CaV3.2 is the major molecular substrate for redox regulation of T-type Ca\(^{2+}\) channels in the rat and mouse thalamus

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Although T-type Ca\(^{2+}\) channels in the thalamus play a crucial role in determining neuronal excitability and are involved in sensory processing and pathophysiology of epilepsy, little is known about the molecular mechanisms involved in their regulation. Here, we report that reducing agents, including endogenous sulfur-containing amino acid L-cysteine, selectively enhance native T-type currents in reticular thalamic (nRT) neurons and recombinant CaV3.2 (\(\alpha_1\)H) currents, but not native and recombinant CaV3.1 (\(\alpha_1\)G)- and CaV3.3 (\(\alpha_1\)I)-based currents. Consistent with this data, T-type currents of nRT neurons from transgenic mice lacking CaV3.2 channel expression were not modulated by reducing agents. In contrast, oxidizing agents inhibited all native and recombinant T-type currents non-selectively. Thus, our findings directly demonstrate that CaV3.2 channels are the main molecular substrate for redox regulation of neuronal T-type channels. In addition, because thalamic T-type channels generate low-threshold Ca\(^{2+}\) spikes that directly correlate with burst firing in these neurons, differential redox regulation of these channels may have an important function in controlling cellular excitability in physiological and pathological conditions and fine-tuning of the flow of sensory information into the central nervous system.

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It has been well established that thalamic neurons are capable of gating the flow of sensory information depending on various states of the sleep and wake cycle. During transition toward quiescent sleep, thalamic nuclei generate rhythmic oscillations that progressively recruit the entire thalamocortical system to produce synchronized rhythmic activity termed spindling (reviewed in McCormick & Bal, 1997; Steriade, 2005). In this mode, flow of sensory information is diminished, and pathologial increases of this rhythmic activity are correlated with brief losses of consciousness such as during absence seizures. This state-dependent gating in the thalamus critically depends on the ability of the T-type of low-voltage activated (LVA) Ca\(^{2+}\) channels to generate low-threshold Ca\(^{2+}\) spikes (LTS), which underlie burst of action potentials in thalamocortical and reticular thalamic (nRT) neurons.

Cloning the \(\alpha_1\) subunits of T-type channels has revealed the existence of at least three subtypes with different kinetic and pharmacological properties. These subtypes, \(\alpha_1\)G (Ca3.1; Perez-Reyes et al. 1998), \(\alpha_1\)H (Ca3.2; Cribbs et al. 1998), and \(\alpha_1\)I (Ca3.3; Lee et al. 1999) are likely to provide a molecular basis for heterogeneity of T-type currents observed in native cells (Herrington & Lingle, 1992; Todorovic & Lingle, 1998). Recent data indicate that the CaV3.1 and CaV3.2 isoforms of T-type channels may be regulated by post-translational mechanisms such as phosphorylation (Welsby et al. 2003; Leresche et al. 2004) and redox modification (Todorovic et al. 2001). Furthermore, selective modulation of particular isoforms of T-type channels that shape excitability among different neurons or even within the same neurons (Kozlov et al. 1999; Chemin et al. 2002) may endow different patterns of burst firing in thalamic nuclei, thus allowing precise control of the flow of sensory information through the central nervous system (CNS).

In recent years, several studies have suggested that the function of some ion channels can be modulated by redox...
agents, presumably by oxidation and reduction of thiol groups present in the channel or neuronal membrane (reviewed by Lipton et al. 2002). We have recently reported that reducing agents such as dithiothreitol (DTT) and the endogenous amino acid l-cysteine selectively and potently enhance T-type currents in rat sensory neurons and recombinant CaV3.2 channels, whereas, oxidizing agents such as 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) inhibit these channels (Todorovic et al. 2001). This form of T-type channel modulation may be important in amplifying pain transmission (Todorovic et al. 2001; Nelson et al. 2005). However, despite the essential role of T-type channels in generating thalamocortical oscillations, little is known about their regulation by endogenous redox substances. Such agents could provide an important intrinsic mechanism for the control of neuronal excitability in both physiological and pathological conditions.

Here, we have used redox modifying agents previously found to alter the function of T-type channels in peripheral sensory neurons to evaluate the possible mechanisms and importance of their action in regulating thalamic neuronal activity. Our results, demonstrating that CaV3.2 (α1H) T-type channels are the major molecular substrate for redox modulation of T-type currents and LTS in the thalamus, identify a novel function of redox agents, including endogenous amino acid l-cysteine, in the cellular excitability of CNS neurons.

**Methods**

**In vitro tissue slice preparation**

We performed most of the experiments on transverse rat brain slices cut through the middle anterior portion of the nRT (Paxinos & Watson, 1994) at a thickness of 250–300 μm. Gravid Sprague-Dawley rats were housed in a local animal facility in accordance with protocols approved by the University of Virginia Animal Use and Care Committee. We adhered to the guidelines in the NIH Guide for the Care and Use of Laboratory Animals.

Rats (age 7–14 days) or mice (age 9–11 days) of either sex were used in this study. α1H (CaV3.2) null mice was generated as previously described (Chen et al. 2003). The animals were briefly anaesthetized with 5% halothane and decapitated. The brains were rapidly removed and placed in chilled (4°C) cutting solution consisting of (m): 2 CaCl2, 130 NaCl, 2.5 MgCl2, 10 glucose, 26 NaHCO3, 1.25 NaH2PO4, 2 MgCl2, 10 glucose and 2 CaCl2, equilibrated with a mixture of 95% O2 and 5% CO2. Slices were maintained at room temperature in the recording chamber, where they remained viable for at least 1 h. In some experiments slices were incubated in air-bubbled solution. We did not detect any differences in viability of cells and nature of the response to redox agents regardless whether slices were incubated in oxygenated or regular saline (data not shown).

**Recombinant cell preparation**

HEK-293 cells (A293; ATCC, Manassas, VA, USA) were transfected with linearized plasmid (pcDNA3, Clontech) containing the rat Ca3.3b isoform (IIb; AY128644; Murbartian et al. 2002) using the calcium phosphate method (CalPhos Maximizer Transfection Kit; Clontech). Stable colonies were isolated in DMEM media supplemented with 1 mg ml−1 G418 (Invitrogen, Carlsbad, CA, USA). The generation of cell lines containing human CaV3.1a or CaV3.2a cDNA, and their electrophysiological properties, have been previously described (Cribbs et al. 2000 and Gomora et al. 2002; respectfully). Cells were typically used 1–2 days after plating. Average cell capacitance (Cn) was 13 ± 6 pF (mean ± s.d.); the average series resistance (Rs) was 5 ± 2 Ω.

