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Redox mechanism of S-nitrosothiol modulation of neuronal Ca_v3.2 T-type calcium channels

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Abstract

T-type calcium channels in the dorsal root ganglia (DRG) have a central function in tuning neuronal excitability and are implicated in sensory processing including pain. Previous studies have implicated redox agents in control of T-channel activity; however, the mechanisms involved are not completely understood. Here we recorded T-type calcium currents from acutely dissociated DRG neurons from young rats and investigated the mechanisms of Ca_v3.2 T-type channel modulation by S-nitrosothiols (SNOs). We found that extracellular application of S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl-penicillamine (SNAP) rapidly reduced T-type current amplitudes. GSNO did not affect voltage-dependence of steady-state inactivation and macroscopic current kinetics of T-type channels. The effects of GSNO were abolished by pretreatment of the cells with N-ethylmaleimide, an irreversible alkylating agent, but not by pretreatment with 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one (ODQ), a specific soluble guanylyl cyclase inhibitor, suggesting a potential effect of GSNO on putative extracellular thiol residues on T-type channels. Expression of wild type Ca_v3.2 channels or a quadruple Cys-Ala mutant in human embryonic kidney (HEK) cells revealed that Cys residues in repeats I and II on the extracellular face of the channel were required for channel inhibition by GSNO. We propose that SNO-related molecules *in vivo* may lead to alterations of T-type channel-dependent neuronal excitability in sensory neurons and in the central nervous system (CNS) in both physiological and pathological conditions such as neuronal ischemia/hypoxia.

Introduction

The Ca_v3.2 isoform of neuronal T-type or low-voltage-activated (LVA) currents is abundantly expressed in neurons in both the peripheral nervous system, such as in the dorsal root ganglion (DRG), and in the central nervous system (CNS), such as in the spinal dorsal horn (DH) and nucleus reticularis thalami (nRT) [1]. These channels are crucial in regulating the neuronal excitability that underlies peripheral sensory processing including nociception and rhythmic thalamo-cortical oscillations, respectively [2-4]. Molecular mechanisms for the regulation of neuronal Ca_v3.2 channel activity are not yet well described; however, nitric oxide (NO) and related compounds have been implicated in one form of regulation. NO, an important signaling molecule in the peripheral as well as in the

central nervous system is known to signal via widely studied increases in cGMP generation [5,6]. More recently, however, a parallel signaling network has been described in which endogenous redox-activated NO congeners signal via regulated S-nitrosylation of critical cysteine residues of proteins (forming S-nitrosothiols or SNOs). This relatively novel, redox-responsive signaling pathway has been demonstrated in neurons [5]. There is good evidence that NO is produced in peripheral nociceptors and is up-regulated in sensory neurons after nerve injury [7]; however, its function in sensory transmission and peripheral nociception is uncertain. For example, some studies suggest that NO can act as a proalgesic agent facilitating prostaglandin-induced hyperalgesia [8] while other studies have shown that NO may have peripheral antinociceptive effects [9,10]. These contrasting effects of application of NO donors may result from different metabolic conditions in tissues that can favor generation of different forms of NO. The mechanisms by which redox-activated forms of NO (particularly SNOs) alter neuronal signaling in pain pathways have not been studied previously. SNOs could provide an important intrinsic mechanism for the control of neuronal excitability in both physiological and pathological conditions such as neuronal ischemia, and thus could serve as novel targets for therapeutic interventions.

We have demonstrated previously that several NO-generating compounds and thiol-modifying agents modulate activity of T-type channels and low-threshold calcium spikes (LTS) in nRT neurons in intact brain slices [4]. Our results demonstrated that these agents down-regulate function of T-type channels in nRT neurons and underlie burst firing via a direct neuronal membrane effect that is independent of cGMP and likely involves S-nitrosylation of critical cysteine residues on the external side of the channel. However, in our previous work we did not identify critical cysteine residues that mediate effects of SNOs on neuronal T-type channels.

