

Triple Cysteine Module within M-Type K^+ Channels Mediates Reciprocal Channel Modulation by Nitric Oxide and Reactive Oxygen Species

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We have identified a new signaling role for nitric oxide (NO) in neurons from the trigeminal ganglia (TG). We show that in rat sensory neurons from the TG the NO donor, *S*-nitroso-*N*-acetyl-DL-penicillamine, inhibited M-current. This inhibitory effect was blocked by NO scavenging, while inhibition of NO synthases increased M-current, suggesting that tonic NO levels inhibit M-current in TG neurons. Moreover NO increased neuronal excitability and calcitonin gene-related peptide (CGRP) release and these effects could be prevented by perturbing M-channel function. First, NO-induced depolarization was prevented by pre-application of the M-channel blocker XE991 and second, NO-induced increase in CGRP release was prevented by incubation with the M-channel opener retigabine. We investigated the mechanism of the effects of NO on M-channels and identified a site of action of NO to be the redox modulatory site at the triplet of cysteines within the cytosolic linker between transmembrane domains 2 and 3, which is also a site of oxidative modification of M-channels by reactive oxygen species (ROS). NO and oxidative modifications have opposing effects on M-current, suggesting that a tightly controlled local redox and NO environment will exert fine control over M-channel activity and thus neuronal excitability. Together our data have identified a dynamic redox sensor within neuronal M-channels, which mediates reciprocal regulation of channel activity by NO and ROS. This sensor may play an important role in mediating excitatory effects of NO in such trigeminal disorders as headache and migraine.

Introduction

Nitric oxide (NO) is a molecular mediator with diverse physiological roles, including blood pressure regulation, clotting, and nociception. Within the somatosensory system NO is released from dural mast cells and nerve fibers following inflammation and contributes to pain (Berger et al., 1994). The cell bodies of sensory neurons that innervate the face and head reside in the trigeminal ganglia (TG); increased excitability of TG neurons is interpreted as pain and in this way NO can induce delayed headaches in migraine sufferers (Akerman et al., 2002). A role for calcitonin gene-related peptide (CGRP), released from trigeminal afferents, has also been implicated in migraine pain. CGRP levels are higher in the plasma of migraine sufferers compared with controls (Ashina et al., 2000) and during migraine attacks activation of trigeminal sensory fibers causes CGRP release (Goadsby et al., 1990). Experimentally migraines can be induced by NO donors (Thomsen et al., 1993) and reduced with CGRP

antagonists (Wei et al., 1992). Further evidence links these signaling molecules as NO promotes the release of CGRP from afferent fibers (Messlinger et al., 2000). However NO-mediated increases in CGRP are not caused by increases in CGRP mRNA (Eberhardt et al., 2009) and a mechanism for increased trigeminal neuronal activity has yet to be identified.

Expression of M-type K^+ channels (encoded by *KCNQ* genes) in sensory ganglia limits neuronal excitability, exerting an antinociceptive effect (Passmore et al., 2003). Accordingly, inhibition of M-current via pro-inflammatory G_q -protein-coupled receptors (G_q -PCRs) contributes to peripheral sensitization and inflammatory pain (Linley et al., 2008; Liu et al., 2010). A triple cysteine pocket within neuronal M-channel subunits (Kv7.2–7.5) can be oxidatively modified by H_2O_2 (Gamper et al., 2006) and O_2^- (Linley et al., 2012a) both causing augmentation of M-current. Here we have identified a reactive redox module within M-channels that is responsible for dynamic M-current modulation by gasotransmitters and contributes to NO-induced increased excitability in trigeminal neurons.

Materials and Methods

Compounds. NO formation from *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; Sigma-Aldrich) is linear (100 μM yields 1.4 μM NO/min). Retigabine (RTG; a gift from Professor Hailin Zhang, Hebei Medical University, People's Republic of China) is a specific M-channel opener, enhancing currents of Kv7.2–7.5 (EC_{50} 2–5 μM ; Tatulian et al., 2001). XE991 (Tocris Bioscience) is a selective M-channel blocker (IC_{50} 0.3 μM ; Passmore et al., 2003). At higher concentrations XE991 blocks HERG channels (IC_{50} 100 μM ; Elmedyby et al., 2007); however, at the concentra-

Received Sept. 6, 2012; revised Feb. 11, 2013; accepted Feb. 24, 2013.

