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Millisecond infrared laser pulses depolarize and elicit action potentials on *in-vitro* dorsal root ganglion neurons

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Abstract: This work focuses on the optical stimulation of dorsal root ganglion (DRG) neurons through infrared laser light stimulation. We show that a few millisecond laser pulse at 1875 nm induces a membrane depolarization, which was observed by the patch-clamp technique. This stimulation led to action potentials firing on a minority of neurons beyond an energy threshold. A depolarization without action potential was observed for the majority of DRG neurons, even beyond the action potential energy threshold. The use of ruthenium red, a thermal channel blocker, stops the action potential generation, but has no effects on membrane depolarization. Local temperature measurements reveal that the depolarization amplitude is sensitive to the amplitude of the temperature rise as well as to the time rate of change of temperature, but in a way which may not fully follow a photothermal capacitive mechanism, suggesting that more complex mechanisms are involved.

OCIS codes: (140.3070) Infrared and far-infrared lasers; (170.0170) Medical optics and biotechnology; (170.1530) Cell analysis; (350.5340) Photothermal effects.

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1. Introduction

Infrared laser light has been shown to stimulate neurons and nerves [1]. Using such a light has real advantages for controlling neuron activities: contactless, good spatial and temporal resolution. Infrared (IR) neural stimulation has been demonstrated to activate numerous nerves such as sciatic nerves [2,3], cavernous nerves [4], auditory nerves [5]. Stimulations were also achieved on cortex [6]. Although promising, clinical developments of such approaches have been restrained by the lack of information regarding the underlying mechanisms. IR light is absorbed by water [7]. Hence, IR laser pulses induce transient heat pulses in tissue. Among the possible biophysical mechanisms underlying the laser-tissue interaction, such as photochemical, photothermal, photomechanical, photoablation [8], the photothermal effect appears to be the most likely [9].

To advance in the understanding of the mechanism or of the mechanisms at the cellular and molecular levels, the IR laser stimulation has been applied on isolated neurons or cells. Infrared laser pulses have been shown to induce intracellular calcium transients implicating mitochondria in neonatal cardiomyocytes [10] and in neonatal spiral and vestibular ganglion neurons [11] and to depolarize membranes in HEK293 cells [12], dorsal root ganglion neurons [13], oocytes, HEK cells and artificial layers [14], retinal and vestibular primary neurons [15,16], hippocampal neurons [17], spiral ganglion neurons [18], brain slices [19] and in vestibular hair cells and afferent neurons [20]. What remains unclear is whether an universal photothermal mechanism exists and how the transient heating induced by the IR laser pulse elicits membrane depolarization of neurons and action potentials or modulates intracellular signalling. Several mechanisms of action have been suggested, including the generation of transient capacitive currents [14,17], the stimulation of temperature sensitive ion channels [15,16], the generation of small pores in the plasma membrane [21] or the generation of laser-generated pressure pulses [18].

This work focuses on the stimulation of dorsal root ganglion (DRG) neurons by a laser at 1875 nm. We show that a few millisecond laser pulse at 220 mW induced transient membrane potential variations, which were observed using whole cell patch-clamp recordings. This stimulation led to action potentials firing beyond an energy threshold, but on a minority of neurons only. A depolarization was observed for the majority of DRG neurons even beyond the action potential energy threshold. Our previous works have shown that action potentials on retinal and vestibular neurons were obtained by the activation of the TRPV4 channels and with a constant temperature rise [15,16]. Our goal in this study was to check whether the same mechanism was involved to fire action potentials or to depolarize the neuron membrane without action potentials on DRG neurons. In particular, we wanted to check whether temperature sensitive ion channels were involved in the membrane potential changes even for laser energy below the action potential energy threshold. Ruthenium red, a general inhibitor of transient receptor potential (TRP) channels stopped the action potential generation, but had no effects on membrane depolarization. Local temperature measurements reveal that the depolarization amplitude is sensitive to the amplitude of the temperature rise (ΔT) as well as to the time rate of change of temperature (dT/dt), which may be coherent with a capacitive mechanism that does not need the presence of temperature-sensitive ion channels. However, the rate of change may not fully follow the trend expected for such a mechanism, suggesting that more complex mechanisms are involved.