**Recording procedures**

The extracellular saline solution typically used for recording Ca2+ currents in whole-cell and nucleated patch experiments consisted of (mm): 2 CaCl2, 130 NaCl, 2.5 MgCl2, 10 glucose, 26 NaHCO3, 1.25 NaH2PO4, and 0.001 TTX. For recording Ca2+ currents in outside-out configuration, we replaced the 2 mm CaCl2 with 10 mm BaCl2. The standard extracellular saline for recording recombinant Ca2+ current contained (mm): 160 tetraethylammonium chloride (TEA-Cl), 10 Heps and 2 BaCl2, adjusted to pH 7.4 with tetraethylammonium hydroxide (TEA-OH) (316 mmol l−1). The extracellular solution for current-clamp experiments was same as the incubating solution plus in some experiments included 20 μM bicuculline, 200 μM picrotoxin, 50 μM dl-2-amino-5-phosphonovaleric acid (APV) and 5 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX). In some experiments 1 μM TTX was used to isolate LTS. For recording T-type currents in brain slices, we used an internal solution (solution 1) of (mm): 135–140 tetramethylammonium hydroxide (TMA-OH), 10 EGTA, 40 Heps and 2 MgCl2, titrated to pH 7.15–7.25 with hydrofluoric acid (HF) (Todorovic & Lingle, 1998). For some experiments we altered this

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internal solution by adding (mm): 3 MgATP, 0.3 Tris-GTP, 45 caesium methane sulfonate (decreasing TMA-OH to 90) titrated with HF to pH 7.15–7.25 (solution 2). For recording high-voltage-activated (HVA) Ca\(^{2+}\) currents in brain slices and recombinant Ca\(^{2+}\) currents from HEK cells, the internal solution contained (mm): 110 caesium methane sulfonate, 14 phosphocreatine, 10 Hepes, 9 EGTA, 5 MgATP and 0.3 Tris-GTP, adjusted to pH 7.15–7.20 with CsOH (solution 3). Recording electrodes for current-clamp studies contained (mm): 130 KCl, 5 MgCl\(_2\), 1 EGTA, 40 sodium Hepes, 40, 2 MgATP and 0.1 Na\(_3\)-GTP (pH 7.2). For the data presented, membrane potential values were corrected for the measured liquid junction potential of −10 mV (solution 1), −2 mV (solution 2) and −3 mV (solution 3) in voltage-clamp experiments, as well as −5 mV in current-clamp experiments. For inside-out recordings of Ca\(^{2+}\) currents, electrodes contained: 140 mm TEA-OH, 10 mm BaCl\(_2\), 2 mm MgCl\(_2\), 1 mm CsCl\(_2\), 3 mm 4-aminopyridine, 1 μm TTX, and 10 mm Hepes (pH 7.2 adjusted with Tris-base solution) (Joksovic \textit{et al.} 2005).

All recordings were obtained from thalamic neurons visualized with an infrared differential interference contrast camera (C2400: Hamamatsu, Hamamatsu City, Japan) on the Zeiss 2 FS Axioscope (Jena, Germany) with a ×40 lens and patch-clamp pipette using a Sutter micromanipulator MP-285 (Sutter Instrument Co., Novato, CA; USA).

Electrophysiological recordings

We recorded Ca\(^{2+}\) currents in thalamic slices from a total of 245 visually identified rat nRT neurons and a total of 29 mouse nRT neurons (17 from wild-type C57/BL6 and 12 from Ca\(_3\)3.2 KO mice). Recordings were made with standard whole-cell, inside-out, and outside-out voltage-clamp techniques (Hamill \textit{et al.} 1981) or the nucleated patch technique (Sather \textit{et al.} 1992). Electrodes were fabricated from thin-walled microcapillary tubes (Drummond Scientific, Broomall, PA, USA) and had final resistances of 3–6 MΩ. We recorded membrane currents with an Axoclamp 200B patch-clamp amplifier (Molecular Devices Corp., Union City, CA, USA). Voltage commands and digitization of membrane currents were done with Clampex 8.2 of the pCLAMP software package (Molecular Devices Corp.). Neurons were typically held at −100 mV and depolarized to −50 mV every 10–20 s to evoke inward Ca\(^{2+}\) currents. Data were analysed using Clampfit (Molecular Devices Corp.) and Origin 7.0 (OriginLab Corp., Northampton, MA, USA). For whole-cell recordings, we filtered currents at 5–10 kHz, and for inside-out, outside-out, and nucleated patch recordings, at 2–5 KHz. We typically compensated for 50–80% of R\(_i\). In some experiments, a P/5 protocol was used for on-line leakage subtraction.

Since voltage control is compromised in whole-cell recordings from slices due to the presence of extensive cell processes, we included in our results only cells in which voltage-dependent current activation was smooth; we observed no excessive delay in the onset of current; the onset and offset kinetics depended on voltage, but not on the amplitude of current. In the kinetic study, we included only cells in which adequate clamp conditions were obtained using these criteria (Fig. 5). Because intact nRT neurons have long processes, in whole-cell experiments rapid components of recorded current, such as fast-activation kinetics or tail currents, are not likely to reflect the true amplitude and time course of Ca\(^{2+}\) current behaviour. However, all our measurements of amplitudes from holding, peak and steady-state currents were made at time points sufficient to ensure reasonably well-clamped current conditions.

To include data from the nucleated and cell-free patch recordings in our analysis, recordings had to be stable for at least 5 min; electrode capacitance had to be sufficiently well compensated and seal resistance sufficiently high (range, 3–25 GΩ) to allow unambiguous identification of ensemble channel currents. T-type channel activity was recognized by characteristic near-complete inactivation of current at negative voltages which could be well described with a single exponential time course. The steps we used to activate T-type channels in the inside-out patches were similar to those used in whole-cell experiments and nucleated patch experiments. T-type currents were presented conventionally as inward currents.

Analysis of current

Current waveforms or extracted data were fitted with the ClampFit program (Molecular Devices Corp.) or Origin 7.0 (OriginLab Corp.).

The voltage dependence of steady-state activation was described with a single Boltzmann distribution:

\[
G(V) = G_{\text{max}}/(1 + \exp[-(V - V_{50})/k])
\]

where \(G_{\text{max}}\) is the maximal conductance, \(V_{50}\) is the half-maximal voltage, and \(k\) (units of millivolts) represents the voltage dependence of the distribution.

The voltage dependence of steady-state inactivation was described with a single Boltzmann distribution:

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

where \(I_{\text{max}}\) is the maximal current.

The time course of current inactivation was fitted using a single exponential equation, \(f(t) = A_1 \exp(-t/\tau_1)\), or a biexponential equation, \(f(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)\), yielding two time constants (\(\tau_1\) and \(\tau_2\)) and their amplitudes (\(A_1\) and \(A_2\)).
The amplitude of T-type current was measured from the peak, which was subtracted from the current at the end of the depolarizing test potential to avoid small contamination with residual HVA currents. For all current–voltage ($I–V$) curves and steady-state inactivation curves, fitted values were typically reported with 95% linear confidence limits. Input resistance was determined from the slope of the peak voltage versus the current plot that resulted from injecting current that was 80–160 ms long, ranging from 100 to 500 pA. Fitting was done with Origin 7.0 (OriginLab Corp.). Statistical analysis was performed with either paired Student $t$ test or ANOVA, with statistical significance determined with $P<0.05$.

**Drugs and chemicals**

TTX was obtained from Alomone Laboratory (Jerusalem, Israel). All other salts and chemicals were obtained from Sigma (St Louis, MO, USA). All final concentrations of redox agents were freshly made.

