

Article

Lipid Peroxidation Products 4-ONE and 4-HNE Modulate Voltage-Gated Sodium Channels in Neuronal Cell Lines and DRG Action Potentials

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Abstract

Oxidative stress-induced lipid peroxidation products (LPPs), particularly 4-hydroxy-nonenal (4-HNE) and 4-oxo-nonenal (4-ONE), have recently gained attention for their direct regulation of ion channels essential for pain signaling. In this study, we investigated how these two LPPs affect the electrophysiological properties of neurons, specifically voltage-gated sodium (Na_V) channels, thereby influencing sensory neuron excitability and pain pathways. Using human neuroblastoma (SH-SY5Y) and ND7/23 cells (a fusion cell line exhibiting partial sensory neuron properties), we measured changes in Na_V channel-mediated sodium currents following treatment with 4-HNE or 4-ONE. Whole-cell patch-clamp experiments showed that 4-ONE (10 μM) and 4-HNE (100 μM) did not significantly alter the peak sodium current amplitude in SH-SY5Y cells. However, in ND7/23 cells, both 4-HNE and 4-ONE induced a negative shift in Na_V channel activation voltage dependence, enabling sodium channel activation at lower membrane potentials. Furthermore, current-clamp recordings in primary mouse dorsal root ganglion neurons demonstrated that treatment with 4-ONE and 4-HNE reduced the current threshold required to elicit action potentials and significantly increased action potential firing frequency. These findings indicate that LPPs enhance pain sensitivity by modulating Na_V channels, which play a crucial role in pain transmission. In conclusion, 4-HNE and 4-ONE shift the voltage-dependent activation of sodium channels toward more negative potentials, thereby increasing the excitability of primary sensory neurons and amplifying pain signals. This study provides molecular insights into how oxidative stress-related lipid peroxidation contributes to sensory mechanisms and offers potential avenues for developing new treatments for oxidative stress- or inflammation-associated pain.



Academic Editors: Alessandra Napolitano and Cinzia Domenicotti

Received: 29 December 2025

Revised: 21 January 2026

Accepted: 3 February 2026

Published: 4 February 2026

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Keywords: dorsal root ganglion; voltage-gated sodium channels; 4-hydroxy-nonenal; 4-oxo-nonenal

1. Introduction

Oxidative stress and lipid peroxidation significantly influence both normal physiological functions and disease processes. Among the various lipid peroxidation products (LPPs), 4-hydroxy-nonenal (4-HNE) and malondialdehyde play crucial roles in cellular signaling pathways [1–3]. These products function as key second messengers of reactive oxygen species, profoundly affecting cellular activity.

4-HNE is generated by the oxidation of polyunsaturated fatty acids (PUFAs) containing n-6 acyl groups, such as arachidonic and linoleic acids. Despite its role in signaling, 4-HNE is considered toxic due to its rapid reactivity with thiols and amino groups [4,5]. Similarly, degradation of n-6 PUFAs leads to the formation of another highly reactive compound, 4-oxo-nonenal (4-ONE), which is more reactive than 4-HNE and can cause severe cellular damage [6].

LPPs, including 4-HNE and 4-ONE, play a critical role in modulating pain pathways by interacting with ion channels in nociceptive neurons. Notably, the transient receptor potential ankyrin 1 (TRPA1) channel is directly regulated by these aldehydes via covalent modification of thiol and amine residues [7,8]. This interaction leads to neurogenic inflammation, mechanical hyperalgesia, and a dose-dependent increase in intracellular calcium levels. Additionally, 4-ONE exhibits greater sensitivity and neurotoxicity at lower concentrations than 4-HNE, likely due to its higher thiol reactivity [6,9]. Elevated 4-HNE levels are associated with increased nociceptive responses in conditions, such as tissue injury, neurodegenerative diseases, and chronic pain [10]. This effect is particularly significant in the TRPA1- and TRPV1-expressing neurons, highlighting the role of 4-ONE in sensory hypersensitivity [11].

Apart from the effect on TRPA1, previous studies have reported that these compounds also modulate cardiac ion channels, including $K_v11.1$ (hERG), $Ca_v1.2$ (L-type calcium channels), and $Na_v1.5$ (cardiac voltage-gated sodium) channels. Specifically, 4-ONE induces late sodium currents in $Na_v1.5$ channels, significantly increasing the net inward current during action potentials and the risk of arrhythmia [12–14]. These findings highlight the importance of understanding the interaction between reactive aldehydes and pain-related ion channels to elucidate the molecular basis of oxidative stress-induced pain and inflammation.

Voltage-gated sodium (Na_v) channels such as $Na_v1.6$ – 1.9 are predominantly expressed in sensory neurons and are associated with genetic pain disorders, including paroxysmal extreme pain disorder, congenital insensitivity to pain, and innate pain syndromes [15–17]. *Gain-of-function* mutations in these ion channels are thought to contribute to pain development. In this context, investigating the modulation of neuronal sodium channels by 4-HNE and 4-ONE could provide insight into their roles in altering neuronal excitability and action potential dynamics.

We aimed to explore the interplay between 4-HNE and 4-ONE and neuronal sodium channels and their impact on action potential regulation. Using neuronal sodium channel-expressing cell lines, such as ND7/23 and SH-SY5Y, along with primary mouse dorsal root ganglion (DRG) neurons, we sought to advance our understanding of how oxidative stress modulates neuronal pathways.

2. Materials and Methods

2.1. Experimental Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (approval number: SNU 191119-1-6, dated

1 March 2020). Male C57BL/6 mice (aged 9–10 weeks) were obtained from Koatech (Seoul, Republic of Korea).

2.2. Dorsal Root Ganglia Isolation and Primary Culture

Primary mouse DRG neurons were obtained according to the protocol described by Lin et al. [18]. DRGs were harvested in Mg^{2+} - and Ca^{2+} -free Hanks' Balanced Salt Solution (Gibco, Waltham, MA, USA) containing 20 mM HEPES buffer. The samples were digested using collagenase (1 mg/mL)/dispase (2.4 U/mL) for 60 min, followed by an additional 7 min digestion in 0.25% trypsin at 37 °C. After digestion, the samples were washed twice with Dulbecco's Modified Eagle Medium (DMEM; Welgene, Seoul, Republic of Korea) with or without the fetal bovine serum (FBS; Hyclone, Logan, UT, USA).