2. Materials and methods

2.1 Cell culture

Adult Swiss mice (Centre d'Élevage Janvier) were used as described previously in Burland et al. [21]. Neuron cultures were established from lumbar (L4–L5) DRG of non injured mice. Dissociated neurons were plated on D-polyornithine (0.5 mg/ml)-laminin (5 g/ml)-coated glass coverslips and kept at 37 °C in an incubator with a humidified 95% air and 5% CO₂

atmosphere. All procedures were carried out in accordance with the French/European Communities Council Directive 86/609/EEC.

2.2 Electrophysiological recordings

Whole cell patch-clamp recordings in current-clamp configuration [22] were performed at room temperature using an Axopatch 200B amplifier (Axon Instruments, Molecular Devices) digitized by a Digidata 1320A. All experiments were performed after an incubation time ranging from 18 to 48 hours with a single electrode (5-6 MOhms, microhematocrit tubes, Bris Vitrex Medical), filled with an intracellular solution (KCl 140 mM, EGTA 0.5 mM, HEPES 5 mM and Mg-ATP 3 mM). Neurons were bathed in an extracellular solution composed of NaCl 140 mM, KCl 3 mM, MgCl₂ 2 mM, CaCl₂ 2 mM, HEPES 10 mM and Glucose 7 mM [23]. Neurons were first selected on morphological criteria. Only neurons with a diameter ranging from 15 to 45 microns and presenting a resting membrane potential below -40 mV were studied. The membrane potential of neurons was not adjusted by injection of a DC current. The ability to fire action potentials (AP) in response to a depolarizing current (200 pA for 2s) was then checked. Electrically evoked AP generation was also tested after the laser stimulation for all the neurons under pharmacological investigation. A total of 80 functional neurons were tested. Data were analyzed on Clampfit software (Molecular Devices). No filtering was applied for data processing unless specified.

2.3 IR laser stimulation

A pigtailed laser diode (Scheumann Laser) emitting at 1875 nm was used. A multimode fiber of 105 microns core diameter (0.22 NA) (Thorlabs) was cleaved and polished to expose the fiber core on one end while its other end was connected via an SMA connector to the fiber output of the laser diodes as previously explained [15]. The optical fiber was mounted on an x-y-z translator attached to a micromanipulator (Thorlabs). A visible light source was also coupled to the fiber for alignment to the target area (165 ± 10 microns) by positioning the light beam at the border of the neuron soma, in order to avoid direct illumination of the laser beam on the measurement electrode.

A laser diode driver (LDX 32420, ILX Lighthwave) and a control board (DAQ, PCI-6221, National Instruments) were used to tune all laser-stimulation parameters (radiant exposure, pulse duration, and repetition rate) and to simultaneously record the electrophysiological signals with a LabVIEW program. At the bare fiber output, a maximum output power of 220 mW was measured with a Fieldmate powermeter (Coherent) and the duration of the single pulses ranged between 2 ms to 9 ms, corresponding to an energy per pulse ranging from 5.6 to 25.2 J/cm².

2.4 Local temperature measurements

Local temperature measurements were monitored by recording the resistance variations of the tip of the pipette in an open patch pipette configuration under laser irradiation [12,15,25]. Pipettes were filled with solution matching the extracellular solution, with a resistance in the range 2-5 MOhms. A resistance-temperature calibration curve was obtained by heating the bath solution up to 45 °C and by recording the bath temperature changes from an immersed thermometer during the cooling phase.

2.5 Drug

Tetrodotoxine (TTX) and ruthenium red were obtained from Sigma-Aldrich. Drugs were applied to the bath solution in the vicinity of the cell by puff.

3. Results

3.1 IR laser pulses induce transient membrane potential variations in DRG neurons

All the tested DRG neurons were depolarized during the irradiation time by a single laser pulse with energy ranging from 5.6 to 25.2 J/cm² (i.e. 0.44 to 2 mJ) by varying the exposure time from 2 to 9 ms at a constant optical power of 220 mW (at the bare optical fiber output). Detailed laser evoked depolarization responses were investigated on 17 neurons which did not show laser evoked action potentials. One-phase (Fig. 1(a)) or two-phase (Fig. 1(b)) depolarizations were observed during the irradiation time. It was followed by a slower repolarization to the resting membrane potential within a few tens of ms. Both configurations were obtained on a similar number of neurons. For the two-phase configuration, the first phase was similar to the one-phase type and was followed by an inflection point and a sharp increase of the depolarization.