The main purposes of the current study were to investigate mechanisms by which SNOs modulate neuronal $\text{Ca}_v3.2$ channels using putative nociceptive rat DRG cells and to identify molecular substrates of such modulation using recombinant $\text{Ca}_v3.2$ channels expressed in human embryonic kidney (HEK) 293 cells.

Materials and Methods

Acutely isolated DRG neurons

DRG cells from adolescent rats were prepared as previously described [11,12]. All patch-clamp recordings were done at room temperature within 6 hours of acute DRG cell dissociation. Cells were plated onto uncoated glass coverslips, placed in a culture dish, and perfused with an external solution (detailed below).

Human embryonic kidney 293 cells

Human embryonic kidney (HEK) 293 cells were grown in DMEM/F12 medium (Invitrogen, Grand Island, NY) supplemented with fetal calf serum (10%), penicillin G (100 U/ml), and streptomycin (0.1 mg/ml). Generation of $\text{Ca}_v3.2$ mutants C939A, $\text{Ca}_v3.2\text{Cys}(3)$ (C123A, C128A and C133A) as well as $\text{Ca}_v3.2\text{Cys}(4)$ (C939A, C123A, C128A and C133A) was described in details in our previous publication [12]. Cells were transiently cotransfected using Lipofectamine 2000 (Invitrogen) at a 10:1 molar ratio with a plasmid encoding CD8 antigen, then incubated with polystyrene microbeads coated with anti-CD8 antibody (Invitrogen). After 48 h, cells with bound microbeads were selected for recording.

Electrophysiology

Recording electrodes were pulled from borosilicate glass microcapillary tubes (Drummond Scientific, Broomall, PA); when filled with internal solution, they had resistances between

1–4 M. We made recordings using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Foster City, CA). Digitization of membrane voltages and currents was controlled using a Digidata 1322A interfaced with Clampex 8.2 or 9.0 (Molecular Devices). We analyzed data using Clampfit 8.2 or 9.0 (Molecular Devices) and Origin 7.0 (Microcal Software, Northampton, MA). Currents were low-pass filtered at 2–5 kHz. Series resistance and capacitance values were taken directly from readings of the amplifier after electronic subtraction of the capacitive transients. Series resistance was compensated to the maximum extent possible (usually 50%–80%). Multiple independently controlled glass syringes served as reservoirs for a gravity-driven perfusion system.

Recording solutions

The external solution for voltage-clamp experiments in DRG cell experiments contained (in mM), 152 tetraethyl-ammonium chloride (TEA)-Cl, 2 CaCl₂, and 10 N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)(HEPES), adjusted to pH 7.4 with TEA-OH. External solution was in most experiments supplemented with the nonspecific metal chelator diethylenetriaminepentaacetic acid (DTPA) at 100 μM. The external solution for recordings from HEK-293 cells was identical to that for DRG cells except that 10 mM BaCl₂ was used as a charge carrier instead of 2 mM CaCl₂. To allow studies of well-isolated and well-clamped T-type currents in acutely isolated DRG cells, we used only fluoride (F[−])-based internal solution to facilitate high voltage-activated (HVA) Ca²⁺ current rundown [11]. This internal solution for voltage-clamp experiments with DRG neurons contained (in mM) 135 tetramethyl-ammonium hydroxide (TMA-OH), 40 HEPES, 10 ethylene glycol tetraacetic acid (EGTA), and 2 MgCl₂, adjusted to pH 7.2 with hydrogen fluoride (HF). Typically, T-currents were evoked from the holding potential (V_h) of −90 mV with depolarization to test potential (V_t) of −30 mV. The amplitude of the T-current at any given potential was measured from the end of the pulse to its peak.