The DRGs were gently dissociated in FBS-free DMEM using a fire-polished Pasteur pipette and then subjected to stratification on bovine serum albumin, followed by centrifugation. The supernatant was removed, and a second centrifugation was performed. Cells were plated on round glass coverslips coated with 0.1 mg/mL poly-D-lysine in 12-well plates. The culture medium consisted of Neurobasal Medium (Gibco) supplemented with 2% of B-27, 1 mM L-glutamine, 1% of a 100X penicillin-streptomycin, 10 μ M Ara-C, 50 ng/mL nerve growth factor 2.5S, and 2 ng/mL glial cell-derived neurotrophic factor. Cells were incubated at 37 °C in a 5% CO₂ atmosphere. The neurons were used for electrophysiological experiments within 48 h of isolation.

2.3. Cell Culture

ND7/23 cells (mouse neuroblastoma [N18 tg 2] $\times$ rat dorsal root ganglion neuron hybrid cell line, ECACC General Cell Collection #92,090,903) were obtained from the UK Health Security Agency (Porton Down, Salisbury, UK). Cells were maintained in DMEM supplemented with 10% FBS (PAN Biotech, Aidenbach, Germany), 2 mM L-glutamine, and 1% penicillin-streptomycin (Welgene, Gyeongsan, Republic of Korea) in a humidified incubator at 37 °C with 10% CO₂.

SH-SY5Y human neuroblastoma cells were obtained from the Korean Cell Line Bank and maintained in RPMI medium supplemented with 10% FBS and 2 mM L-glutamine under similar conditions. For differentiation, SH-SY5Y cells were treated with 10 μ M retinoic acid for 5–7 d. Cells exhibiting neurite outgrowth were identified using microscopy (Nikon, Tokyo, Japan) and selected for patch-clamp experiments after seeding onto 35 mm culture dishes. For patch-clamp recordings, detached ND7/23 and SH-SY5Y cells were plated at low density onto 35 mm dishes to promote single-cell growth and used within 24–48 h.

2.4. Electrophysiological Recording

Whole-cell voltage and current-clamp recordings were performed to measure ionic currents and action potentials (APs). Electrophysiological signals were amplified using an Axopatch 200 B amplifier (Axon Instruments, Union City, CA, USA) and digitized using a Digidata 1550 B system (Molecular Devices, San Jose, CA, USA). Data analysis was conducted using pClamp 10.1 software (Molecular Devices) and Origin 2016 (OriginLab, Northampton, MA, USA).

As described in previous studies [12,19], patch-clamp recordings were performed under the following conditions: giga-seal resistance (>2 G Ω), low series resistance (<10 M Ω), and 80% series resistance compensation to minimize voltage-clamp errors. Sodium channel currents were recorded using a holding potential of -95 mV with a 2 s prepulse hyperpolarization to -120 mV, followed by depolarizing steps from -100 mV to $+80$ mV in 10 mV increments at 10 s intervals. Signals were acquired at a sampling rate of 10 kHz using an Axopatch 200 B amplifier with analog low-pass filtering at 1 kHz without additional software-based filtering. All recordings were performed without leak subtraction and only

when leakage currents remained stable below 200 pA. Drug effects were analyzed during the final 2 min (between 3 and 5 min) of the LPP perfusion period, after confirming that leakage currents had stabilized, ensuring consistent baseline conditions for accurate current measurement (Supplementary Figure S1). The external bath solution for Na⁺ current recordings in ND7/23 and SH-SY5Y was composed of 130 mM NaCl, 10 mM HEPES, 4 mM CsCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 0.1 mM CdCl₂ to block calcium currents, with the pH adjusted to 7.4 using NaOH. The internal pipette solution contained 117 mM CsCl, 20 mM NaCl, 1 mM MgCl₂, 5 mM HEPES, 5 mM EGTA, 5 mM MgATP, and 0.4 mM Tris-GTP (pH adjusted to 7.3 with CsOH).

Voltage-dependent activation of the Na_v channels was assessed using conductance (G_{Na}) calculated from the recorded Na⁺ currents (I_{Na}) using the following equation:

$$G_{Na} = \frac{I_{Na}}{(V_m - V_{rev})}$$

where V_m represents the membrane voltage, and V_{rev} is the reversal potential for Na⁺ currents.

G_{Na} was normalized to the maximum conductance ($G_{Na,max}$) to generate an activation curve, which was fitted using the Boltzmann equation:

$$y = \frac{1}{1 + \exp\left\{-\frac{(V_m - V_{0.5})}{k}\right\}}$$

where k is the slope factor, V_m is the membrane voltage, and $V_{0.5}$ is the voltage at which conductance reaches half its maximum.

Whole-cell current clamp techniques were used to record APs from DRG neurons, which were selected as small-diameter cells based on their capacitance (<15 pF). APs were evoked by injecting depolarizing currents (50–250 pA in 10 pA increments) at a stimulation frequency of one pulse every 5 s. For single action potential experiments, a small basal current injection was applied to maintain the membrane potential at approximately −70 mV (holding potential). In contrast, during multi-spike action potential experiments, no basal current injection was applied, and the resting membrane potential was recorded without adjustment. Signals were acquired at a sampling rate of 10 kHz using an Axopatch 200 B amplifier (Molecular Devices) with analog low-pass filtering at 10 kHz without additional software-based filtering. The internal pipette solution contained 130 mM K-gluconate, 7 mM KCl, 10 mM HEPES, 2 mM MgATP, 0.1 mM EGTA, 1 mM MgCl₂, 2 mM NaCl, and 0.3 mM Na-GTP, (pH adjusted to 7.25 with KOH). The external bath solution consisted of 140 mM NaCl, 3.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, and 1.25 mM NaH₂PO₄, (pH adjusted to 7.4 with NaOH).

