TREK-1 and TRAAK Are Principal K⁺ Channels at the Nodes of Ranvier for Rapid Action Potential Conduction on Mammalian Myelinated Afferent Nerves

Graphical Abstract

Highlights

- TREK-1 and TRAAK are clustered at nodes of Ranvier of myelinated afferent nerves

- They are required for rapid action potential (AP) regeneration at nodes of Ranvier

- They permit high-speed and high-frequency AP conduction on afferent nerves

- Suppressing these channels retards nerve conduction and impairs sensory functions

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In Brief

Kanda et al. studied ion channels at the nodes of Ranvier (NRs) on rat myelinated afferent nerves. They discovered that thermally sensitive K₂P channels, including TREK-1 and TRAAK, are clustered at NRs to secure high-speed and high-frequency nerve conduction.

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TREK-1 and TRAAK Are Principal K⁺ Channels at the Nodes of Ranvier for Rapid Action Potential Conduction on Mammalian Myelinated Afferent Nerves

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SUMMARY

Rapid conduction of nerve impulses is critical in life and relies on action potential (AP) leaps through the nodes of Ranvier (NRs) along myelinated nerves. While NRs are the only sites where APs can be regenerated during nerve conduction on myelinated nerves, ion channel mechanisms underlying the regeneration and conduction of APs at mammalian NRs remain incompletely understood. Here, we show that TREK-1 and TRAAK, the thermosensitive and mechanosensitive two-pore-domain potassium (K2P) channels, are clustered at NRs of rat trigeminal Aβ-afferent nerves with a density over 3,000-fold higher than that on their somas. These K2P channels, but not voltage-gated K⁺ channels as in other parts of nerves, are required for rapid AP repolarization at the NRs. Furthermore, these channels permit high-speed and high-frequency AP conduction along the myelinated afferent nerves, and loss of function of these channels at NRs retards nerve conduction and impairs sensory behavioral responses in animals.

INTRODUCTION

Rapid conduction of nerve impulses is achieved by action potentials (APs) leaping along myelinated nerves via the nodes of Ranvier, the tiny myelin-sheath gaps on myelinated nerves (Boulnerne, 2016; Huxley and Stampfli, 1949; Lillie, 1925; Ranvier, 1871, 1872; Tasaki, 1939). AP propagation on myelinated nerves, also known as saltatory (hop or leap in Latin) conduction (Huxley and Stampfli, 1949; Lillie, 1925; Tasaki, 1939), can reach speeds over 100 m/s and frequencies over hundreds of hertz, 100 times more rapid than impulse conduction on unmyelinated nerves in mammals (Waddell and Lawson, 1990). Saltatory conduction ensures timely sensory responses with high acuity and rapid muscle movement with fine control. Impairments of saltatory conduction at nodes of Ranvier (NRs) occur in many neurological diseases, including multiple sclerosis, spinal cord injury, and inflammatory demyelinating polyneuropathy (Arancibia-Carcamo and Attwell, 2014; Devaux et al., 2012; Stathopoulos et al., 2015; Waxman, 1992, 2006), leading to sensory and motor dysfunctions. NRs are the only places where APs are regenerated to permit saltatory conduction, and voltage-gated Na⁺ channels clustered at NRs (Caldwell et al., 2000; Ritchie and Rogart, 1977) are partially responsible for the AP regeneration by driving AP depolarization. However, the ion channels responsible for driving rapid AP repolarization at NRs and for securing high-speed as well as high-frequency AP conduction on mammalian myelinated nerves remain to be unknown.

Regeneration of APs at NRs of myelinated nerves has long been thought to be the same mechanistically as that on the other parts of nerves; i.e., AP depolarization is driven by voltage-gated Na⁺ channels, and AP repolarization is driven mainly by voltage-gated K⁺ channels (Bean, 2007). This classical ion channel mechanism is responsible for AP regeneration at amphibian NRs (Huocho et al., 1979). However, in mammalian species, including rats and rabbits, Chiu and Ritchie failed to detect significant amount of K⁺ currents at intact NRs except when the myelin sheaths of the nerves were loosen chemically (Chiu and Ritchie, 1980), and they suggested that K⁺ channels may be not present at NRs for AP regeneration. On the other hand, Binah and Palti detected large K⁺ currents at NRs of rat myelinated nerves (Binah and Palti, 1981). Since then, there have been long debates whether or not K⁺ channels are present at NRs for AP repolarization and conduction. This fundamental issue remains unsolved, because patch-clamp recordings, the most appropriate technique for addressing this question, have never been successfully applied to intact NRs of mammalian myelinated nerves. Much of our knowledge about ion channels at mammalian NRs is based on immunohistochemical studies, which provide useful but limited information about the functions of ion channels at NRs. Immunohistochemical studies on mammalian NRs have shown that voltage-gated Na⁺ channel Nav1.6 and Nav1.2 are clustered at NRs (Caldwell et al., 2000; Waxman, 2006). These voltage-gated Na⁺ channels are believed to drive AP depolarization during saltatory conduction. The voltage-gated K⁺ channels Kv7.2 (Devaux et al., 2004) and Kv3.1b (Devaux et al., 2003) were also detected in mammalian NRs, and Kv1.1 and Kv1.2 were detected in juxtaparanodal...
regions immunohistochemically (Rhodes et al., 1997). However, present, there is no information as to whether these voltage-gated K\(^+\) channels at NRs or juxtaparanodal regions are involved in AP repolarization at NRs. Subsequently, alternative hypotheses have been proposed for AP repolarization at NRs such as inactivation of voltage-gated Na\(^+\) channels and/or a periaxial K\(^+\) pathways (Barrett and Barrett, 1982; Li, 2015; Rash et al., 2016). However, none of these putative mechanisms have been tested functionally with the patch-clamp recording technique.

A more important issue about NRs is whether and how ion channels at NRs may play a key role in securing salutatory conduction at high speed and high frequency. Very few studies have investigated mechanisms responsible for high-frequency AP conduction on myelinated nerves. For high-speed AP conduction on myelinated nerves, previous studies have mainly focused on effects of myelination on conduction velocity (Castelfranco and Hartline, 2016). Interestingly, based on studies with computational modeling, it has been hypothesized that the speed of membrane charge for AP initiation at NRs may be a rate-limiting factor for salutatory conduction (Castelfranco and Hartline, 2015, 2016). Biophysically, the speed of membrane charge is faster with smaller membrane capacitance and also with larger membrane input conductance. Intriguingly, myelinated axons are substantially narrowed at each NR, a structural feature thought to be an evolutionary change to reduce membrane capacitance thereby to increase the speed of membrane charge at NRs (Castelfranco and Hartline, 2016; Halter and Clark, 1993). However, a reduction in nodal membrane areas with narrowed nodal axons would normally result in a substantial decrease of membrane input conductance, which would slow membrane charge at NRs (Moore et al., 1979). Nevertheless, if the narrowed nodal axon is co-evolved with the clustering of ion channels that substantially increase membrane input conductance at NRs, this would greatly increase the speed of nodal membrane charge and provide a novel mechanism to secure high-speed AP conduction through NRs in myelinated nerves (Castelfranco and Hartline, 2015, 2016; Halter and Clark, 1993). This putative mechanism has so far never been tested experimentally since, except voltage-gated Na\(^+\) channels, no other principal ion channels have been identified at NRs of mammalian myelinated nerves.

