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Neuroprotective role of the TREK-1 channel in decompression sickness

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Vallee N, Meckler C, Risso J-J, Blatteau J-E. Neuroprotective role of the TREK-1 channel in decompression sickness. *J Appl Physiol* 112: 1191–1196, 2012. First published February 9, 2012; doi:10.1152/japplphysiol.01100.2011.—Nitrogen supersaturation and bubble formation can occur in the vascular system after diving, leading to death and nervous disorders from decompression sickness (DCS). Bubbles alter the vascular endothelium, activate platelets, and lead to focal ischemia with neurological damage mediated by the mechanosensitive TREK-1 neuronal potassium ion channel that sets pre- and postsynaptic resting membrane potentials. We report a neuroprotective effect associated with TREK-1. C57Bl6 mice were subjected to decompression from a simulated 90 msw dive. Of 143 mice that were wild type (WT) for TREK-1, 51.7% showed no DCS, 27.3% failed a grip test, and 21.0% died. Of 88 TREK-1 knockouts (KO), 26.1% showed no DCS, 42.0% failed a grip test, and 31.8% died. Mice that did not express TREK-1 had lower DCS resistance and were more likely to develop neurological symptoms. We conclude that the TREK-1 potassium channel was neuroprotective for DCS.

neuroprotection; knockout

NEUROLOGICAL DAMAGE in the spinal cord and brain underlies the most serious symptom of decompression sickness (DCS) (16). Even after standard treatment with hyperbaric oxygen, 20–30% of victims suffer from sequelae after a neurological DCS (5). Our study describes a novel neuroprotective mechanism following nervous system damage in DCS.

The formation of bubbles in the tissues and vessels during decompression is what causes DCS (4). Two mechanisms are considered. First, bubbles cause mechanical disruption of spinal tissue. Second, these bubbles activate the endothelium, stimulate prothrombotic phenomena, and induce inflammation; leukocyte and platelet activation have been documented, accompanied by rises in the production of vasoactive effectors and stimulators of cell adhesion (14, 27). We suspect that prothrombotic phenomena lead to focal ischemia, which underlies the neurological symptoms of DCS. It is now accepted that ischemia in the spinal cord results in neurological damage (1, 10, 16). Neuroproctection processes should be engaged in both case.

Experiments on spinal cord ischemia in mice have shown that the mechanosensitive TREK-1 channel has neuroprotective properties (24). This potassium channel, the product of the *kcnk2* gene, modulates the neuron's resting membrane potential (15). On the one hand, hyperpolarization induced by the opening of this channel inhibits activation of presynaptic, voltage-dependent Ca^{2+} channels that induce the release of glutamate (Fig. 1), and, at the same time, the blocking of postsynaptic NMDA receptors by Mg^{2+} is enhanced. In this way, TREK-1 regulates the excitability of the cell and restricts NMDA-dependent glutamatergic excitotoxicity. Glutamatergic toxicity leading to NMDA receptor hyperactivation and neuron death has been observed in many experiments on spinal cord ischemia (2, 13, 11, 32), and it has been shown that opening of TREK-1 channels (which are found throughout the central nervous system) inhibits NMDA receptors (19, 25). This means that the TREK-1 channel affords some degree of neuroprotective activity (in addition to its other roles) when it becomes activated on a membrane that has been mechanically deformed by air depression (24). We suspect that this mechanical deformation may be triggered during decompression.

Dallas et al. (8) recently showed that human TREK-1 is modulated by nitric oxide, which seems to play an important role in preconditioning procedures designed to inhibit bubble formation and the risk of DCS (17).

All the various properties of the TREK-1 channel suggest that it may have a neuroprotective role in DCS. We propose the hypothesis that transgenic mice in which the TREK-1 channel has been knocked out (KO) would be more sensitive to the effects of decompression than wild-type mice (WT). The aim of these experiments was to investigate the neuroprotective activity of the TREK-1 channel in a mouse model of DCS.