2.5. RNA Extraction

Total RNA was extracted from cells cultured in a 6-well plate using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, 1 mL of TRIzol was added to each well, and the lysate was transferred to a 1.5 mL microcentrifuge tube. After adding 200 µL of chloroform, the mixture was vortexed vigorously for 10 sec and incubated at room temperature for 5 min. Phase separation was achieved by centrifugation at 12,000 rpm for 15 min at 4 °C. The aqueous phase was collected, mixed with an equal volume of isopropanol, and incubated for 10 min at room temperature. RNA precipitation was performed by centrifugation at 12,000 rpm for 15 min at 4 °C. The pellet was washed three times with 1 mL of 100% ethanol, followed by centrifugation at 12,000 rpm for 10 min at 4 °C. After air-drying, the RNA was

dissolved in 10 μ L of RNase-free water. RNA concentration and purity were determined using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA).

2.6. cDNA Synthesis

First-strand cDNA was synthesized from 2 μ g of total RNA using the cDNA synthesis kit (CMRTK001, Cosmogentech, Seoul, Republic of Korea) according to the manufacturer's protocol. The reaction mixture included random hexamers to ensure comprehensive reverse transcription.

2.7. Quantitative Real-Time PCR (qPCR) Analysis

qPCR was performed using SYBR Green chemistry on a real-time PCR system (Light-Cycler, Roche, Indianapolis, IN, USA). Each 20 μ L reaction contained: 4 μ L of 5 $\times$ A-Taq buffer, 0.4 μ L of dNTPs, 0.1 μ L of Taq DNA polymerase, 1 μ L of SYBR Green, 2 μ L of primer mixture (forward and reverse primers), 1 μ L of diluted cDNA template (1:10), and distilled water to 20 μ L. The thermal cycling conditions were as follows: 95 $^{\circ}$ C for 2 min; 40 cycles of 95 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 15 s, and 72 $^{\circ}$ C for 25 s (fluorescence data collection). Melting curve analysis (65 $^{\circ}$ C to 95 $^{\circ}$ C, ramp rate of 0.05 $^{\circ}$ C/s) was performed to confirm amplification specificity. All reactions were run in triplicate, and relative gene expression was calculated using the 2 $^{-\Delta\Delta C_t}$ method with normalization to GAPDH.

2.8. Chemicals

4-ONE and 4-HNE were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Stock solutions (20 and 30 mM in DMSO for 4-ONE and 4-HNE, respectively) were aliquoted and stored at -20° C. Chembridge-5861528 was purchased from MedChem Express (Monmouth Junction, NJ, USA) as a 10 mM stock solution in DMSO. Prior to experiments, the stocks were freshly diluted in extracellular solution to achieve final working concentrations (10 and 100 μ M, respectively). For drug application, after establishing a whole-cell configuration and obtaining stable sodium currents, the compounds were delivered via a perfusion system for 5 min, and measurements were conducted between 3 and 5 min after the start of perfusion, once stable electrophysiological responses were confirmed.

2.9. Statistical Analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical significance was set at $p < 0.05$. Differences between two groups were assessed using the paired t -test and repeated measures ANOVA followed by Sidak's multiple comparisons test for current-voltage (I–V) curve analysis. Statistical analyses and graphing were performed using Origin 2016 and GraphPad Prism 8.0.1 (GraphPad Software, Boston, MA, USA).

3. Results

3.1. Effect of 4-ONE on I_{NaV} in SH-SY5Y and ND7/23

Since 10 μ M of 4-ONE was previously found to increase the late current of the $Na_v1.5$ channel [10], its impact on neuronal Na_v channel current (I_{NaV}) was investigated. A human neuroblastoma cell line (SH-SY5Y) and a sensory neuron-like cell model (ND7/23, a fusion cell line of embryonic rat DRG and mouse neuroblastoma cells) were used [20].

Robust inward I_{NaV} , showing transient activation, was recorded in SH-SY5Y cells by applying multi-step depolarizing pulses preceded by a 150 ms prepulse to -120 mV from a holding potential of -70 mV. After recording control currents, 10 μ M of 4-ONE was perfused into the recording chamber. The application of 4-ONE did not change the peak amplitudes of I_{NaV} (Figure 1A–E, -44.2 ± 3.15 pA/pF and -42.7 ± 2.55 pA/pF at -10 mV for control and 4-ONE, respectively, $n = 8$).