The internal solution for voltage-clamp experiments with HEK-293 cells contained (in mM), 110 Cs-MeSO₄, 14 creatine phosphate, 10 HEPES, 9 EGTA, 5 Mg-ATP, and 0.3 Tris-GTP, adjusted to pH 7.3 with CsOH.

All drugs were prepared as stocks and freshly diluted to the final concentrations in the external solution at the time of experiments. S-nitroso-glutathione (GSNO) and S-nitroso-N-acetyl penicillamine (SNAP) were prepared as 100 mM stock in distilled water; 10 mM 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one (ODQ) and 100 mM N-ethylmaleimide (NEM) were prepared as stock solutions in dimethylsulfoxide (DMSO). The final concentrations of DMSO had no significant effect on T-current amplitude in DRG and HEK cells (data not shown). Stock solutions of GSNO were kept at −20°C for up to one week.

Analysis

Statistical comparisons were made using paired or unpaired *t*-tests where appropriate. All data are expressed as means ± standard error of the mean (SEM); *p* values are reported only when statistically significant (<.05). The percent reductions in peak current at various concentrations of GSNO were used to generate concentration-response curves. Mean values were fit to the following Hill-Langmuir function:

$$PI([GSNO]) = PI_{\max} / (1 + (IC_{50} / [GSNO])^h) \quad (1)$$

where $PI_{\max}$ is the maximal percent inhibition of peak current by GSNO, IC_{50} is the concentration that produces 50% inhibition, and h is the apparent Hill-Langmuir coefficient for inhibition. The fitted values are reported with > 95% linear confidence limits. The

voltage dependence of steady-state inactivation was described with single Boltzmann distribution of the following form:

$$I(V) = I_{\max} / (1 + \exp[(V - V_{50})/k]) \quad (2)$$

where $I_{\max}$ is the maximal activatable current, V_{50} is the voltage where half the current is inactivated, and k is the voltage-dependence (slope) of the distribution.

To study the effects of GSNO on steady-state inactivation of T-channels (Fig. 2), currents are evoked by test steps to -30 mV after 3.5-sec pre-pulses to potentials ranging from -110 mV to -45 mV in 5 mV increments.

Results

DRGs contain somas of small-diameter primary afferent sensory fibers that originate as pain endings in the periphery and terminate in the dorsal horn of the spinal cord. We used whole-cell recordings from smaller (<35 μm average diameter) acutely dissociated DRG neurons because it generally is accepted that the majority of these cells are likely to be involved in nociceptive processing *in vivo*. To this end, our previous studies have established that these cells express exclusively $\text{Ca}_v3.2$ isoform of T-currents and respond to capsaicin and/or have intense staining with IB_4 , two principal markers of nociceptive function of DRG cells [13,14]. We previously have identified several redox agents that modulate function of DRG T-currents [13] and we continue here by investigating SNOs as possible modulators of $\text{Ca}_v3.2$ currents in putative nociceptive DRG cells. The traces in Figure 1A and the temporal record in Figure 1B illustrate a representative DRG cell in which application of 0.3 mM GSNO in the external solution initially increased peak T-current amplitude by about 20% and then inhibited peak T-current by about 35%. On average, 0.3 mM GSNO increased peak T-current in these cells by $26 \pm 6\%$ ($n = 11$, $p < 0.001$) and inhibited peak T-current in these cells by $42 \pm 2\%$ ($n = 5$, $p < 0.001$). Next, we examined the sensitivity of T-current inhibition by testing several concentrations of GSNO in different DRG cells after applying GSNO until an apparent steady-state inhibition was achieved. Maximal inhibition of current of about 44% was achieved with 1 mM GSNO and an IC_{50} of 110 ± 1 μM was calculated (Fig. 1C).

