De novo expression of Trpm4 initiates secondary hemorrhage in spinal cord injury

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The role of transient receptor potential M4 (Trpm4), an unusual member of the Trp family of ion channels, is poorly understood. Using rodent models of spinal cord injury, we studied involvement of Trpm4 in the progressive expansion of secondary hemorrhage associated with capillary fragmentation, the most destructive mechanism of secondary injury in the central nervous system. Trpm4 mRNA and protein were abundantly upregulated in capillaries preceding their fragmentation and formation of petechial hemorrhages. Trpm4 expression *in vitro* rendered COS-7 cells highly susceptible to oncotic swelling and oncotic death following ATP depletion. After spinal cord injury, *in vivo* gene suppression in rats treated with Trpm4 antisense or in Trpm4^{-/-} mice preserved capillary structural integrity, eliminated secondary hemorrhage, yielded a threefold to fivefold reduction in lesion volume and produced a substantial improvement in neurological function. To our knowledge, this is the first example of a Trp channel that must undergo *de novo* expression for manifestation of central nervous system pathology.

The mammalian transient receptor potential (Trp) superfamily includes 28 nonselective cation channels, most of which conduct both monovalent and divalent cations. Trp channels have numerous critical roles in diverse homeostatic functions involving transport of ions across cell membranes^{1,2}. Two members of the melastatin family, Trpm4 and Trpm5, are unusual in that they conduct monovalent cations exclusively and are impermeable to divalent cations. So far, the only role suggested for Trpm4 is that, when activated, it protects against Ca²⁺ overload by depolarizing the cell, thereby decreasing the driving force for Ca²⁺ influx^{3–5}.

Some Trp channels have been linked to various disease processes^{1,6}, with most Trp “channelopathies” being associated with loss of constitutive function. Trpm2 and Trpm7 have been implicated in Ca²⁺-mediated anoxic death of neurons *in vitro*^{7,8}. However, to our knowledge, no Trp channel has previously been identified that must undergo *de novo* expression for manifestation of central nervous system (CNS) pathology.

Contusion of the CNS involving brain or spinal cord injury (SCI) is frequently complicated by “blossoming” or expansion of the hemorrhage^{9,10}. Although sometimes erroneously attributed to continued bleeding of vessels fractured by the original trauma, this phenomenon represents a secondary pathological process, as suggested by delayed appearance of distinct islands of hemorrhage segregated from the original site of impact. Expansion of secondary hemorrhage occurs during the first several hours after a traumatic insult. It results

from progressive catastrophic failure of the structural integrity of capillaries¹¹ and is characterized by formation of small, discrete satellite (petechial) hemorrhages in spinal cord tissues surrounding the site of primary injury¹². With time, petechial hemorrhages increase in number and eventually coalesce into a hemorrhagic lesion that encompasses a volume far greater than the site of primary injury¹³. Secondary hemorrhage is particularly damaging because it greatly expands the volume of neural tissue destroyed by the primary injury. The capillary dysfunction implicit with secondary hemorrhage causes tissue ischemia and hypoxia¹⁴, and the blood is exquisitely toxic to CNS cells^{15,16}, further injuring neural tissues owing to oxidative stress and inflammation. Together, these processes render secondary hemorrhage the most destructive mechanism of secondary injury involving the CNS.

The molecular antecedents of secondary hemorrhage have not been elucidated, although in SCI, a role for nonselective monovalent cation channels has been postulated¹⁷. Here we identified Trpm4 up-regulation and activation as key molecular events responsible for secondary hemorrhage.