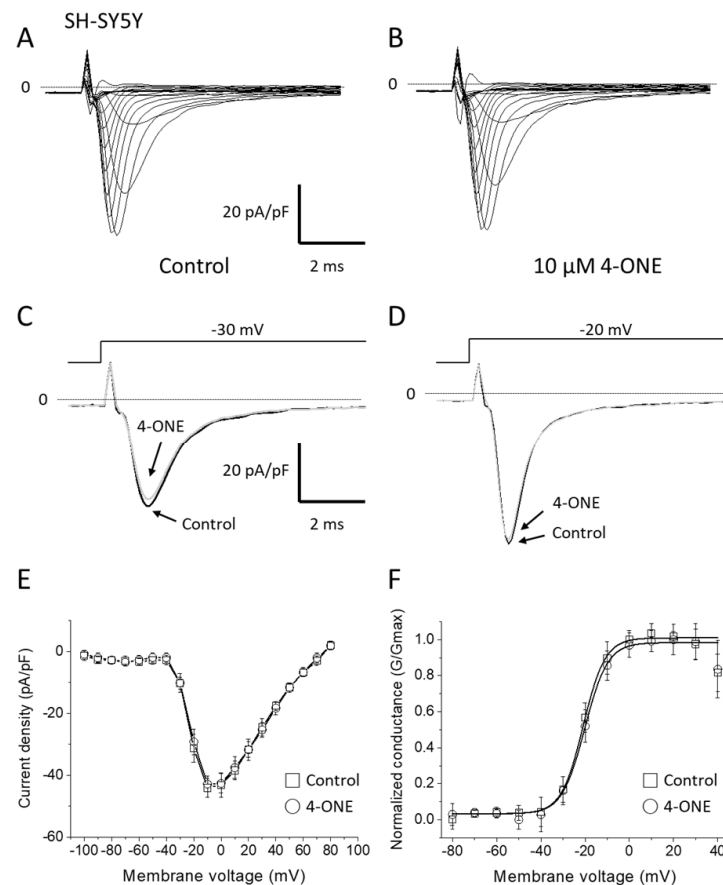


Figure 1. Effects of I_{NaV} induced by 4-oxo-nonenal (4-ONE) in SH-SY5Y cells. (A,B) Representative traces and I-V relationship recorded from the same SH-SY5Y cell. I_{NaV} from SH-SY5Y cell lines were elicited by a 100 ms square test pulse ranging from -100 to 80 mV in 10 mV steps, preceded by a 150 ms prepulse to -120 mV from a holding potential of -70 mV. (C,D) I_{NaV} at -30 and -20 mV test pulses in control (black) and 4-ONE-treated (gray) conditions. (E,F) Current-voltage (I-V) curves and voltage-dependent activation of the channels were plotted using peak current density and conductance. Peak current densities at -10 mV were -44.2 ± 3.15 pA/pF for control and -42.7 ± 2.55 pA/pF for 4-ONE ($n = 8$). Half-activation voltages ($V_{0.5}$) were -20.9 ± 1.05 mV for control and -20.2 ± 0.75 mV for 4-ONE ($n = 8$). Data are presented as the mean $\pm$ SEM.

We further investigated the effect of 4-ONE on the threshold of voltage-dependent activation of I_{NaV} in SH-SY5Y cells, which affects the threshold for action potential generation. The G_{Na} values were plotted according to the depolarizing voltages (see *Methods*) to compare the voltage-dependent activation parameters, such as the half-activation voltage ($V_{0.5}$) of the Na_V channels. No change was observed in the G - V curve for activation (Figure 1F, $V_{0.5}$: -20.9 ± 1.05 and -20.2 ± 0.75 mV for control and 4-ONE, respectively, $n = 8$).

Then we investigated the effect of 4-ONE on I_{NaV} in the ND7/23-derived cells closer to sensory neurons than the SH-SY5Y-derived cells. Apparently, 4-ONE did not alter peak amplitudes of I_{NaV} (Figure 2A,B,E, -57.1 ± 8.00 pA/pF and -56.2 ± 6.59 pA/pF at -20 mV for control and 4-ONE, respectively, $n = 8$) or persistent current amplitudes (Supplementary Figure S2). Interestingly, 4-ONE treatment increased I_{NaV} at -30 mV, which was close to the threshold of voltage-dependent activation in ND7/23-derived cells (Figure 2C,D). The voltage-dependent activation curve (G - V curve) showed a leftward shift to more negative voltage range by 4-ONE treatment (Figure 2F and Table 1, $V_{0.5}$: -28.8 ± 1.67 and -30.5 ± 1.08 mV for control and 4-ONE, respectively, $n = 8$). The most significant current increase induced by 4-ONE was observed at -30 mV (Figure 2D-F).