Two-pore-domain K\(^+\) (K2P) channels are a family of 15 members that were originally known as “leak” or “background” K\(^+\) channels (Enyedi and Czirjak, 2010; Goldstein et al., 2005; Lotshaw, 2007). K2P channels are distinct from voltage-gated K\(^+\) channels in their structure, biophysical and pharmacological properties, and electrophysiological functions. K2P channels are expressed at low levels on neurons and are involved in setting membrane input conductance and resting membrane potentials (Enyedi and Czirjak, 2010). Of the 15 K2P subtypes, tandem of P domains in weak inward rectifier K\(^+\) channel (TWIK)-related K\(^+\) channel (TREK)-1, TREK-2, and TWIK-related arachidonic acid stimulated K\(^+\) channel (TRAAMK) are three closely related subtypes displaying both thermal and mechanical sensitivity (Enyedi and Czirjak, 2010; Kang et al., 2005; Kim et al., 2001; Maingret et al., 1999, 2000). K2P channels in neurons have been suggested to play roles in regulation of excitability, sensory transduction, and neuroprotection (Lotshaw, 2007). Mutations in K2P channels such as TRAAK missense mutations result in abnormal functions of central and peripheral nervous systems in humans (Bauer et al., 2018). TREK channels have recently been found to be inhibited by migraine-associated TWIK-related spinal cord K\(^+\)channel (TRESK) mutations to result in neuronal hyperexcitability (Royal et al., 2019). However, it is entirely unknown whether any K2P channels may be highly expressed at NRs of mammalian myelinated nerves, and if so, whether they play a critical role in regeneration and rapid conduction of APs at NRs. Here, we developed an in situ pressure-patch-clamp recording technique, and using a rat ex vivo trigeminal nerve preparation, we made patch-clamp recordings at intact NRs of mammalian myelinated nerves for the first time. Using this key technique in combination with immunohistochemical, genetic, and pharmacological approaches and in vivo sensory behavioral assessments, we have addressed these key questions.

**RESULTS**

**Nodes of Ranvier of Myelinated Afferent Nerves Display Unconventional APs and High Leak K\(^+\) Conductance**

Whole-cell patch-clamp recording is the most direct approach to study ion channels and their potential functions in salutatory conduction at NRs, but it has never been applied to an intact NR of mammalian myelinated nerves due to technical challenges. This is because NRs are tiny axonal segments surrounded by tough perineural tissues, which not only makes it impossible to visualise nodal axons but also prevents recording electrodes from forming quality membrane seals for patch-clamp recordings. Structurally intact NRs are essential in the present study, since a structural alteration at NRs such as demyelination could alter ion channels and impair salutatory conduction (Waxman, 2006). We developed an in situ pressure-patch-clamp recording technique and for the first time directly made patch-clamp recordings at intact NRs of trigeminal A\(^{\beta}\)-afferent nerves in a rat ex vivo trigeminal afferent nerve preparation (Figures 1A, 1B, and S1). The in situ pressure-patch-clamp recording technique relied on a pressure-clamp device to help patch-clamp electrodes to approach tiny invisible nodal axons and to facilitate the formation of cell-attached and whole-cell patch-clamp recording modes (Figure S1). This new recording technique allowed us for the first time to investigate the function of ion channels at NRs in salutatory conduction. Using recording electrodes that contained fluorescent dye Alexa Fluor 555, we also were able to visualise and characterize the morphological properties of nodal, paranodal, and inter-nodal axons of the fresh trigeminal A\(^{\beta}\)-afferent nerves recorded under the whole-cell patch-clamp configuration (Figure 1B; Table S1). Nodal and paranodal axons were much narrower than inter-nodal axons and had diameters of ~1.88 μm and total length of ~4.2 μm, and inter-nodal axons had diameters of ~5.4 μm and length of ~736 μm (Figure 1B; Table S1). The much narrower axonal segments at NRs shown in our rat trigeminal A\(^{\beta}\)-afferent nerves resemble the structural feature commonly seen in myelinated axons of peripheral nerves in mammals (Castelfranco and Hartline, 2016).

By using patch-clamp recordings at NRs of trigeminal A\(^{\beta}\)-afferent nerves, our first finding was that APs at NRs were...
unconventional in that AP repolarization was totally independent of voltage-gated K⁺ channels. This was evidenced by the lack of effects on AP repolarization (AP width) by high concentrations of extracellular tetraethylammonium (TEA; 20 mM) plus intracellular Cs⁺ (135 mM), two blockers of voltage-gated K⁺ channels (Figure 1C). For conventional APs such as those recorded at somas of trigeminal Aβ-afferent nerves, AP repolarization largely depends on voltage-gated K⁺ channels, and blocking these channels with TEA plus Cs⁺ retarded AP repolarization and severely prolonged AP widths (Figure 1D). Thus, repolarization at NRs of myelinated afferent nerves is mechanistically distinct from AP repolarization at other places in neuronal cells, a finding that settles the decade-long controversies on the mechanisms of AP repolarization in mammalian myelinated nerves (Barrett and Barrett, 1982; Li, 2015; Rash et al., 2016).

We next examined ionic currents at NRs of trigeminal Aβ-afferent nerves to provide insights into the unconventional APs. The direct whole-cell patch-clamp recordings at intact NRs allowed us for the first time to quantitatively determine ionic currents and their characteristics at NRs of the myelinated afferent nerves. Large and transient inward currents were evoked following voltage steps and the inward currents were completely blocked by tetrodotoxin (TTX; Figure S2), consistent with the clustering of the voltage-gated Na⁺ channels Nav1.6 at NRs required for driving AP depolarization during saltatory conduction (Caldwell et al., 2000; Waxman, 2006). A striking finding following the depolarizing voltage steps was the appearance of strong non-inactivating outward currents that were not significantly inhibited by voltage-gated K⁺ channel blockers 135 mM Cs⁺ (intracellular) plus 20 mM TEA (Figure 1E) or the Kv7.2 inhibitor linopirdine (Figure S2). In contrast, in the somas of trigeminal Aβ-afferent nerves non-inactivating outward currents were largely inhibited by Cs⁺ plus TEA (Figure S3). Thus, different from other parts of nerves where voltage-gated K⁺ channels mediate most of outward currents (Bean, 2007), voltage-gated K⁺ channels did not significantly contribute to the outward currents at intact NRs of myelinated afferent nerves. This result is consistent with the lack of significant effects on APs by voltage-gated K⁺ channel blockers at NRs of trigeminal Aβ-afferent nerves (Figure 1C).

Since the non-inactivating outward currents at NRs were the most prominent ionic currents whose nature was completely unknown, we next determined the ionic basis of the outward currents at NRs of trigeminal Aβ-afferent nerves. Outward currents...
of a cell could be mediated by influx of Cl⁻ ions through membrane Cl⁻ channels. However, the outward currents at NRs were not due to Cl⁻ ion influx via Cl⁻ channels, since the outward currents were not affected when altering Cl⁻ concentrations or applying Cl⁻ channel blockers (Figure S4). Nevertheless, altering extracellular K⁺ concentrations shifted the reversal potentials, consistent with Goldman-Hodgkin-Katz equation for K⁺-permeable channels (Figure 1F). Thus, the outward currents at the NRs fell into the class of “leak” or “background” K⁺ (IKleak) currents (Enyedi and Czirják, 2010; Goldstein et al., 2005; Lotshaw, 2007). In contrast to NRs, non-inactivating outward currents in the somas of trigeminal Aβ-afferent nerves were mainly mediated by classical voltage-gated K⁺ channels, since they were largely inhibited by Cs⁺ + TEA (Figure S3). The unexpectedly large IKleak currents at NRs of trigeminal Aβ-afferent nerves have unusually high leak K⁺ conductance mediated by large-conductance leak K⁺ channels.

**Leak K⁺ Conductance at the Nodes of Ranvier of Myelinated Afferent Nerves Are Thermosensitive and Mechanosensitive and Identified as TREK-1 and TRAAK Channels**

The unexpectedly large IKleak currents at NRs of trigeminal Aβ-afferent nerves led us to further investigate molecular identities mediating these currents at NRs. K2P channels, a family of at least 15 members, have been cloned and shown to be responsible for mediating IKleak currents (Enyedi and Czirják, 2010; Goldstein et al., 2005; Lotshaw, 2007). However, none of these K2P channels have been reported to be present at NRs. To determine if members of K2P channels may mediate the large IKleak currents at NRs, we tested the thermal sensitivity of IKleak currents (Enyedi and Czirják, 2010; Goldstein et al., 2005; Lotshaw, 2007). Thus, NRs of trigeminal Aβ-afferent nerves have unusually high leak K⁺ conductance mediated by large-conductance leak K⁺ channels.
currents recorded at NRs were significantly increased in numbers and probabilities of channel openings in responses to negative pressures (Figures 2G–2I; n = 6), indicating that IK_leak channels are mechanosensitive.

