1-deficient mice, we used the response to kainic acid (KA, an agonist of glutamate receptor) and to pentylenetetrazol (PTZ, a $GABA_A$ receptor antagonist), as an overall index of neuronal network excitability. Trek1$^{+/+}$ and Trek1$^{-/-}$ mice were injected intraperitoneally with epileptogenic doses of KA (22 mg/kg) or PTZ (40–55 mg/kg) and the degree of seizures was scored (Tsirka *et al*, 1995). Trek1$^{-/-}$ mice were much more vulnerable to KA-induced seizures than Trek1$^{+/+}$ mice as assessed by either seizure score or mortality rate (Figure 4A). More than 75% of the mutant mice died within 3 days of KA administration, compared with 3% of Trek1$^{+/+}$ mice, and the average maximum intensity of seizures observed in Trek1$^{-/-}$ mice increased by 33%. A comparison of electroencephalogram (EEG) patterns in the hippocampus of Trek1$^{+/+}$ and Trek1$^{-/-}$ mice is shown in Figure 4F. A spectral analysis of EEG activity shows that 45 min following KA treatment (22 mg/kg), Trek1$^{-/-}$ mice developed generalized convulsive seizures with the appearance of bilateral spike-wave discharges with spike frequencies and amplitudes higher than in Trek1$^{+/+}$ mice (Figure 5A and B).

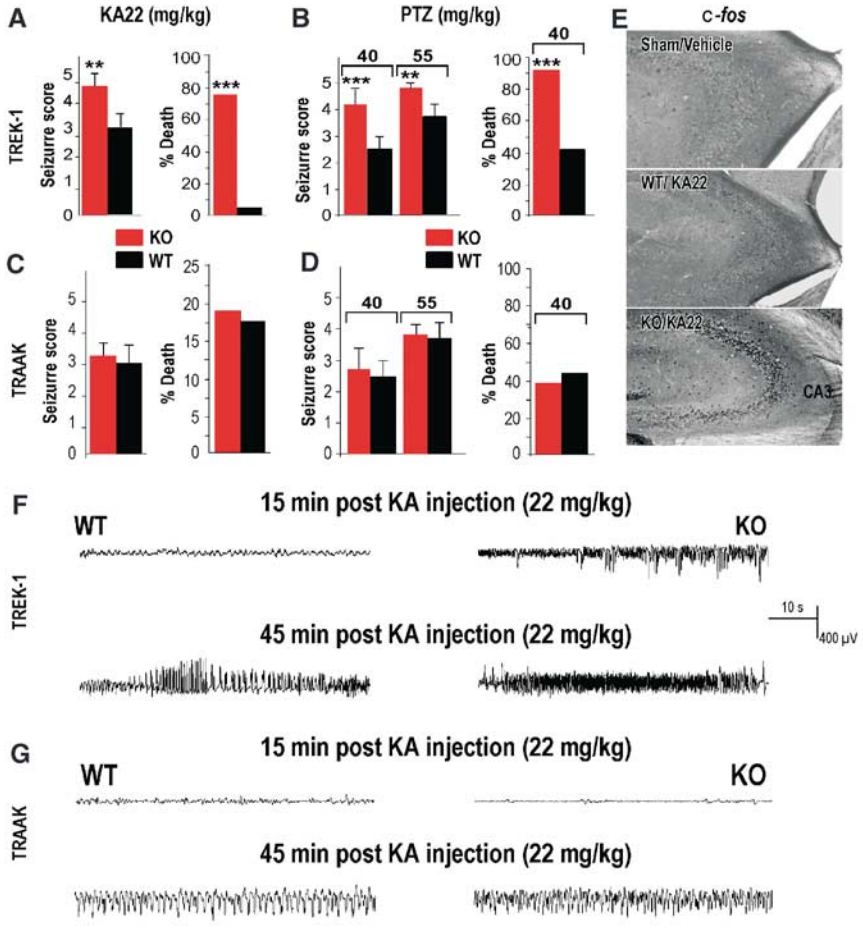

Figure 4 Increased susceptibility to epileptic agents in TREK-1-deficient mice. (**A, B**) Seizure behavior and mortality rate in wild-type and mutant TREK-1 mice after KA (A) or PTZ injection (B). (**C, D**) Seizure behavior and mortality rate in wild-type and mutant TRAAK mice after KA (C) or PTZ (D) injection. Seizures were scored for 2 h after intraperitoneal injection with KA (22–28 mg/kg) or PTZ (40–55 mg/kg). Seizures were ranked as follows: 1, immobility; 2, myoclonic jerks of the neck and head with brief twitching movements; 3, unilateral clonic activity; 4, bilateral forelimb tonic and clonic activity; 5, generalized tonic–clonic activity with loss of postural tone including death from continuous convulsions. Values represent mean ± s.e.m. of the maximum seizure intensity recorded for each mouse ($n = 20$ per genotype). *Significantly different from vehicle-treated wild type (KA treatment 22 mg/kg), $^{**}P < 0.001$, $^{***}P < 0.0001$, ANOVA followed by Tukey's multiple comparison test. (**E**) Increased expression of c-*fos* protein in CA3 pyramidal neurons in Trek1$^{-/-}$ mice 120 min after KA treatment (22 mg/kg). (**F**) EEG following KA (22 mg/kg) showing the increased KA susceptibility of Trek1$^{-/-}$ mice as compared to Traak$^{-/-}$ mice (**G**) ($n = 10$ per genotype).

Trek1$^{-/-}$ mice also showed an increased sensitivity to PTZ-induced seizures (Figure 4B). Unlike the slow progression of motor symptoms observed in the KA-induced seizures, PTZ induced abrupt general tonic–clonic seizures within 5 min of injection. At a dose of 55 mg/kg, more than 90% of Trek1$^{-/-}$ mice died from continuous tonic–clonic convulsions, whereas 60% of Trek1$^{+/+}$ mice survived (Figure 4B).