RESULTS

Trpm4 is upregulated after SCI

The rat model of SCI that we studied involved a ‘severe’ unilateral spinal cord injury at C5 (ref. 17). We chose a hemicord contusion injury because block of contralateral expansion of a unilateral

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Figure 1 *Trpm4* is upregulated in capillaries after SCI. (a) Immunohistochemical localization of *Trpm4* in an uninjured control (CTR) and 24 h after SCI, with montages constructed from multiple individual images, and positive labeling shown in black pseudocolor. Circle marks the impact site; asterisks show sampling area for b and c. (b) Magnified views of *Trpm4* immunolabeled sections taken from uninjured control (CTR) and from the penumbra after SCI. (c) Double immunolabeling of capillaries for *Trpm4* (red) and for vonWillebrand factor (green). (d) *In situ* hybridization for *Trpm4* in the penumbra 24 h after SCI; uninjured control spinal cord tissues (CTR) probed with antisense and post-SCI tissues probed with sense (SE) showed no labeling. (e) RT-PCR of spinal cord tissues 24 h after SCI from three regions—the impact site (SCI) and rostral (R) and caudal (C) to the impact site—with β -actin as control; bar graph shows normalized densitometric analysis of RT-PCR data from uninjured spinal cords (CTR) and cords 24 h after SCI (three rats per group); $^{**}P < 0.01$. Images of immunohistochemistry and *in situ* hybridization are representative of findings in three rats per group.

injury is easier to detect functionally¹⁷ than block of rostro-caudal expansion of a midline injury¹⁸.

Very low levels of *Trpm4* expression were found in uninjured controls, but 24 h after SCI, *Trpm4* was heavily upregulated in tissues surrounding the injury, extending to tissues distant from the actual impact site, including to the contralateral hemicord (Fig. 1a). In the core of the lesion, *Trpm4* upregulation was apparent in all cells, but in penumbral tissues, *Trpm4* upregulation was especially prominent in elongated structures that co-labeled with von Willebrand factor, which suggests that these structures were capillaries (Fig. 1b,c). Study of tissues obtained at earlier times after injury showed no change in *Trpm4* at 1.5 h, but at 3 h and later, increasingly prominent regions of *Trpm4* upregulation were observed near the injury site, with most of the labeling observed in capillaries.

In situ hybridization confirmed that *Trpm4* was upregulated after injury, especially in microvessels in the penumbra, with control spinal cord tissues showing no comparable labeling (Fig. 1d). RT-PCR performed on tissues from the injury site and on tissues rostral and

caudal to the injury showed that *Trpm4* mRNA doubled within the site of injury (Fig. 1e), with this value markedly underestimating the increase in endothelial cells, which comprise only 0.1% of CNS volume.

Trpm5 protein and mRNA were essentially absent both before and after SCI.

Trpm4 antisense blocks *Trpm4* expression

We used antisense oligodeoxynucleotide (*Trpm4*-AS) to assess the role of newly expressed *Trpm4* after SCI. We reasoned that an antisense gene suppression strategy would be particularly effective, given that *Trpm4* is transcriptionally upregulated after SCI. Sense oligodeoxynucleotide (*Trpm4*-SE) was used as control. The *Trpm4*-AS that we used was very effective in reducing *Trpm4* mRNA in cultured endothelial cells (Fig. 2a).

We studied cellular localization of fluorescent CY3-conjugated *Trpm4*-AS (CY3-*Trpm4*-AS) administered after SCI. After 24 h of infusion, followed by washout of intravascular contents, CY3-*Trpm4*-AS was abundant in the liver and kidney, as expected¹⁹, as well as in

Figure 2 Antisense oligodeoxynucleotide downregulates *Trpm4* expression and reduces secondary hemorrhage and capillary fragmentation after SCI. (a) RT-PCR for *Trpm4* mRNA in bEnd.3 cells loaded with *Trpm4*-SE or *Trpm4*-AS; β -actin shown as control; ladder, 100 base pair intervals starting at 200 base pairs. (b) Fluorescence images of the penumbra 24 h after SCI in a rat administered CY3-conjugated *Trpm4*-AS (red) by constant infusion after SCI; rat perfused to remove intravascular contents; nuclei labeled with DAPI (blue); arrows point to capillaries. (c) RT-PCR for *Trpm4* mRNA, immunohistochemistry and immunoblots for *Trpm4* protein in tissues obtained 24 h after SCI from rats administered *Trpm4*-SE or *Trpm4*-AS; positive control (PC) is from COS-7 cells transfected with hamster *Trpm4*; β -actin shown as control; densitometric analysis of immunoblots, normalized for β -actin, is also shown; $^{**}P < 0.01$.