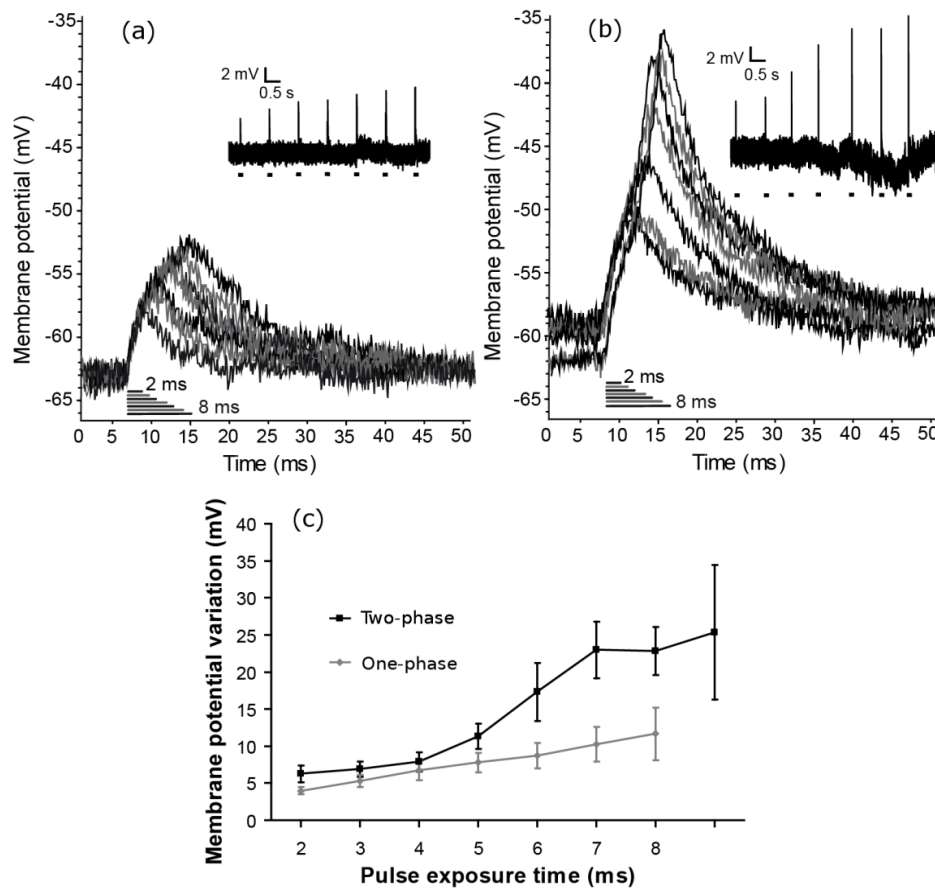


Fig. 1. Membrane potential variation of DRG neurons in current-clamp configuration under irradiation with a laser pulse from 2 to 9 ms presenting (a) a one-phase depolarization and (b) a two-phase depolarization. The inserts show the whole response for a single trace. The irradiation time of successive pulses were increased from 2 to 9 ms at constant laser power (220 mW at the optical fiber tip). (c) Membrane potential amplitude for neurons presenting a one-phase depolarization ($n = 8$) and a two-phase depolarization ($n = 9$). Error bars are \pm s.e.m.

An increase of energy induced an increase of the depolarization amplitude. The typical amplitude increases for the one-phase and the two-phase types are plotted in Fig. 1(c). For neurons presenting a one-phase depolarization, a linear increase is obtained ranging from 4 to 12 mV (R squared coefficient of 0.996 with a slope of 1.2 ± 0.4 V/s). Three domains are

observed for neurons presenting the two-phase depolarization. At the lowest energy levels, stable potentials are obtained. For exposure time between 4 and 7 ms, neurons depolarized from their resting potential from 7 to 23 mV. Finally a plateau is reached at the highest energy corresponding to a maximum depolarization of 23-25 mV. For exposure time higher than 9 ms, damages of neurons were observed, with noticeable swelling of soma and with resting potentials above -40 mV.