Author contributions: L.O. and N.G. designed research; L.O., S.G., and L.P. performed research; L.O., S.G., and L.P. analyzed data; L.O. and N.G. wrote the paper.

This work was supported by grants from the Wellcome Trust (080593/Z06/Z) and the Medical Research Council (G0700966 and G1002183) to N.G. We thank Katarzyna Marszalek for expert technical assistance.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.4275-12.2013

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tion used in this study ($3 \mu\text{M}$) it is M-channel selective. L-Nitroarginine (L-NNA) (Cayman) is a competitive inhibitor of nitric oxide synthase (NOS) [Ki values in the nanomolar range for the neuronal (nNOS) and endothelial (eNOS) isoforms and in the micromolar range for inducible (iNOS); Furfine et al., 1993]. Carboxy-PTIO (Cayman) reacts with NO, inhibiting S-nitrosation ($\text{IC}_{50} 100 \mu\text{M}$; Pfeiffer et al., 1997).

Cell cultures and transfections. TG from P7 rats of either sex were removed and dissociated and neurons and Chinese hamster ovary (CHO) cells were cultured according to the methods described previously (Linley et al., 2008). Plasmids encoding human Kv7.4 wild-type and mutant (CCC156–158AAA) were a gift from Professor Mark Shapiro (University of Texas Health Science Center at San Antonio, San Antonio, Texas). All experimental procedures were approved by and in compliance with the University of Leeds ethical review committee.

Electrophysiology. Recordings were performed as described previously (Linley et al., 2012a). In TG neurons M-current amplitude was measured from the deactivation current (I_{deac}) elicited by a 500 ms square voltage pulse to -60 mV from a holding potential of -30 mV . M-current was quantitated as the XE991-sensitive I_{deac} . CHO cells expressing M-channels were held at 0 mV with 500 ms steps to -60 mV and 600 ms pulses back to 0 mV with 3 s intervals. The amplitude of the current was defined as the XE991-sensitive current at 0 mV (I_0). Current-clamp recordings were performed using current injections (between -0.5 and $+1 \text{ nA}$, increment 0.025 nA , duration 600 ms, interval 5 s). Linear regression of the number of action potentials (APs) versus injected current (nA) in the initial linear range was used to calculate the slope of neuronal firing. This parameter provides a quantitative excitability index describing the dependence of action potential firing frequency on the amplitude of injected current.

Biotin switch assay and Western blot. CHO cells were transfected with pcDNA3.1 encoding myc-tagged Kv7.4 wild-type, CCC156–158AAA mutant or a transfection control (pmaxGFP). The biotin switch assay to visualize S-nitrosylated proteins was performed as in (Jaffrey and Snyder, 2001), in which S-nitrosylated cysteines were labeled with biotin. Protein samples were immunoprecipitated with anti-biotin-labeled protein G Dynabeads (Invitrogen), following the manufacturer's protocol. Protein extracts ($20 \mu\text{g}$) in nonreducing sample buffer (10 mM Tris-HCl, pH 6.8, 10% glycerol, 0.02% bromophenol blue) were separated through 8% native polyacrylamide gels. Proteins were transferred to membranes and screened with anti-myc antibody (Santa Cruz Biotechnology; 1:1000) in PBS, 0.1% Tween 20, 5% milk powder, followed by 1:2000 secondary horseradish peroxidase-conjugated anti-mouse antibody. For the denaturing Western blot, input proteins (collected from the same samples before immunoprecipitation) were boiled for 10 min in SDS sample buffer (as above plus 4% SDS, 200 mM dithiothreitol; DTT), separated on polyacrylamide gels and blotted as above.

CGRP release assay. To determine basal release levels, TG cultures were incubated at 37°C for 10 min in $500 \mu\text{l}$ of standard bathing solution (SBS).

