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Reactive species modify $\text{Na}_V1.8$ channels and affect action potentials in murine dorsal root ganglia neurons

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Abstract

Dorsal root ganglia (DRG) neurons are important relay stations between the periphery and the central nervous system and are essential for somatosensory signaling. Reactive species are produced in a variety of physiological and pathophysiological conditions and are known to alter electric signaling. Here we studied the influence of reactive species on the electrical properties of DRG neurons from mice with the whole-cell patch-clamp method. Even mild stress induced by either low concentrations of chloramine-T (10 μM) or low-intensity blue-light irradiation profoundly diminished action potential frequency but prolonged single action potentials in wild-type neurons. The impact on evoked action potentials was much smaller in neurons deficient of the tetrodotoxin (TTX)-resistant voltage-gated sodium channel $\text{Na}_V1.8$ ($\text{Na}_V1.8^{-/-}$), the channel most important for the action potential upstroke in DRG neurons. Low concentrations of chloramine-T caused a significant reduction of $\text{Na}_V1.8$ peak current and at higher concentrations progressively slowed down inactivation. Blue light had a smaller effect on amplitude but slowed down $\text{Na}_V1.8$ channel inactivation. The observed effects were less apparent for TTX-sensitive Na_V channels. $\text{Na}_V1.8$ is an important reactive-species-sensitive component in the electrical signaling of DRG neurons, potentially giving rise to loss-of-function and gain-of-function phenomena depending on the type of reactive species and their effective concentration and time of exposure.

Keywords

Sodium channel; Pain; *Scn10a*; Aging; Radical stress; Reactive oxygen species

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Introduction

Dorsal root ganglia (DRGs) are relay stations between peripheral organs and the central nervous system, and they harbor the cell bodies of afferent sensory neurons that transduce signals in the periphery and conduct them to the spinal cord [20]. Each DRG comprises a heterogeneous population of neurons [46], differing in size of the cell bodies, diameter and myelination of the axons, and axonal conduction velocity. Small-diameter sensory neurons, most of which are nociceptors, typically form slower-conducting nerve fibers: the unmyelinated C fibers and thinly myelinated A δ fibers. DRG neurons of larger diameter are found in the fast conducting, well myelinated A α and A β fibers. The various functions of these nerve fibers, such as nociception and the sensing of touch and temperature changes, are mediated by specific sets of sensory proteins, including G protein-coupled receptors, tyrosine kinase receptors, and ion channels that are localized in the peripheral transduction zones of sensory neurons [20].

Like in many other neurons, voltage-gated Na⁺ (Na_V) channels play a key role in the generation and propagation of action potentials in DRG neurons. Large myelinated neurons abundantly contain tetrodotoxin-sensitive (TTX-s) subtypes Na_V1.1, Na_V1.6, and Na_V1.7 [4, 14] while small-diameter neurons mainly express the TTX-resistant (TTX-r) subtypes Na_V1.8 and Na_V1.9 in combination with Na_V1.7 and minor contributions of other TTX-s channels [4, 8, 14, 36]. However, this widely-held dichotomy is not absolute; Na_V1.8 channels are also found in subpopulations of the larger myelinated A-fiber neurons [14, 35, 43], for example.

Reactive oxygen species (ROS) are generated under physiological conditions such as mitochondrial metabolism or NADPH oxidase (Nox) activity and they can serve as signaling molecules [9, 27, 45]. However, excess ROS under pathophysiological conditions like in diabetic neuropathy [39, 47], axonal degeneration [33] or spinal cord injury [18], can lead to oxidative stress and cellular dysfunctions [6, 25]. The accumulation of oxidative modifications caused by ROS distorts electric signaling [10, 28, 41] and excess ROS has been implicated in neuropathic and inflammatory pain [38, 50]. In the pathogenesis of neuropathic pain, one of the contributing factors for the excess ROS is the Nox activity in macrophages that are recruited to DRGs in response to peripheral nerve injury [19]. During inflammation, DRG neurons themselves can be ROS producers as well, causing augmented pain sensitivity (hyperalgesia) as inflammatory signals cause upregulation of Nox1 and elevate ROS levels in DRG neurons, a mechanism impeded in neurons of mice deficient in Nox1 [16].

Reactive species may attack and modify multiple signaling proteins including ion channels in DRG neurons altering the propagation of nociceptive and other sensory signals. Oxidizing agents like chloramine-T (ChT) or 2,2'-dithio-bis(5-nitropyridine) modulate the inactivation of voltage-gated K⁺ channels in DRG neurons [15] and other cell types [34]. The main component of the ROS-sensitive A-type K⁺ current found in small DRG neurons most likely is K_V1.4 [15], a channel known to lose inactivation upon cysteine oxidation in the N-terminal ball domain [37]. Other K⁺ channels, such as Kv7 (KCNQ), are augmented by ROS acting as a second messenger in response to neurokinin stimulation [27]. Endogenously

produced ROS were shown to increase Na⁺ currents [49], but thus far it is not clear if all Na_V channel subtypes in DRG are sensitive to oxidative stress. Heterologously-expressed TTX-s subtypes Na_V1.2, Na_V1.4, Na_V1.5, and Na_V1.7 are modified by ChT (200 μM) leading to impaired inactivation [21]. However, the reactive-species sensitivity of Na_V1.8, which is the dominant contributor to the rising phase of the action potential in small diameter DRG neurons [36] and thus a key player for nociception [17], is unknown.