MATERIALS AND METHODS

Animals. All procedures involving experimental animals were in line with European Union rules (Directive 86/609) and French law (Decree 87/848). $TREK1^{-/-}$ mice were very kindly supplied by Pr. Lazdunski and the Institute of Molecular and Cellular Pharmacology (Sophia-Antipolis, Valbonne, France; without whom it would not have been possible to develop this hyperbaric murine model). In these mice, the gene encoding the TREK-1 channel has been knocked out by Cre-Lox recombination (24). The comparator animals were analogous C57black/6 mice (Charles River Laboratory, Arbresle, France). To preclude phenotypic variation between different strains (40), crosses were made every 11 generations. WT ($kcnk2^{+/+}$) and KO ($kcnk2^{-/-}$) mice were produced for this study. Only males were used in this experiment to avoid fluctuations due to female hormone cycles. All mice were housed in a common cage and kept, during both rest and the experiments, on a regular day (0600–1800)/night (12 h) cycle. Food (AO3, UAR) and water were ad libitum, and the temperature was kept at 22 ± 1°C.

A total of 231 mice (6–9 wk of age) was exposed to compressed air to induce DCS.

Hyperbaric procedure. Each numbered mouse was weighed 30 min before the dive. Batches of 20 mice (10 per cage) were subjected to the hyperbaric protocol in a 200-liter tank fitted with three ports for observation. The mice were free to move around the cage.

The compression protocol involved a rise of 0.1 atm/min up to 1 atm, then 1 atm/min up to 9 atm, at which pressure the animals were kept for 45 min before decompression. The decompression rate was 20 atm/min up to the surface. The decompression was automatically controlled by a computer-linked analogical/digital converter (NIUSB-

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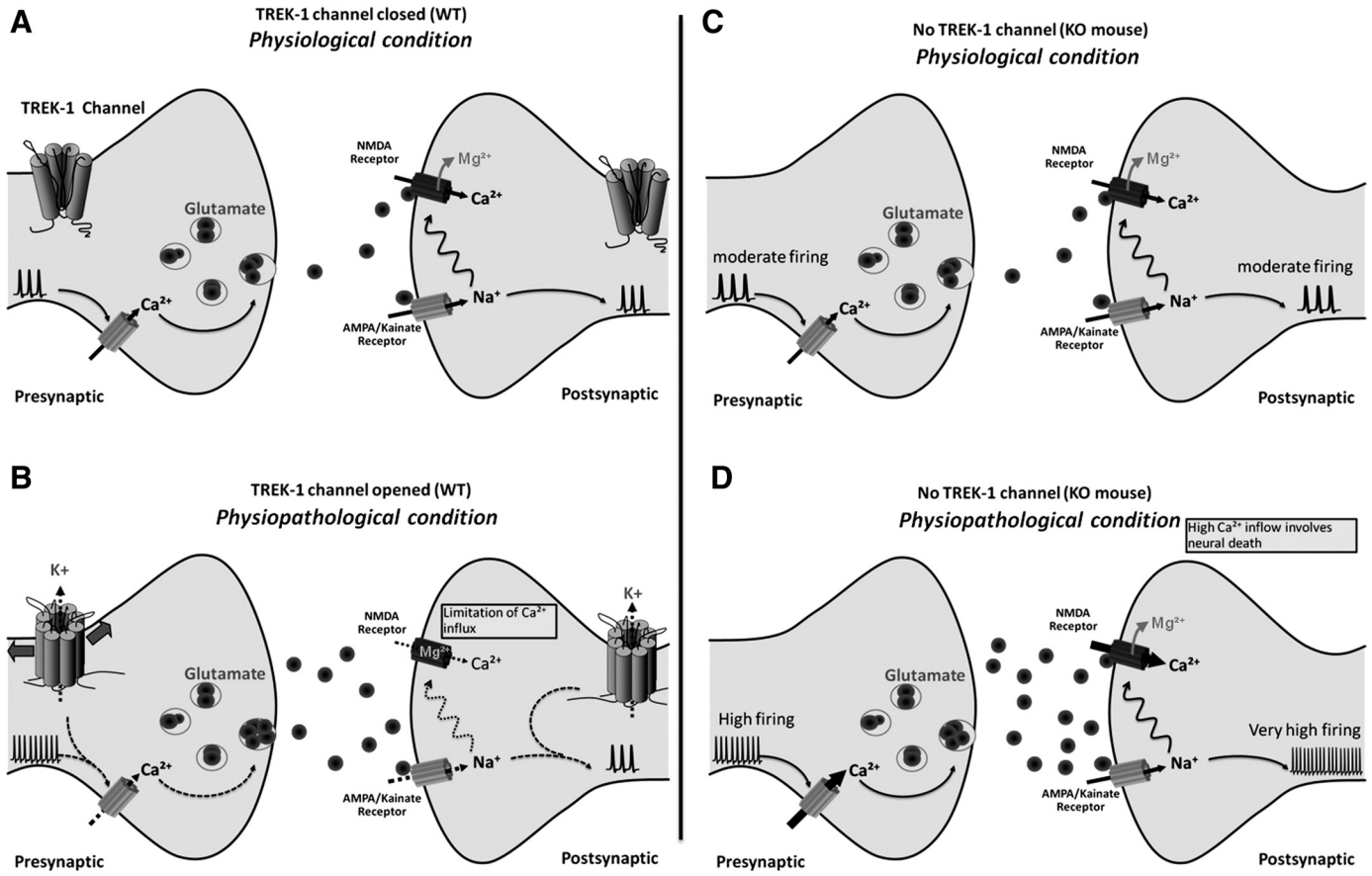