The enhancement of T-current induced by brief exposures to GSNO (about 1 minute) was largely reversible upon washout; there was a nearly complete recovery of baseline current. In contrast, the inhibitory effect was only partially reversible in these cells. Likewise we found that the inhibition of baseline T-currents in DRG cells by a related agent, SNAP, was incompletely reversible, with 1 mM SNAP inhibiting $44 \pm 3\%$ of baseline T-current in these cells ($n=7$, $p < 0.001$, data not shown). Unlike with GSNO, we have not observed any increase in T-current amplitudes with applications of SNAP, suggesting that different mechanisms may mediate two effects. Interestingly, in the presence of a nonspecific metal chelator such as 100 μM DTPA, we did not observe any increase in T-current amplitudes with GSNO (data not shown). Hence, most of the ensuing experiments in our study were performed with 100 μM DTPA in the external solution to study the inhibitory effect of GSNO on T-currents.

To investigate mechanisms for the channel inhibition by GSNO, we first characterized its effect on activation and inactivation of macroscopic currents. We found that 1 mM GSNO had little effect on macroscopic current kinetics. For example, the time for T-current activation to rise from 10 to 90% ($V_h -90$ mV, $V_t -30$ mV) was 6.3 ± 1.0 msec in control conditions and 7.8 ± 1.0 msec ($n = 5$, $p > 0.05$) in the presence of 1 mM GSNO (data not shown). Likewise, T-current inactivation time constants ($V_h -90$ mV, $V_t -30$ mV) that were

obtained by fitting single exponentials to decaying portions of current waveforms were 22.1 ± 1.6 msec in control conditions and 21.4 ± 1.4 msec ($n = 5$, $p > 0.05$, data not shown) in the presence of 1 mM GSNO. Figure 2 shows that 1 mM GSNO similarly inhibited peak T-currents over the range of membrane potentials. Half maximal inactivation occurred at -65 ± 2 mV in control conditions (solid symbols) and at -66 ± 2 mV in the presence of 1 mM GSNO (open symbols, $n = 4$, $p > 0.05$). We conclude that application of GSNO did not significantly affect voltage-dependence of T-channel steady-state inactivation in these cells.

GSNO is a redox-reactive compound capable of modulating thiol groups on other proteins in tissues [5]. To determine whether irreversible thiol modification of T-channels in DRG neurons alters the effects of GSNO on T-currents, we pretreated cells with N-ethylmaleimide (NEM), which covalently modifies protein thiol groups by alkylation and thus may block thiol-based redox reactions on regulatory cysteines in proteins of interest [5]. We reasoned that if GSNO changes a cell's redox state by acting on putative thiol groups on the DRG T-channels, it should be possible to eliminate or greatly attenuate its effect with a prior bath application of NEM. Figure 3A shows the time course for a representative experiment in which we first applied 0.3 mM NEM to the cells for 5-6 minutes until an apparent steady-state effect was achieved. On its own, NEM at this concentration slowly and apparently irreversibly blocked baseline T-currents by about 50% ($45 \pm 8\%$, $n=9$). Using the same cells, we then applied 1 mM GSNO for 100 seconds after NEM had been washed out for 100 seconds, the point being to avoid direct chemical interaction between these agents. Figure 3B shows that the effect of 1 mM GSNO measured after NEM application was virtually abolished. The $44 \pm 4\%$ inhibition ($n=8$) caused by 1 mM GSNO alone was reduced to $2.0 \pm 10\%$ inhibition ($n = 9$, $p < 0.001$) when DRG cells were pre-treated with NEM. The time record in Fig. 3 C and the bar histograms in Fig. 3D summarize results with ODQ, a specific soluble guanylyl cyclase inhibitor. At concentrations of 10 μ M, ODQ minimally affected baseline T-type current when given alone ($96.5 \pm 9.0\%$ of control, $n=6$, $p > 0.05$). Administration of ODQ in combination with GSNO, did not significantly affect the inhibitory response of DRG cells to GSNO; 1 mM GSNO caused $44.0 \pm 4.0\%$ inhibition of T-current when administered alone ($n=8$), and $45.5 \pm 9.9\%$ inhibition of T-current in the presence of 10 μ M ODQ ($n=6$, $p > 0.05$).