In this study we explore the acute impact of mild reactive species stress induced by ChT and blue light on action potentials in murine DRG neurons. We show that both stressors prolong single action potentials and affect Na⁺ currents – both of which are largely mediated via the functional modulation of Na_V1.8 channels.

Materials and methods

Chemicals

Solutions were made of high-grade chemicals obtained from Sigma Aldrich (Taufkirchen, Germany), Carl Roth (Karlsruhe, Germany), and FLUKA (St. Gallen, Switzerland). Tetrodotoxin (TTX) was obtained from International Clinical Service GmbH (Munich, Germany). Chloramine-T (ChT), H₂O₂, and tert-butyl hydroperoxide (tBHP) were diluted in the external solution to the desired concentration right before application; they were locally applied with a fine-tipped glass pipette in close proximity to the cell under consideration or by complete replacement of the bath chamber solution. In some experiments, dithiothreitol (DTT) was either supplemented to the bath solution or tip-filled in the patch pipettes.

Preparation of mouse DRG neurons

DRG neurons were prepared from wild-type mice as well as from mice lacking functional *Scn10a* (Na_V1.8^{-/-}) or *Scn11a* (Na_V1.9^{-/-}) genes [1,2]. The animals were anesthetized with ether and sacrificed by cervical dislocation according to protocols approved by the local animal welfare committee. Subsequently, the spinal cord was removed and longitudinally bisected. After removal of grey and white matter, DRGs from all levels of the spinal cord were extracted and stored in ice-cold buffer containing (in mM) 137 NaCl, 5.3 KCl, 5.3 MgCl₂, 3 CaCl₂, 25 sorbitol, 10 HEPES, pH 7.2 (with NaOH). DRG neurons were isolated by a combination of enzymatic treatment with liberase enzymes TH (20 min) and TM (10 min) (both from Roche Life Science, Mannheim) at 37 °C and mechanical trituration. Subsequently, neurons were suspended in DRG medium (89% DMEM/F12 high glucose plus glutamax, 10% fetal bovine serum, 4% penicillin/streptomycin) and seeded on glass cover slips coated with poly-*L*-ornithine. Cells were cultivated in a humidified incubator at 37 °C with 5% CO₂ until used for electrophysiological recordings.

Electrophysiological data acquisition and analysis

Whole-cell patch-clamp experiments were performed at room temperature (19–21 °C) up to 2 days after plating the cells, using small to medium-sized neurons (mean cell capacitance 12.48 ± 4.29 pF, mean ± SD, *n* = 286). Patch pipettes were fabricated from borosilicate glass, coated with silicone elastomer (RTV 615, KVD, Bad Wimpfen, Germany) and fire polished. Ground electrodes were protected from contact with bath solution by agar bridges.

All data were acquired with an EPC10 patch-clamp amplifier, operated with PatchMaster software (HEKA Elektronik, Lambrecht, Germany).

Current clamp—Action potentials were recorded in the current-clamp mode. Patch pipettes with resistances of 1.5 to 3 MΩ were used. Using a low-frequency voltage clamp, cells were kept at a holding potential around −83 mV to warrant stability and to ensure activity of most Na_V channel components, which otherwise might be inactivated. Trains of action potentials were elicited with 1-s current injections (Fig. 1), single action potentials with current injections of 10 ms (Figs. 2 and 3, Supplementary Fig. 1). The injected current was chosen such as to reliably trigger trains at a rate of 5–10 Hz or single action potentials. Stimulations were repeated every 10 s. The external solution contained (in mM) 140 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, and 10 HEPES, pH 7.3 with NaOH; the internal solution contained (in mM) 140 KCl, 0.5 EGTA, 5 HEPES, and 3 Mg-ATP, pH 7.3 with KOH. Data were corrected for the liquid junction potential of −3 mV; sampling rate was 20 kHz.

Voltage clamp—Patch pipettes with resistances of 1–2 MΩ were used. The holding and leak holding potential was set to −126 mV for Na_V1.8 currents and to −106 mV for TTX-s currents. Leak currents were subtracted using a p/6 leak correction protocol. The external solution contained (in mM) 35 choline-Cl, 100 NaCl, 3 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, and 10 HEPES, pH 7.3 with NaOH; when Na_V1.8 currents were to be recorded, the bath solution additionally contained 300 nM TTX. The internal solution contained (in mM) 140 CsF, 10 NaCl, 2 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, and 10 HEPES, pH 7.2 with CsOH. Data were corrected for the liquid junction potential of −6 mV. Currents mediated by Na_V1.8 channels were measured from DRG neurons of Na_V1.9^{−/−} mice in the presence of 300 nM TTX; TTX-sensitive Na_V currents were measured from DRG neurons of Na_V1.8^{−/−} mice, where cells with marked contributions from Na_V1.9 channels were discarded. Sampling rate was 20 kHz, and an anti-aliasing Bessel filter set to 5 kHz was used.