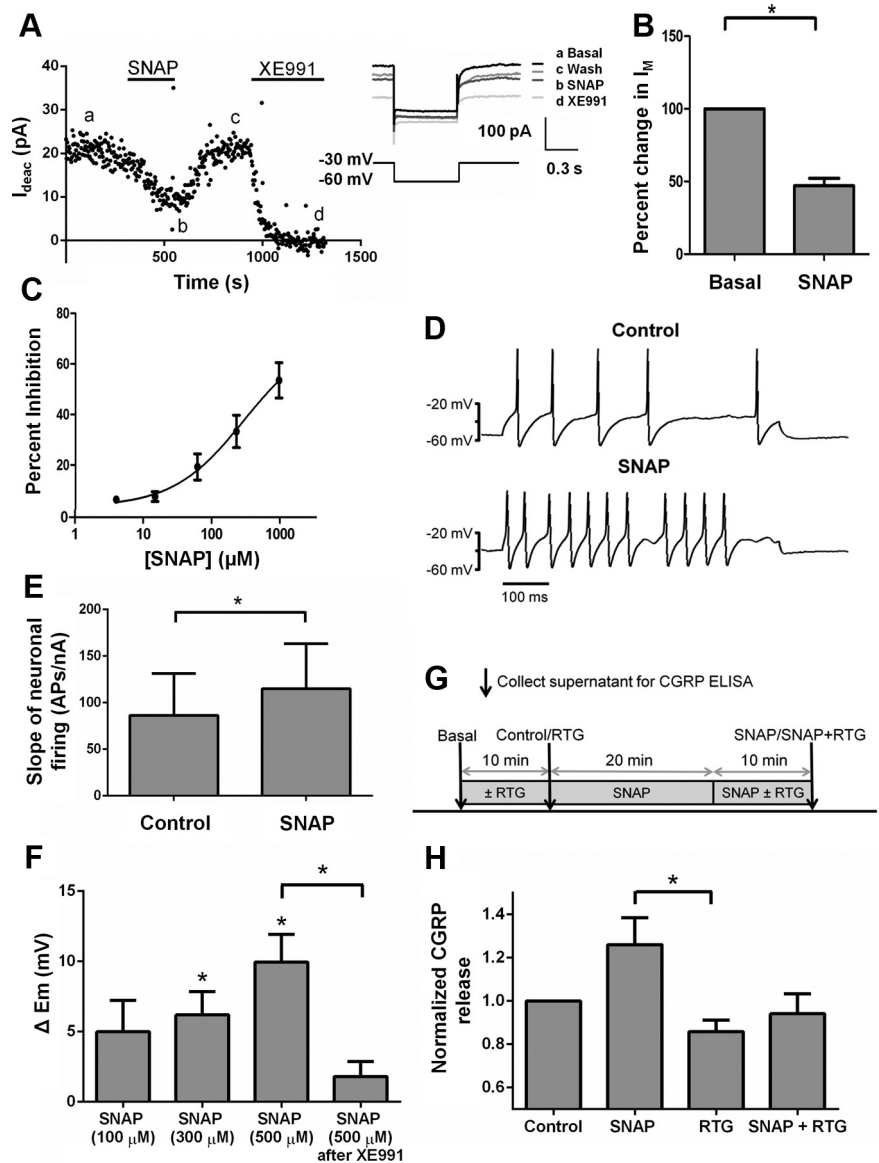


Figure 1. NO inhibits M-current and increases neuronal excitability. **A**, Exemplary perforated patch voltage-clamp recording from a TG neuron. The magnitude of the deactivating tail current (I_{deac}) when stepping from -30 to -60 mV was plotted against time. Bath application of SNAP (1 mM) and the M-channel inhibitor XE991 ($3 \mu\text{M}$) is indicated by the black bars. The voltage protocol and exemplary current traces are also shown. **B**, Mean data from experiments as in **A**, expressed as percentage change in M-current from basal ($n = 12$). **C**, Concentration dependence of SNAP-induced M-current inhibition in TG neurons. SNAP was applied at concentrations of $10 \mu\text{M}$, $30 \mu\text{M}$, $100 \mu\text{M}$, $300 \mu\text{M}$, or $1000 \mu\text{M}$ under conditions of perforated patch voltage clamp; an IC_{50} of $370 \pm 14 \mu\text{M}$ was obtained from the fit shown. **D**, Exemplary voltage trace of a TG neuron in control conditions and in the presence of SNAP ($500 \mu\text{M}$) during a perforated patch current-clamp recording. The traces show voltage responses to $+0.125 \text{ nA}$ current injections (600 ms). **E**, Linear regression of the number of APs versus injected current (nA) was used to calculate the slope of neuronal firing (this parameter characterizes the input–output relationship of a neuron) in the absence (control) or presence of SNAP (100 – $500 \mu\text{M}$, $n = 6$). **F**, Mean data of the change in resting membrane potential (ΔE_m) following