Fig. 1. *A–D*: TREK-1 potassium channels are likely to modulate the strength of the glutamatergic response. TREK-1 is expressed at both presynaptic and postsynaptic sites in wild-type (WT) mice. *A*: a synapse with a TREK-1 channel closed. In the synaptic space, glutamate is released in a proportional amount to the strength of the presynaptic neural pulse. At the postsynaptic level, glutamate activates AMPA receptors and then the NMDA ones (when the membrane depolarization reaches the threshold, Mg^{2+} is released) to generate a new burst of action potentials. *B*: a WT mouse synapse with a TREK-1 channel opened. In contrast with *A*, the opening of TREK-1 channels (*B*) at the presynaptic level allows the reduction of glutamate release by closing voltage-dependent Ca^{2+} channels. At the postsynaptic level, membrane hyperpolarization will tend to antagonize AMPA/kainate receptor-induced depolarization. This hyperpolarization will also decrease NMDA receptor activation (Mg^{2+} blocks the channel) and slow down the Ca^{2+} inflow, leading to a lower excitability of the neuron. In this way, action potentials burst firing is weakened. As such, TREK-1 opening could represent a neuroprotective process against high extracellular glutamate level [inspired from Francks and Honoré (15)]. *C* and *D*: TREK-1 channels are not present on these synapses, which could represent knockout (KO) mice synapses. In moderate firing rate condition (*C*), a moderate burst firing at the presynaptic level induces a glutamate release, itself translates into an equivalent postsynaptic burst. *D*: the same synapse with high burst firing at the presynaptic level, which first induces a high glutamate release and consequently stimulates glutamate receptors (both NMDA and AMPA) in an excessive way, potently pathologic.

6211, National Instrument) plugged to a solenoid valve (Belino LR24A-SR) and a pressure transmitter (Pressure Transmitter 8314, Burket Fluid Control System). The program was designed on DasyLab (DasyLab National Instrument) by our engineer.

Compressed air was generated using a diving compressor (Mini Verticus III, Bauer) coupled with a 100-liter tank at 300 bar. The oxygen analyzer was based on a MicroFuel electrochemical cell (G18007 Teledyne Electronic Technologies/Analytical Instruments). Water vapor and CO_2 produced by the animals were captured with soda lime (<300 ppm captured by the soda lime) and seccagel (relative humidity: 40–60%). Gases were mixed by an electric fan. The day/night cycle was respected throughout. The temperature inside the tank was measured using a platinum-resistance temperature probe (Pt 100, Eurotherm). All these variables were controlled by a special computer.

Behavior and clinical observations. At the end of decompression, the mice were transferred to individual cages and observed for 30 min. All signs were recorded together with their time of onset: death, respiratory distress, convulsions, paralysis, or difficulty moving. Problems with fore and rear limbs and convulsions were classified as being due to neurological problems. Death was defined as the cessation of all movement.

Grip tests [motor/sensory tests adapted from Hall (20)] were used to quantify forelimb involvement 15 and 30 min after the end of decompression: this test uses a 60-cm-long cord suspended at a height of 40 cm. The mouse is placed in the middle of the cord hanging from its front paws, and its performance is timed. Mice that escape by climbing up and then walking along the cord are given the highest score of 30 s. Mice that fail at least one test are considered as symptomatic. The results of this behavioral test are used to define DCS and distinguish the following groups: dead (fatal DCS), mice that failed at least one grip test (Grip−), and mice that passed the grip tests (Grip+).