Blue-light stimulation—Cells were exposed to blue light from a 100-W mercury arc lamp (HBO 100 light source) directed to the objective of an upright fluorescence microscope (AxiObserver.A1, Carl Zeiss AG, Oberkochen, Germany) via the epifluorescence channel equipped with a filter set suited for the visualization of green fluorescent protein: excitation, BP 470/40; beam splitter, FT 495, thus providing excitation in the band of about 450–490 nm, here termed “blue light”. Excitation intensity was varied by using either a 20× dry objective (Objective LD A-Plan 20×/0.3 Ph1, NA 0.3, “low intensity”) or a 63× oil objective (Objective Plan-Apochromat 63×/1.40 Oil M27, NA 1.4, “high intensity”). The total power of the light passed through the 20× objective was ~4 mW.

Data analysis

Data analysis was performed with FitMaster (HEKA Elektronik) and IgorPro (WaveMetrics, Lake Oswego, OR, USA). Data traces shown in the figures were digitally low-pass filtered at 3 kHz. Ion channel or action potential parameters were determined in a time interval of 20 s before and between 140 and 160 s after application of stressors. The averaged relative changes are presented as means ± SEM (*n* = number of independent measurements) unless specified otherwise. Averaged data were tested for normal distribution. Subsequently, groups

of data were compared with paired or unpaired two-sided Student's *t*-tests ($\alpha = 0.05$) or with the Wilcoxon signed rank test, when applicable; the resulting *P* values are given explicitly.

Current clamp—The shapes of stimulated action potentials were characterized by four parameters: (1) the peak amplitude, (2) the “rise time” was measured from the first crossing of a threshold at -33 mV to the maximum amplitude; this threshold was chosen to eliminate the slow initial rise in potential, which is largely determined by the injected current and the capacitance of the cell. (3) The “decay time” is the time from the maximum amplitude to a threshold set at -63 mV. (4) The absolute area of the action potential between these thresholds at -33 mV and -63 mV, relative to the -63 -mV level, is referred to as “integral”.

Voltage clamp—Currents were evoked by depolarizing 20-ms pulses to -26 and 4 mV, applied at intervals of 5 s. The maximal peak inward currents and the degree of open-state inactivation were analyzed. The latter was characterized by an inactivation indicator, R_i , relating the peak current (I_{peak}) to the mean current integral following the peak time (t_{peak}) until the end of the 20-ms pulse:

$$R_i = \frac{\int_{t_{\text{peak}}}^{20 \text{ ms}} I(t) dt}{I_{\text{peak}} \times (20 \text{ ms} - t_{\text{peak}})} \quad (1)$$

Before and about 10 min after stressor application of 5 min, currents were measured at various voltages ranging from -86 to 24 mV, in steps of 10 mV. Mean peak currents as a function of test potential were described with a Hodgkin-Huxley formalism with three independent gates according to

$$I(V) = \frac{\gamma (V - E_{\text{rev}})}{\left(1 + e^{-(V - V_m)/k_m}\right)^3} \quad (2)$$

with the absolute conductance Γ , the reversal potential E_{rev} (constrained to 53 mV), the half-maximal gate activation voltage V_m , and the slope factor k_m expressing the voltage dependence.

Results

Mild oxidative stress affects action potentials of small DRG neurons

We followed two strategies to investigate the dependence of electrical signaling in DRG neurons on stress conditions. Chloramine-T (ChT) was selected as a mild oxidant with some preference to oxidize methionine residues [3, 42] and because chloramines are also physiologically generated by means of myeloperoxidase and implicated in inflammatory

diseases [23, 32]. From previous experiments on recombinant $\text{Na}_V1.4$ channels ChT at 1 mM was expected to have a profound effect on the time course of inactivation, primarily because the methionine in the inactivation domain (IFM, isoleucine-phenylalanine-methionine) is oxidized [21]. It was also shown that 100 μM ChT has a negligible effect on $\text{Na}_V1.4$ channels when applied for 200 s [21]. We therefore chose 10 μM ChT to simulate mild stress conditions, and investigated its impact on the action potential firing of mouse DRG neurons after an application period of 150 s. As shown in Fig. 1a, weak current injections in the current-clamp mode evoked trains of action potentials and the application of ChT did not have a major impact on the action potential shape but reduced their frequency about twofold while leaving the resting membrane potential unchanged (Fig. 1b). As an alternative source of stress we chose to illuminate the cells with blue light (450–490 nm) because it was previously shown that visible light results in the generation of reactive species [24] and causes oxidative modifications even when applied at relatively low intensity [31]. In addition, the use of light as stressor bears the advantage of avoiding any mechanical agitation that might affect the cell under study, e.g. by activating mechano-sensitive cation channels expressed in DRG neurons [5, 29]. To achieve low-intensity excitation, we illuminated the cells for 150 s via a 20 $\times$ dry objective. As shown in Fig. 1c and d, the action potential frequency was diminished significantly, while the resting membrane potential was not altered. Thus, very mild stress via ChT or blue light has a significant impact on the electrical excitation of mouse DRG neurons.