(d) Spinal cord sections and homogenates from untreated control rats (CTR) and rats administered *Trpm4*-SE, *Trpm4*-AS or flufenamic acid (FFA); arrows point to petechial hemorrhages distant from the impact sites (circles); also shown is spectrophotometric quantification of extravasated blood at 1, 15 and 24 h after SCI in spinal cord homogenates of untreated controls (CTR, $n = 6-12$), and in rats administered *Trpm4*-SE ($n = 4$), *Trpm4*-AS ($n = 5$) or FFA ($n = 5$) after SCI. (e) Sections of penumbra immunolabeled for vimentin showing fragmentation of capillaries in untreated rats (CTR) and rats administered *Trpm4*-SE versus elongated intact capillaries in rats administered *Trpm4*-AS or FFA; images representative of findings in three rats per group.

Figure 3 Cells expressing Trpm4 are highly susceptible to ATP-depletion—induced oncotic cell death. **(a)** Sodium azide (1 mM) plus 2-deoxyglucose (10 mM) (NaAz + 2DG), which produced a rapid drop in cellular ATP, had no effect in cells transfected with EGFPN1 plasmid alone, but caused activation of an inward current that reversed at ~ 0 mV and was blocked by *N*-methyl-D-glucamine (NMDG) in COS-7 cells transfected with EGFPN1 + Trpm4 plasmid; the data shown are representative of 11 and 17 cells, with current densities of -0.91 ± 0.24 and -29.8 ± 7.8 pA pF $^{-1}$ (holding potential, -50 mV), without and with Trpm4, respectively. **(b)** Oncotic blebbing, swelling and nuclear labeling with propidium iodide (PI, red) induced by ATP depletion, as above, in COS-7 cells transfected with EGFPN1 + Trpm4 plasmid, but not in cells transfected with EGFPN1 plasmid alone. **(c)** Quantification of propidium iodide–positive oncotic cell death induced 10 min after ATP depletion in COS-7 cells transfected with EGFPN1 + Trpm4 plasmid or with EGFPN1 plasmid alone; values represent the percentage of the transfected cells (green cytoplasm) with nuclear propidium iodide labeling; experiments were performed in triplicate, with data from > 100 cells per experiment; **** $P < 0.0001$.

the core of the injury, in association with destroyed spinal cord tissue. Uptake of CY3-*Trpm4*-AS was prominent in capillaries of the penumbra, but almost no CY3-*Trpm4*-AS appeared to traverse capillaries and label parenchymal cells (Fig. 2b). No fluorescent label was detected in more distant normal capillaries of the spinal cord or brain.

RT-PCR, immunohistochemistry and immunoblots of tissues 24 h after SCI confirmed that *Trpm4*-AS was highly effective in reducing *Trpm4* mRNA and protein expression following SCI, and independently validated the antibody we used (Fig. 2c).

Block of Trpm4 reduces secondary hemorrhage

Spinal cord tissue of untreated control rats or control rats administered *Trpm4*-SE examined 24 h after SCI showed prominent bleeding at the surface as well as internally, with internal bleeding consisting of a central region of hemorrhage at the impact site plus numerous petechial hemorrhages in regions remote from the impact site (Fig. 2d). Treatment with *Trpm4*-AS resulted in a notable reduction in petechial hemorrhages, which was confirmed by examining homogenates of injured spinal cord tissues (Fig. 2d). Quantifying the amount of hemoglobin in spinal cord homogenates reaffirmed that bleeding increased with time after injury, and that *Trpm4*-AS was highly effective in reducing hemorrhage at 24 h; *Trpm4*-AS completely eliminated secondary hemorrhage (Fig. 2d). We also tested the effect of flufenamic acid, an open-channel blocker of nonselective cation channels including Trpm4 (ref. 20). As with *Trpm4*-AS, flufenamic acid was highly effective in reducing hemorrhage (Fig. 2d), which suggests that newly upregulated Trpm4 was acting as a functional membrane channel.