SNAP application at concentrations of $100 \mu\text{M}$ ($n = 7$), $300 \mu\text{M}$ ($n = 8$), or $500 \mu\text{M}$ ($n = 6$) \pm pre-application of XE991 ($3 \mu\text{M}$, $n = 9$). **G**, Schematic showing the experimental protocol for CGRP release assay and cell treatments. **H**, Mean data from CGRP release assay ($n = 3$). In all parts error bars indicate SEM and significant difference indicated by $*p \leq 0.05$.

Cells were incubated with a further $500 \mu\text{l}$ of SBS in the presence or absence of RTG ($10 \mu\text{M}$) for 10 min at 37°C , followed by incubation with or without SNAP ($100 \mu\text{M}$) for 20 min. SBS was replaced with either SNAP alone or SNAP and RTG for a further 10 min. The SBS was collected and CGRP release was measured according to the manufacturer's instructions (SPBio).

Statistics. All data are given as mean \pm SEM. Differences between groups were assessed by Student's *t* test or one-way ANOVA with Bonferroni *post hoc* test.

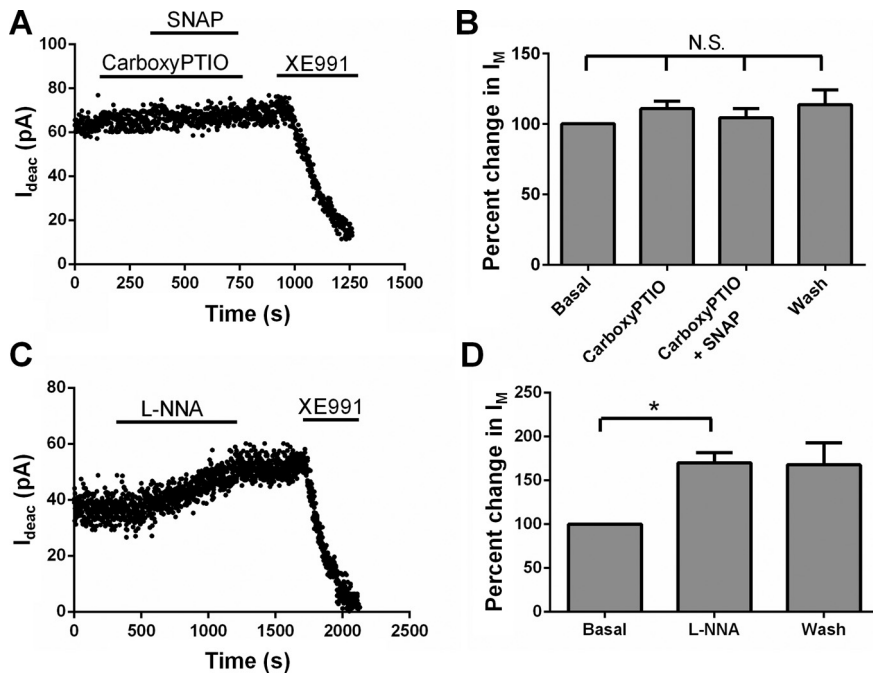


Figure 2. Manipulating NO levels with a scavenger or by blocking NO synthase affects M-current. **A**, Example time course of the effect of carboxy-PTIO (1 mM) incubation before SNAP application (1 mM) on M-current in TG neurons. **B**, Mean data from experiments as in **A**. **C**, Example time course of the effect of L-NNA (1 mM) on M-current in TG neurons. **D**, Mean data from experiments as in **A**, expressed as percentage change in M-current from basal ($n = 5$). Significant difference indicated by $*p \leq 0.05$.