Action potentials in DRG neurons of $\text{Na}_V1.8^{-/-}$ mice are less sensitive to stress conditions

The shape of the action potentials was investigated in more detail by evoking them individually with 10-ms current injections adjusted in amplitude to reliably elicit an action potential per pulse. The application of ChT (10 μM) consistently and noticeably affected the shape of the action potentials (Fig. 2a, *top, left*). Although not statistically significant (paired t -test, $P = 0.093$), there was a trend to increase the action potential rise time (by $10.7 \pm 5.1\%$). The decay time from the action potential peak to a threshold at -63 mV was significantly increased by $15.1 \pm 3.6\%$ ($n = 12$; $P < 0.001$). A similar increase was observed for the action potential integral between the first and second threshold: $12.4 \pm 2.3\%$ ($P < 0.001$). Furthermore, ChT application caused a minor reduction of the peak amplitude by -2.4 ± 1.0 mV ($P = 0.039$). Slow-down of action potential upstroke and broadening of action potentials were much more apparent after application of 100 μM ChT (Fig. 2a, *top, right*): rise time, $+23.1 \pm 8.3\%$ ($P = 0.049$); decay time, $+51.8 \pm 11.1\%$ ($P < 0.001$); integral, $+43.5 \pm 8.0\%$ ($P < 0.001$). The peak amplitude was decreased by 2.2 ± 1.0 mV ($P = 0.044$).

Various lines of evidence suggest that Na_V channels are potential targets of oxidative stress [13, 21, 31, 49, 50]. We therefore investigated action potentials in DRG neurons from mice with *Scn10a* genes disrupted, i.e. lacking functional $\text{Na}_V1.8$ channels ($\text{Na}_V1.8^{-/-}$). Although $\text{Na}_V1.8$ channels substantially contribute to the upstroke of the action potentials in DRG neurons [36], using a slow voltage clamp to hold the cells most of the time at -83 mV ensured that current injection into DRG neurons of $\text{Na}_V1.8^{-/-}$ mice also reproducibly evoked single action potentials. Application of 10 μM ChT had a much weaker impact on the shape of the action potentials compared with wild-type neurons (Fig. 2a, *bottom*). The rise time was not significantly altered, while the decay time was increased by $5.4 \pm 1.6\%$ (P

= 0.005) and the integral by $4.7 \pm 1.7\%$ ($P = 0.014$). With 100 μM ChT: decay time, $+15.2 \pm 6.5\%$ ($P = 0.039$), integral, $+16.2 \pm 4.7\%$ ($P = 0.005$), peak amplitude, $-8.2 \pm 1.5 \text{ mV}$ ($P < 0.001$). When results from wild-type cells and DRG neurons of $\text{Na}_V1.8^{-/-}$ mice are compared (Fig. 2b), it becomes evident that the lack of $\text{Na}_V1.8$ channels does not change the impact of ChT on the rise time but significantly reduces the prolongation of the action potential as evidenced by the decay time and the integral, thus identifying $\text{Na}_V1.8$ channels as a potential target of ChT-induced modification.

In a separate series of experiments we triggered reactive species production in DRG neurons by illuminating cells with blue light. As shown in Fig. 3a (*top, left*), low-intensity light applied via a 20 $\times$ objective modestly increased the rise time, the decay time, and the integral (Supplementary Tab. 1), while the same stimulation had no effect on action potentials in DRG neurons from $\text{Na}_V1.8^{-/-}$ mice (Fig. 3a, *bottom, left*). Comparison of the results from wild-type and $\text{Na}_V1.8^{-/-}$ neurons clearly shows that the action potentials of the latter are significantly less sensitive to blue-light stress with respect to the duration (Fig. 3b). The absolute difference in sensitivity became even more apparent when blue light of higher intensity (63 $\times$ objective) was applied (Fig. 3a, *right*, Fig. 3b). Thus, also when blue light is chosen as reactive species source, the primary impact is a broadening of the action potentials, and this effect is strongly diminished when $\text{Na}_V1.8$ channels are absent.

Action potentials in DRG neurons are not only sensitive to ChT or blue light but also to H_2O_2 or tBHP, both of which significantly broadened evoked action potentials (Supplementary Fig. 1), albeit to a smaller extent.

Reactive species preferentially affect $\text{Na}_V1.8$ channels in mouse DRG neurons

The action potential measurements suggest that $\text{Na}_V1.8$ channels are particularly sensitive to the stress conditions applied. We therefore assayed the function of Na_V channels in DRG neurons under conditions where the contribution of other channels (e.g. K^+ and Ca^{2+} channels) was largely eliminated. $\text{Na}_V1.8$ -mediated currents were measured in DRG neurons from $\text{Na}_V1.9^{-/-}$ mice in the presence of 300 nM TTX to suppress TTX-s current components, such as $\text{Na}_V1.7$. TTX-s Na_V channels were assayed in select DRG neurons from $\text{Na}_V1.8^{-/-}$ mice that additionally lacked noticeable contributions of $\text{Na}_V1.9$ channels.