We studied the structural integrity of capillaries in spinal cord sections from rats administered *Trpm4*-SE or *Trpm4*-AS. In untreated control rats and rats administered *Trpm4*-SE, broken fragments of capillaries were prominent in penumbral tissues adjacent to the site of injury, which is consistent with catastrophic failure of capillary integrity, and which accounts for high levels of extravasated blood (Fig. 2e). By contrast, in rats treated with *Trpm4*-AS or flufenamic acid, intact elongated capillaries predominated in penumbral tissues, thus accounting for the significant ($P < 0.01$) reduction in extravasated blood (Fig. 2e).

Trpm4 expression predisposes to cell death

Opening of Trpm4 channels is known to result in cell-protective depolarization²¹. However, the capillary fragmentation observed in

association with Trpm4 expression suggested that Trpm4 might be injurious under certain conditions. We hypothesized that sustained opening of Trpm4 channels, as would arise with the severe ATP depletion associated with SCI (refs. 22–24), could lead not only to depolarization but also to continuous influx of Na⁺, which, if unchecked, could induce oncotic swelling and oncotic death of endothelial cells, resulting in capillary fragmentation.

We tested this hypothesis in COS-7 cells heterologously expressing Trpm4, with expression of functional channels being verified electrophysiologically. In control cells, ATP depletion had no effect on membrane currents, but in cells expressing Trpm4, ATP depletion resulted in activation of a sustained inward cationic current that reversed near 0 mV, and that was blocked by *N*-methyl-D-glucamine, which indicates that the channels were Trpm4 (Fig. 3a).

In COS-7 cells expressing Trpm4, but not in controls, ATP depletion caused marked cell blebbing, oncotic swelling and membrane leakage, resulting in nuclear labeling by propidium iodide, which is consistent with oncotic cell death (Fig. 3b). In control cells, ATP depletion was well tolerated, with only 7% of cells exhibiting propidium iodide–positive cell death. By contrast, 50% or more of cells expressing Trpm4 died within 10 min of ATP depletion (Fig. 3c). These findings indicate that Trpm4 expression renders cells highly susceptible to ATP-depletion–induced oncotic cell death. Moreover, these data suggest that the fragmentation of capillaries that express Trpm4, as observed *in vivo* after SCI (see Figs. 1b,c and 2e), was due to oncotic death of endothelial cells associated with opening of Trpm4 channels.

Block of Trpm4 improves outcome after SCI

We further evaluated the role of Trpm4 in SCI by studying neuro-behavioral function and lesion volume in rats treated with intravenous *Trpm4*-AS for only 24 h, beginning shortly after injury.

Rats were evaluated serially over the course of one week. We quantified vertical exploration, a complex exercise that requires balance, truncal stability, bilateral hindlimb dexterity and strength, and at least unilateral forelimb dexterity and strength, which in combination are excellent markers of cervical spinal cord function¹⁷. We also evaluated performance in two other standard tests, beam walking and inclined plane, the latter requiring more and more dexterous function of the limbs and paws as the angle of the plane is increased²⁵. Performance on all three functional tests was consistently better with *Trpm4*-AS. By day 7, *Trpm4*-AS–treated rats exhibited near-normal performance, which was never achieved by control