These experiments with NEM and ODQ strongly suggest that the effect of GSNO on T-current is due to alterations of extracellular thiol groups on Ca_v3.2 channels in membranes and is not due to G-protein modulation. Since GSNO cannot readily cross the cell membrane, it is unlikely that these putative thiol groups are located in the cytosolic (intracellular) face of the channel. To begin to address the molecular basis for GSNO modulation of T-channels, and specifically, to test the hypothesis that GSNO modulates extracellular thiol groups on the Ca_v3.2 channels, we used recombinant wild-type and mutated channels expressed in HEK-923 cells. Earlier knockdown and knockout studies have implicated Ca_v3.2 as the main T-type isoform in smaller DRG cells [15,16]. We assessed the effects of GSNO on the peak current and current kinetics of recombinant Ca_v3.2 T-currents in stably transfected HEK 293 cells. The results were much like those we obtained in DRG cells, with 1 mM GSNO significantly reducing recombinant Ca_v3.2 T-current amplitudes by $48 \pm 13\%$ ($n=6$, $p<0.05$, first bar on Fig. 4A), but having no significant effect on the kinetics of macroscopic current activation or inactivation (data not shown). As expected, there was a similar inhibition of currents arising from wild-type Ca_v3.1 ($26 \pm 2\%$, $n = 5$, second bar on Fig. 4A) and wild-type Ca_v3.3 constructs ($38 \pm 4\%$, $n = 5$, third bar on Fig. 4A) by 1 mM GSNO.

Cysteine residues frequently are paired and often stabilize structure in proteins by forming covalent disulfide bonds. We sought candidate extracellular cysteines that are conserved across T-channel isoforms and that are in loops with odd numbers of cysteine molecules

such that at least one might be unpaired. We focused on two regions, repeat II, where there is a single highly conserved cysteine (C939) in the loop connecting S5 to the pore loop, and repeat I, where there are three conserved cysteines in the loop connecting S1 to S2 (C123, C128, C133). These cysteines are schematically depicted in Fig. 4B. We performed site-directed mutagenesis of these candidate Cav3.2 isoform extracellular cysteine residues into alanines, as shown in Figure 4B, and tested the effect of GSNO. In Cav3.2 channels in which all four of the critical cysteines were mutated, [Cav3.2 Cys (4)], the inhibitory effect of 1 mM GSNO was completely eliminated ($102 \pm 5\%$ of baseline current, $n = 5$, $p > 0.05$; Fig. 4A, last bar on the right). Previously we have shown that these mutations do not affect kinetic and pharmacological properties of Cav3.2 channels [12]. Thus, we conclude that one or more of these extracellular cysteine residues is required for the inhibitory effect of GSNO on Cav3.2 currents.

Discussion

T-channels first were described in sensory neurons [17] where they play a crucial role in regulation of the excitability of nociceptors [14]. Their function in nociception has been reasonably well established [13,15,18]. However, the mechanisms that regulate the function of these channels still are not well understood. The major finding of this study is that SNOs inhibit native and recombinant Cav3.2 T-currents via a mechanism that depends on thiol group oxidation and at least one of four cysteine residues on the extracellular face of the channel. Inhibition of Cav3.2 T-channels, and the ensuing decrease in cellular excitability in nociceptive DRG cells, in turn, could diminish pain signaling.