Because it is expected that reactive species ultimately cause severe modifications in any protein, differences in sensitivity towards reactive species will primarily be reflected in the kinetics of modification in response to stressor application. Therefore, we measured Na^+ currents at -26 and 4 mV in a repetitive manner where 100 μM ChT was applied at time zero. In Fig. 4a and b the results of such experiments are summarized, clearly demonstrating that the peak inward current of $\text{Na}_V1.8$ channels is strongly diminished within about a minute after ChT application, while currents through TTX-sensitive channels initially increased, followed by a slow reduction in amplitude (Fig. 4b). Similar results were obtained for application of blue light (Fig. 4c, d), although the exact time courses differed from those obtained with ChT and the differences between $\text{Na}_V1.8$ and TTX-s channels were less pronounced. These experiments show that the functional properties of $\text{Na}_V1.8$ channels are more strongly dependent on the stressors applied and that reactive species from different sources cause different types of functional modification. A prominent observation is that the

change of peak current with stressor application is composed of multiple phases (e.g. Fig. 4b), already indicating that several processes must occur. One reason is the additional impact of stressors on rapid channel inactivation as documented in Supplementary Fig. 2. Overall, long-term exposure decreases the peak current amplitude in all cases and at both voltages investigated.

To gain insight into the mechanisms involved, similar experiments were performed with 2 mM of the reducing agent DTT, which is slowly membrane permeable, in the patch pipette. For blue light exposure the effect on the peak current amplitude was greatly diminished by DTT, both for Na_v1.8 channels and for TTX-s currents (Fig. 4c, d). However, intracellular DTT potentiated the impact of ChT on the peak current for Na_v1.8 channels (Fig. 4a). A likely explanation is provided in Supplementary Fig. 4, which illustrates that intracellular DTT strongly diminished the impact of (extracellularly applied) ChT on Na_v1.8 inactivation, while it has only a marginal effect on the loss of inactivation induced by blue light. Extracellular DTT, however, effectively “protected” Na_v1.8 channels from modification by blue light. Taken together, these experiments not only demonstrate that ChT and blue light exert effects on the channels observed via intermediates that can be scavenged with DTT, it also illustrates that the sidedness of reactive species occurrence (intracellular vs. extracellular) has an important influence on the quality and magnitude of the functional changes observed.

Given the results shown in Fig. 4, we analyzed the current signals in more detail before and 150 s after stressor application, also including the weak stimuli. As shown in Fig. 5a and b, the major apparent effect of 10 μM ChT was a reduction of the peak current, while TTX-s currents were unaffected ($P < 0.001$ for a comparison of Na_v1.8 and TTX-s channels). At 100 μM ChT, the reduction of peak current was less pronounced (presumably because of loss of inactivation, see below), and the differences between Na_v1.8 and TTX-s channels were less apparent. Low concentrations of ChT (10 μM) had no significant impact on the speed of inactivation; at 100 μM ChT, inactivation became consistently slower (expressed as change in inactivation index, R_i , estimated with Eq. (1)), but the effect on Na_v1.8 channels was not significantly greater than that on TTX-s channels (Fig. 5c). Thus, at low ChT stress, the peak amplitude of Na_v1.8 channels is more strongly reduced than that of TTX-s channels, while at higher concentrations of ChT the progressive loss of inactivation, which happens in both channel types, partially compensates the peak current reduction and, hence, overwrites the differences between Na_v1.8 and TTX-s channels.

The same analysis was performed for experiments in which blue light was applied. Unlike for low concentration of ChT, low-intensity blue light did not significantly affect the peak amplitude of currents mediated by Na_v1.8 and TTX-s channels (Fig. 6a, b). However, inactivation was slowed down in both cases; at -26 mV this latter effect was even more pronounced in Na_v1.8 channels (Fig. 6c). At high light intensity, the peak current was consistently reduced for both channel types at low voltage; at 4 mV, peak current loss was stronger for Na_v1.8 channels. The slow-down of inactivation, however, was significantly ($P < 0.001$) stronger in Na_v1.8 channels than in TTX-s channels (Fig. 6c). Taken together, under both forms of reactive species stress, the functional consequences for Na_v1.8 channels appear larger than for TTX-s channels, thus corroborating the inferences from the action

potential measurements, which already indicated a prominent role of Na_v1.8 channels as elements sensitive to reactive species in the electrical signaling via DRG neurons.

As already anticipated from the results shown in Fig. 4, long-term exposure to reactive species inevitably results in some loss of function. Analysis of current-voltage relationships before and 10 min after application of stressors (100 μ M ChT or high-intensity blue light) revealed a reduction of peak current of about 25–40% with only marginal changes in the voltage dependence of the remaining currents (Supplementary Fig. 3).

Discussion

Radical stress, caused by reactive species under physiological and pathophysiological conditions, is implicated in diseases of the peripheral nervous system such as persistent pain following spinal cord injury [18] and diabetic neuropathy [47]. Given the wide range of molecular targets, reactive species have the propensity to damage cells in multiple ways. However, low concentrations of reactive species might also have physiological regulatory influences on neuronal excitability [27]. Here we aimed at elucidating the impact of low concentrations of reactive species, i.e. those that do not obviously lead to an unspecific deterioration of the cellular components, on the electrical properties of murine DRG neurons, particularly on Na_v1.8 channels.