Figure 4 *Trpm4*-AS improves neurobehavioral performance and reduces lesion volume in rats after SCI. (a) Rearing 24 h after SCI in rats administered either *Trpm4*-AS or *Trpm4*-SE for 24 h after SCI. (b) Quantified rearing, performance on up-angled and down-angled planes, and performance on beam walking tests at various times after SCI in rats administered *Trpm4*-AS, *Trpm4*-SE or scrambled (SCR) oligodeoxynucleotide for 24 h after SCI; 5–6 rats per group; values for uninjured rats (No inj) or perfect scores are shown as dotted lines; ** $P < 0.01$. (c) Hematoxylin and eosin-stained sections from representative rats 7 d after SCI that were administered *Trpm4*-AS or *Trpm4*-SE for 24 h, along with average lesion areas and lesion volumes; data are from the same rats as in b.

rats treated with scrambled oligodeoxynucleotide, even after 6 weeks of recovery (Fig. 4a,b). In the same animals, treatment with *Trpm4*-AS for only 24 h resulted in a threefold reduction in lesion volume when measured 7 d after injury (Fig. 4c).

Trpm4 knockout improves outcome after SCI

To confirm our findings from the experiments with transient gene suppression, we also studied SCI in *Trpm4* knockout (*Trpm4*^{−/−}) mice. For these experiments, we used a unilateral spinal cord injury at T9.

As in rats, SCI in wild-type (WT) mice was associated with marked upregulation of *Trpm4* in the vicinity of the injury (Fig. 5a). Spinal

cords of WT mice examined 24 h after injury invariably showed more hemorrhage than those harvested 30-min after injury, which is consistent with progressive hemorrhage (Fig. 5b). By contrast, spinal cords of *Trpm4*^{−/−} mice showed no more blood at 24 h than those of WT mice at 30 min after injury (Fig. 5b). At 24 h after SCI, *Trpm4*^{−/−} mice showed less than half the amount of extravasated blood observed in WT mice (Fig. 5c), which is in agreement with our data on parenchymal hemorrhage in rats treated with *Trpm4*-AS (see Fig. 2).

We evaluated functional performance using a standardized, reliable instrument commonly used in SCI research on mice, the BMS scale²⁶, modified to assess individual hindlimb function separately. *Trpm4*^{−/−}

Figure 5 *Trpm4*^{−/−} reduces secondary hemorrhage and improves neurobehavioral performance in mice after SCI. (a) Immunohistochemical localization of *Trpm4* 24 h after SCI in a WT mouse, with montage constructed from multiple individual images, and positive labeling shown in black pseudocolor; circle denotes impact site. (b) Spinal cords from WT mice harvested 30 min and 24 h after SCI, as indicated, showing progressive hemorrhage, and spinal cords from *Trpm4*^{−/−} mice harvested 24 h after SCI, as indicated, showing reduced hemorrhage compared to WT mice; intravascular blood removed by saline perfusion. (c) Spinal cord homogenates from WT mice and *Trpm4*^{−/−} mice and quantitative analysis for hemoglobin 24 h after SCI; five mice per group; ** $P < 0.01$. (d) Photographs of WT (red mark on tail) and *Trpm4*^{−/−} (blue mark on tail) mice showing truncal posture, ipsilateral (black arrow) and contralateral (white arrow) hindlimb position, and muscle tone in tail following left-sided hemicord contusion at T9. Ipsilateral and contralateral functional performances quantified using unilateral BMS scores are also shown (score in uninjured rat, 9); Fisher's exact test; * $P < 0.05$; ** $P < 0.01$. (e) Low-power and high-power views of sections stained with hematoxylin and eosin from uninjured control (CTR) and 7 d after SCI in WT and *Trpm4*^{−/−} mice; lesion areas and lesion volumes are also shown. *** $P < 0.001$.