Activation of c-*fos*, in regions susceptible to kainate injection, is routinely used as a biochemical marker of neuronal excitability (Smeyne *et al*, 1992). The expression of the c-*fos* protein was drastically enhanced in Trek1$^{-/-}$ mice compared to Trek1$^{+/+}$ mice, particularly in CA3 subfield at 120 min after KA injection (Figure 4E).

A comparative study was carried out with the TRAAK channel. TRAAK-deficient mice did not display an increased sensitivity to epilepsy (Figures 4C, D and G and 5C). Taken together, all these results show that, unlike TRAAK null mice, TREK-1-deficient mice are hypersensitive to kainate and PTZ-induced seizures and point to TREK-1 as a key target for epileptogenesis.

TREK-1 channel in brain and spinal chord ischemia and its major role in the neuroprotection provided by PUFAs and LPLs

Linolenic acid (LIN) or lysophosphatidylcholine (LPC) at a dose of 500 nmol/kg injected 30 min before the KA administration induced a potent decrease of the seizure activity in Trek1$^{+/+}$ mice but had no effect in Trek1$^{-/-}$ mice (Figure 6A and B). The seizure score or the mortality rate shows that LIN- or LPC-injected Trek1$^{+/+}$ mice were much less vulnerable to KA-induced seizures than vehicle-injected Trek1$^{+/+}$ mice, while LIN- or LPC-injected Trek1$^{-/-}$ mice were not protected (Figure 6A). More than 78% of the mutant mice treated with LIN or LPC died within 3 days of KA22 administration, compared with 3% of LIN- or LPC-injected Trek1$^{+/+}$ mice, and the average maximum intensity of seizures observed in treated Trek1$^{-/-}$ mice increased by 38%. EEG

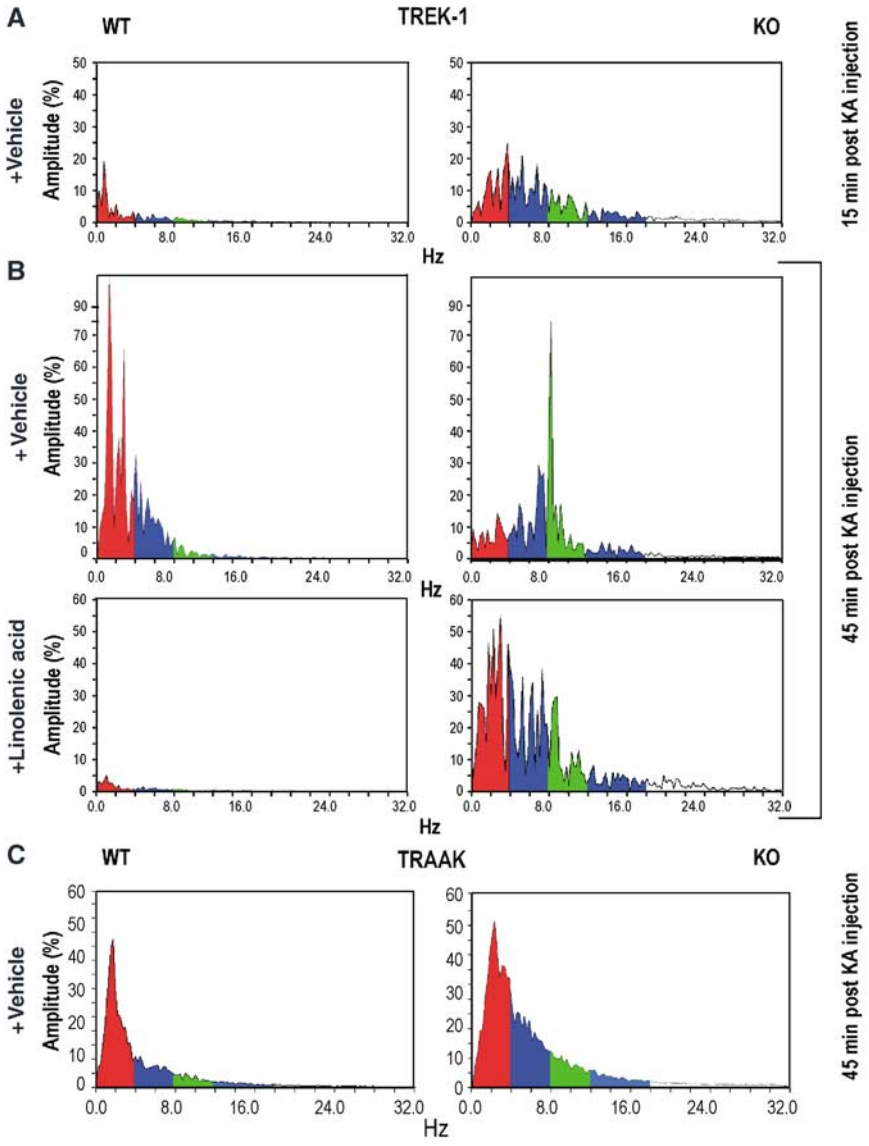

Figure 5 Spectral profiles of EEG recordings following KA (22 mg/kg) injection in Trek and Traak mice. (**A**) Increased KA susceptibility of Trek1$^{-/-}$ mice. (**B**) No anticonvulsive effect of LIN injection in Trek1$^{-/-}$ mice. (**C**) No difference in KA susceptibility between vehicle-treated Traak$^{-/-}$ (KO) and Traak$^{+/+}$ (WT) mice. Spectral profiles of EEG recordings ($n = 10$ per genotype and treatment) are shown 15 and 45 min following KA injection in vehicle-treated Trek1$^{+/+}$ and Trek1$^{-/-}$ mice and 45 min following KA injection in LIN (500 nmol/kg)-treated Trek1$^{+/+}$ and Trek1$^{-/-}$ mice. Spectral profiles of EEG recordings in vehicle-treated Traak mice are shown 45 min following KA injection.

patterns in the hippocampus of Trek1$^{+/+}$ and Trek1$^{-/-}$ mice treated with LIN (Figure 6B) and their spectral analysis of EEG activity (Figure 5B) confirm the lack of efficiency of LIN treatment in null mutant mice. The same protocol applied to TRAAK mice showed no difference in the neuroprotective effect of LIN or LPC between Traak$^{+/+}$ and Traak$^{-/-}$ mice (data not shown). This strongly suggests that the antiepileptic effect of PUFAs or LPLs is directly related to the activation of the TREK-1 channel.

