Opioid-induced hypernociception is associated with hyperexcitability and altered tetrodotoxin-resistant Na\(^{+}\) channel function of dorsal root ganglia


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Ross GR, Gade AR, Dewey WL, Akbarali HI. Opioid-induced hypernociception is associated with hyperexcitability and altered tetrodotoxin-resistant Na\(^{+}\) channel function of dorsal root ganglia. Am J Physiol Cell Physiol 302: C1152–C1161, 2012. First published December 21, 2011; doi:10.1152/ajpcell.00171.2011.—Opiates are potent analgesics for moderate to severe pain. Paradoxically, patients under chronic opiates have reported hypernociception. In conclusion, chronic morphine-induced hypernociception has become a critical problem. This phenomenon of opiate-induced hypernociception is also demonstrated in several experimental animal models such as thermal and tactile nociception in mice (56) as well as in a rat model of narcotic bowel syndrome (1). Rats develop visceral hyperalgesia to colorectal distension after day 6 of morphine administration (1). In mice, chronic morphine administration produced thermal as well as tactile hypersensitivity 7 days after morphine pellet implantation (56). The mechanisms involved in the development of opiate-induced hypernociception are not clearly known, but it appears to be a multifactorial neurobiological process, including 1) primary afferent sensitization, 2) altered production, release, and uptake of excitatory neurotransmitters, 3) second-order neuron sensitization to excitatory neurotransmitters, and 4) increased descending facilitation due to rostral ventromedial medullary neuroplastic changes leading to enhanced primary afferent neurotransmitter release and pain (6, 23). Probable changes in the first-order neurons that carry pain signals from visceral organs could also be involved in amplification of the pain signals, since recent reports show evidence for both central and peripheral sensitization mechanisms causing narcotic bowel syndrome (1), and also changes at the dorsal root ganglion (DRG) level with respect to transient receptor potential vanilloid 1 (TRPV1) channels were suggested to be involved in thermal and tactile hypersensitivity. Therefore, it is highly likely that any opioid-induced change in the excitability status of the sensory neurons could contribute to opioid-induced hypernociception.

DRG harbor cell bodies of the first order of neurons that carry pain signals from the abdominal organs (61). Several pathological states, including inflammatory bowel disease (33), colitis (9), gastric ulcer (11), neuropathic pain (54), and local inflammation (59), can enhance excitability and firing rate of DRG neurons. Interestingly, in all the above-mentioned hyperexcitability conditions, the tetrodotoxin (TTX)-resistant Na\(^{+}\) channels (TTX-R Na\(^{+}\)) were upregulated. Moreover, expression of TTX-R channels Na\(_{V}1.8\) and Na\(_{V}1.9\) has been implicated in nociception (2, 42, 46, 63). TTX, a guanidinium toxin from puffer fish, binds and blocks TTX-sensitive Na\(^{+}\) channels. DRGs express both TTX-R Na\(^{+}\) subtypes at nanomolar concentrations. In contrast, TTX-R Na\(^{+}\) channels are resistant to TTX, even in the micromolar range (7, 34). DRGs express both TTX-R Na\(^{+}\) channels and TTX-sensitive channels that are important in the electrogenesis of action potentials (APs; see Refs. 14, 15, and 47). Although it is clear that TTX-R Na\(^{+}\) channels have profound influence on neuronal excitability (36), the status of excitability of DRGs as well as the changes, if any, in the biophysical properties of TTX-R Na\(^{+}\) channels are not known following chronic mor-
In this study, we confirmed chronic morphine-induced hypernociception in mice and tested the hypothesis that chronic morphine induces hyperexcitability and remodels the TTX-R Na$^+$ channel function, along with TRPV1 channels in mouse DRGs.

MATERIALS AND METHODS

All protocols and procedures that were performed in this study were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University Medical Center and comply with the recommendations of the International Association for the Study of Pain.

Materials

Papain and collagenase type II were purchased from Worthington (Lakewood, NJ); Dulbecco’s modified Eagle medium, neurobasal media, B27 supplement, glutamax, and penicillin/streptomycin were purchased from Invitrogen. Hank’s balanced salt solution (HBSS) and FBS were from VMR (Batavia, IL). Glass cover slips were from Leo (Lakewood, NJ). Dulbecco’s modified Eagle medium, neurobasal medium, 5% FBS, 1% B27, 100 U/ml penicillin/streptomycin, and 2 mM glutamax. Isolated cells were plated on 12-mm glass cover slips and maintained at 37°C in a 95% air-5% CO$_2$ incubator overnight.