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**Figure 1. DTT modulates native thalamic T-type currents only in the nucleus reticularis (nRT), and not in ventrobasal (VB) or laterodorsal (LD) neurons**

Left, traces represent T-type current (black line) elicited from $V_h = -100$ mV to $V_t = -50$ mV recorded in different thalamic regions, and single exponential fit (grey line) representing different inactivation properties of T-type currents in nRT, VB and LD neurons. Photomicrographs on the right show transverse brain slices displaying biocytin-filled neurons (black arrows) from nRT (top), VB (middle) and LD (bottom) in relation to internal capsule (IC) (scale bars 200 μm) and enlarged pictures of the same neurons as inserts (scale bars 50 μm). A, B and C, traces on the left represent typical T-type currents recorded from nRT, VB and LD neurons, respectively. nRT (A) and VB (B) currents are reasonably well fitted with a single exponential function showing slow inactivation in nRT (inactivation $\tau = 62$ ms) and fast inactivating current in VB ($\tau = 26$ ms). In contrast, T-type current in LD (C) was poorly described with a single exponential fit, as indicated by a grey line; a better fit is achieved with a double exponential function revealing slow ($\tau = 93$ ms) and fast ($\tau = 21$ ms) inactivation time constants. Time courses in the centre panels depict the reversible effect of 0.2 mM DTT on T-type current in nRT (top) and a lack of effect of DTT on T-type current in VB (middle) and LD (bottom). Horizontal bars represent duration of application of DTT. Bars indicate calibration.
from stock solutions of 10 mM l-cysteine, (tris(2-carboxyethyl)phosphine) (TCEP) and DTT in H2O; 100 mM 2-(trimethylammonium) ethyl methanethiosulfonate (MTSET) and oxidized glutathione in H2O, and 600 mM DTNB in DMSO. The maximal final concentration of DMSO used in our experiments was 0.5%, which did not significantly affect native thalamic concentration of DMSO used in our experiments was confirmed by using the atlas of Paxinos & Jokovic et al. 2005a).

Solutions
Multiple independently controlled glass syringes attached to the thin PVC tubing served as reservoirs for a gravity-driven perfusion system. Switching between solutions was accomplished by manually controlled valves. All experiments were done at room temperature (20–24°C). All drugs were prepared as stock solutions and freshly diluted to appropriate concentrations at the time of the experiment to avoid any chemical interaction of redox agents with trace metal ions in external solutions. During an experiment, solution was removed from the end of the chamber opposite the tubing by constant suction. Changes in Ca2+ current amplitude in response to rapidly acting drugs or ionic changes were typically complete in 1–2 min.

Immunohistochemistry
After recording T-type current from biocytin-filled neurons, we fixed thalamic slices overnight in 4% paraformaldehyde and then washed them with 0.1% phosphate buffer (PB). Neurons were revealed using the avidin–biotin–peroxidase complex method (ABC elite kit; Vector Laboratories, Burlingame, CA, USA). After blocking endogenous peroxidase with 0.3% H2O2 in 0.4% Triton X-100 and PB, we incubated slices in the ABC for 2 h. Incubated slices were washed several times in PB before immersion in a solution containing 0.05% of 3,3′-diaminobenzidine tetrahydrochloride (DAB reagent) in 0.1% Tris buffer and 0.02% H2O2. After several washes in PB, slices were mounted on gelatin-coated slides and dehydrated through alcohol to xylene for light microscopic examination. The position of labelled neurons within the thalamus was confirmed by using the atlas of Paxinos & Watson (1944).

Results
Nucleus-specific redox alteration of native T-type channels in the thalamus
Previous studies indicated the existence of diverse T-type currents in thalamic nuclei that may underlie different properties of burst firing associated with small membrane depolarizations in reticular thalamic neurons (nRT) (Domich et al. 1986; Huguenard & Prince, 1992) and in ventrobasal (VB) (Coulter et al. 1989; Huguenard & Prince, 1992) and laterodorsal (LD) neurons (Tarasenko et al. 1997). More recent molecular studies have revealed that three isoforms of T-type channels probably underlie these observed differences in the properties of native thalamic T-type currents (Talley et al. 1999).

Thus, we recorded whole-cell T-type currents in brain slices in the regions of the thalamus containing mRNA for three different isoforms: nRT nucleus (CaV3.2 and CaV3.3), VB nucleus (CaV3.1) and LD nucleus (CaV3.1 and CaV3.3). Having identified different nuclei according to the rat brain stereotaxic atlas, we confirmed the position of neurons within the thalamus by including biocytin in the recording electrodes, fixing slices overnight and visualizing biocytin-filled neurons using the avidin–biotin–peroxidase complex (ABC) method (right panels, Fig. 1A–C). The left panels in Fig. 1A–C show that, based on the different

Figure 2. Redox regulation of different T-type currents in the thalamus
A and B, T-type currents recorded from nRT neurons (Vh = −100 to V, of −50 mV) showing the effect on T-type current of 0.2 mM l-cysteine (A) and 1 mM 5,5′dithio-bis(2-nitrobenzoic acid) (DTNB) (B). Note speeding of inactivation kinetics indicated by criss-crossing of the decaying portions of the current waveforms in control conditions (inactivation r 54 ms) and in the presence of l-cysteine (inactivation r 36 ms). C, the average effect of separate applications of 0.2 mM l-cysteine, 0.2 mM dithiothreitol (DTT), and 1 mM DTNB on T-type currents in nRT, VB and LD thalamic nucleus. l-Cysteine (l-cys) and DTT exhibited a selective effect only on T-type currents in nRT neurons, with an average increase of 29 ± 6% (l-cys, n = 9 cells) and 42 ± 11% (DTT, n = 13 cells) (P < 0.001); they had virtually no effect on T-type current in the VB and LD nucleus (n = 5 cells each, P > 0.05). DTNB inhibited 56 ± 6% of current (n = 10 cells) in nRT, but only 22 ± 3% in VB (n = 5 cells) and 21 ± 6% in LD (n = 5 cells) (P < 0.001 each). *Significant change from control peak current (dotted line); vertical lines indicate s.e.m.
molecular makeup of these neurons, T-type currents (evoked from $V_h = -100 \text{ mV}$, $V_t = -50 \text{ mV}$) in these thalamic nuclei had distinct properties of inactivation, as determined by fitting the decaying portion of the current waveforms. nRT neurons characteristically showed slowly inactivating current that could be fit by a single exponential fit yielding a $\tau$ of $64 \pm 4 \text{ ms} \ (n = 15)$; VB neurons showed a faster inactivating current, with single exponential fits yielding an average inactivation $\tau$ of $21 \pm 3 \text{ ms} \ (n = 7)$; and inactivation of current in LD neurons exhibited biphasic, double-exponential inactivation with a larger component described with a fast $\tau$ (presumably CaV3.1) of $22 \pm 3 \text{ ms}$.