Another important cause of neuronal damage is ischemia. Trek1$^{+/+}$ and Trek1$^{-/-}$ mice were submitted to a transient bilateral occlusion of common carotid arteries (CCAs) during systemic hypotension (mean arterial blood pressure (MABP) 30 ± 3 mmHg) maintained for 30 min. Trek1$^{+/+}$ mice presented no sign of hyperexcitability in the days following a 30 min period of ischemia. In contrast, most of the knockout mice developed seizures of progressive severity during the same time of reperfusion. More than 70% of Trek1$^{-/-}$ mice died in the 3 days after ischemia compared with 34% of Trek1$^{+/+}$ mice (Figure 6C; $P < 0.001$). LIN or LPC (500 nmol/kg) injected 30 min before the induction of global ischemia had no effect in Trek1$^{-/-}$ mice, while it protected the Trek1$^{+/+}$ mice against neuronal death and significantly increased their survival (Figure 6C). This observation strongly suggests that the neuroprotective effect of PUFAs or LPLs against global ischemia is directly related to the activation of the TREK-1 channel. The specificity of the TREK-1 channel in neuroprotection against ischemic injury is strengthened by results obtained with TRAAK-deficient mice, which did not display an increased sensitivity to ischemia (Figure 6C).

We also analyzed the role of TREK-1 in spinal cord ischemia. It is a devastating complication with resulting paraplegia, observed after repair of thoracic or abdominal

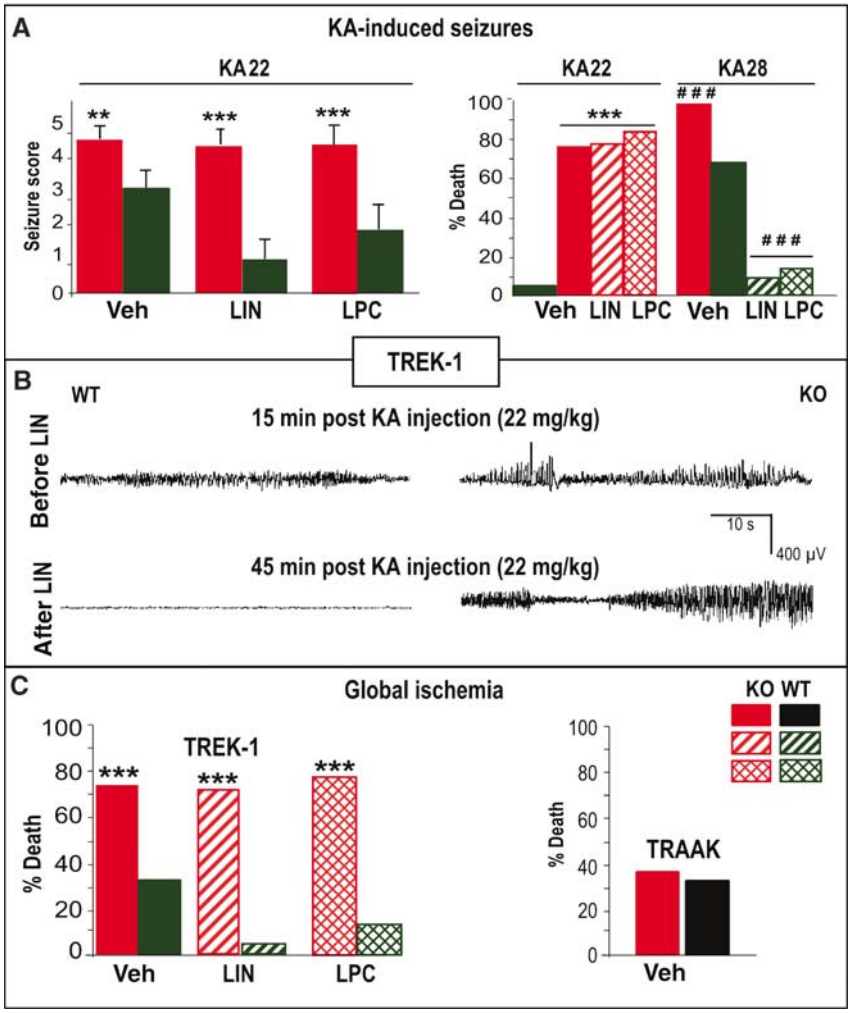

Figure 6 Increased vulnerability of TREK-1-deficient mice to ischemia and loss of the neuroprotective effect of LIN and LPC in Trek1$^{-/-}$ mice. (**A**) Effect of LIN or LPC injection (500 nmol/kg) 10 min before KA treatment. (**B**) EEG recordings (15 and 45 min after KA treatment) in Trek1$^{+/+}$ and Trek1$^{-/-}$ mice with or without LIN (500 nmol/kg). (**C**) Increased mortality rate in vehicle (Veh)-, LIN- or LPC-treated Trek1$^{-/-}$ mice following 30 min global ischemia ($n = 20$ per genotype). LIN and LPC were injected at a concentration of 500 nmol/kg 30 min before ischemia. *Significantly different from vehicle-treated wild type (KA treatment 22 mg/kg), $^{\#}$significantly different from vehicle-treated wild type (KA treatment 28 mg/kg), $^{**}P<0.001$, $^{***}P<0.0001$, $^{\#\#\#}P<0.0001$, ANOVA followed by Tukey's multiple comparison test.