3.2 IR laser pulses elicit action potential in DRG neurons

Action potentials were triggered by laser pulses on 15 neurons from 80 functional studied DRG neurons with an optical radiation beyond an energy threshold (between 14 and 27 J/cm² at the bare output fiber). APs were triggered with an IR laser stimulation at 1 Hz during several minutes, without variations of the resting potential (Fig. 2(a)), i.e. without apparent damages of the neuron. The addition of TTX at 1 μ M, a specific inhibitor of the voltage-gated sodium channels [26], abolished the spike-like component of the laser evoked membrane depolarization, as shown in Fig. 2(a) ($n = 3$). This result identifies the spike-like component as a sodium-based action potential.

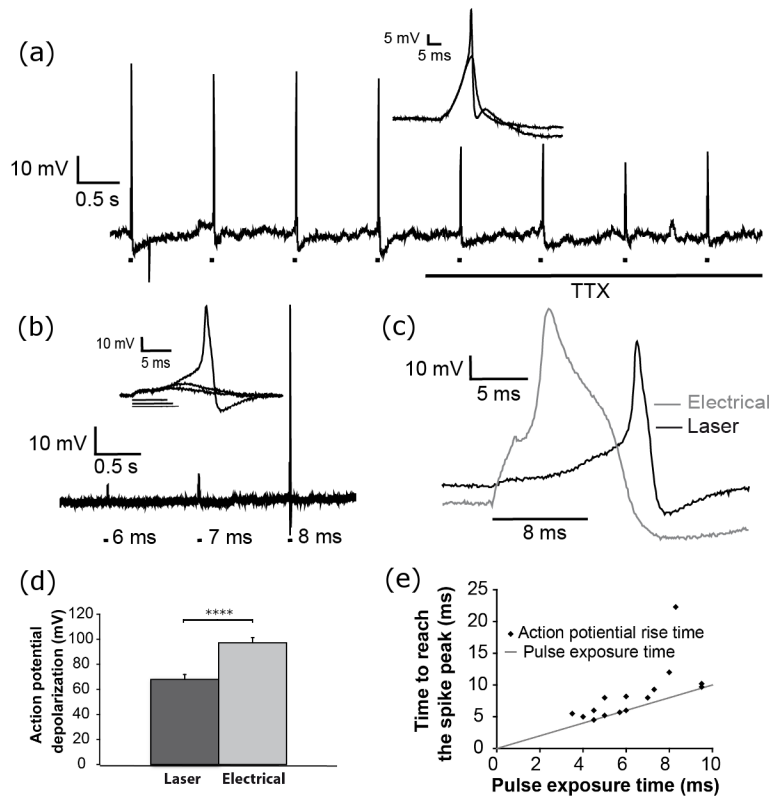


Fig. 2. (a) Membrane potential variations of DRG neurons in whole-cell current-clamp configuration under irradiation with a laser pulse of 8 ms. The addition of TTX at 1 μ M inhibits the action potentials (AP). A residual depolarization is observed. The insert shows the merge of control versus drug tests. (b) Membrane potential variations under laser irradiation with exposure time of 6, 7 and 8 ms at constant power (220 mW). Depolarizations without AP were observed at 6 and 7 ms. An AP was obtained at 8 ms. The insert shows the merge of depolarizations without and with AP. (c) Electrical AP versus the laser evoked AP plotted in (b). (d) Comparison of the mean amplitude of the action potential triggered by laser (67 ± 4 mV) and by a patch-clamp current (97 ± 3 mV) ($n = 15$, $p < 0.0001$). (e) Latency time between the end of the laser pulse and the AP spike peak. Values are means \pm s.e.m.

On neurons exhibiting laser evoked APs, IR pulses at low energy depolarized the membrane without triggering APs. To estimate the stimulation thresholds, the pulse duration was increased by steps at constant power. Figure 2(b) illustrates two membrane potential depolarizations below the AP threshold induced by an IR laser pulse with a duration of 6 and then 7 ms at constant power. The amplitude of the depolarization increased (7 mV and 9 mV, respectively). A pulse duration of 8 ms triggered an AP. For neurons firing laser induced APs, depolarizations below the AP threshold presenting one phase and two phases were observed on 7 and 8 neurons, respectively.

Figure 2(c) shows an electrical AP obtained by a depolarizing current versus the laser evoked AP plotted in Fig. 2(b). The mean amplitude of the laser-evoked APs compared to the amplitude of electrically triggered APs is plotted in Fig. 2(d) ($n = 15$). Electrically triggered APs had higher amplitudes than laser induced APs. A latency time was observed between the end of the laser pulse and the AP spike for 9 neurons of our tests (Fig. 2(e)). The mean latency time was 2.1 ± 0.9 ms. The mean electrically evoked AP duration was 19 ± 3 ms and the mean laser evoked AP duration was 13 ± 2 ms. The variation did not differ significantly (p value of 0.123).