Results

NO inhibits M-current and increases excitability of nociceptive trigeminal neurons

Small-diameter, nociceptive neurons (mean capacitance 25 ± 2 pF) from TG cultures displayed M-currents (mean basal M-current amplitude 42 ± 6 pA). To study the effect of NO on M-current, SNAP (1 mM) was bath applied causing a slow inhibition corresponding to $53 \pm 12\%$ of M-current in 12/15 TG neurons tested ($p \leq 0.05$) (Fig. 1A,B). The M-current inhibiting effects of SNAP were concentration dependent (Fig. 1C) with an IC_{50} of 370 ± 14 μ M. Inhibiting M-current in sensory neurons from dorsal root ganglia (DRG) has previously been shown to induce depolarization, increasing neuronal excitability and causing pain (Linley et al., 2008; Liu et al., 2008); conversely, M-channel enhancers hyperpolarized the membrane potential of cultured neurons (Linley et al., 2012b) and produced antinociceptive effects when injected into the hindpaw of rats (Liu et al., 2010). Thus in the present study the effect of SNAP-mediated M-current inhibition on neuronal excitability was evaluated using perforated patch current clamp. The majority of TG neurons (24/30) produced only one AP with or without SNAP or XE991 during current injections. However, 6/30 neurons produced multiple APs and SNAP application (100–500 μ M) increased AP firing ($n = 6$; Fig. 1D) and the slope of neuronal firing of these neurons (see Materials and Methods; Fig. 1E). Application of SNAP (100 μ M, 300 μ M, or 500 μ M) depolarized the neurons causing an increase in resting membrane potential (ΔE_m) of up to 10.0 ± 2.0 mV (Fig. 1F). However pre-application of XE991 (3 μ M) almost completely abolished SNAP-induced depolarization (ΔE_m 1.8 ± 1.1 mV; ΔE_m for XE991 alone 4.6 ± 1.1 mV; data not shown). In fact the observed ΔE_m during application of SNAP (500 μ M) following XE991 pre-application was significantly smaller than ΔE_m observed during application of SNAP at

300 μ M or 500 μ M without XE991 pre-application ($p \leq 0.05$) (Fig. 1F).

M-current augmentation in cultured DRG neurons correlates with a reduction in CGRP release (Linley et al., 2012a). Increased CGRP release from trigeminal afferents has been implicated in migraine and headache pain, which could be a result of increased neuronal excitability in response to NO signaling. To test if SNAP-mediated inhibition of M-current could contribute to increased CGRP release, we studied the effect of SNAP and the M-channel opener RTG on such release. TG neurons were treated with SNAP (100 μ M), RTG (10 μ M), or both and CGRP release measured. As shown in the experimental scheme (Fig. 1G), basal levels were first collected from each sample and used for normalization to correct for the number of CGRP-positive neurons in each sample. SNAP treatment increased CGRP release ($130 \pm 10\%$ of control, $n = 3$). RTG reduced CGRP release ($80 \pm 10\%$ of control), while RTG prevented SNAP-mediated increases ($100 \pm 10\%$ of control) (Fig. 1H). The effects were small but consistent, though only the SNAP-alone and RTG-alone values were significantly different ($p \leq 0.05$). Thus, preventing M-current inhibition with RTG prevented the SNAP-mediated increase in CGRP release.

These data are consistent with a model whereby inhibition of M-current by SNAP increases neuronal excitability contributing to increased CGRP release.