aortic aneurysms or dissection (Kouchoukos and Dougenis, 1997). Combined occlusion of the aortic arch and left subclavian artery was performed to induce spinal cord ischemia in mice (Lang-Lazdunski *et al*, 2000). Values of mean femoral arterial blood pressure (MABP) recorded for 5 h throughout the procedure did not differ significantly between Trek1$^{+/+}$ and Trek1$^{-/-}$ mice (Table Ia). The susceptibility to spinal cord ischemia was much higher in null allele mice. A total of 75% of Trek1$^{-/-}$ mice died within the first 3 h following 10 min ischemia compared with 14% of Trek1$^{+/+}$ mice up to 24 h after the procedure (Table Ib; $P<0.001$). All surviving Trek1$^{+/+}$ mice recovered without any neurological deficit and failed to develop any form of neurological deficit during the subsequent 48 h. In contrast, surviving Trek1$^{-/-}$ mice developed severe hind limb paralysis at the onset of reperfusion. They remained paralyzed during the first hours of reperfusion and retained deficits in motor function during the subsequent 48 h (Table Ib). Within 5 min following aortic crossclamping, Trek1$^{-/-}$ mice had vesical relaxation with urination, which did not occur in Trek1$^{+/+}$ mice, further indicating a lower tolerance to spinal cord ischemia. Autopsies of Trek1$^{-/-}$ mice did not reveal any severe abnormality in heart, lungs or major vessels.

TREK-1 channel in the mechanism of action of volatile anesthetics in vivo

Another interesting property of the TREK-1 channel concerns its sensitivity to activation by general volatile anesthetics (Patel *et al*, 1999), and we hypothesized (Patel *et al*, 1999) that TREK-1 might be involved in the mechanism of action of these agents. The comparative sensitivity to different volatile anesthetics of Trek1$^{+/+}$ and Trek1$^{-/-}$ mice was assessed by comparing the onset of anesthetic action, the loss of righting reflex (LORR) and the inspired minimum alveolar anesthetic concentration (MAC) values for each anesthetic in both. MAC is the minimum steady-state alveolar concentration of an inhalational anesthetic required to suppress a strong motor reaction to the noxious stimulus of tail-clamping in 50% of mice (Quasha *et al*, 1980). Figure 7A shows that knockout mice had a decreased sensitivity to chloroform and halothane, which are the most potent activators of the TREK-1 channel *in vitro* (Patel *et al*, 1999). Interestingly, the same

Table I Comparison of susceptibility to spinal cord ischemia in wild-type and TREK-1-deficient mice

(a) Physiological variables

Genotype	Mean arterial blood pressure (mmHg)			Rectal temperature (°C)		
	Preischemia	Ischemia	Reperfusion	Preischemia	Ischemia	Reperfusion
$Trek1^{+/+}$	71.9±3.3	16.1±6.2	67.4±4.2	37.5±0.4	37.3±0.5	37.6±0.3
$Trek1^{-/-}$	73.2±2.9	17.2±2.4	69.7±3.8	37.3±0.3	37.2±0.3	37.4±0.2

(b) Number of mice with their neurologic status (MSDI) and death rate at the onset of reperfusion and 1, 3 and 24 h after ischemia

Time after ischemia (h)	Genotype	MSDI							
		0	1	2	3	4	5	6	Death
0	$Trek1^{+/+}$	0	7	0	0	0	0	0	0
	$Trek1^{-/-}$*	0	0	0	0	0	0	8	0
1	$Trek1^{+/+}$	1	6	0	0	0	0	0	0
	$Trek1^{-/-}$*	0	0	0	0	0	1	3	4
3	$Trek1^{+/+}$	7	0	0	0	0	0	0	0
	$Trek1^{-/-}$*	0	0	0	1	1	0	0	2
24	$Trek1^{+/+}$	6	0	0	0	0	0	0	1
	$Trek1^{-/-}$*	0	2	0	0	0	0	0	0

The neurologic score involved a six-point scale (0 (normal function) to 6 (severe paraplegia); Lang-Lazdunski *et al*, 2000). Motor sensory deficit indices (MSDIs) were analyzed with Kruskal–Wallis test followed by Mann–Whitney U-test when significant. *$P<0.05$ versus wild-type mice.

type of results was obtained with sevoflurane and desflurane (Figure 7B), the most widely used agents in clinical anesthesia as well as isoflurane (Supplementary Figure 2C). The period of time necessary for the induction of anesthesia was longer, the concentrations required for LORR lower and the partial pressures of all anesthetics tested (i.e. MAC) were higher in $Trek1^{-/-}$ mice. There was no significant difference in the respiratory rate between either genotype before induction of anesthesia and at the MAC value (Table II). In contrast with volatile anesthetics, no difference was seen between $Trek1^{+/+}$ and $Trek1^{-/-}$ mice upon injection of the barbiturate pentobarbital (Figure 7C), which produces anesthesia by acting on different $GABA_A$ receptor subunits (Yamakura *et al*, 2001) and *in vitro* it has no effect on TREK-1 channel activity (Figure 7C), unlike halothane and sevoflurane (Supplementary Figure 2A and B). Pentobarbital did not affect the latency or the duration of LORR (Figure 7C) in null mutants. This latter result supports the idea that the differences observed are specific to volatile anesthetics and related to the TREK-1 channel.