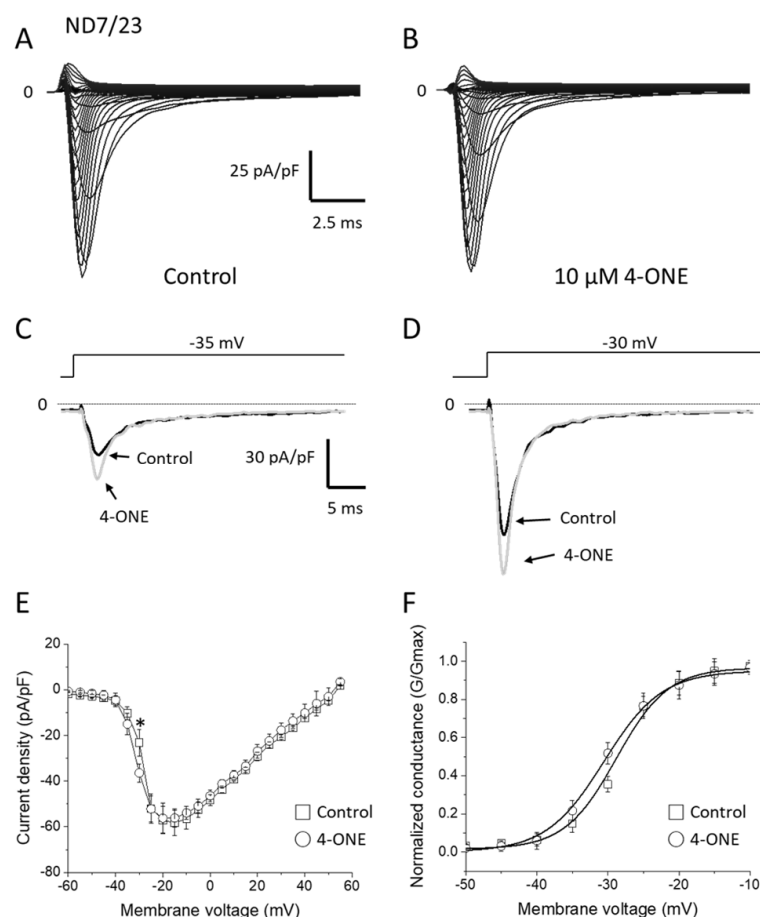


Figure 2. I_{NaV} alterations induced by 4-ONE in ND7/23 cells. (A,B) Representative traces and I-V relationship recorded from the same ND7/23 cell. I_{NaV} from ND7/23 cell lines were elicited by a 100 ms square test pulse ranging from -90 to 50 mV in 5 mV steps, preceded by a 150 ms prepulse to -120 mV from a holding potential of -70 mV. (C,D) I_{NaV} at -35 and -30 mV test pulses in control (black) and 4-ONE-treated (gray) conditions. (E,F) Current-voltage (I-V) curves and voltage-dependent activation of the channels were plotted using peak current density and conductance. Peak current densities at -20 mV were -57.1 ± 8.00 pA/pF for control and -56.2 ± 6.59 pA/pF for 4-ONE ($n = 8$). $V_{0.5}$ were -28.8 ± 1.67 mV for control and -30.5 ± 1.08 mV for 4-ONE ($n = 8$). Data are presented as the mean $\pm$ SEM. All data were analyzed using repeated measures ANOVA followed by Sidak's multiple comparisons test, with $p < 0.05$ considered statistically significant (*).

Table 1. Effects of 4-ONE and 4-HNE on Half-Activation Voltage ($V_{0.5}$). Half-activation voltages ($V_{0.5}$) of voltage-dependent sodium channels were measured in ND7/23 and SH-SY5Y cells following treatment with lipid peroxidation products. Values are expressed as mean $\pm$ SEM. Statistical comparison was conducted using paired t -tests.

Cell Type	Compound, (n)	$V_{0.5}$ (mV, Control)	$V_{0.5}$ (mV, Treated)
ND7/23	4-ONE, ($n = 8$)	-28.80 ± 1.67	-30.50 ± 1.08 *
ND7/23	4-HNE, ($n = 8$)	-26.40 ± 1.04	-28.00 ± 0.85 *
SH-SY5Y	4-ONE, ($n = 8$)	-20.90 ± 1.05	-20.20 ± 0.75

* Significantly different from control ($p < 0.05$), using paired t -test.

3.2. Effect of Chembridge-5861528 on 4-ONE-Induced Changes in Action Potential Generation in Mouse DRG

The shift of $V_{0.5}$ by 4-ONE in ND7/23 cells suggests that the threshold potential for initiating action potentials (APs) in primary sensory neurons might also be lowered. In addition, to examine whether TRPA1 channels contribute to this effect, we conducted

experiments in the presence of the TRPA1 antagonist candidate Chembridge-5861528. To test this hypothesis, we measured the threshold current required to elicit APs using the current-clamp mode of patch-clamp recordings in small-cell neurons isolated from mouse DRGs. Figure 3A–C,G show that 4-ONE reduced the threshold current compared with control (27.5 ± 6.02 pA for control and 18.83 ± 6.27 pA for 4-ONE, $n = 6$), whereas co-treatment with Chembridge-5861528 prevented this reduction (23 ± 5.03 pA, $n = 6$).

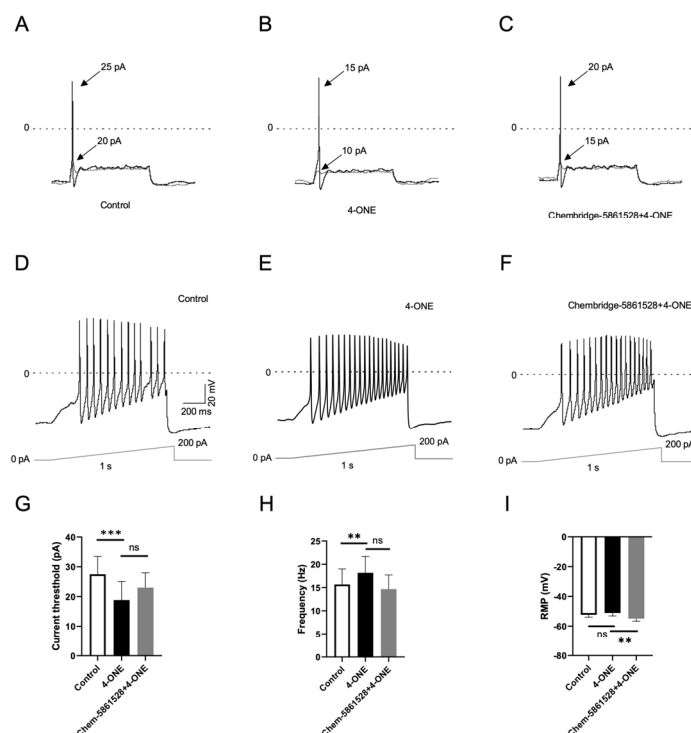


Figure 3. Impact of Chembridge-5861528 on 4-ONE-induced changes in action potential (AP) properties in dorsal root ganglion (DRG) neurons. (A–C) APs were elicited by injecting a series of depolarizing currents (e.g., 5–30 pA in 5 pA steps, 5 ms duration) to measure the threshold level at a holding potential (HP) of -70 mV. (D–F) Multi-spike APs were evoked by injecting a linearly increasing ramp current up to 200 pA for 1 s. (G) Threshold values were 27.5 ± 6.02 pA for control, 18.83 ± 6.27 pA for 4-ONE, and 23 ± 5.03 pA for Chembridge-5861528 + 4-ONE ($n = 6$). (H) Firing frequency was 15.67 ± 3.33 Hz for control, 18.17 ± 3.53 Hz for 4-ONE, and 14.67 ± 3.06 Hz for Chembridge-5861528 + 4-ONE ($n = 6$). (I) Resting membrane potential was -52.27 ± 1.6 mV for control, -51.13 ± 1.95 mV for 4-ONE, and -54.88 ± 1.77 mV for Chembridge-5861528 + 4-ONE ($n = 6$). Data are presented as the mean $\pm$ SEM. All data were analyzed using the paired *t*-test, with $p < 0.01$ (**) and $p < 0.001$ (***) considered statistically significant. ns = not significant.