Blood tests. Blood tests were carried out in an automatic analyzer (ABCvet, SCIL) on samples taken before the dive and again 30 min afterward. Leukocytes and platelets were counted in 20-μl samples taken from the tip of the tail and diluted in an equivalent volume of 2 mM EDTA (Sigma). At the end of the experiment, mice were anesthetized with halothane (5% in oxygen, Halothane, Belamont) and then killed by injection of pentobarbital sodium (200 mg/kg ip,

Sanofi Santé). A biopsy was taken to determine the animal's genotype.

Genotyping. To blind the experiments, genotyping (KO/WT) was only carried out post mortem. DNA for PCR was extracted from cells from the tip of the tail (5 mm) after overnight digestion at 56°C with protease K (200 μg/ml; Promega, Charbonnière, France), freshly added in a buffer solution containing 100 mM Tris (pH 8.5), 200 mM NaCl, 5 mM EDTA, and 0.2% SDS. The protease K was then heat-inactivated (95°C for 5–10 min). The lysate was diluted 20-fold in ultrapure water before amplification.

PCR was carried out on 5 μl of lysate added to 20 μl of reaction mixture. For the negative controls, water was substituted for the lysate. The reaction mixture contained a pair of primers (10 pM/μl; 1–2 or 1–3) with an amplification mixture (GoTaq Green Master Mix 2X, Promega). DNA primers (MWG-Opéron Biotech) corresponding to loci of interest in the *kcnk2* gene [*primer 1* (5′-GGT GCC AGG TAT GAA TAG AG-3′); *primer 2* (5′-TTC TGA GCA GCA GAC TTG G-3′); *primer 3* (5′-GTG TGA CTG GGA ATA AGA GG-3′)] were used with the following thermocycler (MultiGene Gradient, Labnet International) settings: initialization step 94°C/3 min>> (denaturation step 94°C/25 s>> annealing step 61°C/25 s>> elongation step 72°C/35 s)×35 cycles.

Amplified DNA sequences were resolved by electrophoresis (Biorad Generator, Powerpac 200; 90 V 45 min) on buffered 1.2% (Tris acetate EDTA) agar gel supplemented with BET for ultraviolet detection (Geneflash, Syngene Bioimaging). PCR-detected bands at both 680 bp (1–2) and 1,870 bp (1–3) characterize the homozygous WT ($kcnk2^{+/+}$), and a single band at 650 bp (1–3) characterizes the homozygous KO ($kcnk2^{-/-}$).

Statistical analyses. Blood cell counts were expressed as the percentage change vis-à-vis the control reading. The reference value (100%) was based on measurements before hyperbaric exposure. Data were then compiled. Numerical data points were expressed as mean and standard deviation. A contingency table was used for independence and association tests, coupled with a χ^2 test of significance. Different groups were compared using a Mann-Whitney (MW) test, and matched comparisons within groups were analyzed using a Wilcoxon (W) test. The significance threshold was 95% with an α-risk of 5%.

RESULTS

A total of 231 mice (143 WT and 88 KO) were subjected to the hyperbaric protocol to induce DCS. In the two groups, weight was similar [$weight_{WT}$: mean = 27.1 ± 1.7 g (range: 23.0–31.0 g); $weight_{KO}$: mean = 27.6 ± 2.4 g (range: 23.7–32.9 g); $MW_{WT/KO}$: n = 143/88, α = 0.05, P = 0.137].

Fatal DCS. Statistical analysis of the probability of fatal DCS and the different symptoms shows a significant difference between the two populations (MW: n = 143/88, α = 0.05, $P <$ 0.001). More KO mice died from the consequences of DCS (contingency table, $\chi^2_{KO\text{-}WT}$ = 2.082 vs. 1.282, n = 143/88, α = 0.05, $P <$ 0.001): 31.8% of the KO mice died vs. 21.0% of the WT mice. However, the WT mice tended to die sooner (MW: n = 30/28, α = 0.05, P = 0.004): WT mice died within 5.9 ± 2.7 min of the end of decompression compared with 9.5 ± 5.3 min for the KO mice.