Immunoreactivity to TREK-1 (TREK-1-ir) and TRAAK (TRAAK-ir), but not TREK-2 (TREK-2-ir), was observed in the axonal regions of trigeminal Aβ-afferent nerves lacking myelin basic protein (MBP) immunoreactivity (Figures 3A–3C). Furthermore, TREK-1-ir or TRAAK-ir on trigeminal Aβ-afferent nerves was present between the immunoreactivity of contactin-associated protein 1 (CASPR), a glycoprotein specifically enriched in paranodal axonal regions (Figures 3A, 3B, and S5). The specificity of the antibodies for TREK-1 and TRAAK were validated using their blocking peptides (Figure S5) and also validated by the specific immunoreactivity to TREK-1 and TRAAK heterologously expressed in HEK293 cells (Figure S6). TREK-1 immunoreactivity, but not TRAAK immunoreactivity, was also observed at NRs of myelinated nerve fibers in the spinal dorsal column, ventral column, and motor nerves (Figure S7). For trigeminal Aβ-afferent nerves, 87% and 91% of their nodes were TREK-1-ir and TRAAK-ir positive, respectively (Figure 3D), indicating that TREK-1 and TRAAK are mostly co-expressed at NRs of myelinated afferent nerves.

The co-expression of TREK-1 and TRAAK at NRs of trigeminal Aβ-afferent nerves raised a question as whether they formed heteromeric TREK-1/TRAAK channels. Previous studies have shown that mouse TREK-1 and TRAAK each formed homomeric channels and their co-expression formed heteromeric channels.
(Blin et al., 2016; Levitz et al., 2016). However, a recent study has shown that TREK-1 and TRESK form heteromeric channels in trigeminal nerves and that the activity of these channels is poten-
tiated by intracellular Ca\(^{2+}\) elevation (Royal et al., 2019). Never-
theless, we found that IK\(_{\text{leak}}\) currents at NRs were not affected by the Ca\(^{2+}\) ionophore ionomycin (Figure S8), suggesting that functional K2P channels at NRs of trigeminal A\(\beta\)-afferent nerves are unlikely to be heteromeric channels formed by TREK-1 and TRESK. We expressed rat TREK-1 in HEK293 cells, which re-
sulted in homomeric TREK-1 channels with single-channel conductance of 67 ± 6 pS (n = 6) at 80 mV and 88 ± 10 pS (n = 5) at −80 mV (Figures 3E, 3F, and 3H). We also expressed TRESK in HEK293 cells, which resulted in homomeric TRESK channels with single-channel conductance of 100 ± 12 pS (n = 6) at 80 mV and 67 ± 12 pS (n = 6) at −80 mV (Figures 3E, 3F, and 3H). In contrast, when TREK-1 and TRESK channels were co-expressed, a new type of unitary currents appeared that had single-channel conductance of 87 ± 7 pS (n = 5) at 80 mV and 125 ± 6 pS (n = 8) at −80 mV (Figures 3E, 3G, and 3H). The new channels are distinct from the homomeric channels formed by either TREK-1 or TRESK (Figures 3E–3H), indicating that co-expression of TREK-1 and TRESK proteins resulted in the formation of heteromeric TREK-1/TRESK channels. In the HEK293 cells co-transfected with TREK-1 and TRESK, we also observed a single-channel type with conductance of 51 ± 3 pS at 80 mV (n = 5) and 87 ± 4 pS at −80 mV (n = 12), similar to ho-
omeric TREK-1 channels (TREK-1-like; Figures 3G and 3H). For the heteromeric TREK-1/TRESK channels formed in HEK293 cells, their single-channel conductance was similar to the single channels recorded at the NRs (nodal K2P; Figure 3H). In HEK293 cells co-expressing both TREK-1 and TRESK, whole-cell patch-clamp recordings showed large outward currents following depolarizing voltage steps, and the currents were temper-
ature sensitive and TEA insensitive (Figure S9), similar to the whole-cell IK\(_{\text{leak}}\) currents recorded at NRs (Figures 1 and 2). Homomeric TREK-1 channels expressed in HEK293 cells were also not sensitive to TEA or to Zn\(^{2+}\) (Figure S9). Thus, at NRs of trigeminal A\(\beta\)-afferent nerves, functional leak K\(^+\) channels were most likely formed as heteromeric TREK-1/TRESK channels, but the presence of homomeric TREK-1 and homomeric TRESK cannot be excluded.

TREK-1 and TRESK Channels Are Required for Rapid AP Repolarization at the Node of Ranvier of Myelinated Afferent Nerves

To explore the role of TREK-1 and TRESK channels at NRs of myelinated afferent nerves, we designed small hairpin RNAs (shRNAs) to knock down TREK-1 and TRESK channels. The shRNAs were inserted in adeno-associated viral (AAV) plasmids containing EGFP or mCherry reporter genes, packed into AAV particles, and microinjected into maxillary (V2) branches of tri-
geminal afferent nerves (Figure 4A). After 4 weeks, EGFP and mCherry were observed in trigeminal afferent nerve somas (Figure S10) and their peripheral axons (Figure 4B). In whole-cell patch-clamp recordings, shTREK-1-expressing and shTRESK-expressing NRs of trigeminal A\(\beta\)-afferent nerves showed signifi-
cantly smaller IK\(_{\text{leak}}\) conductance compared to the shScramble group (Figures 4C and 4D). A greater reduction of IK\(_{\text{leak}}\) conduc-
tance (by ~30%) was observed with shTREK-1+shTRESK double knockdown (Figures 4C and 4D). Consistently, in EGFP-positive trigeminal afferent nerve somas, strong TREK-1 immunoreactive neurons was significantly less in shTREK-1 group than in the shScramble group (Figure S10); in mCherry-positive trigeminal afferent nerve somas, the number of strong TRESK immunoreactive neurons was significantly less in the shScramble group than in the shTRESK group (Figure S10).

Pharmacologically, IK\(_{\text{leak}}\) currents at NRs were also significantly inhibited by barium (Ba\(^{2+}\)), norfluoxetine (NF), and ruthenium red (RR) (Figure 4E), three IK\(_{\text{leak}}\) channel inhibitors (Lotshaw, 2007). In contrast, BL1249 (BL), intracellular arachidonic acid (AA), and low intracellular pH of 5 ([pH]i 5), three activators of thermo-
sensitive and mechanosensitive K2P channels (Lotshaw, 2007), significantly potentiated IK\(_{\text{leak}}\) currents at 24°C (Figure 4F) and 15°C (Figure S11).

TREK-1 and TRESK channels may play a pivotal role in driving rapid AP repolarization at the NRs of myelinated afferent nerves. To test this hypothesis, we determined whether AP repolariza-
tion at NRs may be retarded by gene knockdown or pharmaco-
logical inhibition of TREK-1 and TRESK channels. AP repolarization at NRs of trigeminal A\(\beta\)-afferent nerves was rapid with AP widths of ~0.7 ms (Figure 4G). However, AP widths at NRs were significantly prolonged in shTREK-1, shTRESK, or shTREK-1 + shTRESK groups, which decreased TREK-1, TRESK, or both TREK1 and TRAAK, respectively (Figure 4G). AP widths at NRs were also significantly prolonged following Ba\(^{2+}\), NF, and RR applications to inhibit the leak K\(^+\) channels (Figure 4H). We performed AP-dynamic clamp recordings at NRs and showed that AP repolarization phase was aligned with the outward currents, and the outward currents were insen-
sitive to the voltage-gated K\(^+\) channel blocker TEA (Figure 4I) but inhibited by Ba\(^{2+}\), which blocked IK\(_{\text{leak}}\) currents (Figure 4J).

These results further support the role of leak K\(^+\) channels in driving rapid AP repolarization at NRs and suggest that TREK-1 and TRESK channels are required for the formation of rapid APs at NRs of myelinated afferent nerves for saltatory conduction.

Knockdown and pharmacological inhibition of TREK-1 and TRESK channels also increased nodal membrane input resis-
tance and depolarized resting membrane potentials (Table S2) but had no effects on voltage-activated sodium currents at NRs of trigeminal A\(\beta\)-afferent nerves (Figure S12). The effects on nodal membrane input resistance and resting membrane po-
tentials (Table S2) also revealed a role of TREK-1 and TRESK channels in controlling intrinsic membrane properties. The very negative resting membrane potentials at NRs of trigeminal A\(\beta\)-af-
ferent nerves (near ~82 mV; Table S2) were thus a result of the high expression of TREK-1 and TRESK channels.