mice exhibited significantly ($P < 0.05$) better performance than WT mice (Fig. 5d). In WT mice 24 h after injury, the hindlimb ipsilateral to the injury typically exhibited flaccid paralysis (no ankle movement), whereas in *Trpm4*^{-/-} mice, the ipsilateral hindlimb exhibited ankle movement without plantar placement. More notably, in WT mice 24 h after injury, the contralateral hindlimb typically exhibited only minor ankle movement without plantar placement, whereas in *Trpm4*^{-/-} mice, the contralateral hindlimb exhibited consistent plantar stepping with at least some coordination. Quantitative evaluation of BMS scores over the course of one week after injury confirmed the marked protective effect of *Trpm4*^{-/-} and demonstrated the durability of this effect (Fig. 5d). Near-total recovery of function on the contralateral side by day 7 attested to minimal spread of progressive hemorrhage in *Trpm4*^{-/-} mice with unilateral injury. The excellent neurological outcome in the same *Trpm4*^{-/-} mice was attributable to a significant ($P < 0.001$) reduction in lesion size (Fig. 5e), which exceeded that observed with *Trpm4*-AS in rats.

DISCUSSION

Trpm4 is one of only two molecularly identified nonselective cation channels that conducts monovalent but not divalent cations³⁻⁵. Although Trpm4 is constitutively expressed at high levels in some native tissues, normally the CNS shows only low levels of Trpm4 expression²⁷. However, Trpm4 is upregulated under certain pathological conditions: (i) Trpm4 is expressed in greater abundance in cardiac myocytes from hypertensive rats compared with normotensive controls, where a role in delayed after-depolarization has been postulated²⁸; (ii) as we show here, Trpm4 is greatly upregulated in capillary endothelial cells of the spinal cord following SCI, where we demonstrated a critical role in capillary fragmentation, formation of petechial hemorrhages and secondary hemorrhage.

In electrically nonexcitable cells such as T cells^{4,29}, mast cells⁵ and endothelial cells³⁰ that do not express voltage-dependent Ca²⁺ channels, and where Ca²⁺ entry occurs via store- or receptor-operated channels, depolarization of the cell membrane decreases the rate of Ca²⁺ influx³. The probability of opening Trpm4 increases with high Ca²⁺ and low ATP in cytoplasm²¹. Notably, both of the pathological conditions in which Trpm4 is upregulated, myocyte hypertrophy³¹ and endothelial cell activation^{32,33}, are associated with elevated levels of cytosolic Ca²⁺. These cells appear to be upregulating a Ca²⁺-activated mechanism that can act as negative feedback on Ca²⁺ influx. It is thus tempting to consider that upregulation of Trpm4 may be evolutionarily favored in metabolically injured cells because it can serve the important protective role of promoting depolarization and thereby defending against Ca²⁺ overload³⁴.

Normally, cytoplasmic concentrations of ATP are such that Trpm4 channel activity can be modulated by physiological changes in Ca²⁺ (ref. 21). However, in the context of severe ATP depletion, Trpm4 channel opening may persist indefinitely^{4,5}, with no “run-down” and no mechanism for inactivating or deactivating the channel except restoration of ATP or depletion of phosphatidylinositol-4,5-bisphosphate^{29,35}. Thus, under pathological conditions such as in SCI, when ATP depletion may be severe²²⁻²⁴, the normal physiological modulation of Trpm4 channel activity may be replaced by near-maximal activation. Unchecked activation of Trpm4 may lead not only to complete cell depolarization, but also to unrestricted influx of sodium, resulting eventually in oncotic cell swelling and oncotic cell death. Trpm4 expression *per se* is not deleterious, as long as ATP levels are adequate to limit channel opening. However, as we show here, cells expressing Trpm4 are highly susceptible to oncotic cell death following severe ATP depletion. Severe ATP depletion apparently converts what

may be an adaptive mechanism of Trpm4 upregulation that defends against Ca²⁺ overload into a maladaptive mechanism of oncotic cell death when ATP is depleted.

The histopathological sequelae of crush and contusion injury to the spinal cord have been reviewed^{10,36}. Within minutes to hours after injury, individual petechial hemorrhages form around the site of injury as well as in more distant areas^{37,38}. Petechial hemorrhages continue to form, and the lesion gradually enlarges, with a characteristic region of hemorrhage “capping” the advancing front of the lesion¹⁰. A small hemorrhagic lesion involving primarily the capillary-rich central gray matter observed 15 min after injury typically enlarges progressively over 3–24 h³⁸⁻⁴⁰. Overall, the pattern and extent of secondary hemorrhage would not be predicted based simply on the volume of tissue involved in the primary injury or on the physical proximity to the injury site.