As sources for reactive species we used ChT, a known oxidant and preferential modifier of cysteines and methionines, in concentrations of 10 and 100 μ M; the concentrations were chosen according to previous experiments on Na_v1.4 channels for which even 100 μ M ChT exerts only marginal functional alterations [21]. In addition, reactive species were generated by blue-light irradiation [12, 22, 31]. This type of stress induction is particularly useful as it can be timed precisely and does not involve any mechanical agitation that may otherwise confound with mechano-sensitive components in DRG neurons [5, 29]. We therefore also used blue light (450–490 nm) as a source to generate reactive species, directed to the cells under consideration via 20 $\times$ or 63 $\times$ microscope objectives. ChT and blue light both diminished the frequency of action potentials, thus presumably reducing the transmitter release at the neuronal terminal *in vivo*. On the other hand, the same stressors consistently altered the shape of evoked action potentials, mainly by prolonging the duration. This effect is reminiscent of an action potential prolongation previously found in aged mice [40]. The observed prolongation might suggest an increase in overall signal strength, a stronger Ca²⁺ influx and, hence, an elevated release of neurotransmitters per single action potential, likely partially compensating for the observed reduction in action potential frequency. However, some caution is warranted for conclusions regarding the *in-vivo* situation as the ion channel composition of dissociated cultured neurons investigated here may deviate from those of the nerve terminals and the synapses.

Although action potentials in small DRG neurons are mainly driven by Na_v1.8 channels, individual action potentials can also be evoked in cells from Na_v1.8^{-/-} mice when the resting membrane potential is kept hyperpolarized enough to keep TTX-s channels in a non-inactivated state. Such action potentials showed a much smaller response to the same

stressor treatments than action potentials of wild-type cells, indicating a particular importance of Na_v1.8 channels for ROS-sensitive signal transmission in DRG neurons.

The anticipated importance of Na_v1.8 was further substantiated by investigating Na_v1.8 currents in Na_v1.9^{-/-} DRG neurons in the presence of TTX. Previous studies have shown that inflammatory conditions increase resurgent currents of TTX-r sodium channels in DRG neurons [44], indicating a contribution of Na_v1.8 channels. In the present study, reactive species, known to be one of the key players in inflammatory responses, seem to have different effects that strongly depended on the type of reactive species present: ChT mostly reduced the peak current and slowed down inactivation at higher concentrations. In contrast, blue light illumination predominantly slowed down inactivation of Na_v1.8 and reduced the peak current only at higher intensities.

In particular after short durations of stress exposure, functional impacts on Na_v1.8 channels were significantly stronger than on TTX-s channels, both with respect to peak current amplitude and subsequent loss of inactivation. The opposing effects of “decrease of peak current” and “loss of inactivation” have the consequence that the total outcome for the overall current mediated by Na_v1.8 channels chiefly depends on the exact dose and timing of stress application: a rapid reduction in inward current amplitude is followed by an overcompensation via loss of inactivation until, ultimately, reactive species stress results in loss of function. Since only mild stimuli will be relevant for physiological processes, the initial decrease of Na_v1.8 amplitude might be the most important factor for regulating the electrical activity of DRG neuronal firing. In particular under such conditions, the types of reactive species presumably will be decisive for the overall outcome.

The intricate dependence of the functional outcome on the precise experimental or physiological circumstances is particularly well illustrated by experiments in which the impact of extracellular ChT application is compared with or without the reducing agent DTT in the patch pipette: DTT strongly augmented the ChT-induced time-dependent decline of peak amplitude (Fig. 4a); the most likely explanation is that intracellular DTT diminishes the impact of reactive species originating from externally applied ChT on channel inactivation (Supplementary Fig. 2a). Given that the molecular elements of the Na_v channel protein important for fast inactivation, at the first place the inactivation motif “IFM” in the domain III–IV linker, are accessible from the cytosolic side of the membrane and that oxidative modification of the methionine in this motif confers oxidation sensitivity to inactivation [31], intracellular DTT scavenges intracellular reactive species, thus effectively protecting cytosolic structures of the Na_v1.8 channel from being modified. With unmodified inactivation in place, the effect of extracellular ChT on other channel parameters (conductance combined with number of activated channels) becomes fully visible. The molecular mechanism still needs to be elucidated, but extracellularly accessible sulfur-containing amino acids, such as cysteines or methionines in the linkers of the pore-forming S5–S6 linkers, are likely targets.