Mice that passed all grip tests (Grip+). More mice in the WT group passed all grip tests: 51.7% (WT) vs. 26.1% (KO) (contingency table, $\chi^2_{KO\text{-}WT}$ = 5.741 vs. 3.533, n = 143/88, α = 0.05, $P <$ 0.001).

Mice that failed a grip test (Grip−). Fewer mice in the WT group failed at least one grip test: 27.3% (WT) and 42.0% (KO) (contingency table, $\chi^2_{KO\text{-}WT}$ = 2.237 vs. 1.377, n = 143/88, α = 0.05, $P <$ 0.001).

Concerning timed performance in the grip tests (Fig. 2), a significant difference was observed between the two populations in the duration of the first grip test ($MW_{WT/KO}$: n = 39/37, α = 0.05, P = 0.030) but not in that of the second ($KW_{WT/KO}$: n = 39/37, α = 0.05, P = 0.826).

No difference in performance (Fig. 2) was observed between the first and second tests in the KO population (W_{KO}: n = 37, α = 0.05, P = 0.746, 15.6 ± 11.0 vs. 16.0 ± 10.4 s), whereas in the WT mice, the mean time spent suspended from the cord was significantly (W_{WT}: n = 39, α = 0.05, P = 0.015) shorter in the first test (10.8 ± 11.5 s) than in the second, 15 min later (15.8 ± 11.8 s).

Clinical observations. Compared with WT mice, the KO mice showed no abnormal behavioral or phenotypic sign.

Following decompression, most of the mice were prostrate, suggesting that they were in physiological distress. Symptoms of DCS or death occurred after return to the surface. Mice died from convulsions and/or respiratory distress. Mice expressed essentially neurological symptoms of varying seriousness with motor and locomotor impairment (paraplegia, paraparesis) and, in some cases, convulsions.

Platelet counts. No significant difference in platelet count emerged between the populations ($KW_{WT/KO}$: n = 92/47, α = 0.05, P = 0.322; Fig. 3).

Following the dive, mean platelet consumption was 8.4 ± 31.5% in the WT mice: WT_{Grip-} mice consumed 15.1 ± 36.2% of their platelets (W: n = 33, α = 0.05, $P <$ 0.001), whereas no change was observed in the WT_{Grip+} group (−2.9 ± 26.7%; W: n = 64, α = 0.05, P = 0.808). Thus the difference between the WT_{Grip+} and WT_{Grip-} groups is significant (MW: n = 33/64, α = 0.05, P = 0.024).

In the KO mice, only the KO_{Grip-} animals consumed a significant number of platelets: −16.7 ± 30.7% ($W_{KOGrip-}$: n = 32, α = 0.05, P = 0.003; $W_{KOGrip+:}$ n = 13, α = 0.05, P = 0.807). The difference between the two groups is insignificant ($MW_{KOGrip-\ vs.\ Grip+}$: n = 32/13, α = 0.05, P = 0.113).

Leukocyte counts. In WT mice, the leukocyte count (Fig. 3) went down by 19.5 ± 33.6% in the Grip+ group (W: n = 33,

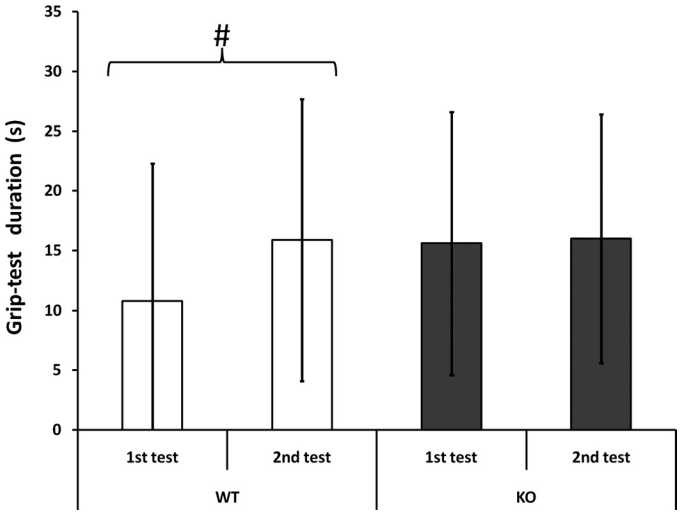

Fig. 2. Grip durations after a provocative dive in Grip-WT (open bars) or Grip-KO (shaded bars) mice. Grip tests were used to quantify forelimb involvement 15 and 30 min. Scores were expressed in seconds. Only Grip− groups, with mice that failed at least one grip test (Grip−) are displayed on the graphic. #Significant difference (α = 0.05, Wilcoxon test).