Discussion

Potassium channels play a major role in the control of K^+ homeostasis and in physiological and pathological functions that are associated with modifications of the electrical membrane potential. Many subtypes of K^+ channels have been cloned in the past decades (Salkoff *et al*, 1992; Jan and Jan, 1997; Pongs, 1999; Kurachi *et al*, 1999). The mammalian two-pore-domain K^+ channel family (Lesage and Lazdunski, 2000; Patel and Honoré, 2001; Lesage, 2003), and particularly the TREK-1 channel, has been proposed to play a key role in brain and spinal chord injuries (Lauritzen *et al*, 2000; Blondeau *et al*, 2002; Lang-Lazdunski *et al*, 2003). The lipid and mechano-gated TREK-1 channel is closely related to pathophysiological conditions, such as ischemia and epilepsy. It is activated by arachidonic acid and other PUFAs, LPLs, cell volume expansion and internal acidosis. During the process of ischemia, arachidonic acid is released from the plasma and intracellular pH is decreased. These condition changes could potently activate the lipid-sensitive mechano-gated K_{2P} channels, an activation that would occur to protect the neuronal cell against excessive and deleterious neuronal excitability and Ca^{2+} entry. On the other hand, the TREK-1 channel is inhibited by the activation of group I metabotropic glutamate receptors, known to be involved in brain disorders, including ischemia, epilepsy and neurodegenerative disorders (Bockaert *et al*, 1993; Bordi and Ugolini, 1999; Fagni *et al*, 2000). Group I metabotropic glutamate receptor antagonists are neuroprotectors, while agonists amplify the excitotoxic neuronal degeneration induced by glutamate (Nicoletti *et al*, 1996; Gasparini *et al*, 2002). In fact, injections of PUFAs and LPLs protect against brain and spinal chord ischemia as well as epileptic seizures (Lauritzen *et al*, 2000; Blondeau *et al*, 2001, 2002; Lang-Lazdunski *et al*, 2003). Riluzole, another activator of TREK-1 channel (Duprat *et al*, 2000), is also neuroprotective against ischemia (Pratt *et al*, 1992; Ettaiche *et al*, 1999; Lang-Lazdunski *et al*, 1999). Although the opening of lipid-sensitive mechano-gated K_{2P} channels has been presumed to be the significant factor in neuroprotection, the lack of specific blockers did not allow until now a direct demonstration of this property. Using mice with disrupted TREK-1 and TRAAK genes, the present study provides evidence for a major role of the TREK-1 channel in surviving excessive neuronal excitability and in resistance to forebrain and spinal cord ischemia. The absence of an increased sensitivity to ischemia and epilepsy in $Traak^{-/-}$ mice demonstrates that the extreme vulnerability of $Trek1^{-/-}$ mice is not a nonspecific effect due to the lack of an important K^+ channel on neuronal excitability. Consequently, the TREK-1 channel can be considered to play a key role in the regulation of neuronal excitability. The high expression of the TREK-1

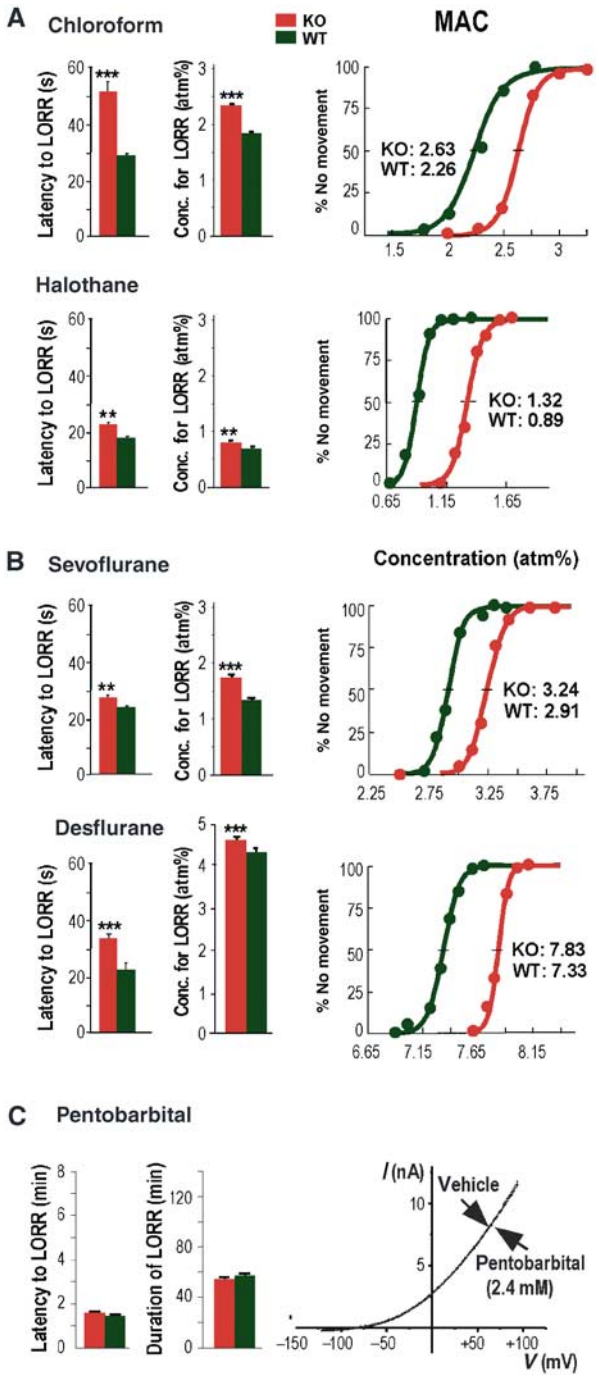

Figure 7 Effects of different anesthetics on LORR and MAC in Trek1$^{+/+}$ and Trek1$^{-/-}$ mice. LORR measurements after inhalation of volatile anesthetics. Latency to LORR is defined as the period of time (s) from inhalation to the LORR. Concentration for LORR corresponds to average concentrations of volatile anesthetics (**A**) chloroform and halothane and (**B**) sevoflurane and desflurane for the recovery from LORR. (**C**) LORR measurements (latency and duration of LORR expressed in minutes) after pentobarbital injection (30 mg/kg). Lack of effect of pentobarbital (2.4 mM) on TREK-1 channel expressed in transfected COS cells. *I–V* curves in steady-state control condition and after a 5 min application of pentobarbital (2.4 mM). *I–V* curve was elicited by a voltage ramp (1 s duration from −130 to +100 mV). Data represent mean±s.e.m. ($n=20$ per genotype and anesthetic agent). Statistical significance (Student's *t*-test): $^{**}P<0.001$, $^{***}P<0.0001$. Logistic regression probability of no movement fitted for volatile anesthetic concentrations. MAC and its 95% confidence interval (horizontal line) are shown on each graph.