We present several findings that argue for oxidation by GSNO of extracellular cysteines on native and recombinant T-channels. First, the effects of GSNO on native DRG currents were attenuated by the relatively specific thiol-alkylating agent, NEM. Second, the effect of GSNO on DRG T-current was not altered by applications of ODQ, a soluble guanylyl cyclase inhibitor. Third, site-directed mutation of 4 critical cysteine residues in recombinant Cav3.2 completely abolished the inhibitory effect of GSNO on recombinant Cav3.2 T-current. We previously have shown that mutation of these 4 cysteines also specifically eliminated inhibitory effects of other thiol-oxidizing agents like lipoic acid and dithionitrobenzoic acid (DTNB) on Cav3.2 currents while single cysteine mutation (C939A) and triple cysteine mutation (C123A, C128A, C133A) of these channels only partially diminished inhibition of Cav3.2 currents by lipoic acid [12]. Thus, in this study we examined the effect of GSNO only on quadruple cysteine mutant. We also reported previously that GSNO partially inhibits T-currents in nRT cells in acute brain slices [4]. However, unlike the inhibition of DRG T-current, GSNO inhibits thalamic T-currents in a voltage-dependent manner. A possible explanation for the different GSNO effects in the different tissues is that DRG T-currents are comprised entirely of Cav3.2 currents whereas nRT T-currents are comprised of Cav3.3 and Cav3.2 T-channel isoforms [1,19]. Furthermore, regardless of the precise mechanisms, even partial inhibition of T-currents is sufficient to inhibit burst-firing of DRG cells [12]. Additional molecular studies will be needed to identify other critical cysteine residues on T-channels that are modulated by various thiol-oxidizing agents.

Together, these results strongly suggest that endogenous SNOs such as GSNO modify the sulfhydryl (thiol) moiety of key regulatory cysteines in native T-channels via trans S-nitrosylation reactions, resulting in inhibition of the calcium currents. Yoshimura *et al.* [20] found that N-type high-voltage-activated (HVA) calcium currents in peripheral sensory neurons are modulated by SNAP via a cGMP-dependent signaling pathway. It is unlikely that cGMP is involved in the effects of GSNO on DRG T-currents since ODQ did not affect the response to GSNO (Figure 3). Additionally, our recordings from native DRG cells are

performed using fluoride-based internal solutions, which would be expected to block most of the G-protein-dependent pathways. Thus, different subtypes of voltage-gated calcium channels may be differently regulated by SNOs either by S-nitrosylation or by cGMP-dependent signaling pathways. Indeed, recent data indicate that S-nitrosylation may play an important role in regulation of neuronal excitability by affecting multiple targets such as voltage- and ligand-gated ion channels as well as release of intracellular calcium by interaction with ryanodine receptors (reviewed in [21]).

Thus, it is possible that generation of NO-related molecules *in vivo* may lead to alterations of T-type channel-dependent neuronal excitability in sensory neurons and in the CNS in both physiological and pathological conditions. NO serves as a key signaling molecule in physiological processes as diverse as host-defense reactions, neuronal communication, and vascular tone regulation [5,22]. Disturbance of the balance among different NO-related species has been implicated in inflammatory processes, ischemia of CNS neurons, and neurodegenerative disorders [6]. Various NO donors, including SNOs, can exert neuroprotective effects in cortical cell cultures *in vitro* [23] and GSNO reduces inflammation and protects the brain against focal cerebral ischemia in an experimental stroke model in rats [24]. Furthermore, it has been shown that ischemia/hypoxia *in vitro* up-regulates T-type channels [25,26]. Since ischemic strokes often are accompanied by abnormal neuronal excitability, which can aggravate neuronal damage and cause pain episodes, it would be a desirable therapeutic intervention to down-regulate the function of T-type channels in peripheral sensory and CNS neurons during ischemic episodes. Thus, exogenously applied SNOs could be useful for dampening the T-type channel-dependent neuronal excitability in conditions such as ischemic pain. Further *in vivo* studies are necessary to confirm this notion.

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Figure 1. Effects of GSNO on isolated T-currents in acutely dissociated rat DRG cells

A. Original traces of T-current in a representative DRG cell before (black trace), after (light gray trace), as well as during bath application of 0.3 mM GSNO (dark gray trace). GSNO reversibly increased peak inward current about 20% and then inhibited it about 35%. Bars indicate calibration.