Manipulating NO levels with a scavenger or by blocking NO synthase affects M-current

To confirm that the effects of SNAP were due to NO release, an NO scavenger, carboxy-PTIO was used in conjunction with SNAP in perforated patch-clamp experiments. Carboxy-PTIO reacts stoichiometrically with NO to inhibit its physiological effects. Incubation of neurons with carboxy-PTIO (1 mM) prevented SNAP-mediated inhibition of M-current in 6/8 cells ($104 \pm 6\%$ of basal amplitude) and, furthermore, in 2/8 neurons carboxy-PTIO increased basal M-current (109 and 111% of basal), suggesting the inhibitory effect of SNAP on M-current is indeed mediated by NO (Fig. 2A,B). Endogenous NO is generated from L-arginine by NOS enzymes. The competitive inhibitor of NOS, L-NNA (1 mM), increased M-current in 4/8 neurons ($170 \pm 12\%$ of basal, $p \leq 0.05$), presumably via actions through nNOS inhibition (Fig. 2C,D). This suggests that manipulating NO levels directly affects M-current and implies there is a level of tonic NO production that maintains partial inhibition of M-current in TG neurons.

NO-mediated inhibition opposes oxidation-mediated augmentation of Kv7.4 currents

Oxidative modification of cysteines in Kv7.2–7.5 augments M-current (Gamper et al., 2006). Since NO can modulate protein function via modification of cysteines (Lipton et al., 1993), we hypothesized that the redox-sensitive cysteine triplet may be involved in NO-sensitivity. A cell line model (CHO) transiently expressing M-channels was used to investigate the effect of NO on M-current. Application of SNAP (1 mM) inhibited Kv7.4 cur-

rents (mean inhibition $32 \pm 5\%$, $p \leq 0.05$) (Fig. 3A,B). Washout of SNAP did not return currents to basal levels. Ascorbate allows NO to combine with superoxide ($O_2^{\cdot-}$) to generate the oxidative free radical peroxynitrite ($ONOO^-$) (Pryor and Squadrito, 1995). Ascorbate (2 mM) applied after SNAP caused strong augmentation of Kv7.4 current, overriding the initial inhibition by SNAP. This channel augmentation was reversed by the selective sulfhydryl reducing agent DTT, suggesting that the augmenting effects of ascorbate are due to a reversible oxidative modification. Thus, NO and oxidative modification oppose one another functionally and the triple cysteine module provides for a dynamic and redox-dependent control of M-channel activity.

NO site of action is cysteines 156–158 of Kv7.4

The site of oxidative modification of Kv7 channels has been mapped to the conserved triple cysteines within the S2–S3 linker (amino acids 156–158 in Kv7.4) (Fig. 4A) (Gamper et al., 2006). To determine the contribution of these cysteines to the effects of NO, voltage-clamp experiments were performed in CHO cells expressing the Kv7.4 wild-type or a mutant channel in which all three cysteines were substituted by alanines. Application of SNAP to wild-type Kv7.4 caused inhibition of M-current, whereas mutation of the triple cysteines to alanines (CCC156–158AAA) abrogated the inhibiting effect of NO (Fig. 4B,C). One possible effect of NO is cysteine S-nitrosylation (the addition of a nitrosyl group to protein thiol groups), a modification that can regulate protein function (Stamler et al., 1992). A biotin switch assay was performed to test whether SNAP can cause S-nitrosylation of Kv7.4. CHO cells expressing myc-tagged wild-type Kv7.4 or CCC156–158AAA were treated with SNAP; wild-type and mutant protein were identified on nondenaturing gels using anti-myc antibodies. Monomeric (77 kDa) and multimeric forms of wild-type Kv7.4 were strongly biotin labeled (Fig. 4D, top), consistent with S-nitrosylation. In contrast the mutant CCC156–158AAA showed a dramatic reduction of biotin signal that was not due to a lack of protein expression, since input protein fractions showed high levels of both wild-type and mutant protein (Fig. 4D, bottom). The remaining S-nitrosylation signal may be underscored by additional cysteines; however, the functional data (Fig. 4B) support the conclusion that CCC156–158 are important for Kv7 channel modulation by NO.