The situation appears more complex for channel modification induced by blue light. While the effect on the peak current was smaller than for ChT application, internal and external DTT both diminished the blue-light impact on peak current (Fig. 4c), and only external DTT

efficiently reduced the loss of channel inactivation induced by blue light (Supplementary Fig. 2c). One obvious difference is the nature of reactive species produced: blue light is expected to specifically produce hydrogen peroxide, superoxide and singlet oxygen, some of which can easily cross the membrane [24], which make the association to a particular sidedness complicated. Furthermore, while ChT is applied from the outside and reactive components will only subsequently enter the cell, the site of blue-light generated reactive species is not so clear. One would assume that the density of absorbers for blue light, such as flavonoids, is high inside the cell and the cell membrane, thus yielding the highest production rate of reactive species inside cells. This, however, would not explain why extracellular DTT so efficiently counteracts the blue-light effect on channel inactivation. Therefore, one has to consider the extended extracellular glycocalyx with its associated proteins as site of photon absorption. In addition, the volume of the extracellular solution in the focus of the objective, although not absorbing light at high efficiency, is several orders of magnitude larger than the volume of the cell. Furthermore, DTT can cross the membrane and, hence, an excess of extracellular DTT may result in a significant intracellular DTT concentration in the whole-cell recording configuration.

The molecular mechanism underlying the particular sensitivity of $\text{Na}_V1.8$ channels remain to be elucidated, but it is quite likely that the unique relationship of kinetics and voltage dependence of $\text{Na}_V1.8$ channel activation and inactivation play an important role. A possible contribution of such oxidative regulation to the diversity of observed $\text{Na}_V1.8$ channel kinetics can be inferred from Supplementary Fig. 2c: extracellular DTT already significantly accelerated $\text{Na}_V1.8$ channel inactivation before additional stress application. This implies that the degree of $\text{Na}_V1.8$ inactivation may already fluctuate under “control” conditions owing to differences in the extracellular milieu.

$\text{Na}_V1.8$ is particularly sensitive towards different types of reactive species and particularly important for ROS-sensitive signal transmission. The complex kinetics and voltage dependence of the functional changes observed for $\text{Na}_V1.8$ currents – ranging from loss-of-function to gain-of-function – anticipate an intricate relationship between type and dose of the stressor on the one hand and electrical activity of DRG neurons on the other. This is obvious from the results on Na_V channels shown here, but it will be supported further by other ROS-sensitive signaling elements in DRG neurons, such as A-type potassium channels, which are expected to diminish neuronal activity via ROS-induced loss of N-type inactivation [15, 37]. Moreover, M-type K^+ channels – important for setting the resting membrane potential in DRG neurons – may be oxidatively augmented [11], resulting in a hyperpolarized membrane potential. However, the resting membrane potential was not altered in our experiments, indicating no significant contribution from M-type K^+ channels under the conditions examined here. Although the contribution of Slo1 BK channels to DRG action potentials are still not fully understood [26], the oxidative inhibition of these channels may lead to prolonged action potentials, thus indirectly augmenting the influence of depolarizing Na^+ currents [7]. The low-voltage-activated Ca^{2+} channel $\text{Ca}_V3.2$ is predominantly expressed in small DRG neurons and is involved in setting the threshold for action potentials and contributes to the shoulder of the action potential decay phase. The sensitivity of this channel may thus contribute to a reactive-species-dependent modulation of DRG electrical signaling as well [30].

Because $\text{Na}_V1.8$ channels are emerging as a target for the treatment of pain [51], the results reported herein may shed some light on various pain conditions, known to be caused or exacerbated by oxidative stress, such as in diabetic neuropathy. In a physiological context, however, it is also conceivable that $\text{Na}_V1.8$ acts as an ROS sensor to indicate various physiological and potentially pathophysiological conditions to upstream components in the nervous system and the brain. In particular the initial reduction of $\text{Na}_V1.8$ current amplitude when exposed to mild stress may constitute a protection mechanism against overexcitation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ChT	chloramine-T
DRG	dorsal root ganglion
DTT	dithiothreitol
NADPH	Nicotinamide adenine dinucleotide phosphate
Nox	NADPH oxidase
ROS	reactive oxygen species
tBHP	tert-butyl hydroperoxide
TTX	tetrodotoxin
TTX-r	tetrodotoxin-resistant
TTX-s	tetrodotoxin-sensitive
wt	wild type

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Fig. 1.

Reactive species lower action potential frequency in mouse DRG neurons. **a** Representative trains of action potentials recorded from mouse DRG neurons before (Ctrl, *top*) and 150 s after application of 10 μ M of the mild oxidant ChT to the bath solution (*middle*). The current stimulus used to evoke the action potentials is shown at the *bottom*. Dashed lines indicate 0 mV (*top*) and 0 pA (*bottom*), respectively. **b** Mean action potential frequency and resting membrane potential before and after ChT application. **c** Data as in **a**, but reactive species were generated by illuminating the cell under consideration with blue light (450–490 nm, 150 s) through a 20 $\times$ dry objective. **d** Mean action potential frequency and resting membrane potential before and after illuminating the cells. Data in **b** and **d** are mean $\pm$ SEM with n indicated in parentheses. Wilcoxon signed rank test: n.s., not significant; ***, $P < 0.001$.

Fig. 2.