TREK-1 and TRESK Channels Are Required for High-Frequency and High-Speed Saltatory Conduction on Myelinated Afferent Nerves in Vivo Sensory Behavioral Responses

Sensory information is encoded by nerve impulses in a broad range of frequencies up to hundreds of hertz, and TREK-1 and TRESK channels at NRs may be critical for high-frequency im-
pulses conducted on myelinated afferent nerves. To test this...
idea, we first compared APs conducted through somas and through NRs of trigeminal Aβ-afferent nerves following increased stimulation frequencies (Figures 5A and 5B).

At somas where voltage-gated K+ channels drive AP repolarization, APs were broadened in a frequency-dependent manner (Figure 5B). In sharp contrast, APs showed no AP broadening at NRs of trigeminal Aβ-afferent nerves in response to high-frequency stimulation up to 200 Hz (Figure 5B). We next examined AP success rates at different stimulation frequency. APs conducted through somas typically failed at frequencies ≥50 Hz and mostly failed at 200 Hz (Figures 5C and S13). In contrast, APs conducted through NRs of trigeminal Aβ-afferent nerves showed no significant failures at frequencies up to 200 Hz (Figures 5C and S13). Quantitatively described by the frequency at which AP conduction success rate was 50% (FS50), FS50 was 74.2 ± 23.7 Hz (n = 6) and 458.1 ± 58.3 (n = 7) Hz for APs conducted through somas and NRs, respectively (Figures 5C and S13).

AP success rates at NRs were significantly reduced in the shTREK-1, shTRAAK, and shTREK-1 + shTRAAK groups (Figures 5D and S14). Similarly, AP success rates at NRs were significantly reduced by Ba2+, NF, and RR, which inhibit IKleak currents (Figures 5E and S14). These results indicate that TREK-1 and TRAAK channels play a key role in high-frequency AP conduction on mammalian myelinated afferent nerves.

TREK-1 and TRAAK channels may be required for high-speed nerve conduction along myelinated afferent nerves. To test this hypothesis, we determined whether AP conduction velocity at NRs of trigeminal Aβ-afferent nerves was reduced following knockdown of TREK-1 and/or TRAAK channels. AP conduction velocity recorded at NRs of trigeminal Aβ-afferent nerves was 48.3 ± 1.8 m/s (n = 6) in the control group injected with shScramble and was significantly reduced to 30.4 ± 1.9 m/s (n = 6), 28.5 ± 1.6 m/s (n = 6), and 24.5 ± 0.7 m/s (n = 6) in the shTREK-1, shTRAAK, and shTREK-1 + shTRAAK groups, respectively.
Cited by tactile stimuli are conducted by trigeminal A
of the whisker tactile behavioral tests was because impulses eli-
nerves. As shown in Figures 6H and 6I, microinjection of
stimulation to whisker hairs (Figure 6G). The reason for the use
manner for their avoidance behaviors in responses to tactile
shRAN-AAV preparations, animals were tested in a blinded
resulted in large leak K+ currents at NRs at cooling
temperatures (Figures 2A and 2B). Furthermore, BL, AA, and
intracellular pH of 5, three activators of thermosensitive and me-
chanosensitive K2P channels, reversed the cold-induced reduc-
tion of AP conduction velocity (Figure 6E). The conduction veloc-
ity recorded at NRs was not affected in the presence of a mixture
of voltage-gated K+ channel blockers TEA (20 mM), Cs+ (135 mM,
intracellular), and 4-AP (1 mM, intracellular) (Figure 6F). These re-
results indicate that high-speed nerve conduction on mammalian myelinated afferent
nerves are essential for in vivo sensory behavioral responses and that loss of function of these channels at NRs impairs in vivo sensory behavioral responses.

DISCUSSION

In this study, we have shown that TREK-1 and TRAAK are principal ion channels clustered at NRs of myelinated afferent nerves of rats. These thermosensitive and mechanosensitive K2P channels (not classical voltage-gated K+ channels as in other parts of nerves) drive rapid AP repolarization at NRs of myelinated afferent nerves. More importantly, we have demonstrated that these K2P channels at NRs are required for high-speed and high-frequency saltatory conduction along myelinated afferent nerves as well as in vivo tactile behavioral responses.

By applying our newly developed in situ pressure-patch-clamp recording technique to intact NRs of trigeminal afferent nerves, we have detected for the first time large leak K+ currents at NRs of trigeminal A-afferent nerves. This indicates that leak K⁺ currents are specifically clustered and anchored at mammalian NRs of myelinated afferent nerves as principal ion channels. The leak K⁺ channels at NRs of trigeminal afferent nerves are the thermosensitive and mechanosensitive

(Figures 6A and 6B). Consistently, AP conduction velocity at NRs of trigeminal A-fferent nerves was also significantly reduced following the applications of Ba²⁺, NF, and RR to inhibit the K2P channels at NRs (Figure 6C). These results indicate that TREK-1 and TRAAK channels at NRs play a key role in securing high-speed AP conduction on mammalian myelinated afferent nerves. Conduction velocity was also progressively reduced with temperature decreases from 35°C to 15°C (Figure 6D), consistent with the inhibition of IKneak currents at NRs at cooling temperatures (Figures 2A and 2B). Furthermore, BL, AA, and intracellular pH of 5, three activators of thermosensitive and me-
chanosensitive K2P channels, reversed the cold-induced reduc-
tion of AP conduction velocity (Figure 6E). The conduction veloc-
ity recorded at NRs was not affected in the presence of a mixture
of voltage-gated K+ channel blockers TEA (20 mM), Cs⁺ (135 mM,
intracellular), and 4-AP (1 mM, intracellular) (Figure 6F). These re-
results indicate that high-speed nerve conduction along myelinated afferent nerves relies on the activity of TREK-1 and TRAAK channels at NRs.

The role of TREK-1 and TRAAK channels in AP conduction on myelinated afferent nerves should impact in vivo sensory behavioral responses. To test this idea, tactile behavioral tests were performed in animals (Figure 6G) that were microinjected with the aforementioned shRNA-AAV preparations into individual D2 whisker hair follicles (Figure 6H) or individual D2 whisker afferent nerves (Figure 6I). 4 weeks after microinjections of the shRAN-AAV preparations, animals were tested in a blinded manner for their avoidance behaviors in responses to tactile stimulation to whisker hairs (Figure 6G). The reason for the use of the whisker tactile behavioral tests was because impulses eli-
cited by tactile stimuli are conducted by trigeminal A-fferent nerves. As shown in Figures 6H and 6I, microinjection of