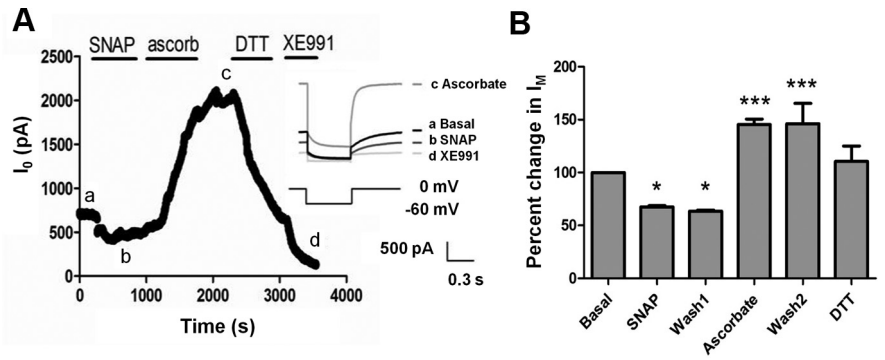


Figure 3. Ongoing effects of SNAP and oxidative modification on M-current. **A**, Example time course of the effect of SNAP (1 mM), ascorbate (2 mM), and DTT (2 mM) on M-current in CHO cells transfected with a plasmid encoding Kv7.4. Bath application of SNAP (1 mM), ascorbate (2 mM), DTT (2 mM), and XE991 ($3 \mu\text{M}$) is indicated by the black bars. **B**, Mean data from experiments as in **A**, expressed as percentage change in M-current from basal ($n = 5$). Wash 1 followed SNAP application, Wash 2 followed ascorbate application. Significant difference from basal indicated by $*p \leq 0.05$ or $***p \leq 0.001$.