protein both pre- and postsynaptically in the cortex and thalamic nuclei is consistent with a potential role for this channel in prevention of epileptic seizures. The high levels of TREK-1 expression in the hippocampus, a structure susceptible to damage during ischemia, and its modulation by neurotransmitter receptor activation are supplementary arguments for a major role of this channel in the control of excitotoxicity. Its activation in the neurons would be expected to hyperpolarize synaptic terminals, decreasing glutamate release and/or producing a postsynaptic hyperpolarization, which would favor the blockade of the NMDA receptor-associated channel by Mg^{2+} and also counterbalance glutamate-induced depolarization on other types of ionotropic glutamate receptors (Lauritzen *et al*, 2000). Without excluding a localization of the TREK-1 protein in glutamatergic neurons (Lauritzen *et al*, 2000), the TREK-1 channel has been described to be colocalized in GABAergic interneurons, specifically from striatum (this work), cerebellum, cortex and hippocampus (Hervieu *et al*, 2001). The phenotype of extreme vulnerability of TREK-1 null mutants against epilepsy and ischemia is consistent with the absence of TREK-1 channel in GABAergic interneurons, known to serve inhibitory functions in CNS and be involved in ischemic and epileptic disorders (Treiman, 2001; Wang, 2003). In the light of the role of TREK-1 channels in setting resting membrane potential, this is suggestive that TREK-1 may set the membrane potential of interneurons and thereby contribute to their often distinctive neurophysiological properties.

The beneficial effects of PUFAs on human health have long been advocated (Leaf and Kang, 1996; Nair *et al*, 1997; Leaf *et al*, 1999; Nordoy, 1999; Stoll *et al*, 1999) and indeed the effects of PUFAs on neuroprotection against epilepsy and ischemic paradigms in animals are spectacular (Lauritzen *et al*, 2000; Lang-Lazdunski *et al*, 2003). The results presented here strengthen the idea that neuroprotection induced by PUFAs (and LPLs) against seizures and ischemia is related to their action on the TREK-1 channel since this neuroprotection disappears in Trek1$^{-/-}$ mice and open the way for a novel neuroprotective strategy.

The possibility that a significant part of the effects of general anesthetics might result from potassium channel activation and especially K_{2P} channels has been previously suggested (Patel *et al*, 1999). This work definitively shows that the deletion of the TREK-1 gene induces a resistance to volatile anesthetics. This resistance is actually the greatest found for any ion channel knockout tested, including knockouts of $GABA_A$ receptors (Campagna *et al*, 2003), also believed to be potential targets of volatile anesthetics. One might of course wonder why the deletion of TREK-1 does not completely abolish sensitivity to volatile anesthetics. An important reason is that volatile anesthetics such as halothane, desflurane and sevoflurane also activate other K_{2P} channels such as TREK-2 and TASK channels (Patel *et al*, 1999; Lesage *et al*, 2000), which are still expressed in the Trek1$^{-/-}$ mice. It will be important in the future to analyze multiple K_{2P} channel knockouts, which would then be expected to display extreme resistance to volatile anesthetics. Further experiments using selective Cre mice to abolish specifically the gene in a tissue or a cell type will also permit a more detailed analysis of the cellular mechanisms that underlie the behavioral responses.

Table II Respiratory rate (beats/min) of wild-type and TREK-1-deficient mice before the induction of anesthesia and at the MAC value

	Chloroform	Halothane	Isoflurane	Sevoflurane	Desflurane
Wild-type preanesthesia	152±4	160±1	159±2	155±3	163±1
Wild-type MAC	166±4	141±2	84±4	105±3	106±3
Knockout preanesthesia	154±2	156±2	161±2	152±3	160±2
Knockout MAC	174±3	148±4	96±5	116±3	111±3

Data were expressed as mean±s.e.m. Statistical significance between wild-type and knockout mice was set at $P<0.05$.

In conclusion, this work provides evidence for a major involvement of the TREK-1 channel in the control of the neuronal excitability and neuroprotective effects induced by PUFAs and LPLs against ischemia and epileptic seizures. TREK-1 appears to be an innovative target for the development of novel therapeutic neuroprotective strategies for brain pathologies.

Materials and methods

All experiments were conducted according to the policies on the care and use of laboratory animals of the Society of Neurosciences.

Generation of TREK-1-deficient mice

Trek-1 genomic clones were isolated from a 129 mouse genomic library by using a TREK-1 cDNA probe and subcloned into pBluescript SK (Stratagene). The floxed targeting vector was generated from a 7.5 kb *Bgl*II/*Eco*RI restriction fragment containing exons 1–3 of the KCNK2 gene. The vector was designed to allow CRE-mediated deletion of exon 3, which encodes the TM1 domain of the channel. The first loxp sequence was inserted in the 5′ flanking intron of exon 3. Similarly, the PGK-neomycin resistance cassette (neo) was inserted together with a second loxp sequence in the 3′ flanking intron of exon 3. Both loxp sequences were in the same orientation to allow CRE-mediated simultaneous excision of Exon 3 and neo cassette. A copy of the diphteric toxin gene was subcloned adjacent to the homologous region for negative selection of the ES clone. The targeting vector (50 μg) was linearized prior to electroporation into 129-derived embryonic stem cells. After drug selection (G-418, 350 μg/ml), one positive clone (1/288) was identified by Southern blot and PCR analysis. Five highly chimeric males were generated by injection of the targeted ES cells into C57Bl/6J blastocysts. They were mated with C57Bl/6J females and germline transmission was assessed by Southern blot and PCR analysis of tail DNA from the agouti pups. TREK-1 floxed mice were then crossed with mice carrying the CRE recombinase gene under the control of the ubiquitous CMV promoter (D Metzger). Heterozygous TREK-1-deficient mice were then backcrossed with C57Bl/6J congenic mice over 11 generations. All animals (+/+ and −/−) were 8- to 10-week-old males of N6F2 to N11F2 backcross generation.