3.3 Temperature ΔT and rate of temperature change dT/dt measurements

The rise in temperature during IR pulses with the same increase in temperature (23 °C to 40 °C) for duration times of 6, 8 and 10 ms is shown in Fig. 3(a). Optical powers were decreased accordingly to the increase of the duration time to keep a constant energy per pulse, i.e. by using powers of 220, 200 and 178 mW inducing rates of change of temperature dT/dt of 2.8, 2.3 and 1.7 °C/ms. The rise in temperature during the IR pulses was almost linear since the laser pulse was shorter than the thermal diffusion of water.

To estimate the effect of a temperature change ΔT versus the effect of dT/dt on the amplitude of the depolarization, the stimulation characteristics of pulses (duration time and optical power) were selected to perform tests at three ΔT (14, 17 and 22 °C ± 1 °C corresponding to a temperature rise from 23°C to 37, 40 and 45 °C ± 1 °C, respectively) and at four dT/dt (2.8, 2.3, 1.7 and 1.2 °C/ms), as shown in Fig. 3(b).

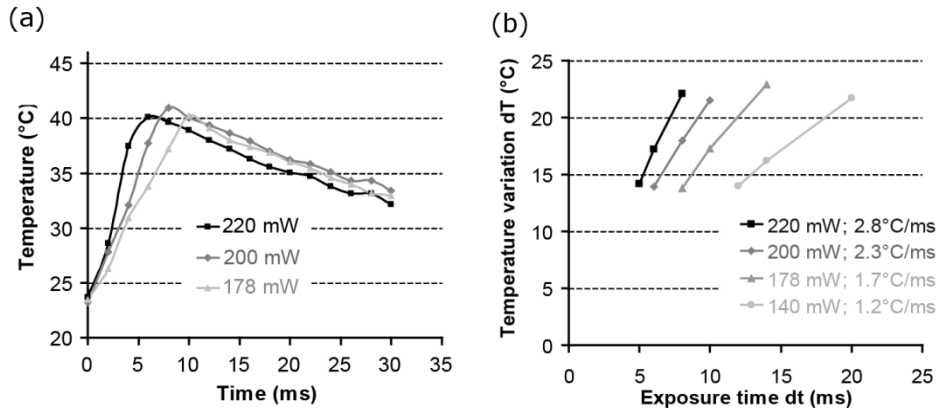


Fig. 3. (a) Constant temperature variation T (from 23 °C to 40 °C, i.e. $\Delta T = 17$ °C) obtained under a pulse laser irradiation with 3 different laser powers. The exposure time was adjusted to ensure a constant energy. (b) Stimulation characteristics (duration time and optical power) to induce a temperature change ΔT of 14, 17 and 22 °C with a rate of temperature dT/dt of 2.8, 2.3, 1.7 and 1.2 °C/ms.

3.4 IR laser depolarization amplitude below the AP threshold depends on ΔT and on dT/dt

To determine whether the laser-induced depolarizations observed on neurons below the AP threshold changed with the temperature variation or with the time rate of change of temperature or both, the stimulation conditions presented in Fig. 3(b) were applied on DRG neurons. Maximal membrane depolarization amplitudes for temperatures ΔT of 14, 17 and 22 °C obtained at rates of change of temperature of 2.8, 2.3, 1.7 and 1.2 °C/ms are shown in Fig. 4 for 4 neurons.

Figure 4(a) shows that the depolarization amplitude increased while increasing the stimulation temperature independently of the rate of change of T. The average change in membrane potential has been estimated by calculating the mean of the slopes from the four cells and from the four rate dT/dt conditions function of the temperature change shown in Fig. 4(a). The mean variation of membrane potential function of ΔT is 0.7 ± 0.2 mV/°C. The general trend shown on 4 neurons is that the depolarization amplitude decreased while increasing the temperature rate dT/dt independently of the temperature variation ΔT as shown in Fig. 4(b). The average change in potential was also estimated by calculating the mean of the slopes from the four cells and from the three temperature rise conditions function of the rate dT/dt as shown in data in Fig. 4(b). The mean variation of membrane potential function of dT/dt is -3 ± 1 mV/(°C/ms).