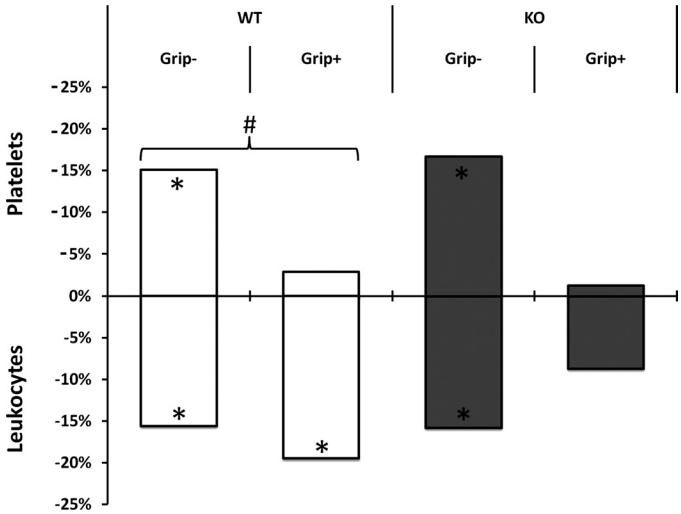

Fig. 3. Fall in blood platelet and leukocyte count (%) after decompression in WT (open bars) or KO for the TREK-1 channel (shaded bars) population of mice. Blood test were carried out on the Grip− group, mice that failed at least one grip test, and the Grip+ group, mice that passed the grip tests. *Significant difference ($\alpha = 0.05$, Wilcoxon test) between pre- and postdecompression count in a group of a population. #Significant difference ($\alpha = 0.05$, Mann-Whitney test) between groups (Grip+ vs. Grip−) of a population.

$\alpha = 0.05$, $P < 0.001$) and 15.6 ± 30.0% in the Grip− group (W: $n = 64$, $\alpha = 0.05$, $P = 0.025$). The difference between the groups is insignificant (MW: $n = 33/64$, $\alpha = 0.05$, $P = 0.264$).

In the KO mice, no drop in leukocyte count was observed in the KO_{Grip+} group (−8.7 ± 37.6%, W: $n = 32$, $\alpha = 0.05$, $P = 0.311$) but there was a significant drop in the KO_{Grip-} (−8.6 ± 37.6%, W: $n = 13$, $\alpha = 0.05$, $P = 0.004$) although this difference is not significant (MW: $n = 32/13$, $\alpha = 0.05$, $P = 0.287$).

Finally, no significant difference emerged between the different genotypes vis-à-vis leukocyte count ($MW_{WT/KO}$: $n = 92/47$, $\alpha = 0.05$, $P = 0.841$).

DISCUSSION

The decompression protocol used in this study is comparable to that used in other studies on mice of similar weight (3). This protocol induces neurological DCS with motor and locomotor impairment and convulsions, suggesting damage to the spinal cord or brain.

The main finding in this experiment is that WT mice are more resistant to the neurological consequences of decompression than mice with impaired TREK-1 channel function. This suggests that this potassium channel may have a neuroprotective effect in neurological DCS.

Neuroprotective activity of TREK-1. Absence of the TREK-1 channel in KO mice is significantly predictive of a higher failure rate in a grip test (odds ratio = 3.1). A similar but less marked correlation is seen for mortality (odds ratio = 1.9).

Although the survival rate was higher in the WT group after exposure to a DCS-inducing protocol, the individuals that died ($WT_{DCS\ fatal}$) did so very quickly, on average. In contrast, in the KO population, survival was low but the individuals that died survived for a longer time (on average). We propose that the excess mortality in KO mice is consistent with the absence of a neuroprotective effect of TREK-1. In addition, neither improvement nor deterioration was seen in the performance of the KO mice between the first and second grip tests: significant improvement in terms of reduced suspension time in the second test was only seen in the WT population. This could be attributed to physiological recovery due to TREK-1 channels. Finally, mice expressing TREK-1 may benefit from the channel's neuroprotective properties with better neurological recovery and a lower mortality rate. In contrast, motor recovery is not enhanced in TREK-1 KO mice between the grip tests and their initial mortality rate is higher.