As previously reported, approximately 50% of primary DRG neurons exhibit repetitive firing in response to prolonged stimuli [21,22]. To further examine this effect, we used a linearly increasing ramp current up to 200 pA for 1 s. Figure 3D–F,H show that the firing frequency increased from 15.67 ± 3.33 Hz (control) to 18.17 ± 3.53 Hz (4-ONE-treated neurons) ($n = 6$). Chembridge-5861528 modestly reduced the firing frequency (14.67 ± 3.06 Hz, $n = 6$), but this change was not significant compared with 4-ONE alone. Furthermore, the resting membrane potential did not differ significantly between control and 4-ONE groups (-52.27 ± 1.6 mV vs. -51.13 ± 1.95 mV). However, co-treatment with Chembridge-5861528 slightly hyperpolarized the membrane potential compared with 4-ONE alone (-54.88 ± 1.77 mV; Figure 3I). Therefore, 4-ONE lowered the current threshold and increased firing frequency in DRG neurons. Chembridge-5861528 did not significantly alter these effects, but induced a hyperpolarization of the resting membrane potential compared with 4-ONE.

3.3. Effect of 4-HNE on Neuronal I_{NaV} and AP

Since the 4-ONE-induced Na^+ current increase was not observed in SH-SY5Y cells but was detected exclusively in ND7/23 cells, the effect of 4-HNE was investigated using ND7/23-derived neuronal cells. An application of $100\ \mu\text{M}$ 4-HNE did not significantly alter the peak amplitude of I_{NaV} at $-20\ \text{mV}$ and higher voltages (Figure 4A,B,F, $-102.8 \pm 3.24\ \text{pA/pF}$ and $-102.7 \pm 4.32\ \text{pA/pF}$ for control and 4-HNE, respectively, $n = 8$). 4-HNE also had no significant effect on persistent current amplitude (Supplementary Figure S2). Similar to the effects of 4-ONE, 4-HNE induced a negative shift in the voltage-dependent activation curve of Na_V channels (Figure 4F and Table 1, activation $V_{0.5}$: $-26.4 \pm 1.04\ \text{mV}$ for control and $-28.0 \pm 0.85\ \text{mV}$ for 4-HNE, $n = 8$). Also, the amplitudes of I_{NaV} at -30 , -25 , and $-20\ \text{mV}$ were increased by 4-HNE (Figure 4C,D).

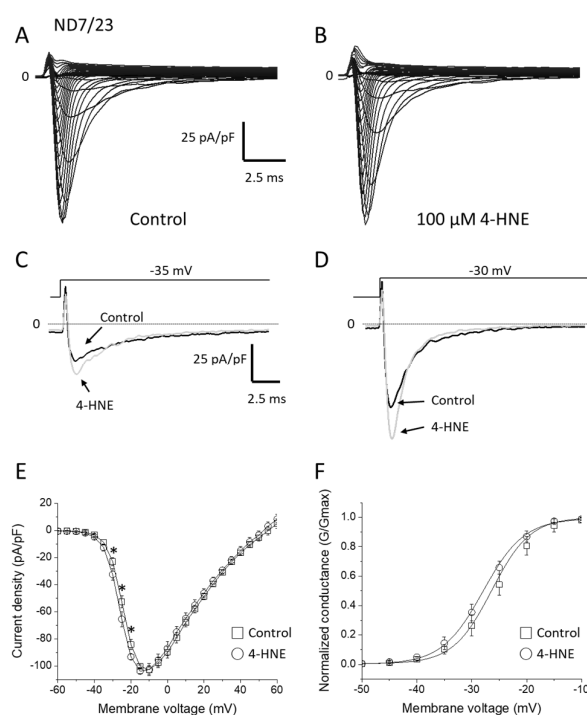


Figure 4. 4-HNE-induced changes in I_{NaV} in ND7/23 cells. (A,B) I_{NaV} from ND7/23 cell lines were elicited by a $100\ \text{ms}$ square test pulse ranging from -100 to $80\ \text{mV}$ in $5\ \text{mV}$ steps, preceded by a $150\ \text{ms}$ prepulse to $-120\ \text{mV}$ from a holding potential of $-70\ \text{mV}$. (C,D) I_{NaV} at -35 and $-30\ \text{mV}$ test pulses in control (black) and 4-HNE (gray). (E,F) Current-voltage ($I-V$) curves and voltage-dependent activation of the channels were plotted using peak current density and conductance. Peak current densities at $-10\ \text{mV}$ were $102.8 \pm 3.24\ \text{pA/pF}$ for control and $-102.7 \pm 4.32\ \text{pA/pF}$ for 4-HNE ($n = 8$). $V_{0.5}$ were $-26.4 \pm 1.04\ \text{mV}$ for control and $-28.0 \pm 0.85\ \text{mV}$ for 4-HNE ($n = 8$). Data are presented as mean $\pm$ SEM. All data were analyzed using repeated measures ANOVA followed by Sidak's multiple comparisons test, with $p < 0.05$ considered statistically significant (*).

To evaluate the effect of 4-HNE on neuronal excitability, the threshold current for AP initiation and firing frequency were analyzed in mouse DRG neurons. The potential contribution of TRPA1 channels was also examined using the TRPA1 antagonist Chembridge-5861528. Treatment with 4-HNE markedly reduced the threshold current required to elicit an AP (Figure 5A,B,G; $87.5 \pm 16.57\ \text{pA}$ for control and $43.33 \pm 7.6\ \text{pA}$ for 4-HNE, $n = 6$). Co-treatment with Chembridge-5861528 slightly increased the threshold current (Figure 5C,G; $56.67 \pm 7.92\ \text{pA}$, $n = 6$), but this change was not statistically significant compared with 4-HNE alone. Additionally, the repetitive firing frequency in response to a ramp current stimulus ($1\ \text{s}$, up to $200\ \text{pA}$) was significantly increased by 4-HNE treatment, rising from $2.17 \pm 1.58\ \text{Hz}$ (control) to $9 \pm 1.46\ \text{Hz}$ (4-HNE-treated

neurons) (Figure 5D,E,H; $n = 6$). Chembridge-5861528 did not alter the firing frequency (9.17 ± 1.35 Hz, $n = 6$) compared with 4-HNE alone (Figure 5F,H). No significant difference was observed in resting membrane potential between the control and 4-HNE-treated groups; however, Chembridge-5861528 slightly hyperpolarized the membrane potential compared with 4-HNE alone (Figure 5G). Taken together, these findings indicate that 4-HNE lowers the current threshold and enhances firing frequency in DRG neurons, similar to the findings with 4-ONE. Chembridge-5861528 did not significantly suppress these excitability changes but induced a modest hyperpolarization of the resting membrane potential.