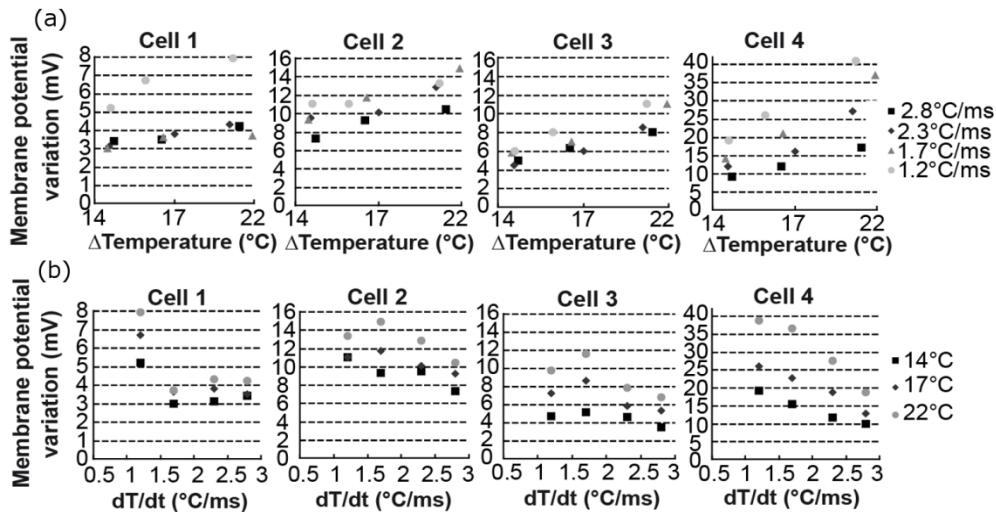


Fig. 4. Membrane potential variation (a) function of ΔT for four different rates of temperature change dT/dt and (b) function of dT/dt for three different temperature variations ΔT . A lowpass filter (-3 dB cutoff at 1000 Hz) and a filter at 50 Hz were applied on data to measure the potential variations.

3.5 IR laser pulses depolarize the DRG neuron membrane without the involvement of TRP channels

Bath applications of ruthenium red (10 μ M), a general TRP channel blocker [27], did not change the depolarization amplitude induced by IR laser pulses ($n = 5$) for depolarizations below the AP threshold (Fig. 5(a)). This was observed for both types of depolarization (one and two phases). It indicated that TRP channels are not involved in the increase of membrane potential below the AP threshold induced by laser pulses. Similar results were obtained for different depolarization amplitudes.

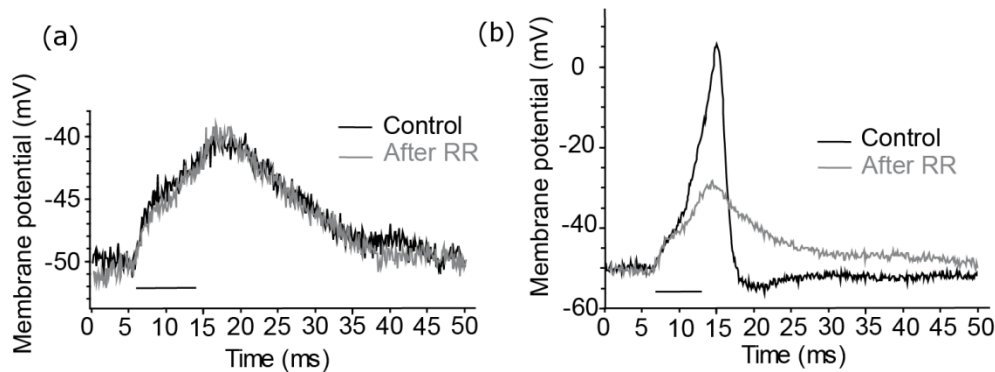


Fig. 5. (a) Depolarization induced by a laser pulse under the AP threshold without and with the use of ruthenium red, a general blocker of TRP channels (optical power of 220 mW, exposure time of 8 ms). (b) Effect of the use of RR on an AP induced by a laser pulse (optical power of 220 mW, exposure time of 7 ms).