Figure 3. Redox regulation of nRT T-type currents in nucleated and cell-free patches

A, effect of 0.2 mM L-cysteine in a nucleated patch recording. B, time course of the effect on peak T-type current before, during and after application of L-cysteine from the same cell. A depolarizing pulse was applied every 10 s; the horizontal bar indicates time of the application. The effect was fast and easily reversible, similar to the effects in whole-cell recordings. C, effect of 1 mM DTNB on T-type current recorded in nucleated patch mode from another nRT neuron ($V_h = -100 \text{ mV}$, $V_t = -50 \text{ mV}$). D, effects of 0.2 mM L-cysteine, 0.2 mM DTT, and 1 mM DTNB on T-type current recorded in nucleated patch configuration. L-cysteine and DTT increased T-type current by 50 $\pm$ 3 and 41 $\pm$ 6%, respectively ($P < 0.001$ each); DTNB decreased peak current by 46 $\pm$ 4% ($P < 0.005$). * Significant change from control peak current (dotted line). E, the lack of effect of 0.2 mM DTT on ensemble T-type current in inside-out configuration. F, at the same concentration (0.2 mM), DTT increased peak ensemble T-type current in outside-out configuration by about 30% and increased inactivation $\tau$ by about 20%. All displayed traces are averages of 5–10 consecutive sweeps. Bars indicate calibration, and pertain to all panels.

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and a smaller component described with a slow $\tau$ (presumably $\text{CaV}_{3.3}$) of $88 \pm 8 \text{ ms}$ ($n = 8$).

Figure 1A (middle panel) shows the time course in an experiment in which DTT (0.2 mM), a representative thiol-reducing agent, caused a rapid and reversible increase in peak T-type currents in nRT neurons. The average increase over control level was $42 \pm 11\%$ (mean $\pm$ s.e.m., $n = 13$, $P < 0.005$), and the effect reached an apparent steady-state within 1–2 min of application. In contrast, 0.2 mM DTT did not significantly affect currents in VB (2 ± 3%, $n = 6$, $P > 0.05$) or LD (4 ± 2%, $n = 5$, $P > 0.05$) neurons even with longer applications (middle panels of Fig. 1B and C). The effect of L-cysteine, an endogenous sulfur-containing amino acid and redox agent,

was similar to that of DTT in nRT neurons. Overall, 0.2 mM L-cysteine increased peak T-type current an average of $29 \pm 6\%$ ($n = 9$, $P < 0.005$), while 0.1 mM L-cysteine did so by an average of $22 \pm 3\%$ ($n = 4$, $P < 0.01$) (data not shown). Figure 2A shows representative traces of the effects of L-cysteine on T-type current in an nRT neuron. It is evident that L-cysteine increased the peak current and accelerated current inactivation kinetics. On average, both DTT and L-cysteine caused a small but significant increase in the inactivation rate of nRT T-type currents, by $21 \pm 6\%$ ($P < 0.05$) and $15 \pm 7\%$ ($P < 0.05$), respectively. Like DTT, L-cysteine had no significant effect on current in VB and LD neurons ($n = 5$–6).

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**Figure 4. Reducing agents are $\text{CaV}_{3.2}$ isoform-selective in recombinant HEK cells**

$A$, $B$ and $C$ (top), representative traces ($V_h = -90$, $V_t =-40 \text{ mV}$) recorded from recombinant $\text{CaV}_{3.1}$, $\text{CaV}_{3.2}$ and $\text{CaV}_{3.3}$ channels stably expressed in HEK cells in control conditions (black trace) and after application of L-cysteine (grey trace). L-Cysteine at 0.1 mM increased the peak of the T-type current more than twofold only in $\text{CaV}_{3.2}$ isoform ($B$), having at 1 mM virtually no effects on T-type currents recorded in $\text{CaV}_{3.1}$ ($A$) and $\text{CaV}_{3.3}$ ($C$) isoforms. Note speeding of inactivation with L-cysteine in $\text{CaV}_{3.2}$ ($B$) from control $\tau$ of 37 ms to 24 ms, and no apparent change in inactivation kinetics in the presence of L-cysteine of T-type currents in $\text{CaV}_{3.1}$ (inactivation $\tau$ of 30 ms) and $\text{CaV}_{3.3}$ (inactivation $\tau$ of 80 ms) recombinant isoforms. $A$, $B$ and $C$ (bottom), average effects of redox agents L-cysteine, DTT and DTNB on different T-type channel isoforms in HEK cells. Middle histogram, 0.1 mM L-cysteine increased only $\text{CaV}_{3.2}$ currents by $108 \pm 23\%$ (L-cys); 0.1 mM DTT did so by $157 \pm 50\%$ ($n = 7$–12 cells, $P < 0.001$). At 1 mM, L-cysteine and DTT had little effect on $\text{CaV}_{3.1}$ (left) and $\text{CaV}_{3.3}$ (right) currents ($n = 6$ cells each, $P > 0.05$); $\text{CaV}_{3.1}$ 1 mM DTT: $3 \pm 2\%$, $n = 6$, $P > 0.05$; 1 mM L-cysteine: $3 \pm 1\%$, $n = 6$, $P > 0.05$; $\text{CaV}_{3.3}$ 1 mM DTT: $1 \pm 3\%$, $n = 6$, $P > 0.05$; 1 mM L-cysteine: $2 \pm 1\%$, $n = 6$, $P > 0.05$). In contrast, the oxidizing agent DTNB at 1 mM inhibited T-type current in every cell line, giving an average decrease of $58 \pm 13\%$ of $\text{CaV}_{3.1}$ ($n = 7$, $P < 0.001$), 50 ± 4% of $\text{CaV}_{3.2}$ ($n = 6$, $P < 0.001$) and 40 ± 12% of $\text{CaV}_{3.3}$ currents ($n = 8$, $P < 0.01$). *Significant change from control peak current; vertical lines are s.e.m.
Figure 5. The effects of redox agents on steady-state inactivation and activation of T-type currents in nRT neurons and recombinant CaV3.2 currents

A and B, average current–voltage (I–V) curves in control conditions (●) and in the presence of 0.2 mM l-cysteine (▲) or 1 mM DTNB (○) in whole-cell recordings from native nRT neurons (A, n = 9 cells each) and recombinant CaV3.2 HEK cells (B, n = 6 cells each). All controls are pooled. l-Cysteine increased, while DTNB depressed, peak T-type current similarly at all tested potentials. C and D, apparent peak conductance values defined as $I_{\text{peak}}/(V - E_r)$ plotted against command potentials in whole-cell recordings from native nRT cells (C) and from HEK cells expressing recombinant CaV3.2 channels (D) calculated from the experiments depicted in A and B. All fits are done using Boltzmann equation. The extrapolated reversal potential ($E_r$) was taken to be $+55$ mV. The estimates of $V_{50}$ and $k$ show only small shifts with up to 20 mV differences in assumed $E_r$ values. DTNB (○) had essentially no effect on half-activation in native nRT neurons ($V_{50} = -61 \pm 1$ mV, $k = 5.9$ mV, in control conditions; and $V_{50} = -61 \pm 1$ mV, $k = 6.3$ mV with DTNB) nor recombinant cells (control $V_{50} = -51 \pm 1$ mV, $k = 6.2$ mV, and with DTNB $V_{50} = -51 \pm 1$ mV and $k = 7.2$ mV). l-Cysteine (▲) shifted half-activation in recombinant cells to the left by 4 mV ($V_{50} = -55 \pm 1$ mV, $k = 6.2$ mV, $P < 0.01$), while having no significant effect on half activation in native nRT neurons ($V_{50} = -62 \pm 1$ mV and $k = 5.9$ mV, $P > 0.05$).