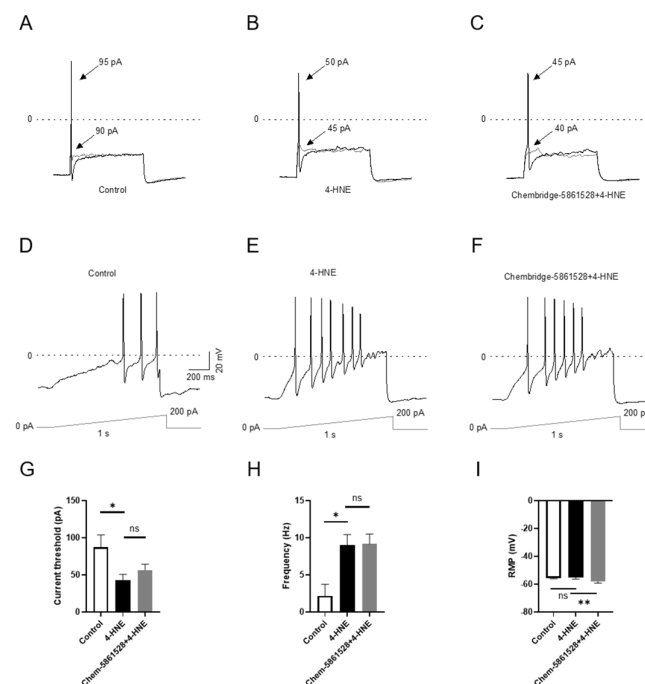


Figure 5. Influence of Chembridge-5861528 on 4-HNE-induced changes in AP properties in DRG neurons. (A–C) APs were elicited by injecting a series of depolarizing currents (e.g., 40–100 pA in 5 pA steps, 5 ms duration) to measure the threshold level at a HP of -70 mV. (D–F) Multi-spike APs were evoked by injecting a linearly increasing ramp current up to 200 pA for 1 s. (G) Threshold values were 87.5 ± 16.57 pA for control, 43.33 ± 7.6 pA for 4-HNE, and 56.67 ± 7.92 pA for Chembridge-5861528 + 4-HNE ($n = 6$). (H) Firing frequency was 2.17 ± 1.58 Hz for control, 9 ± 1.46 Hz for 4-HNE, and 9.17 ± 1.35 Hz for Chembridge-5861528 + 4-HNE ($n = 6$). (I) Resting membrane potential was -55.5 ± 0.54 mV for control, -55.13 ± 1.2 mV for 4-HNE, and -57.88 ± 1.28 mV for Chembridge-5861528 + 4-HNE ($n = 6$). Data are presented as the mean $\pm$ SEM. All data were analyzed using the paired t -test, with $p < 0.05$ (*) and $p < 0.01$ (**) considered statistically significant. ns = not significant.

4. Discussion

4.1. Oxidative Stress, Lipid Peroxidation, and Ion Channel Modulation

Oxidative stress is a critical factor in various pathological conditions including neurodegenerative diseases, inflammation, and cardiovascular disorders [23–25]. Among its consequences, ischemic pain, such as angina pectoris and trigeminal neuralgia, is an example of how oxidative damage contributes to nociception [26,27]. A major molecular mechanism underlying oxidative stress-induced cellular dysfunction is lipid peroxidation, which generates reactive aldehydes such as 4-HNE and 4-ONE. These electrophilic lipids covalently modify proteins including ion channels, thereby altering their function.

Previous studies have identified transient receptor potential (TRP) channels, particularly TRPA1 and TRPV1, which have been extensively studied as key mediators of oxidative stress-induced nociceptive signaling [7,11]. However, our findings suggest that

Na_V channels serve as an important molecular target in this process. Specifically, LPPs induce a negative shift in the voltage-dependent activation of Na_V channels and enhance repetitive action potential firing in DRGs. This finding suggests that LPPs not only modulate receptor potentials via TRPA1 activation but also increase the intrinsic excitability of sensory neurons by lowering the threshold for action potential initiation. This dual mechanism may be particularly relevant in ischemic pain syndromes, where oxidative stress sensitizes nociceptive pathways. The enhancement of intrinsic excitability through Na_V channel modulation suggests that LPPs contribute to nociceptive hypersensitivity not only by amplifying incoming sensory signals, but also by reducing the threshold for spontaneous neuronal firing, which may be a key factor in persistent pain states.

4.2. Differential Electrophysiological Effects of 4-HNE and 4-ONE

The distinct electrophysiological effects of 4-HNE and 4-ONE and the requirement of a higher concentration of 4-HNE for similar effects observed in this study can be attributed to differences in their chemical reactivity and interaction mechanisms [11,12,14]. Both LPPs can modify Na_V channels; however, the extent and nature of these modifications differ. 4-HNE undergoes Michael addition, forming covalent adducts with nucleophilic residues, such as cysteine, lysine, and histidine, leading to moderate and relatively slow electrophysiological effects [6]. In contrast, 4-ONE contains a β -carbonyl group that enables more rapid and potent modifications, including both Michael addition and Schiff base formation, which can result in stronger and more sustained alterations in protein function.