Effect of chloramine-T (ChT) on evoked action potentials. **a** Superposition of representative action potentials evoked with the indicated current injections (*bottom*) before (black) and 150 s after application of 10 μ M (*left*) or 100 μ M ChT (*right*) for DRG neurons from wild-type (*top*) or NaV1.8^{-/-} mice (*bottom*). The thresholds used to measure the time to peak, the decay time, and the integral between both thresholds are indicated with dotted lines in the first panel (*top, left*); dashed lines indicate 0 mV (*top*) and 0 pA (*bottom*), respectively. **b** Fold change (on a logarithmic scale) of the indicated action potential parameters induced by

10 or 100 μ M ChT for the wild type (*open circles*) or $\text{Na}_v1.8^{-/-}$ (*filled circles*). Data are mean $\pm$ SEM for $n = 12$ –14; statistics indicators refer to unpaired two-sided t -tests between wild-type and the corresponding knockout data: n.s., not significant; *, $P < 0.05$; **, $P < 0.01$.

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Fig. 3.

Effect of blue light on evoked action potentials. **a** Superposition of representative action potentials evoked with the indicated current injections (*bottom*) before (black) and 150 s after illuminating the cells with blue light (450–490 nm) of low intensity (20× dry objective, *left*) or high intensity (63× oil objective, *right*) for DRG neurons from wild-type (*top*) or $\text{Na}_V1.8^{-/-}$ mice (*bottom*). The thresholds used to measure the time to peak, the decay time, and the integral between both thresholds are indicated with dotted lines in the first panel (*top, left*); dashed lines indicate 0 mV (*top*) and 0 pA (*bottom*), respectively. **b** Fold change

(on a logarithmic scale) of the indicated action potential parameters induced by low- or high-intensity blue light for the wild type (*open circles*) or $\text{Na}_v1.8^{-/-}$ (*filled circles*). Data are mean $\pm$ SEM for $n = 12\text{--}14$; statistics indicators refer to unpaired two-sided t -tests between wild-type and the corresponding $\text{Na}_v1.8^{-/-}$ data: n.s., not significant; *, $P < 0.05$; **, $P < 0.01$.

Fig. 4.

Kinetics of peak current changes of voltage-gated Na^+ channels in DRG neurons. Peak inward Na^+ currents, elicited by depolarization to -26 mV and 4 mV, were measured in DRG neurons from $\text{Na}_v1.9^{-/-}$ mice in the presence of TTX ($\text{Na}_v1.8$, **a**, **c**) and from $\text{Na}_v1.8^{-/-}$ mice in the absence of TTX (TTX-s, **b**, **d**) to assay the kinetics of effects exerted by application of $100 \mu\text{M}$ ChT (**a**, **b**) or irradiation with blue light via a $63\times$ objective (**c**, **d**). Black data points were obtained under control condition, grey data points with 2 mM DTT added to the pipette (internal) solution. Light blue data points in panel **c** additionally represent experiments with 2 mM DTT in the bath solution. Data are mean $\pm$ SEM with n values indicated in the panels.

Fig. 5.

Influence of chloramine-T (ChT) on voltage-gated Na^+ channels in DRG neurons. **a top**, Superposition of current traces at 4 mV recorded from a DRG neuron of a $\text{Na}_V1.9^{-/-}$ mouse in the presence of 300 nM TTX before (black) and 150 s after application of 10 μM (left) and 100 μM ChT (right). **a bottom**, Equivalent to the top panels but for TTX-sensitive Na_V currents recorded from DRG neurons of $\text{Na}_V1.8^{-/-}$ mice in the absence of TTX. **b** Mean percent change of peak current at -26 and 4 mV for $\text{Na}_V1.8$ currents and TTX-sensitive Na_V currents for the indicated ChT concentrations. **c** Change in inactivation indicator (see Eq. 1)

induced by ChT application, for the indicated voltages and current types. Data are mean $\pm$ SEM for $n = 14$ and 15 (Na_v1.8) and $n = 12$ and 12 (TTX-sensitive); statistics indicators refer to unpaired two-sided t -tests between Na_v1.8 and TTX-sensitive current data: n.s., not significant; *, $P < 0.05$; ***, $P < 0.001$.

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Fig. 6.

Influence of blue light on voltage-gated Na^+ channels in DRG neurons. **a top**, Superposition of current traces at 4 mV recorded from a DRG neuron of a $\text{Na}_V1.9^{-/-}$ mouse in the presence of 300 nM TTX before (black) and 150 s after application of blue light via a 20× objective (*left*) or a 63× objective (*right*). **a bottom**, Equivalent to the top panels but for TTX-sensitive Na_V currents recorded from DRG neurons of $\text{Na}_V1.8^{-/-}$ mice in the absence of TTX. **b** Mean percent change of peak current at -26 and 4 mV for $\text{Na}_V1.8$ currents and TTX-sensitive Na_V currents for the indicated illuminations. **c** Change in inactivation

indicator (see Eq. 1), induced by blue light, for the indicated voltages and current types. Data are mean $\pm$ SEM for $n = 14$ and 15 (Na_v1.8) and $n = 14$ and 14 (TTX-sensitive) for 20 $\times$ and 63 $\times$, respectively; statistics indicators refer to unpaired two-sided t -tests between Na_v1.8 and TTX-sensitive current data: n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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