3.6 IR laser pulses trigger action potential on DRG neurons with the involvement of TRP channels

The effect of ruthenium red (10 μ M) was tested on neurons on which laser evoked APs were observed. The addition of this general inhibitor of TRP channels suppressed the laser evoked APs ($n = 4$) (Fig. 5(b)). The laser pulse induced a similar depolarization at the start but failed to initiate the spike, resulting in a membrane depolarization of 21 ± 2 mV during the duration time, followed by a slower recovering. These results indicated that TRP channels mediate the laser-evoked APs in DRG neurons.

4. Discussion

Our results show that IR laser stimulations induce transient membrane potential changes on DRG neurons. Two types of kinetic of depolarizations were observed during the irradiation time. The two-phase configuration which presents an inflection point and a sharp increase was obtained on a similar number of neurons than the one-phase configuration. At the end of the laser pulse, the decay to the resting potential was achieved in few tens of ms. No correlation was found between the size of the neurons and the number of phase during the depolarization step (data not shown). For a minority of neurons, action potentials were triggered by IR laser pulses above an energy threshold.

Since the recordings of action potential in current-clamp mode were made with a patch-clamp amplifier instead of a microelectrode bridge, inaccuracies in the recording of action potential may have occurred [28]. Raw values of AP amplitudes, hyperpolarizing afterpotentials and depolarizing and repolarizing slopes may be deformed and may differ from recordings obtained with a microelectrode amplifier. However, our study did not focused on the AP shape analyses. The general spike firing observation, the relative changes of the membrane potential and the pharmacological test responses should not be noticeably affected by the amplifier.

Our previous works on the IR laser stimulation of retinal and vestibular primary neurons have shown that APs were triggered on almost all functional neurons for both cell types when sufficient optical energy was used [15,16]. The same laser at 1875 nm and a similar configuration have been used for this study. A similar damage threshold was obtained on DRG neurons, i.e. for exposure time above 9 ms at 220 mW [15]. This threshold has not been investigated at higher or at lower optical power. Damages were considered when the resting potential was not recovered after the pulse and when noticeable cellular morphological changes were observed. A similar energy threshold (same optical power (220 mW) and same exposure time range (5-9 ms)) have been found to trigger APs for DRG neurons compared to

retinal and vestibular neurons. However, less than 20% of DRG neurons were able to elicit APs. Our previous studies have shown that thermosensitive TRPV channels were involved in the AP activation, in particular TRPV4 [15,16]. TRPV channels are characterized by their temperature activation: TRPV4 > 27 °C, TRPV3 > 32 °C, TRPV1 > 43 °C and TRPV2 > 53 °C [29,30]. Temperatures reached during our tests suggest that such channels could be triggered by the IR laser. However, it has been demonstrated that only 10% of DRG neurons in mice had TRPV4 channels [31]. TRPV1 expression was found in 32% of rat cutaneous DRG neurons [32] and in 37% of nodose ganglion neurons in mouse [33]. TRPV2 expression was detected in 7% of nodose ganglion neurons in mouse [33]. These results could explain the low rate of AP activation of DRG neurons compared to retinal and vestibular neurons.

The use of ruthenium red abolished APs induced by laser pulses. In DRG neurons, our results suggest that TRPV channels are required to elicit APs although they are not involved in the depolarization process without action potential. Suh et al. have used TRPV1 knockout mice and they have shown that TRPV1 channels were playing a role in the generation of APs [34]. Thermal TRP ion channels have been activated by a temperature increase induced by an IR laser pulse with millisecond exposure time as shown by Yao et al. on transfected HEK cells [12]. Hence, such thermosensitive channels are likely to be involved at some level under IR illumination. Further pharmacological tests are required to determine which types of channels are involved for DRG neurons.

Katz et al. have previously shown that IR laser pulses at 1889 nm were able to induce membrane depolarizations as well as action potentials on DRG neurons [13]. A similar configuration has been used in this study. The laser light was coupled to an optical fiber which was positioned at 100 μm of the neuron. The exposure time was chosen in the same range than our study: 5-10 ms. Main differences concern the size of the optical fiber (600 μm versus 105 μm) and the energy range (0.2 to 1.8 J/cm^2 versus 5.6 to 25.2 J/cm^2). The higher level of energy used in our study may partially be explained by the lower value of the absorption coefficient of water at the wavelength we used ($\lambda_{1875} = 30 \text{ cm}^{-1}$ and $\lambda_{1886} = 48 \text{ cm}^{-1}$ [7]). Katz et al. have shown that IR laser pulses produced membrane depolarizations of 18 mV exhibiting long time to recover the resting potential (100 ms to few seconds). Although we found in our study similar levels of membrane depolarization induced by laser pulses, the difference in the decay time has no clear explanation. A very long decay time could be explained by damaged neurons although authors claimed that no damage was induced. Measurements of the reversal potential of laser evoked depolarization have also shown that it does not correspond to the TRPV1 reversal potential alone and they suggest that several distinct membrane ionic channels may be operated by IR laser irradiation. Further experiments on the measurement of the reversal potentials of laser evoked depolarization responses in DRG neurons would help to determine its nature and the ionic channels involved.