Figure 5. TREK-1 and TRAAK Channels Are Required for High-Frequency Saltatory Conduction on Myelinated Afferent Nerves
(A) Experimental setting.
(B) Traces illustrate APs recorded from the soma (left) and NR (right) of a trigeminal A-fferent nerve following 50-Hz stimulation. Traces were 1st, 10th, and 20th APs. Bar graph, AP widths of 20th APs at somas (n = 7, black) or NRs (n = 7, green) following stimulation at 1–200 Hz. Stimulation duration, 20 s. (C) Left: two traces illustrate APs recorded at a soma (top) and an NR (bottom) following stimulation at 50 Hz. Tall spikes, successful APs. Right: AP success rates at somas (n = 7) and NRs (n = 7) with stimulation from 1 to 1,000 Hz. Dashed lines indi-
cate the frequency at which AP conduction success rate is 50% (FS50).
(D) AP success rates at NRs of the shScramble (n = 6), shTREK-1 (n = 6), shTRAAK (n = 6), and shTREK-1 + shTRAAK (n = 6) groups. (E) AP success rates at NRs before (control, n = 6) and after application of 5 mM Ba²⁺ (n = 7), 50 μM NF (n = 6), or 2 mM RR (n = 6). Recording duration at each frequency was 20 s. Data represent mean ± SEM. *p < 0.05, **p < 0.01, one-way ANOVA with the Tukey post hoc test. See also Figures S13 and S14.
K2P channels most likely formed by TREK-1 and TRAAK. This conclusion is based on several lines of evidence, including the presence of immunoreactivity of TREK-1 and TRAAK at the NRs of trigeminal Aβ-afferent nerves, the whole-cell currents and single-channel properties recorded from the nodal membranes, and pharmacology and gene knockdown experiments. Immunostaining results of the present study indicated that most NRs of trigeminal Aβ-afferent nerves co-expressed both TREK-1 and TRAAK channels. We show that the co-expression of rat TREK-1 and TRAAK in HEK293 cells resulted in the formation of heteromeric TREK-1/TRAAK channels, with electrophysiological properties consistent with previous studies with mouse TREK-1 and TRAAK channels (Blin et al., 2016). At the NRs of trigeminal Aβ-afferent nerves, single-channel properties of our nodal K2P channels suggest that these channels may be heteromeric TREK-1/TRAAK channels. The clustering of high-density TREK-1 and TRAAK channels at NRs of trigeminal Aβ-afferent nerves may require anchoring proteins, the nature of which has not been determined in the current study. Ankyrin-G and neurofascin have been shown to be anchoring proteins for the clustering of high-density voltage-gated Na+ channels at NRs (Arancibia-Carcamo and Attwell, 2014; Jenkins et al., 2015). Nodal anchoring proteins may enrich K2P channels such that their immunoreactivity is detectable at NRs but may or may not be detectable at their somas. It should be noted that TREK-1, but not TRAAK, was detected immunocytochemically at NRs of motor nerves as well as central projection nerves in the spinal dorsal and ventral columns, raising the possibility that TREK-1 may be functional K2P channels clustered at NRs of these myelinated nerves. At NRs of trigeminal Aβ-afferent nerves, the high-density leak K+ currents mediated by TREK-1 and TRAAK channels well matches the high-density voltage-gated Na+ channels at NRs (Arancibia-Carcamo and Attwell, 2014; Jenkins et al., 2015). This can permit sufficiently large outward currents flowing through the leak K+ channels to rapidly drive AP repolarization at NRs. This pivotal role of TREK-1 and TRAAK channels in AP regeneration at the NRs of myelinated afferent nerves is evidenced by significant prolongation of AP widths following gene knockdown or pharmacological inhibition of these channels. Thus, the NR of myelinated afferent nerves is a unique site on an afferent neuron where leak K+ channels rather than conventional voltage-gated K+ channels drive AP repolarization. It should be noted that in the present study, under the condition with gene knockdown or pharmacological inhibition, AP repolarization was significantly retarded, but not completely impaired. This partial effect on AP repolarization may be due to the partial suppression of the activity of TREK-1 and TRAAK channels under our experimental conditions with gene knockdown or pharmacological inhibition. In addition, inactivation of voltage-gated Na+ channels at NRs may also partially contribute to AP repolarization. Previous immunochemical studies have shown the expression of the voltage-gated K+ channel Kv7.2 (Devaux et al., 2004) at NRs and the expression of Kv1.1 and Kv1.2 in juxtaparanodal regions (Rhodes et al., 1997) of mammalian peripheral nerves. However, outward currents carried by these
voltage-gated K⁺ channels may be too small to be detected in the present study due to the overwhelmingly large outward currents carried by leak K⁺ channels. The contribution of voltage-gated K⁺ channels to AP repolarization at intact NRs may be also too small to be detected in the present study. The functions of these channels in salutatory conduction are not the scope of the present study, but previous studies have proposed that these nodal and extra-nodal voltage-gated K⁺ channels may fine-tune the excitability of nodal axons (Zhou et al., 1999). In addition, these voltage-gated K⁺ channels may be upregulated to compensate the loss of function of K2P channels at NRs in mice with the global deletion of TREK-1, TREK-2, and TRAAK genes (Mirkovic et al., 2012). However, K2P channels are better suited than voltage-gated K⁺ channels to drive AP repolarization and regeneration at NRs of myelinated afferent nerves, since these leak K⁺ channels activate and inactivate nearly instantaneously (Renigunta et al., 2015).

We have shown that TREK-1 and TRAAK channels at NRs are essential in securing salutatory conduction at high frequency on myelinated afferent nerves. Several mechanisms may underlie this important role of these leak K⁺ channels. First, with the use of the leak K⁺ channels for AP repolarization, each AP at NRs is brief and frequency-dependent AP broadening (Liu et al., 2017) will not take place at NRs. Second, instantaneous channel activation and deactivation of K2P channels (Renigunta et al., 2015) minimizes the inter-spike interval. Third, the very negative resting membrane potentials due to high leak K⁺ conductance at NRs should promote a quick recovery of voltage-gated Na⁺ channels from inactivation following each AP (Bean, 2007). These effects collectively would permit salutatory conduction at a high frequency through NRs of myelinated afferent nerves.

TREK-1 and TRAAK channels at NRs are shown in the present study to be essential for salutatory conduction at a high speed along myelinated afferent nerves. This important role of the leak K⁺ channels may be due to the enhanced nodal membrane input conductance or the decreased nodal membrane input resistance, a result of the clustering of the high-density TREK-1 and TRAAK channels at NRs. Previous theoretical studies by computational modeling have predicted that the addition of ion channels to enhance membrane input conductance at NRs would accelerate the membrane charge at NRs and boost the speed of salutatory conduction (Casteel Franco and Hartline, 2015, 2016; Halter and Clark, 1993). Our study for the first time provides experimental evidence supporting this novel mechanism responsible for securing high-speed salutatory conduction on mammalian myelinated afferent nerves. Thus, NRs are not only the sites of AP regeneration during salutatory condition but control the speed of AP conduction on myelinated afferent nerves in mammals.

We show that temperature sensitivity of TREK-1 and TRAAK channels at NRs of myelinated afferent nerves significantly contribute to temperature-dependent salutatory conduction with conduction velocity reduced in colder temperatures (Franz and Iggo, 1968). TREK-1 and TRAAK channels are more sensitive to temperature changes than voltage-gated Na⁺ channels (Hodgkin and Huxley, 1952; Kang et al., 2005; Maingret et al., 2000). Therefore, these thermosensitive and mechanosensitive K2P channels may account more for temperature-dependent salutatory conduction than voltage-gated Na⁺ channels. We show that cooling temperatures impair salutatory conduction by inhibiting the thermosensitive and mechanosensitive K2P channels at the NRs of myelinated afferent nerves. This finding may have clinical implications for sensory impairment such as numbness in cold temperatures in patients with peripheral neuropathy.

Our in vivo tactile behavioral assessments performed in a blinded manner demonstrate the impairment of tactile sensory responses in animals following the knockdown of TREK-1 and TRAAK channels in whisker afferent nerves. Tactile behavioral assessment is well suited for the present study, since impulses evoked by whisker displacements are conducted by trigeminal Aβ-afferent nerves. These impulses are generated from several different types of low-threshold mechanoreceptors, including rapidly adapting types and slow-adapting types in whisker hair follicles (Gottschaldt et al., 1973). All impulses generated from these low-threshold mechanoreceptors are conducted at a high speed on trigeminal Aβ-afferent nerves, and impulses generated from slowly adapting types of low-threshold mechanoreceptors in whisker hair follicles are conducted at a high frequency (Chang et al., 2016; Ikeda et al., 2014). Therefore, it is conceivable that knockdown of TREK-1 and TRAAK channels, which compromises rapid salutatory conduction on the trigeminal Aβ-afferent nerves, would impair the in vivo tactile behavioral responses.