E and F, steady-state inactivation kinetics of T-type currents in native nRT (E) and recombinant HEK CaV3.2 (F) currents were examined by paired-pulse protocols obtained at different conditioning potentials. Double-pulse protocols to $-50$ mV were used to elicit T-type currents, separated by 3.5 s prepulse to potentials ranging from $-120$ to $-40$ mV before (●) and during application of l-cysteine (▲) and DTNB (○). Inactivation curves were best fitted with a Boltzmann equation, yielding $V_{50}$ of $-80.8 \pm 2.5$ mV in controls ($k = 6.9$ mV), and $-80.3 \pm 0.6$ mV ($k = 7.5$ mV) in the presence of l-cysteine ($n = 7$) and $-93.2 \pm 2.5$ mV ($k = 8.2$ mV) in the presence of DTNB ($n = 6$).
In contrast to the reducing agents that increase T-type currents, the oxidizing agent DTNB at 1 mM reversibly inhibited nRT T-type currents (Fig. 2B) by an average of 56 ± 6% (n = 10, P < 0.005). DTNB also more weakly inhibited both LD and VB T-type currents, by about 20% (n = 5–6, P < 0.01). Again in contrast to the effects of reducing agents, we did not observe any significant kinetic changes in T-type current with the application of 1 mM DTNB either in nRT neurons (3 ± 4% change, n = 5, P > 0.05) or VB and LD neurons (data not shown). The effects of DTNB on T-type currents in nRT neurons were fully mimicked by two other oxidizing agents, oxidized glutathione and MTSET. Oxidized glutathione, an endogenous redox agent, at 1 mM inhibited 35 ± 5% of current (P < 0.01, n = 6, data not shown); MTSET, a larger charged molecule, at 1 mM, inhibited 29 ± 3% of current (P < 0.01, n = 5, data not shown). We summarized the effects of l-cysteine, DTT and DTNB in three adjacent but functionally different nuclei in the histogram in Fig. 2B. These data indicate that redox agents differentially affect T-type channels in the thalamus.

The effects of redox agents were specific for T-type currents since, at the same concentration, DTNB and l-cysteine did not affect HVA Ca2+ current (Vh, −60 mV, Vc, −10 mV) in nRT neurons. DTNB at 1 mM had very little affect on peak HVA current (average 1 ± 1%, n = 4, P > 0.05, data not shown). Similarly, l-cysteine at 0.2 mM did not significantly affect peak HVA current (2 ± 1%, n = 5, P > 0.05, data not shown). In neither of the thalamic neurons did the application of redox agents significantly change leak current.

Direct effects of redox agents on T-type channels in nRT neurons

Our data are consistent with the existence of putative redox-sensitive sites on neuronal membrane that can modulate T-type channel behaviour in nRT neurons. Thus, we undertook to determine whether redox modulation of T-type currents was indirect, occurring in response to the release of some other mediators or neurotransmitters from the neighbouring neurons or glial cells in intact slices.

In nucleated patch recordings, after establishing whole-cell configuration, the recording electrode is slowly withdrawn until a small piece of somatic membrane with the cell’s nucleus and surrounding cytoplasm is pulled out (Sather et al. 1992; Joksovic et al. 2005b). Figure 3A and B shows that l-cysteine in nucleated patches increased rapidly, and reversibly peaked T-type current by about 50% (average, 51 ± 3%, n = 7, P < 0.01). Similarly, DTT increased peak T-type current by 40 ± 5% (n = 6, P < 0.01). In contrast, 1 mM DTNB reversibly inhibited T-type current by an average of 45 ± 4% (n = 5, P < 0.01; Fig. 3C). The histogram in Fig. 3D summarizes the effects of redox agents on nRT T-type currents in nucleated patches that are qualitatively similar to the effects seen in whole-cell recordings in intact slices.

Next we studied the effects of DTT in cell-free patches of the somatic membrane of nRT neurons. To record ensemble channel currents, we used barium-rich solution and voltage protocols similar to those we used in whole-cell and nucleated patch experiments. In the experiments in inside-out configuration, we found a lack of effects on the average ensemble T-type current peak and kinetics (2 ± 3% change, n = 4, P > 0.05; Fig. 3E). In contrast, in outside-out configuration, DTT fully mimicked the effects observed in whole-cell and nucleated patch experiments. Overall, DTT (0.2 mM) increased peak ensemble T-type current by 38 ± 8% (n = 3, P < 0.01) and significantly increased inactivation kinetics by 14 ± 7% (n = 3, P < 0.05; Fig. 3F). These data strongly suggest that the effects of redox agents on T-type currents in nRT neurons are direct and that redox sites on T-type channels that mediate the effects of redox agents are confined to the external side of the cell membrane. Consistent with the external membrane site of action for reducing agents, we found that membrane-impermeant reducing agent TCEP (tris(2-carboxyethyl)phosphine) (Cline et al. 2004) at 1 mM increased peak T-type current in nRT neurons in whole-cell recordings by 27 ± 6% (n = 5 cells, P < 0.01, data not shown).

Cav3.2-isoform-specific effects of reducing agents

To test the idea that direct modulation of specific T-type channel isomers underlies the selective modulation by redox agents in the thalamus, we recorded Ca2+ currents from the three recombinant T-type channel isoforms stably expressed in human embryonic kidney (HEK) cells. Since both native DRG and nRT cells express abundant mRNA for Cav3.2 (Talley et al. 1999), we initially examined the redox sensitivity of Cav3.2 channels. Consistent with our previous report (Todorovic et al. 2001), 0.1 mM DTT increased peak currents by an average of 2.5-fold, while 1 mM DTNB inhibited Cav3.2 currents by about 50% (Fig. 4B, lower panel). L-Cysteine mimicked the effects of DTT, significantly increasing Cav3.2 currents at 0.01 mM (n = 3, data not shown) and, at 0.1 mM achieving a near-maximal effect with an increase of

\[ n = 5 \) from a control value of \(-74.5 ± 0.2\) mV (k 6.9 mV), while DTNB (n = 5) shifted steady-state inactivation to more negative potentials by 5 mV (−79.8 ± 0.2 mV, k 8 mV). All points are averages from multiple cells; vertical lines are ±s.e.m.
Figure 6. The effect of redox agents on T-type current in wild-type and CaV3.2 knock-out mice