The mechanism of IR stimulation of neurons is still not fully understood. Due to the high value of the water absorption coefficient at these wavelengths, there is no doubt that IR laser pulses induce transient heating of the tissue. What remains unclear is how this transient heating elicits membrane depolarization of neurons and action potentials. Several mechanisms of action have been suggested, including the generation of transient capacitive currents [14,17], the stimulation of temperature sensitive ion channels [15,16], the generation of small pores in the plasma membrane [20] or the generation of laser-generated pressure pulses [18]. Our results show that IR laser pulses depolarize DRG neurons without the involvement of thermo-sensitive TRPV channels since the use of ruthenium red, a general blocker of TRP channels did not modify the depolarization amplitude. A similar effect was reported on other cell types: ruthenium red did not affect the depolarization currents induced by IR laser pulses on oocytes [14] and on neuromuscular junction of *Caenorhabditis elegans* [17].

As shown in Fig. 4, the depolarization amplitude was linearly dependent of the magnitude of the temperature change ΔT . It was also sensitive to the rate dT/dt . This was consistent with the works of Shapiro and Liu showing that such laser heating induces electrical capacitance changes of the plasma membrane with the key variable being the rate of change of temperature. Surprisingly, our results have shown a decrease of the membrane potential amplitude as a general trend while increasing dT/dt . An increase would be expected in the photothermal capacitive effect. Our rate dT/dt was between 1100 and 2800 °C/s, i.e. higher than the rates presented in Liu's work (less than 1000 °C/s), but lower than the rates in Shapiro's work on oocytes (2300 and 14000 °C/s). The photothermal capacitive effect is expected to be universal and is also predicted by classical models of the plasma membrane electrochemical double-layer. Our results raise questions about whether DRG neurons follow the trends observed for a photothermal capacitive effect. Membrane capacitance increases induced by the laser pulse were reported to produce capacitive currents in experiments and theoretical modeling [14,17,20]. To our knowledge, experimental results of the depolarization amplitude responses function of dT/dt have not been previously reported. A recent theoretical work has highlighted the importance of the membrane physical dimension variations induced by the laser pulse, confirming a capacitive current sensitive to the rate dT/dt [35]. However, membrane potential equations presented in this work suggest that the whole cell depolarization depends only on the temperature increase ΔT , not the rate dT/dt . Similar conclusion was reported in Rabbit et al. [20], although Liu et al. predicted an increase of the membrane potential while increasing the rate [17]. This was not observed in our work. In these models, it is assumed that the heat pulse acts on the entire cell membrane which was not the case in our experiments since the optical fiber was positioned to illuminate the neuron soma at its border to avoid direct illumination of the laser beam on the measurement electrode. Further experiments include the measurements of the currents in voltage-clamped configuration. Membrane capacitance of DRG neurons is 12 pF [36] while it is 26 pF for *C. elegans* [17], 200 nF for oocyte [37] and 7 to 8 pF for HEK [38]. Since membrane capacitances from DRG neurons and HEK cells are similar, it is unlikely that this parameter alone is the key value for the decrease of dT/dt observed in our work. Our results suggest that the mechanism(s) underlying the neural stimulation by IR laser is (are) likely more complex than a unique depolarization due to a capacitive change of the membrane. The heating wave reaches the cell membrane but also enters the whole cell body, which may directly interact with intracellular machinery. Beier et al. have shown that IR laser light acts on intracellular pathways [20] and have suggested that the laser-induced Ca^{2+} increase might be activated through generation of nanopores [39]. It is clear that further studies are required to fully understand the interaction of the IR laser light and the intracellular signaling pathways.

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Disclosure

The authors declare that there are no conflicts of interest related to this article.