The use of K2P channels for rapid AP conduction may represent an evolutionary advancement to permit high-frequency and high-speed salutatory conduction on myelinated afferent nerves of mammals. It is conceivable that this advancement may be widely adopted in mammalian nervous systems, including other rapidly conducting somatosensory afferent nerves, motor nerves, and ascending and descending projection nerves in the CNS. Consistently, we have found that TREK-1 channels are not only clustered at NRs of myelinated afferent nerves but also highly expressed at NRs of motor nerves as well as central projection nerves in the spinal dorsal and ventral columns. It remains to be determined whether TREK-1 and/or other K2P channels are used for rapid AP conduction at NRs of all myelinated nerves in the peripheral nervous system and CNS of mammals. Growing evidence has shown that molecular dysfunctions occur at NRs in many neurological diseases such as inflammatory demyelinating neuropathy (Devaux et al., 2012; Stathopoulos et al., 2015), multiple sclerosis (Waxman, 2006), and spinal cord injury (Waxman, 1992), leading to severe sensory and motor impairments. Loss of function of TREK-1 and TRAAK channels at NRs of myelinated afferent nerves in our animals impairs in vivo sensory behavioral responses. It would be interesting to study whether K2P channels at NRs of different myelinated nerves may be targeted by pathological factors such autoimmune antibodies (Devaux et al., 2012; Stathopoulos et al., 2015) to impair nerve conduction, leading to sensory and motor dysfunctions in humans.

**STAR METHODS**

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Supplemental Information can be found online at https://doi.org/10.1016/j.neuron.2019.08.042.

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AUTHOR CONTRIBUTIONS

J.G.G. conceived and designed the experiments and wrote the paper. K.N. conceived experiments of immunostaining. H.K. performed experiments and data analysis. J.L. and S.T. performed experiments for the revised manuscript. S.M. participated in result interpretation and discussions.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


### STAR METHODS

**KEY RESOURCES TABLE**

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LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jianguo Gu (jianguogu@uabmc.edu). There are no restrictions on any data or materials presented in this paper.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
Male Sprague-Dawley rats (Envigo, Prattville, AL, USA) aged at 5-7 weeks were used for experiments. The rats were housed in a temperature-controlled room (23°C - 24°C) and maintained on a 12-hour light/dark cycle. Animal care and use conformed to NIH guidelines for care and use of experimental animals. Experimental protocols (IACUC-10249) were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. For knockdown of TREK-1 and TRAAK gene expression, a glass electrode with a tip size of 5 µm was filled with shRNA/AAV preparations for TREK-1 and/or TRAAK genes and inserted into the nerve bundle of infraorbital nerves or D2 whisker hair follicles. The shRNA/AAV preparations were then slowly microinjected into the nerve bundle or D2 whisker hair follicles. Control groups were microinjected with scrambled shRNA/AAV preparations.

METHOD DETAILS

Ex vivo trigeminal nerve preparation
Rats were euthanized by overdose of isoflurane, and trigeminal nerve bundles (~15 mm) with their ganglia were dissected out and placed in a Petri dish filled with ice cold Leibovitz’s L-15 medium (Corning cellgro®, Manassas, VA, USA). Connective tissues on the
surface of the nerves were removed with a fine forceps under a dissection microscope. Trigeminal nerve bundles with their ganglia were then affixed in a recording chamber by a tissue anchor and submerged in a normal Krebs solution that contained (in mM): 117 NaCl, 3.5 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 1.2 Na$_2$HPO$_4$, 25 NaHCO$_3$, and 11 glucose. The Krebs solution was saturated with 95% O$_2$ and 5% CO$_2$, had pH of 7.35 and osmolarity of 324 mOsm, and at room temperature of 24°C. The recording chamber was mounted on the stage of an Olympus BX51 microscope that was equipped with IR-DIC and fluorescent imaging systems. To facilitate patch-clamp electrode to penetrate perineural tissues, the trigeminal nerve bundles were briefly exposed to a mixture of 0.07% dispase® II (Roche, Indianapolis, IN, USA) and 0.07% collagenase (MilliporeSigma, Billerica, MA, USA) in Krebs solution for 5 min at the room temperature, and the enzymes were then washed off with the Krebs solution. The ex vivo trigeminal nerve preparation was continuously perfused with Krebs bath solution at 24°C, unless otherwise indicated.

**In situ pressure-patch-clamp recordings at nodes of Ranvier**

Nodes of Ranvier in myelinated nerves of ex vivo trigeminal nerve preparations were visualized under a 40x (NA 0.80) water immersion objective and with an infrared CCD camera (IR-1000, DAGE-MTI, USA). Myelinated nerves chosen for all experiments had diameters (including myelin thickness) of 8 to 10 μm. The axons at nodes of Ranvier for these myelinated nerves had diameters of ~2 μm. These myelinated nerves were Aβ-afferent nerve fibers based on their conduction velocity.

Patch-clamp recordings were performed at nodes of Ranvier of trigeminal Aβ-afferent nerve fibers. Recording electrodes were pulled with a Flaming/Brown Micropipette Puller (P-97, Shutter Instruments, CA, USA). The electrode resistance after filling recording electrode internal solutions ranged from 8 to 10 MΩ for patch-clamp recordings from the somas of trigeminal ganglia. For most experiments, recording electrodes were filled with a K$^+$-based internal solution containing (in mM): 105 K-gluconate, 0.5 CaCl$_2$, 2.4 MgCl$_2$, 5 EGTA, 10 HEPS, 5 Na$_2$ATP and 0.33 GTP-TRIS salt; the pH of the solution was adjusted to 7.35 with KOH. The use of high Cl$^-$ intracellular solution was because trigeminal afferent nerves normally have high intracellular Cl$^-$ concentrations. In some experiments, a Cs$^+$-based internal solution was used and the solution contained (in mM): 135 CsCl, 0.5 CaCl$_2$, 2.4 MgCl$_2$, 5 EGTA, 10 HEPS, 5 Na$_2$ATP and 0.33 GTP-TRIS salt; the pH of the solution was adjusted to 7.35 with CsOH. In the Cs$^+$-based internal solution, Cs$^+$ served as major intracellular cation and also as a blocker for voltage-gated K$^+$ channels. To access nodal axon membranes by recording electrodes and achieve high quality membrane seals, a high-speed pressure-clamp device (HSPC-1, ALA Scientific instruments, NY, USA) was connected to patch-clamp recording electrode to fine control internal pressures of patch-clamp recording electrodes. A high positive pressure of 200 mmHg was first applied into recording electrodes to pressure-clean the surface areas around nodes of Ranvier. Intra-electrode positive pressures were then reduced to 80 to 100 mmHg while electrodes were penetrating the perineurium that wrapped on nodal axons. Once the recording electrodes penetrated through the perineurium, intra-electrode pressures were reduced to 5 mmHg to approach nodal axons. Since nodal axons could not be differentiated from perineural tissues under the IR-DIC microscope, optimally accessing nodal axon membranes was judged by the reduction of seal-test currents and the appearance of a small current oscillation. Once the electrode tip optimally assessed nodal membranes, intra-electrode positive pressure was gradually reduced and a negative pressure of ~2 to −10 mmHg was applied into recording electrodes until forming gigaohm seals (usually > 5 GΩ) between the recording electrodes and nodal axon membranes (usually less than 3 min). To achieve whole-cell configuration, nodal membranes were ruptured by a train of short electrical pulses (±200 mV, 20 ms each pulse) delivered through the patch-clamp recording electrodes while intra-electrode pressures were held at a constant negative pressure of ~30 mmHg. After establishing the whole-cell configuration, negative pressure was reduced to −5 mmHg and maintained during recordings. Stable recordings from nodes of Ranvier usually could last for more than 1 hour. For patch-clamp recordings from somas of trigeminal ganglia, cells with large diameters (~50 μm) were chosen because they were usually the somas of trigeminal Aβ-afferent nerves. High positive pressure of 200 mmHg was initially applied into the recording electrode during penetration through satellite glial cell layers that wrapped around individual somas of trigeminal nerves. Once penetrated through satellite glial layers, intra-electrode pressures were reduced to 20 mmHg to approach the somas of trigeminal ganglia. After achieving optimal contact between electrodes and membranes, a negative pressure of approximately −10 mmHg was applied until the formation of gigaohm seals. Signals of voltage-clamp experiments were recorded and amplified using an Axopatch 200B amplifier, filtered at 2 kHz and sampled at 10 kHz using the pCLAMP 10 software (Molecular Devices, Sunnyvale, CA, USA). Signals of current-clamp recordings for action potentials at nodes of Ranvier were low-pass filtered at 2 kHz and sampled at 50 kHz.