A, average traces of 7–10 consecutive sweeps from wild-type (WT; left) and knock-out (KO) mice (right) recorded from nRT regions (V_h = 100 mV, V_t = 50 mV) in brain slices. Note faster inactivation of T-type current recorded from the neuron in wild-type (τ = 65.3 ms) than KO mice (τ = 93.6 ms). Bars indicate calibration. B, lack of the CaV3.2 gene in KO mice prolonged inactivation kinetics of T-type currents in the nRT nucleus. The histogram represents average inactivation time constants (τ) of T-type currents recorded from nRT in WT (61.25 ± 5 ms, n = 17 cells) and KO mice (83.6 ± 5.3 ms, n = 12 cells); *P < 0.01. C, representative traces showing the effect of 0.2 mM DTT on T-type current in intact nRT neurons in slices from WT (left) and KO mice (right), respectively (control and wash, black trace; DTT, grey trace). Note that DTT increased peak current for 29% in WT mouse. In contrast, the traces almost overlap in recordings from KO mice. D, the lack of significant difference in T-type current density between the WT (16.18 ± 3.96 pA pF\(^{-1}\), n = 17 cells) and KO mice (13.01 ± 2.96 pA pF\(^{-1}\), n = 12 cells, P > 0.05). E, representative traces of whole-cell T-type currents recorded from nRT neurons in slices showing the similar inhibitory effect of 1 mM DTNB (V_h = 100 mV, V_t = 50 mV) in WT (33% decrease, left) and KO mice (33% decrease, right). Control trace before and after application is in black, and during the application of DTNB the trace is in grey. F, comparative effect of 0.2 mM DTT and 1 mM DTNB on T-type current from nRT WT and KO mice. DTT increased T-type current in the WT by 30 ± 1% (n = 5 cells) and had no significant effect on T-type current in KO mice (n = 8), while DTNB decreased T-type current by 39 ± 5% in WT (n = 7, P < 0.01) and 33 ± 4% (n = 8, P < 0.01) in KO mice. *Significant change from control peak current (dotted line).
108 ± 23% \( (n = 12, P < 0.001; \text{Fig. 4B}) \). Furthermore, both \( \text{CaV}_3.1 \) and \( \text{CaV}_3.3 \) were completely insensitive to even 10-fold higher concentrations of these reducing agents (Fig. 4A–C). In contrast, oxidation of the channel with 1 mM DTNB inhibited 58 ± 12% of \( \text{CaV}_3.1 \) current \( (n = 7, P < 0.001) \) and 40 ± 12% of \( \text{CaV}_3.3 \) current \( (n = 8, P < 0.001) \). The top panels in Fig. 4A–C show representative traces for the differential effects of l-cysteine (l-cys) on three isoforms of T-type channels; the bottom panels show histograms that summarize the effects of redox agents on these channels.

We performed biophysical studies to discern possible similarities in the mechanisms of redox modulation of nRT and \( \text{CaV}_3.2 \) T-type currents. Figure 5A and B compares normalized \( I–V \) curves for isolated T-type currents in the absence and presence of l-cysteine and DTNB. In all experiments, we obtained control data before applying the redox agent. Both l-cysteine and DTNB modulated native and recombinant currents across all test potentials. From these experiments we calculated an apparent voltage dependence of activation depicted in Fig. 5C and D. Neither l-cysteine nor DTNB had a large effect on the mid-point of activation \( (V_{50}) \) in either nRT neurons or recombinant \( \text{CaV}_3.2 \) channels. Similarly reduction of the channels had little influence on voltage-dependent inactivation assessed by the paired-pulse protocol. In contrast, oxidation with DTNB shifted steady-state inactivation to more hyperpolarized potentials by about 12 mV in native nRT channels and 5 mV in recombinant \( \text{CaV}_3.2 \) channels.

The preceding data strongly suggest that the \( \text{CaV}_3.2 \) channel is the main molecular substrate for redox modulation of neuronal T-type channels in the thalamus, and that reducing agents display high selectivity toward \( \text{CaV}_3.2 \)-based currents. To test this hypothesis directly, we used \( \text{CaV}_3.2 \) knockout (KO) mice (Chen et al. 2003) and compared the effects of DTT, l-cysteine and DTNB on whole-cell T-type currents on nRT neurons in slices in wild-type (WT) and \( \text{CaV}_3.2 \) KO mice. We compared average traces obtained at \( V_1 = -50 \text{ mV} \) from these two groups of mice (Fig. 6A). Consistent with the finding that \( \text{CaV}_3.2 \)-based currents exhibit three- to fourfold faster inactivation than do \( \text{CaV}_3.3 \) recombinant currents (Lee et al. 1999; see also upper panels of Fig. 4B and C, the average inactivation \( \tau \) in whole-cell recordings in WT mice was significantly faster (61 ± 5 ms, \( n = 17 \)) than that in KO mice (84 ± 5 ms, \( n = 12, P < 0.01; \text{Fig. 6B} \)). It is interesting that in spite of the changes in current kinetics, the average current density from WT and KO mice was not significantly changed \( (16 ± 4 \text{ pA} \text{ pf}^{-1} \text{ for WT}, n = 17; 13 ± 3 \text{ pA} \text{ pf}^{-1} \text{ for KO mice}, n = 12, P > 0.03; \text{Fig. 6D}) \), reflecting possible compensatory upregulation of the remaining, presumably \( \text{CaV}_3.3 \) currents.

Figure 6C shows representative traces of T-type currents from experiments in WT mice, in which we found that DTT (0.2 mM) similar to rat nRT neurons increased the peak current \( (30 ± 1%, n = 5, P < 0.01) \). In contrast, nRT T-type currents from KO mice were not significantly affected by DTT: there was no significant change in peak current \( (2 ± 7% \text{ change}, n = 8, P > 0.05) \). Similarly, l-cysteine \( (0.2 \text{ mM}) \) increased peak T-type current in nRT neurons of WT mice by \( 30 ± 6\% \) \( (n = 8, P < 0.01) \) but did not significantly affect currents in KO mice \( (4 ± 2% \text{ change}, n = 3, P > 0.05, \text{data not shown}) \). Consistent with our previous findings that DTNB inhibits all T-type channel isoforms non-selectively, oxidation with 1 mM DTNB inhibited about 40 and 30% whole-cell nRT T-type currents in WT \( (n = 7, P < 0.01) \) and KO mice, respectively \( (n = 8, P < 0.01; \text{Fig. 6E}) \). Figure 6F summarizes the effects of DTT and DTNB on nRT T-type currents in WT and \( \text{CaV}_3.2 \) KO mice. Overall, these data validate the results we obtained earlier with native and recombinant isoforms of thalamic T-type channels, and indicate that the \( \text{CaV}_3.2 \) channel in nRT neurons is selectively modulated by reducing agents.

Effects of redox agents on LTS and burst firing in nRT neurons

The ability of thalamic neurons to fire LTS allows burst firing of these neurons with small membrane depolarizations. It has been established that the LTS of nRT neurons is the initial critical event in the cascade of events leading to synchronization of low-amplitude oscillation in the loop of mutually connected nRT, thalamic relay and cortical neurons (Steriade, 2005). Thus, we tested the functional effect of redox modulation on spike firing in nRT neurons.