Kainate and pentylenetetrazol administration

After intraperitoneal injection of KA at 22 or 28 mg/kg, mice ($n=20$ per group) were monitored for 2 h for onset and extent of seizures. Seizure severity was blindly scored (Tsirka *et al*, 1995). PTZ was injected similarly at 40 or 55 mg/kg and seizures were scored based on the highest degree of seizure within 15 min of the PTZ injection. The seizure index was calculated by averaging the points for seizure activity in each group ($n=20$ per genotype and treatment). EEGs were recorded for 2 h on conscious mice ($n=10$ per genotype and treatment) using four small platinum electrodes (diameter 0.28 mm) placed in the hippocampus (1.2 mm lateral, 1.6 mm posterior to the bregma, 1.6 mm inside) and in the anterior neocortex (2 mm lateral, 0.5 mm anterior to the bregma, 1.5 mm inside). The signals were amplified, digitized and quantified using the Galileo system (Sirius BB, Medical Equipment International).

Forebrain ischemia model (2 VO + hypotension)

Global ischemia ($n=20$ per genotype and treatment) was induced by occluding both CCAs with aneurysm clips (Aesculap, Germany) during a 30 min episode of systemic hypotension induced by withdrawal of blood to maintain an MABP of 30±3 mmHg (Sheng *et al*, 1999).

Spinal cord ischemia model

Mice were subjected to crossclamping of the aortic arch, left subclavian artery and internal mammary artery for 10 min (Lang-Lazdunski *et al*, 2000). Motor function was blindly evaluated in the hind limbs using a rating scale of 0 (normal function) to 6 (total absence of movement) (Lang-Lazdunski *et al*, 2000).

Behavioral studies of sensitivity to anesthetic agents

Loss of righting reflex. Unrestrained mice ($n=10$ per genotype and volatile anesthetic) were placed in a chamber maintained at 33–35°C. Carbon dioxide pressure (<0.05 atm) and rectal temperature (36.5±1.2°C) were controlled. Each volatile anesthetic (chloroform, halothane, isoflurane, sevoflurane and desflurane) was administered with a calibrated vaporizer in 100% oxygen as the carrier gas with a fresh gas flow of 2 l/min at initial concentrations of 3.0, 1.2, 1.0, 1.8 and 5%, respectively. Concentrations of the volatile anesthetic were continuously measured by using a calibrated infrared analyzer (RGM 5250, Ohmeda, Louisville). After equilibration for 20 min at each initial anesthetic concentration, mice were blindly scored for LORR. The concentration of the anesthetics was then decreased in 10–20% increments and allowed to re-equilibrate at each concentration. Mice were observed continuously for recovery of the righting reflex. The concentration reported for LORR was calculated by averaging the two concentrations at which the mouse either retained or lost the righting reflex. Data were reported as mean±s.e.m. Differences were evaluated using an unpaired *t*-test.

Tail-clamp/withdrawal assay. MAC was determined using the tail-clamp technique (Quasha *et al*, 1980). Mice ($n=20$ per genotype and volatile agent) were first exposed for 20 min to a constant anesthetic concentration of almost 50% anesthetic induction values used in clinical practice. A hemostatic clamp was applied for 45 s to the midportion of the tail. Mice were scored blind for a motor withdrawal in response to clamping the tail. A mouse was considered to have moved if it made a purposeful muscular movement of the hind limb and/or the body. The anesthetic concentration was decreased in steps of 0.1% for each anesthetic, and the testing sequence was repeated after 20 min of exposure to each concentration. Concentration–response data were fitted to a logistic equation, yielding half-effect concentrations (median MAC values), slopes and estimates of their respective standard errors. Median MAC values were given with their respective 95% confidence interval limits. All *P*-values were two-tailed, and a *P*-value <0.05 was considered significant.

Sleep time assay (i.e. duration of the LORR). Mice ($n=20$ per genotype and anesthetic agent) were blindly tested for the duration of LORR (i.e. sleep time) in response to an intraperitoneal injection of pentobarbital (30 mg/kg). Mean sleep times for each agent were compared in null allele and wild-type mice using an unpaired *t*-test.

Onset of volatile and intravenous anesthetic action (i.e. latency to the LORR). Mice ($n=10$ per genotype and anesthetic agent) were exposed to 8% chloroform, 4% halothane, 8% sevoflurane, 3% isoflurane or 10% desflurane in the same chamber used for LORR and tail-clamp assays. Onset of anesthetic action was defined as the time interval between the beginning of the anesthetic inhalation or the injection of the intravenous agent and the LORR.

Electrophysiology on COS cells

COS cells were seeded at a density of 20 000 cells per 35-mm dish 24 h before transfection. Cells were transiently transfected by the classical DEAE–dextran method with 0.1 μg pCI-mTREK-1 + 0.05 μg pCI-CD8. Transfected cells were visualized 48 h after transfection using anti-CD8 beads. The external solution contained (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$ and 10 HEPES, adjusted to pH 7.4 with NaOH. The pipette solution contained (in mM) 140 KCl, 4 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.2). The cell under study was continuously superfused with a microperfusion system (0.1 ml/min) at room temperature.