To determine the properties of membranes and action potentials of nodal axons, patch-clamp recordings were performed under the whole-cell current-clamp configuration. Step current pulses were injected into nodes of Ranvier through recording electrodes. Step currents were from −100 pA to 1800 pA with increments of 50 pA per step and the duration of each pulse was 1 s. To determine the properties of currents flowing through nodal membranes following voltage steps, recordings were performed under the whole-cell voltage-clamp configuration with nodal membranes held at −72 mV. Voltage steps were applied from −102 mV to +58 mV (voltage command of −90 to +70 mV) with increments of 10 mV each step and a step duration of 500 ms. Unless otherwise indicated, membrane voltages mentioned in the texts have been corrected for the calculated junction potentials of 12 mV.

To record single channel activity of axonal membranes at nodes of Ranvier, the aforementioned **in situ** pressure-patch-clamp recordings were applied to nodes of Ranvier and recordings were performed under the cell-attached configuration. After forming gigaohm seal between patch-clamp recording electrodes and nodal membranes, intra-electrode pressure was adjusted to 0 mmHg using a high-speed pressure-clamp device. Single channel recordings were performed using K$^+$-base recording electrode internal solution.
with the $K^+$ concentration being 135 mM, which yielded symmetrical $K^+$ concentrations across nodal membranes (assuming intranodal $K^+$ concentrations were approximately 135 mM). Therefore, the reversal potentials of single channels that were permeable to $K^+$ would be near 0 mV. Experiments were performed with nodal membranes voltage-clamped at the voltage steps from $-80$ mV to $80$ mV with increments of 10 mV each step. The values of voltages in recording electrodes were in reference to the resting membrane potentials of $-80$ mV at nodes of Ranvier. Single channel activity was recorded and amplified using Axopatch 200B amplifier and signals were low-pass filtered at 2 kHz and sampled at 10 kHz.

**Determination of conduction velocity and success rates of action potential propagation through nodes of Ranvier of trigeminal Aβ-afferent nerves**

To determine conduction velocity of action potentials through nodes of Ranvier of myelinated afferent nerves, action potentials were evoked at a peripheral end of the infraorbital nerve branch using a suction stimulation electrode. The suction stimulation electrode had its tip size about 1 mm in diameter and was fire-polished. The peripheral end of trigeminal afferent nerve (infraorbital branch) was aspirated into the suction stimulation electrode with a tight fitting by a negative pressure. The negative pressure was continuously applied into the suction stimulation electrode to maintain the tight fitting during experiments. To initiate action potentials at the peripheral end of the nerve, monophasic square wave pulses were generated by an electronic stimulator (Master-8, A.M.P.I, Israel) and delivered via a stimulation isolator (ISO-Flex, A.M.P.I, Israel) to the suction stimulation electrode. The duration of the stimulation pulse was 50 μs. Minimum stimulation intensity for evoking action potentials, i.e., stimulation threshold at the peripheral end of the nerves, was first determined. Then stimulation was applied at the intensity of 2 fold of threshold (3.52 ± 0.34 mA, n = 12) throughout the experiments. Conduction velocity was calculated based on the latency of action potentials and the length of axons. The latency of action potentials was measured from the time of stimulation that was marked by stimulation artifacts to the time when action potential was initiated at the nodal recording site. The length of axons was the distance between stimulation site and nodal recording site. In some cases, stimulation artifacts were not completely separated from action potentials due to short length of axons. In these cases, the time of action potential initiation was first resolved by subtraction of artifacts, and then the latency of action potentials was measured for determining conduction velocity. To determine success rates of action potentials at nodes of Ranvier following different stimulation frequencies, stimulation pulses were applied to the trigeminal nerve bundles at frequencies of 1, 10, 50, 100, 200, 500 and 1000 Hz. Stimulation at each frequency was applied for 20 s. Intervals between different tests were 30 s. Success rates of action potential conducted through nodes of Ranvier were the percentage of successfully propagated action potentials through the nodal recording sites during 20 s period of stimulation.

**Thermal and mechanical sensitivity**

Effects of temperatures on membrane and action potential properties at nodes of Ranvier as well as on conduction of action potentials along trigeminal Aβ-afferent nerves were determined by the aforementioned electrophysiological experiments at bath solution temperatures of 35, 24, 15 and 10°C. The temperatures of bath solutions were controlled by a Peltier temperature control system (CL-200A, Warner Instrument, CT, USA), and were continuously monitored with a thermal probe placed in the recording chamber (TA-29, Warner Instrument, CT, USA). The bath solution was applied at 2 ml/min from a short tube (500 μm in internal diameter) whose outlet was positioned 1 cm away from the recording site. The time was less than 1 min for warming from 24°C to 35°C and less than 2 min for cooling from 24°C to 10°C at the recording site. Effects of mechanical stimulation on single channel activity of nodal membrane patches were determined by applications of stepwise negative pressures via patch-clamp recording electrodes. The stepwise pressure pulses were generated by the high-speed pressure-clamp device (HSPC-1, ALA Scientific instruments, NY, USA) that was connected to patch-clamp recording electrodes.

**Short hairpin RNA (shRNA)/AAV preparations for knockdown of TREK-1 and TRAAK channels**

The sequence of shRNAs for targeting a region of rat TREK-1 mRNA (shTREK-1) was 5′- CAAAGTGGAGGACACATTAT-3′, for targeting a region of rat TRAAK mRNA (shTRAAK) was 5′- ACCATCGGCCATCGCATTAT-3′. The scrambled sequence of shRNA that does not target any known rat genes (shScramble) was 5′-CTTAAGGTAACTCGCCCTCG-3′ and was used as control. The sequences of shTREK-1 and shScramble were separately inserted into the adenov-associated viral (AAV) vectors which contain human U6 promoter to drive shRNA expression and a reporter gene for the expression of enhanced green fluorescent protein (eGFP). The sequence of shTRAAK was inserted into the AAV vectors which contain human U6 promoter to drive shTRAAK expression and a reporter gene for the expression of mCherry fluorescent protein (mCherry). The above vector constructors were packed in either adenovirus serotype 5 (AAV5, for shTREK-1 and shTRAAK) or serotype 2 (AAV2, for shScramble). All the above mentioned shRNA/AAV preparations were custom-prepared by VectorBuilder Inc. (Santa Clara, CA, USA) and the titer of each preparation was > $10^{12}$ viral particles.

**Delivery of shRNA/AAV preparations to trigeminal afferent nerves**

Rats were anesthetized with isoflurane and the anesthesia was maintained by continuous administration of isoflurane via a nose cone using an isoflurane anesthesia machine (E-Z Anesthesia, Euthanex Corp., PA, USA). The skin along top of the snout was shaved and a mid-line incision was made to expose nasal and maxillary bone. Infraorbital nerve was exposed 1 mm rostral to infraorbital fissure in the maxillary bone by using a pair of forceps. A glass electrode with a tip size of 5 μm was filled with shRNA/AAV preparations. The
electrode was then mounted on an injection holder of a stereotaxic apparatus. Under a dissection microscope, the glass electrode was inserted into the nerve bundle of infraorbital nerves. The shRNA/AAV preparations were then slowly microinjected into the nerve bundle using a microprocessor-controlled pump (Micro4, World Precision Instruments, Fl, USA). Each shRNA/AAV preparation was microinjected at the volume of 3 μl and the duration of each injection was 2 min and the glass microelectrode remained within the nerve bundle for 10 min before being withdrawn. The microinjections were performed on both sides of infraorbital nerves in each rat. Four weeks after the administration of shRNA/AAV preparations, trigeminal nerves with their ganglia were dissected out from rats. Patch-clamp recordings were then performed at nodes of Ranvier of eGFP-expressing or mCherry-expressing myelinated afferent nerve fibers. Some of these rats were fixed with 4% PFA 4 weeks after microinjections of shRNAs/AAV and immunohistochemistry experiments were then performed on their trigeminal nerves and ganglia. In a different set of experiments, the shTREK-1/AAV and shTRAAK/AAV preparations or shScramble/AAV (control) were microinjected into D2 whisker hair follicles in a similar manner as aforementioned intra-nerve microinjections.