We hypothesize that an advancing front of Trpm4 upregulation in capillary endothelium has a leading role in lesion expansion, and that the autodestruction of activated capillaries is due to molecular events within endothelial cells. Support for these hypotheses is provided by our findings that: (i) Trpm4 is abundantly upregulated in capillaries, initially immediately adjacent to the primary injury and later in more distant penumbra; (ii) Trpm4 expression greatly sensitizes cells to ATP-depletion—induced oncotic death; (iii) Trpm4 upregulation in capillaries is associated with fragmentation of capillaries and results in formation of petechial hemorrhages and (iv) block of Trpm4 eliminates fragmentation of capillaries and formation of hemorrhages.

The findings reported here with Trpm4 parallel recent observations on sulfonylurea receptor 1 (Sur1) after SCI¹⁷. As with Trpm4, Sur1 is upregulated, especially in penumbral capillaries, and block of Sur1 reduces secondary hemorrhage, reduces lesion size and greatly improves functional outcome. It has been conjectured that Trpm4 may comprise the pore-forming subunit of the Sur1-regulated NC_{Ca}-ATP channel⁴¹. The data presented here support this conjecture, although additional molecular evidence will be required to advance this hypothesis.

SCI continues to devastate the lives of millions of people worldwide. Short of total prevention, the best hope for improving outcome from SCI is to minimize the secondary injury that occurs within the first few hours of trauma. So far, the best attempts have yielded modest improvements⁴². The data presented here on block of Trpm4, as well as previous data on block of Sur1 (ref. 17), indicate that in preventing secondary hemorrhage, significant improvements in functional outcome may be expected.

METHODS

Rodent models of SCI. This study was approved by the Institutional Animal Care and Use Committee of the University of Maryland. Adult female Long-Evans rats (275–350 g) were anesthetized (ketamine, 60 mg kg⁻¹, plus xylazine, 7.5 mg kg⁻¹, intraperitoneally) and underwent a hemi-cervical cord contusion as described¹⁷. We exposed the dura at C4–C5 via a left hemilaminectomy and created a hemicervical cord contusion using a blunt force impactor (modified NYU device; 1.3-mm impactor head; 10 g weight dropped 25 mm). After SCI, we gave animals 10 ml of glucose-free normal saline subcutaneously. We maintained rectal temperature at ~37 °C. Typical blood gases (i-STAT, Heska Corp), sampled before injury, were pO₂ > 90 mm Hg and pCO₂ < 47 mm Hg, and glucose levels were 150–200 mg dl⁻¹.

Trpm4^{-/-} mice, obtained as previously described⁵, showed neurological function, gait and spinal cord histology that were indistinguishable from that of WT (see Fig. 5e), and maximum BMS scores of 9. For SCI experiments, female WT (background, C57BL/6) and *Trpm4*^{-/-} mice (backcrossed for 6 generations on the C57BL/6 background), 24–28 g, were anesthetized (ketamine, 60 mg kg⁻¹, plus xylazine, 7.5 mg kg⁻¹, intraperitoneally), the dura at T9 was

exposed via a left hemilaminectomy and a hemithoracic spinal cord contusion was created (modified NYU device; 0.6-mm impactor head; 10 g weight dropped 5 mm).

For gene suppression of *Trpm4*, we used antisense oligodeoxynucleotide (5'-TTGTGGTAACACTCTCCAAA-3') phosphorothioated at four distal bonds. We used sense as control. We verified the functionality of *Trpm4*-AS by measuring *Trpm4* mRNA by RT-PCR in bEnd.3 cells loaded with *Trpm4*-AS or with *Trpm4*-SE (using LipofectAMINE, Invitrogen) and exposed to tumor necrosis factor- α (20 ng ml⁻¹). For SCI rats, a few minutes after injury, we gave a loading dose of 300 μ g of oligodeoxynucleotide in 300 μ l intravenously and implanted mini-osmotic pumps (Alzet; Durect Corporation) with jugular vein catheters (infusion: 40 μ g hr⁻¹ $\times$ 24 h)¹⁷.