B. Temporal record from the same cell presented in panel A of this figure. Black solid bar indicates duration of GSNO application. Dotted horizontal bar denotes baseline T-current level.

C. Concentration-response relationship for GSNO inhibition of T-current in rat DRG cells measured at the time point of an apparent steady-state effect ($n = 6-11$ per data point). Solid line is the best fit (equation # 1, see Methods), yielding an IC_{50} of $110 \pm 1 \mu M$, slope coefficient of 3.3 ± 0.1 , and maximal inhibition of $43.8 \pm 8.1\%$ of the peak of T-current.

Figure 2. Lack of effect of GSNO on voltage-dependent inactivation of T-current in DRG cells
 Normalized peak T-current steady-state inactivation curves from 4 cells. Filled symbols represent the control conditions; open symbols represent the bath application of 1 mM GSNO. Solid black lines are fit using equation #2 (see Methods), and give half-maximal availability (V_{50}) at -65 ± 2 mV with a k of 10 ± 1 mV in control conditions. V_{50} was -66 ± 2 mV with a k of 8 ± 2 mV with application of GSNO.

Figure 3. Redox mechanism of GSNO inhibition of T-current in DRG cells

A. Time course for effect of 0.3 mM NEM on T-current in acutely dissociated DRG cells from a representative experiment. NEM started inhibiting channel activity immediately and achieved maximal channel inhibition of about 60% of the T-current by 200 msec. When 1 mM GSNO was applied after NEM treatment, it induced very little change in the remaining T-current. The black horizontal bars indicate the times of NEM and GSNO applications, as indicated.

B. Effect of GSNO on peak amplitude of T-current in DRG cells (average from 9 different DRG cells tested using the same experimental protocol as depicted in panel A of this figure). There was no statistical difference (n.s.) between the amplitudes of peak T-currents remaining after applications of NEM alone (black bar, $55 \pm 8\%$) vs. after GSNO following NEM (open bar, $53 \pm 10\%$, $p > 0.05$).

C. Time course for effect of 10 μ M ODQ +/- GSNO on T-current from a representative experiment using acutely dissociated DRG cells. ODQ slightly increased the peak T-current over the course of 200 msec. Addition of 1 mM GSNO at 200 msec inhibited rapidly about 50% of the peak T-current observed with ODQ alone. The black horizontal bars indicate the times of ODQ and GSNO exposure.

D. Effect of ODQ on peak amplitude of T-currents (average from 6 different DRG cells tested +/- GSNO using the same experimental protocol as depicted in panel C of this figure). There was a statistically significant difference between the amplitudes of peak T-

currents remaining after applications of ODQ alone (black bar, $96 \pm 9\%$) vs. GSNO with ODQ (open bar, $51 \pm 6\%$, $p < 0.01$).

Figure 4. Molecular mechanisms of inhibition of recombinant Cav3.2 currents by GSNO

A. Effect of 1 mM GSNO on calcium current measured in HEK cells expressing recombinant wild type (WT) Cav3.2 (open bar), WT Cav3.1 (medium crisscrossed bar), WT Cav3.3 (large crisscrossed bar), or a mutant Cav3.2Cys(4) construct with mutation of 4 potentially critical cysteine residues to alanines (black filled bar). After application of GSNO, 52 ± 12 % of Cav3.2 current, 75 ± 2 % of Cav3.1 current, and 62 ± 4 % of Cav3.3 current remained. In contrast, bath application of GSNO to the Cav3.2Cys(4) mutant did not significantly affect peak amplitudes of T-currents (102 ± 5 %). The number of cells in each experiment was 5-6. * indicates a statistically significant difference from baseline T-currents prior to GSNO application as marked with dotted line ($p < 0.05$).

B: Schematic diagram of Cav3.2 showing the position of conserved cysteine residues in the extracellular face of the channel in domains I and II. Designated cysteine residues (green) were mutated to alanine residues (red).