To study the effects of redox modulation of T-type currents on isolated LTS of rat nRT neurons, we recorded in whole-cell current-clamp mode in rat brain slices with a physiological internal solution and 1 \( \mu \text{m} \) TTX in the external solution to block generation of action potentials (APs). Neurons were hyperpolarized by a constant current injection to the membrane potentials at which LTS is prominent. Figure 7A shows a typical current-clamp experiment in which the application of 0.2 mM l-cysteine reversibly enhanced subthreshold membrane response to a depolarizing current and generated LTS in a rat nRT neuron \( (n = 7) \). Our protocol in these cells included five depolarizing pulses every 10 s before, during and after application of l-cysteine. The average probability of firing LTS was significantly increased from 13 ± 1% in control, to 80 ± 1% \( (P < 0.001) \) during application of l-cysteine, and was reversible upon wash of l-cysteine \( (14 ± 1\%) \). Furthermore, if cells were depolarized to membrane

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Figure 7. Redox agents modulate LTS and burst firing in whole-cell current clamp recordings from nRT neurons

A, representative traces recorded in whole-cell current clamp from a rat nRT neuron at the membrane potentials of $-82 \text{ mV}$ induced by a subthreshold current ($160 \text{ ms}$ duration) injection (left). Traces in the middle indicate that $0.2 \text{ mM} \text{ L-cysteine}$ evoked a prominent low-threshold $\text{Ca}^{2+}$ spike (LTS) in the presence of the same depolarizing stimulus. The effect was completely reversible on removal of L-cysteine from the external solution ($n = 7$ cells). B, LTS traces elicited with small depolarizations from a negative potential ($-79 \text{ mV}$) recorded in current-clamp mode. DTNB ($1 \text{ mM}$) reversibly abolished LTS (middle trace) and diminished the amplitude of the membrane response by 50% ($n = 5$ cells, $P < 0.005$). All experiments presented in A–C were recorded in the presence of $1 \mu\text{M}$ TTX in external solution to isolate LTS. C, traces recorded under similar conditions as A, except that the external solution in this and the next experiment (D) did not contain TTX. The trace on the left indicates the membrane response to subthreshold current injection. When the same protocol was repeated in the presence of $0.2 \text{ mM} \text{ L-cysteine}$ (middle trace), it elicited a high-frequency burst of five action potentials crowning the LTS ($n = 5$ cells). The effect was completely reversed by removal of L-cysteine from the external solution (right trace). D, high-frequency burst firing before (left), during (middle), and after (right) application of $1 \text{ mM}$ DTNB was elicited by the injection of a current pulse of $80 \text{ ms}$. DTNB reversibly decreased the number of action potential spikes from 5 to 2. Note: there was little change in the resting membrane potential (dotted lines) in the presence of either L-cysteine or DTNB (A–D). In all experiments, constant current injection was delivered via the recording electrode to achieve membrane potentials at which LTS is most prominent. E, traces before, after and during application of L-cysteine during hyperpolarizing pulse injection ($0.4 \text{ nA}, 285 \text{ ms}$) in an nRT cell. Not that L-cysteine increased rebound LTS amplitude by about twofold, while it had very little effect on resting membrane potential and the amplitude of the preceding hyperpolarizing pulse. F, a similar protocol was used in another nRT cell (current injection $0.3 \text{ nA}$ for $285 \text{ ms}$). DTNB diminished the amplitude of rebound LTS by $68\%$, while it had very little effect on passive membrane properties of the neuron. G, representative recordings from an nRT neuron in WT mouse in external solution not containing TTX. Subthreshold membrane response (left) was enhanced in the presence of L-cysteine into threshold LTS crowned with three spikes. The effect was again reversed after removal of L-cysteine from the bath (right). H and I, representative recordings from the same nRT neuron in $\text{CaV3.2 KO}$ mouse. Threshold current...
potentials above \(-50\) mV, at which T-type channels are inactivated, L-cysteine failed to alter the membrane response to subthreshold current injection \((n = 3, \text{ data not shown})\). In contrast, application of 1 mm DTNB abolished the amplitude of LTS (Fig. 7B) by reducing the membrane response to threshold current injection by \(51 \pm 2\%\) \((n = 5, P < 0.005)\).

In the next set of experiments, aimed at determining whether redox agents modulate burst firing of nRT neurons, the external solution did not contain TTX. Figure 7C shows that L-cysteine \((0.2 \text{ mm})\) at concentrations that significantly enhance T-type currents also reversibly increased the excitability of rat nRT neurons by generating LTS crowned with a burst of five APs. The number of firing APs was increased from an average of 0.0 to 5.5 \pm 1.0 with L-cysteine \((n = 5, P < 0.001)\). In contrast, Fig. 7D shows that 1 mm DTNB reversibly blocked LTS and reduced the number of APs from five to two \((\text{average}, 5.2 \pm 1.0 \text{ in control, } 2.3 \pm 1.0 \text{ with DTNB, } n = 5, P < 0.005)\). In none of these cells did the application of L-cysteine or DTNB significantly change the membrane potential or input resistance \((\text{Fig. 7E and F})\).

Our voltage-clamp studies indicate that effects of L-cysteine are isoform-specific and confined to the CaV3.2 T-type channel. Thus, we tested the hypothesis that effects of L-cysteine on LTS and spike firing in nRT neurons is due to modulation of CaV3.2 channels. We repeated our subthreshold protocol in WT mice \((\text{Fig. 7G})\) and found that similar to experiments in rat, 0.2 mm L-cysteine increased the average probability of firing LTS crowned with repetitive APs in five cells from 36 \pm 10\% in control to 72 \pm 9\% \((P < 0.001)\). Figure 7H depicts that sufficiently high threshold stimulus in nRT neurons from CaV3.2 KO mouse reliably evoked LTS and multiple APs. However, in four tested cells, L-cysteine failed to significantly alter subthreshold excitability as depicted on Fig. 7I. In average, probability to fire LTS in these cells from CaV3.2 KO mice was 25 \pm 9\% in control conditions and 20 \pm 9\% during application of 0.2 mm L-cysteine \((P > 0.05)\). These data directly implicate CaV3.2 T-type channel as the main molecular target for L-cysteine in the control of subthreshold excitability of nRT neurons.

**Discussion**

**Direct selective effect of reducing agents on T-type currents in nRT neurons**

There is increasing evidence that redox agents regulate the function of many proteins, including ion channels (reviewed in Stamler et al. 2001; Lipton et al. 2002).

We present evidence that reducing agents such as DTT and L-cysteine selectively enhance T-type currents in recombinant CaV3.2 channels and intact nRT neurons in brain slices. The lack of effect of DTT and L-cysteine on T-type current in thalamocortical VB and LD neurons correlates with the absence of CaV3.2 isoform in these regions (Talley et al. 1999).

On the other hand, oxidizing agents such as DTNB inhibit T-type currents in nRT, VB and LD neurons in the thalamus, as well as all three isoforms of recombinant T-type channels. We have found similar redox modulation of T-type current in rat sensory neurons, which may have an important function in amplifying peripheral sensory transmission (Todorovic et al. 2001). Furthermore, using intact native thalamic cells that express different isoforms of T-type channels, recombinant isoforms of T-type channels, and KO mice, we have now shown that the CaV3.2 isoform is the major molecular substrate for the effects of redox agents on T-type channels. This implies that redox agents that selectively and potently upregulate CaV3.2-based currents may modulate not only peripheral sensory transmission, but also sensory information flow at the level of the thalamus. These data also indicate that L-cysteine and DTT can be valuable tools for defining native CaV3.2 (α1H)-based T-type currents. Furthermore, our results indicate that human α1H channels respond to DTT and L-cysteine in a manner similar to native rat T-type channels implying that the mechanisms studied in rat and mouse are pertinent to human.