Our results show that 10 μ M of 4-ONE and 100 μ M of 4-HNE were sufficient to induce a negative shift in Na_V channels activation in ND7/23 cells, whereas neither compound significantly altered in SH-SY5Y cells. While physiological or pathological conditions in vivo typically elevate 4-HNE concentrations to 5–100 μ M [28], we selected 100 μ M 4-HNE for our experiments to represent the upper range of pathophysiological relevance. Similarly, based on reports showing 4-ONE accumulation up to 20 μ M in endothelial models under oxidative stress [29], we employed 10 μ M 4-ONE to maintain consistency with observed pathological levels. Given that Na_V channel expression profiles differ between these cell types, specific Na_V channel subtypes may be more susceptible to LPP-induced modulation. Previous studies have indicated that ND7/23 cells predominantly express Na_V1.6 and Na_V1.7 [30], whereas SH-SY5Y cells express Na_V1.7 and Na_V1.8 but lack functional Na_V1.6 [31]. Consistent with previous reports, we confirmed that Nav1.6 and 1.7, rather than Nav1.8, are predominantly expressed in ND7/23 cells by RT-PCR-based mRNA analysis (Supplementary Figure S3 and Table S1). Moreover, in preliminary experiments, a leftward shift in the activation curve was still observed when 4-ONE was applied in the presence of a Nav1.7-specific inhibitor (ProTx-2, 100 nM) (Supplementary Figure S4A–C). In contrast, this shift was not observed following pretreatment with tetrodotoxin (TTX, 5 nM), which has been reported to exhibit an IC₅₀ value of 2.3 nM for Nav1.6 and for 36 nM for Nav1.7 [32] (Supplementary Figure S4D–F). Taken together, these observations suggest that Na_V1.6, a subtype known to play a role in repetitive firing and action potential initiation, may be particularly sensitive to LPP modification. However, given the complexity of Na_V channel expression patterns, the involvement of other subtypes cannot be ruled out, and this question should be addressed in future studies using heterologous expression systems.

4.3. Voltage-Gated Sodium Channels and Pain Pathways

Na_V channels are essential mediators of nociceptive signal transmission, with different subtypes contributing to distinct aspects of pain processing [33,34]. The voltage-dependent activation shift induced by LPPs enhances sodium influx, thereby increasing neuronal excitability. This alteration in Na_V channel function is likely to contribute to pain by

facilitating spontaneous and repetitive neuronal firing. Among Na_V channel subtypes, $\text{Na}_V1.7$ is well known as a threshold channel that amplifies subthreshold depolarizations, while $\text{Na}_V1.8$ supports repetitive firing under sustained depolarization [35,36]. $\text{Na}_V1.6$, which is widely expressed in A-fibers and at lower levels in small-diameter DRG neurons, is particularly important for action potential initiation and repetitive firing [37].

Given the established role of $\text{Na}_V1.7$ and $\text{Na}_V1.8$ in pain signaling, our findings suggest that $\text{Na}_V1.6$ may also contribute to oxidative stress-induced hyperexcitability in sensory neurons. Previous studies demonstrated that $\text{Na}_V1.6$ knockout increases the threshold for action potential initiation and suppresses repetitive firing, whereas gain-of-function mutations lower the activation threshold and enhance neuronal signaling [38,39]. In this context, the LPP-induced negative voltage shift in Na_V channel activation suggests that $\text{Na}_V1.6$ may be a key player in amplifying neuronal transmission under oxidative stress conditions. This may be particularly relevant in chronic pain states where increased Na_V channel activity contributes to spontaneous neuronal firing and pain hypersensitivity.

5. Conclusions

Our findings highlight the critical role of lipid peroxidation products, particularly 4-HNE and 4-ONE, in modulating Na_V channel function and enhancing neuronal excitability. By shifting the voltage-dependent activation of Na_V channels toward more negative potentials, LPPs lower the threshold for action potential initiation and increase firing frequency, thereby amplifying pain transmission. These findings provide mechanistic insights into how oxidative stress contributes to pain and suggest that Na_V channels may serve as potential therapeutic targets for oxidative stress- and inflammation-associated pain. Given the evidence for $\text{Na}_V1.6$ involvement in the observed electrophysiological changes, further studies using selective inhibitors, genetic knockout models, and heterologous expression systems are necessary to elucidate the specific contributions of individual Na_V channel subtypes to oxidative stress-induced pain hypersensitivity.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox15020206/s1>, Figure S1: Stability of Na_V currents during 4-ONE application in SH-SY5Y; Figure S2: Effects of 4-ONE and 4-HNE on persistent Na_V currents in ND7/23 cells; Figure S3: mRNA expression of $\text{Na}_V1.6$ (SCN8A), $\text{Na}_V1.7$ (SCN9A), and $\text{Na}_V1.8$ (SCN10A) in ND7/23 cells; Figure S4: Effects of 4-ONE on Na_V currents in ND7/23 cells in the presence of pharmacological inhibitors targeting $\text{Na}_V1.6$ or $\text{Na}_V1.7$ channels Table S1: Oligonucleotide primers used in RT-PCR (ND7/23).

Author Contributions: Conceptualization: S.J.K. and S.W.C.; Methodology: M.-Z.Y., N.K.P., H.J.K., J.W. and M.S.S.; Formal analysis: M.-Z.Y., N.K.P. and M.S.S.; Investigation: M.-Z.Y., N.K.P. and M.S.S.; Writing—original draft preparation: N.K.P. and S.W.C.; Writing—review and editing: S.W.C., H.J.K., J.W., J.K., J.R.A., M.S.S. and M.Y.; Supervision: M.Y., S.J.K. and S.W.C.; Funding acquisition: S.J.K. and S.W.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) [RS-2024-00456454 and RS-2025-02303718 to S.J.K. and RS-2023-00213304 to S.W.C.].

Institutional Review Board Statement: The animal study protocol was approved by Institutional Animal Care and Use Committee of Seoul National University SNU 191119-1-6 (protocol code SNU 191119-1-6 and date of approval 1 March 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

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