For block of *Trpm4*, we used flufenamic acid, 10 mg kg⁻¹ intraperitoneally, given at 0.1, 6 and 16 h after SCI.

Immunohistochemistry. We immunolabeled cryosections prepared from perfusion-fixed spinal cords^{17,43} with primary antibodies directed against *Trpm4* (1:100; overnight at 4 °C; SC-27540; Santa Cruz Biotechnology), vWf (1:200; F3520, Sigma) and vimentin (1:100; overnight at 4 °C; Sigma).

Immunoblots. 80 μ g of protein from cord lysates were separated on a 3–8% Tris-acetate gel and blotted onto a nitrocellulose membrane blocked with 7% nonfat milk. We visualized *Trpm4* using the same antibody.

RT-PCR. We used forward and reverse primers, designed from different exons, as follows: forward (5'-GTCATCGTGAGCAAGATGATGAA-3') and reverse (5'-GTCCACCTTCTGGGACGTGC-3').

In situ hybridization for *Trpm4* (XM_574447). We processed cryosections with digoxigenin-labeled probes (antisense: 5'-CCAGGGCAGGCCGCGAATGGAATCCCGGATGAGGCTGTAGCGCTGCG-3'; GeneDetect).

Spinal cord tissue blood. We determined the amount of blood in spinal cord tissue as described¹⁷.

***Trpm4* expression.** We recloned the *mTrpm4* insert from *mTrpm4*-GFP plasmid²⁹ into a plasmid containing enhanced green fluorescence protein (EGFPN1; Clontech). We used EGFPN1 alone as control. We transiently transfected COS7 cells with plasmid DNA using FuGENE (Roche) per the manufacturer's instructions. We studied cells 48 h after transfection. We assessed ATP depletion following exposure to Na azide and 2-deoxyglucose using an ATP bioluminescent assay kit (Sigma-Aldrich).

Patch clamp electrophysiology. We performed patch clamp electrophysiology as described^{17,43}.

Oncotic cell death. ATP was depleted using Na azide (1 mM) plus 2-deoxyglucose (10 mM). We collected cells and resuspended them in labeling reagent containing propidium iodide for 10 min and then counted. We divided the number of cells exhibiting a green fluorescent signal (EGFPN) plus red nuclear staining (propidium iodide) by the total number of cells exhibiting a green fluorescent signal.

Neurobehavioral assessment. Blinded evaluators performed all measurements. For vertical exploration (rearing)⁴⁴, we videotaped animals while in a translucent cylinder (20 $\times$ 20 cm), with rearing quantified as the number of seconds spent with both front paws elevated above shoulder height during the first 3-min period of observation. Beam walking, inclined plane²⁵ and BMS scoring were determined as described²⁶.

Lesion volumes. We calculated spinal cord lesion volumes 7 d after SCI as described¹⁷.

Statistical analyses. Statistical significance was evaluated using Student's *t*-test or ANOVA with Bonferroni comparisons, as appropriate, except for BMS scores, which were evaluated using Fisher's exact test. Data are presented as mean $\pm$ s.e.m.

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AUTHOR CONTRIBUTIONS

V.G. and J.M.S. conceived of and directed the study, participated in neurobehavioral assessments, analyzed the data and wrote the manuscript; S.K.W., Z.G. and Z.C. performed molecular and cell death experiments; O.T. performed rodent surgeries; S.I. performed immunolabeling and *in situ* hybridization; A.I. performed patch clamp experiments; R.V., M.F., V.F. and B.N. developed the *Trpm4*^{-/-} mice and contributed to early drafts of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturemedicine/>.

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