Several lines of evidence indicate that the effects of redox agents in our experiments are direct and confined to the postsynaptic targets in thalamic neurons, and are not caused by the release of other mediators from neuronal, glial or vascular tissue in intact slice preparation. We routinely observed the effects in nRT neurons in the presence of TTX and blockers of glutamate and GABA\(_A\) receptors, at concentrations that block synaptic transmission. Second, these effects were relatively fast and did not show any of the depression or facilitation commonly seen with synaptic physiology. Third, the effects were essentially identical in whole-cell recordings and nucleated outside-out patches, which allow direct examination of the modulation of putative redox-sensitive sites on postsynaptic membranes.

Our data with recombinant T-type channels indicate that CaV3.2 (α1H), one of the isoforms expressed in rat nRT neurons, is selectively modulated with reducing agents such as DTT and L-cysteine, indicating the presence of unique redox-sensitive modulatory sites. Similarly,
reduced glutathione enhances recombinant CaV3.2 but not CaV3.1 currents (Fearon et al. 2000). In contrast, oxidizing agents such as DTNB modulate all three isoforms of recombinant and native thalamic T-type channels. Beyond that, the different effects of reducing and oxidizing agents on the kinetics of T-type currents also indicate that multiple redox-sensitive modulatory sites exist on T-type channels in native neurons and recombinant channels. Most of the agents used in our experiments (e.g. DTT, L-cysteine, DTNB and MTSET) have been shown to interact with thiol groups in proteins (Stamler et al. 2001; Lipton et al. 2002). If cells possess a sufficient redox potential, oxidizing agents can react to form adducts on single sulphhydryl groups or, if two free sulphhydryl groups are nearby, more stable disulfide bonds may possibly be formed, which may, in turn, downregulate the function of T-type channels in nRT and, to a lesser degree, those in VB and LD thalamic neurons. In contrast, reducing agents can regenerate free sulphhydryl groups by donating electrons, and increase the current flow through T-type channels only in nRT neurons. Furthermore, our data with DTT in cell-free patches indicate that putative redox-sensitive sites that can be further reduced under physiological conditions are confined to the outside of the CaV3.2 channel membrane. Similarly, the inhibitory effect of extracellularly applied MTSET, a membrane impermeant oxidizing agent, strongly suggests that the putative redox-sensitive sites on T-type channels that can be oxidized are also located on the outside of the channel membrane. Future molecular studies will be necessary to elucidate the exact nature of these multiple redox-sensitive sites on T-type channels.

**Possible functional implications of redox regulation of T-type currents in the thalamus**

A key element in thalamic rhythm generation is the GABA-ergic nucleus reticularis (nRT), which is reciprocally connected to thalamocortical relay neurons of dorsal thalamic nuclei and also receives collateral excitatory connections from corticothalamic fibres within the internal capsule (Jones, 1985). A series of in vivo experiments using anatomical lesions has shown that the LTS of nRT neurons is the most important facet in the generation of spindle rhythmic activity and that this activity can persist even after nRT is anatomically disconnected from the rest of the brain (Steriade et al. 1985).

It has been hypothesized that in addition to their role in slow-wave sleep and loss of consciousness during absence seizure, LTS of nRT neurons and resulting spindling have an important function in the neuronal synaptic plasticity of cortical and thalamic neurons (Llinas et al. 1999; Steriade, 2005). Thus, any substance that may modulate intrinsic burst firing and the LTS properties of nRT neurons may have a widespread influence on the function of the mutually interconnected thalamic and cortical neurons. We found that redox agents regulate T-type currents and LTS and associated burst firing in nRT neurons in brain slices even in the presence of blockers of synaptic transmission, suggesting that they have a direct effect on the intrinsic cellular behaviour of these neurons. Thus, based on our current-clamp experiments with nRT neurons in brain slices in vivo, we speculate that upregulation of T-type currents and underlying LTS with reducing agents favours slow oscillatory rhythms that would dampen the sensory flow of information in vivo. However, downregulation of the function of T-type channels in thalamic neurons by T-type channel blockers and/or oxidizing agents may diminish neuronal excitability and subthreshold membrane oscillations. Moreover, the divergent effects of reducing and oxidizing agents in various regions of the thalamus that express functionally distinct isoforms of T-type channels indicate that local changes in the neuronal redox milieu can be important in fine-tuning of neuronal signalling (e.g. amplification with reducing agents, and dampening with oxidizing agents). Our findings that only CaV3.2 is upregulated by reducing agents, and that these agents increase burst firing of nRT neurons in WT rats and mice but not CaV3.2 KO mice, reveal an important role of this isoform in thalamic signalling. Such a role supports the hypothesis that single nucleotide polymorphisms that increase CaV3.2 channel activity might contribute to childhood absence epilepsy (Vitko et al. 2005).

The redox potential of tissue is maintained in a dynamic equilibrium influenced by local factors such as metabolism, temperature, pH, haemoglobin and oxygen tension. Previous studies have shown that during metabolic stress of CNS neurons induced, for example, by ischaemia or acidosis, the cellular environment moves toward a more reduced state (Auer & Siesjo, 1988; Slivka & Cohen, 1993). This suggests that upregulation of T-type channels during metabolic stress may contribute to an increase in cellular excitability during ischaemic episodes in CNS neurons. It is particularly important that L-cysteine, an endogenous reducing amino acid that is naturally present in the human brain and in the environment, is also a selective, direct and potent modulator of CaV3.2 T-type channels and LTS in nRT neurons. In the plasma, free L-cysteine concentrations reportedly are as high as 140 μM (Suliman et al. 1997). Some pathological conditions such as cerebral ischaemia in vivo are accompanied by even higher extracellular levels of L-cysteine up to 700 μM (Slivka & Cohen, 1993). Because significant enhancement of T-type currents and LTS occurs with L-cysteine at the lower concentrations, it appears that L-cysteine could be an important endogenous modulator of T-type currents and cellular excitability in both peripheral (Todorovic et al. 2001; Nelson et al. 2005) and
central sensory pathways. Previous studies have shown that l-cysteine is released in CNS neurons and may serve as an excitatory neurotransmitter (Do et al. 1986; Keller et al. 1989; Li et al. 1999); it may also function as an endogenous excitotoxin (Olney et al. 1990; Landolt et al. 1992; Schurr et al. 1993; Mathisen et al. 1996). However, all of these studies have attributed the in vitro and in vivo effects of l-cysteine and related compounds to their ability to interact with the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors. However, our data indicate that l-cysteine in vitro modulates native and recombinant CaV3.2 T-type currents and underlying burst firing of nRT neurons at concentrations that are 10- to 20-fold lower than those reported to affect NMDA currents (Pace et al. 1992). Thus, the potential involvement of CaV3.2 T-type channels as a target for this common sulfur-containing amino acid in physiological and pathological functioning of thalamocortical circuits warrants consideration.

References


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