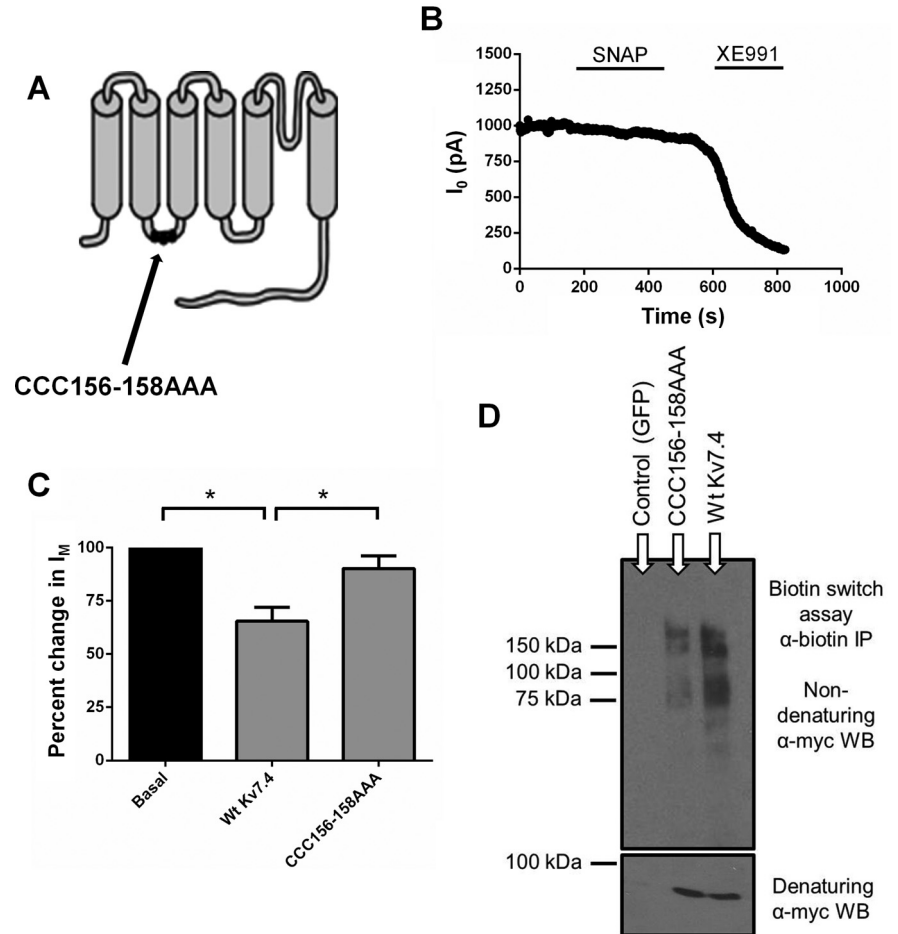


Figure 4. Mutation of cysteines 156–158 of Kv7.4 abrogates inhibiting effect of SNAP and nitrosylation of Kv7.4. **A**, Diagram indicating the position of the CCC156–158AAA residues within the S2–S3 linker. **B**, Example time course of the effect of SNAP (1 mM) on Kv7.4 mutant CCC156–158AAA. Bath application of SNAP (1 mM) and XE991 ($3 \mu\text{M}$) is indicated by the black bars. **C**, Mean data from experiments as in **A**, expressed as percentage change in M-current from basal ($n = 5$). Significant difference indicated by $*p \leq 0.05$. **D**, Biotin switch assay protein samples (top) from CHO cells transfected with pmxGFP, myc-tagged mutant (CCC156–158AAA), or myc-tagged Kv7.4 wild-type (Wt) were immunoprecipitated (IP) with an anti-biotin antibody, separated on a native, nondenaturing polyacrylamide gel and blotted with anti-myc antibody (WB). Input protein fractions from the same samples were separated on a denaturing polyacrylamide gel and the membrane blotted with anti-myc antibody (bottom).

Discussion

Since M-channels have a threshold for activation below -60 mV and are able to remain partially open at the resting membrane potential of a neuron, small changes in M-current magnitude can affect neuronal excitability profoundly. SNAP-induced depolarization was not observed following blockade of M-current, suggesting that NO depolarized neurons via depression of M-current. In large-diameter DRG neurons NO donors activate K_{ATP} channels, reducing excitability (Kawano et al., 2009). However K_{ATP} currents are thought to play a relatively minor role in small-diameter neurons in DRG (Du et al., 2011). In our experiments NO inhibited M-current in small-diameter TG neurons, increasing excitability and CGRP release. Thus it would seem that K_{ATP} currents may also be relatively minor in small-diameter TG neurons. Consistent with this notion, K_{ATP} channel openers do not affect CGRP release in TG neurons (Ploug et al., 2012).

NO can intimately control CNS excitability and synaptic strength by regulating potassium channel function (Steinert et al., 2011). Endogenous NO is generated by nNOS and eNOS in the trigeminovascular system (Ramachandran et al., 2010) and during inflammation via iNOS (Corbett et al., 1993). Inflammatory signaling alters multiple sensory neuron currents (Linley et al., 2010) and many G_q -PCRs evoke an intracellular signaling cascade that stimulates cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2), triggering Ca^{2+} release and ultimately inhibiting M-current (Guo and Schofield, 2002; Zaika et al., 2007; Jia et al., 2008). Interestingly, elevated Ca^{2+} triggers NO production from nNOS (Abu-Soud and Stuehr, 1993), while NOS activity is inhibited by PIP_2 (Yamamoto et al., 2004). This adds an intriguing complexity to G_q -PCR inflammatory signaling, which is thus able to increase NO levels by two means: the removal of PIP_2 -mediated inhibition and increases in Ca^{2+} activating nNOS. It therefore follows that G_q -PCR signaling and NO likely work in concert to ensure robust location- and temporal-specific inhibition of M-current.

Studies using NOS inhibitors in migraine patients suggest that delayed headaches are due to increased nNOS activity in the trigeminal system causing CGRP release (Akerman et al., 2002), most likely due to depolarization of synaptic terminals (Durham, 2006). Our data present a contributing mechanism, whereby M-current inhibition could mediate this increased excitability of TG neurons. Mutations or changes in expression levels of the M-channel genes *KCNQ2* or *KCNQ3* cause neuronal hyperexcitability disorders, including neuropathic pain (Rose et al., 2011) and benign familial neonatal convulsions (BFNC) (Charlier et al., 1998; Singh et al., 1998). It was previously suggested that BFNC and familial hemiplegic migraine cosegregate in some patients (Terwindt et al., 1997). It would be interesting to test if M-channel dysfunction can indeed facilitate the coincidence of migraine and other hyperexcitability disorders.

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