Whisker tactile behavioral assessments

Four weeks after microinjections of the shRNA/AAV preparations into infraorbital nerves or D2 whisker hair follicles, whisker tactile behavioral responses were assessed. Whisker tactile behavioral assessments were performed in a blinded manner in which the examiner did not know the shScramble group and the group microinjected with shTREK-1/AAV and shTRAAK/AAV. In brief, rats were placed in a cage and habituated for 10 min. During habituation and subsequent experiments, the testing room only had a red light on so that animals could not see the examiners and the tactile stimulation filament (300 g von Frey filament, Touch Test, USA). After the habituation, a single whisker hair (right side D2 whisker) was displaced (stroke-through in 1 s) in ventral-dorsal direction by the tactile stimulation filament, and the whisker tactile test was performed 30 times with an interval of ~30 s between trials. The tactile responses were quantified by two methods. One method is based on response scores in that no response is 0, sniffing 1, avoiding head movement 2, avoiding body movement 3. Another one is the percent of the above tactile responses induced by tactile stimulation.

Pharmacology

Electrophysiological properties of nodes of Ranvier were tested with the following pharmacological reagents. Tetraethylammonium (TEA, 20 mM, MilliporeSigma, Billerica, MA, USA), cesium (Cs+, MilliporeSigma, Billerica, MA, USA, 135 mM applied intracellularly, replacing K+ in the recording electrode internal solution) and norfluroxetine (NF, 50 μM, Cayman Chemical, MI, USA) for blocking voltage-gated K+ channels. Barium (Ba2+, 5 mM, MilliporeSigma, Billerica, MA, USA), arachidonic acid (AA, 20 μM, Tocris Bioscience, Bristol, UK) and intracellular low pH of 5 ([pH]i 5) for potentiating leak K+ channel activity. Linopirdine (LP, 10 μM, MilliporeSigma, Billerica, MA, USA) for blocking Kv7.2 channels. These reagents were either applied through bath solution to the recording chambers (TEA, Ba2+, RR, NF, and BL1249) or applied intracellularly through recording electrode internal solutions (Cs+, AA, [pH]i 5). Cs+ was applied in the Cs+-based recording electrode internal solution that contained 135 mM Cs+.

To determine K+ permeability of leak currents recorded at nodes of Ranvier, currents following voltage steps (from −102 mV to +58 mV) were first recorded in normal Krebs solution and then recorded in a high K+ bath solution. The high K+ bath solution contained (in mM): 120.6 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 Na2HPO4, 25 NaHCO3 and 11 glucose. With this high K+ bath solution and recording electrode internal solution had similar K+ concentrations which would shift reversal potentials to ~0 mV if the leak currents were carried by K+ ions. To determine Cl− permeability of leak currents, currents following voltage steps (from −102 mV to +58 mV) were first recorded in normal Krebs solution and then recorded in a low Cl− extracellular bath solution that contained (in mM): 29.9 NaCl, 87.1 sodium-glucuronate, 3.5 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 Na2HPO4, 25 NaHCO3, and 11 glucose. With this low Cl− bath solution, the Cl− concentrations at extracellular and intracellular sides were symmetric with a ratio of 1:1, which would shift the reversal potentials to ~0 mV if the leak currents were carried by Cl− ions. In a different set of experiments, a cocktail of Cl− channel blockers was bath applied to test if leak currents were inhibited by Cl− channel blockers. These experiments were performed under the voltage-clamp configuration in normal Krebs solution. The cocktail of Cl− channel blockers contained 1 mM Zn2+ (MilliporeSigma, Billerica, MA, USA), 100 μM phoretin (MilliporeSigma, Billerica, MA, USA), and 500 μM 4,4′-Diisothiocyanato-2,2′-stilbenedisulfonic acid disodium salt (DIDS, Tocris Bioscience, Bristol, UK).

For all pharmacology tests with bath application of testing compounds, unless otherwise indicated, each compound was perfused to ex vivo nerve preparations for 10 min. The testing compounds were delivered through a pinch valve drug delivery system (VC-6, Warner Instrument, CT, USA) which was controlled by digital signals from pClamp 10 software. For experiments with intracellular applications of testing compounds, each compound was dissolved in recording electrode internal solutions and recordings were performed 20 min after establishing the whole-cell configuration.

Morphology of nodes of Ranvier and inter-nodal axons

To determine lengths and diameters of nodal axons and inter-nodal axons, axons were labeled by the fluorescent dye Alexa Fluor 555 (ThermoFisher Scientific, Waltham, MA, USA). The dye was applied through recording electrode internal solution that contained 85 μM Alexa Fluor 555. Following the establishment of the whole-cell configuration for 30 min, nodal and inter-nodal axons were visualized under a fluorescence microscope. The fluorescence excitation was provided by an LED illumination system (X-Cite 120LED,
Lumen Dynamics, Mississauga, ON, Canada). A filter set with excitation wavelength of 500 - 550 nm and emission wavelength of 565 - 625 nm was used for imaging Alexa Fluor 555 fluorescence. The images of dye-labeled axons were captured with a digital complementary metal-oxide semiconductor (CMOS) camera (ORCA-Flash4.0 LT, Bridgewater, NJ, USA). The images were then analyzed using the ImageJ software (National Institutes of Health, Bethesda, USA) to measure the lengths and diameters of nodal, paranodal and inter-nodal axons.

**Immunohistochemistry**

Rats were anesthetized with isoflurane and perfused with 4% paraformaldehyde (PFA), and trigeminal afferent nerves with their ganglia were dissected out. After post-fixation in 4% PFA for 24 hours, tissues were then transferred to a solution of 30% sucrose. Rats were anesthetized with isoflurane and perfused with 4% paraformaldehyde (PFA), and trigeminal afferent nerves with their ganglia were dissected out. After post-fixation in 4% PFA for 24 hours, tissues were then transferred to a solution of 30% sucrose.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Electrophysiological data were measured using the Clampfit software (Molecular Devices, Sunnyvale, CA, USA). All data analyses were performed using the GraphPad Prism software (version 7, La Jolla, CA, USA). Data are presented as mean ± SEM. Unless otherwise indicated, statistical significance was evaluated using paired, unpaired Student’s t-Test, one-way ANOVA with Tukey’s post hoc tests, or Chi-square test. Differences were considered to be significant with *p < 0.05, **p < 0.01 and ***p < 0.001, and not significant (ns) with p ≥ 0.05. Sample sizes of each experimental group and methods of data analysis were chosen based on standards in the field, and no tests were applied to predetermine sample sizes. All error bars indicate standard error of mean (SEM) unless indicated otherwise in the Figure Legends. Each figure Legend lists the number of recordings or number of animals used in each experiment group and the statistical tests used.

**TREK-1 and TRAAK expression in HEK293 cells and electrophysiological characterization**

Rat TREK-1 subunits (rKcnk2, NM_172042.1) were cloned into pR[Exp]-eGFP-CMV plasmid and rat TRAAK subunits (rKcnk4, NM_053804.2) cloned into pR[Exp]-mCherry-CMV plasmid for expression in HEK293 cells and electrophysiological characterization. HEK293 cells were grown on coverslips in 35-mm dish in 2 mL DMEM (Fisher Scientific, cat#10-013-CV) with 10% fetal bovine serum (HyClone/Fisher Scientific, SH30071.02), 100 units/ml penicillin-streptomycin (Invitrogen, 15140-122). They were then transfected with the TREK-1 plasmid (1 μg per dish), the TRAAK plasmid (1 μg per dish), or the TREK-1 plasmid (1 μg per dish) plus TRAAK plasmid (1-5 μg per dish) using Lipofectamine 2000 reagent (Invitrogen, 11668-027) in 1 mL DMEM medium. Three to five days following the transfection, patch-clamp recordings were performed either under whole-cell or cell-attached configurations. During recordings cells were perfused with normal Krebs bath solution. Recording electrode internal solution contained 105 K-glucosan, 30 KCl, 0.5 CaCl₂, 2.4 MgCl₂, 5 EGTA, 10 HEPES, 5 Na₂ATP and 0.33 GTP-TRIS salt; the pH of the solution was adjusted to 7.35 with KOH.
DATA AND CODE AVAILABILITY

The published article includes all datasets generated or analyzed during this study. All data are available from the Lead Contact upon request.