Electrophysiology on mouse striatal neurons

Primary culture of mouse striata was carried out according to Weiss *et al* (1986). Cells were plated in culture dishes previously coated with polyornithin and 50% fetal calf serum. Culture medium was DMEM plus glucose (1.5 g/l) for the first 24 h, then B27 plus uridine (2 μM) and 5-fluoro-2′-deoxyuridine (2 μM). Patch clamp measurements were performed 2 or 3 days after plating. In outside-out configuration, the internal solution contained (in mM) 155 KCl, 3 $MgCl_2$, 5 EGTA, 10 HEPES and 5 ATP-K^+ (pH 7.2) and the external solution contained (in mM) 120 NaCl, 5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES. We daily prepared and added to the external solutions 10 mM tetra-ethyl-ammonium chloride, 3 mM 4-aminopyridine, 10 μM glibenclamide and 5 mM glucose (pH at 7.4). TREK current anesthetic sensitivity was assessed in striatal neurons and in TREK-1-expressing COS cells (Patel *et al*, 1999).

DNA extraction

Tail biopsy was lysed with proteinase K (200 μg/ml) for 5–12 h at 56°C in buffer containing 100 mM Tris (pH 8.5), 200 mM NaCl, 5 mM EDTA and 0.2% SDS. Proteinase K was heat inactivated at 95°C for 5–10 min and the lysate was then either diluted in water for PCR amplification or centrifuged to get rid of undigested material prior to ethanol precipitation for subsequent digestion by restriction enzymes.

Southern blot

For Southern blotting, genomic DNA was digested overnight with the appropriate restriction enzyme, precipitated, size fractionated on a 0.6% agarose gel and transferred onto a nylon membrane in 0.4 M NaOH. ^{32}P-labelled probe hybridization was carried out overnight at 65°C in 0.5 M Na_2Pi/5% SDS, pH 6.8.

PCR analysis

PCR reactions were performed on 1 μl of a 20–30 times water dilution of the crude tail lysate in 15 μl final volume containing 67 mM Tris–HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 1.5 mM $MgCl_2$, 200 mM dNTP and 0.2 μl *Taq* polymerase (Eurobio). Conditions were as follows: for TREK-1, 94°C/3 min ≫ (94°C/20 s ≫ 58°C/20 s ≫ 72°C/35 s) × 33, oligos (see Figure 1) #1 (5′GGT GCC AGG TAT GAA TAG AG3′), #2 (5′TTC TGA GCA GCA GAC TTG G3′), #3 (5′GTG TGA CTG GGA ATA AGA GG3′); for TRAAK, 94°C/3 min ≫ (94°C/30 s > 63.5°C/25 s > 72°C/35 s) × 35, primers #1 (5′CCCTGCTCCTTCTTCCC3′), #2 (3′ATTCTTCCTTCCTCCCTTCC5′), #3 (5′TGGACGAAGAGCATCAGGG3′), #4 (5′GAGGAGCAGCCAACTT TAGC3′) (see Supplementary Figure 1).

In situ hybridization

Perfused brain sections were hybridized with specific oligonucleotide 3′-end-labelled probes (nucleotides 726–694 and 1536–1504 of the cloned mouse TREK-1; GenBank accesssion number U73488.2).

Immunohistochemistry

Immunostainings were performed on floating brain sections (50 μm) using the anti-rabbit α-TREK-1 (Lauritzen *et al*, 2000) and c-fos (Oncogene) rabbit polyclonal antibodies. Sections were floated in a solution of the primary antibody overnight at 4°C (1:200 dilution). Biotinylated secondary antibodies were amplified using a rabbit IgG Vector Elite ABC kit (Vector laboratories) with 3-diaminobenzidine as substrate.

TaqMan assays (real-time quantitative RT–PCR analysis)

Total RNA from the brain and cerebellum of Trek1$^{-/-}$ and Trek1$^{+/+}$ mice was isolated by using the Trizol method (InVitrogen). Reverse transcription was performed with 2 μg of total RNAs, treated for 30 min with RQ1 DNase I (Promega) and reverse-transcribed with Superscript II reverse transcriptase (InVitrogen). Real-time PCR analysis (SYBR Green Mastermix Plus, Eurogentec) was performed to estimate the level of expression of TREK-1, TREK-2, TRAAK, TASK-1, TASK3, TWIK-1 and GABAα6 subunit in the brain and cerebellum of Trek1$^{-/-}$ and Trek1$^{+/+}$ mice. Primers for the seven different amplicons were as follows:

TREK-1 forward TTTTCCTGGTGGTCGTCCTC;
TREK-1 reverse GCTGCTCCAATGCCTTGAAC;
TREK-2 forward CCGGAATTACTCTCTGGATGAAGA;
TREK-2 reverse CATGGCTGTGCTGGAGTTGT;
TRAAK forward CCCCAGTGAGAATCTGGCC;
TRAAK reverse GGGCACAGCCACGCTC;
TASK-1 forward CGGCTTCCGCAACGTCTAT;
TASK-1 reverse TTGTACCAGAGGCACGAGCA;
TASK-3 forward GACGCCCTCGAGTCGGACCA;
TASK-3 reverse CTCTGAGACGGACTTCTTC;
TWIK-1 forward TGTCCTTCTCCTCCGTCACTG;
TWIK-1 reverse AGGCCACAAAAGGCTCACTTT;
GABAα6 forward CGCCCCCTGTGGCAA;
GABAα6 reverse TACTTGGAGTCAGAATGCACAACA;
CYCLOPHILIN forward GGCTCTTGAAATGGACCCTTC;
CYCLOPHILIN reverse CAGCCAATGCTTGATCATATTCTT.

Real-time PCR assays for each gene target were performed on cDNA samples in 96-well plates on an ABI Prism 7700 Sequence Detection System (PE Biosystems). PCR data were captured using Sequence Detector Software. Data were analyzed using the comparative CT method where the amount of target was normalized to an endogeneous reference (cyclophilin D) and calibrated to the amount of target in wild-type mice (User Bulletin No. 2 Applied Biosystems). Experiments were performed in triplicate. Standard curves were generated for each set of primers using serial dilutions of mouse brain cDNA to ensure a high efficiency of amplification.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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