We observed that KO mice died in a higher proportion than WT, whereas the survival KO mice show very few additional symptoms (such as paraplegia, paresis, or convulsions) compared with WT mice. Although it seems speculative without other supporting data, we propose serious injuries were directly lethal in KO mice, whereas in WT they were overcome due to the protective effects of TREK-1.

Platelet and leukocyte consumption. As observed before (39), platelets were consumed and the leukocyte count dropped after decompression, in both WT and KO mice.

This drop in platelet count is usually attributed to clotting activity following exposure of the collagen under bubble-damaged endothelial cells in the blood vessels (22, 31, 34, 42) or direct interactions between bubbles and platelets (18, 21). Platelet counts did not drop in animals of any genotype that passed all the grip tests. TREK-1 channels are not known to affect clotting function directly although platelet activating factor may be able to activate TREK-1 (29). A similar argument could be made for leukocytes because TREK-1 is not known to interact with these cells either. The drop in leukocyte count after DCS is usually attributed to diapedesis (12, 23, 43–45).

Hypotheses about the activation of TREK-1 channels in DCS. Bubbles, as platelet aggregation (35–38, 41) that leads to the thrombi, can cause ischemia in DCS (6, 21). Such ischemia is known to amplify platelet and leukocyte activation (9) through the action of PAF, which is also known to activate TREK-1 channels (29). In the thrombotic phenomena of DCS, platelet activating factor may trigger the opening of TREK-1 channels, in addition to its activator and mediator of many leukocyte functions, including platelet aggregation and degranulation, inflammation, and anaphylaxis (30).

However, another mechanism that might activate the TREK-1 channel depends on its mechanosensitive properties. During desaturation, bubbles are growing because of a negative delta of pressure in the organism. This negative pressure, depression, is ubiquitous in the organism. Membranes themselves should be impacted by this depression. It has been previously shown that air depression to −60 mmHg (9, 28, 33) by patch-clamping mechanically opens the TREK-1 channel. We could considered this patch clamp experiment as a model of decompression and therefore conclude that desaturation could open TREK-1 channels.

However, the TREK-1 channel could also be indirectly inhibited by bubbles. Buckler and Honoré (7) showed that bubbles in a solution rich in arachidonic acid tend to drag the acid molecules toward the surface of the container, thereby inhibiting their interaction with dissolved materials. Arachidonic acid is required for TREK-1 activation, and a decrease in

its availability could compromise the channel's neuroprotective potential in DCS.

As a rule, a decrease in cellular excitability threshold following the opening of TREK-1 channels could reduce the amount of glutamate released and therefore the activity of glutamatergic receptors. The activation of TREK-1 channels could be modulated mechanically by decompression or by PAF following platelet activation, and this could be seen as a mechanism to limit the effects of ischemia to control glutamatergic excitotoxicity and prevent the death of neurons. Nitric oxide [by protein kinase G-mediated phosphorylation (9)] also activates TREK-1, and it should kept in mind as some preconditioning trainings that prevent risk of DCS noterheological changes and endothelial adaptation attributed to nitric oxide (17).

Conclusion. Mice that do not express the TREK-1 channel are more likely to develop neurological symptoms after a DCS that has damaged the central nervous system. In wild-type mice, the importance of the TREK-1 channel suggests that it mediates a neuroprotective effect that may be designed to control glutamatergic excitotoxicity and thereby prevent neuron death. Blocking glutamatergic receptors at the same time as stimulating TREK-1 channels could represent a promising therapeutic modality in the treatment of DCS.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: N.V. and J.-E.B. conception and design of research; N.V. performed experiments; N.V., C.M., and J.-E.B. analyzed data; N.V., C.M., J.-J.R., and J.-E.B. interpreted results of experiments; N.V., C.M., and J.-E.B. prepared figures; N.V., C.M., J.-J.R., and J.-E.B. drafted manuscript; N.V., C.M., J.-J.R., and J.-E.B. edited and revised manuscript; N.V., C.M., J.-J.R., and J.-E.B. approved final version of manuscript.

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