General electrophysiology. Standard patch-clamp procedures were followed (35). Whole cell voltage- and current-clamp recordings were recorded at room temperature from DRG cells (<30 pF capacitance) using an EPC 10 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany), with patch micropipettes pulled from borosilicate glass capillaries on a Flaming-Brown P97 (Sutter Instruments, Novato, CA) electrode puller. The pipette resistances were 3–5 MΩ when filled with internal solutions. For current-clamp experiments, DRGs were bathed in HEPES-buffered external solution containing (in mM): 160 NaCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 10 HEPES, pH 7.4. The pipette solution contained the following (in mM): 140 KCl, 2 Na$_2$ATP, 0.5 NaGTP, 10 HEPES, and 0.1 EGTA (pH 7.2 by KOH). To determine the threshold for the AP, current steps (300 ms duration) were applied at 10-pA increments from −15 to 300 pA. For voltage-clamp recordings, we isolated the Na$^+$ current using an internal solution containing (in mM): 130 N-methyl-D-glucamine (NMDG), 15 NaCl, 1.8 MgCl$_2$, 9 EGTA, 9 HEPES, 4 Na$_2$ATP, 14 Tris-creatine PO$_4$, and 0.3 Na-GTP, pH 7.4 (with CS$_2$O). The external solution contained the following (in mM): 100 NaCl, 10/TEA-Cl, 4 CsCl, 2 MgCl$_2$, 0.03 CdCl$_2$, 10 glucose, and 10 HEPES, pH 7.4. The TTX-R Na$^+$ currents were isolated using 1 µM of TTX. Direct block of TTX-R sodium channels was minimized by the low concentration of CdCl$_2$ (37, 41). Na$^+$ currents were evoked by depolarizing pulses from −80 mV to +60 mV at an increment of 10 mV. Peak Na$^+$ currents were observed within 5 ms of voltage step, which is an indication of appropriate voltage control. Steady-state inactivation of TTX-R Na$^+$ currents was measured using two-step voltage protocols (prepulse from −80 to 0 mV for 500 ms at an increment of 10 mV and a 10–100 ms, current clamp pulse of −20 mV for 100 ms), whereas voltage dependence of activation was calculated from peak current-voltage relationships assuming a reversal potential of +50 mV. Furthermore, the time course of recovery from inactivation of TTX-R Na$^+$ channels was studied using a double-pulse protocol in which two identical voltage-clamp pulses were delivered from a holding potential of −80 mV to a step potential of −20 mV for 50 ms with increasing interpulse interval (1-ms increment) at 0.2 Hz. For TRPV1 current measurements, the DRGs were bathed in HEPES-buffered physiological solution, and the pipette internal solution contained 100 mM t-aspartic acid, 30 mM CsCl, 1 mM MgCl$_2$, 5 mM HEPES, 2 mM ATP (disodium salt), and 5 mM EGTA, with pH adjusted to 7.2 using CsOH. The cells were held at −50 mV, and capsaicin (10 µM) was applied in the external solution (by continuous perfusion) to induce the TRPV1 currents.

Data acquisition and analysis. Currents and voltages were controlled and sampled using an EPC 10 patch-clamp amplifier (HEKA; Lambrecht/Pfalz) with Patchmaster v2X40 software. Signals were filtered at 5–10 kHz (~3 dB, four-pole Bessel) and digitized at 10–100 µs. Patchmaster data files were converted to axon binary file (ABF) formats using the ABF Utility program (Synaptosoft, Fort Lee, NJ) and were analyzed by Clampfit 10.1 (Molecular Devices, Sunnyvale, CA) or Igor Pro 6.0 (WaveMetrics, Portland, OR). Nonlinear and linear curve fittings were performed with clampfit while Boltzmann fits were performed either in clampfit or Graphpad Prism (La Jolla, CA). Conductance-voltage curves were calculated from the peak current according to the equation $G_{Na} = I_{Na}/(V - E_{Na})$, where $V$ is the test pulse potential and $E_{Na}$ the reversal potential extrapolated from...
the current (I-voltage (V) relationship. The activation curves (G-V) were fitted using the Boltzmann function \( G_{V_{max}} = \frac{I}{1 + \exp(V - V_{1/2})} \), where \( G_{V_{max}} \) is the normalized Na\(^+\) conductance, \( V_{1/2} \) is the potential of half-maximum activation, and \( k \) is the slope factor. Data fittings with exponential functions, \( f = \sum A_i \exp(-x_i t) + C \), were carried out using Clampfit. Data are presented as the means and SE of \( n \) observations (individual cells from at least 3 different animals). Significance was determined by unpaired t-test or one-way ANOVA and Bonferroni posttest at the level of \( P \leq 0.05 \).

RNA extraction and real-time polymerase chain reaction. Total RNA was purified from DRGs (L5–S1) (pooled from 3 animals/group) using the Invitrogen PuriLink Micro to Midi RNA purification system (Invitrogen, Carlsbad, CA). Purified RNA was eluted in 70 \( \mu \)l of total volume with diethyl pyrocarbonate-treated water. Equal concentrations of RNA were used in all of the samples as determined by an ultraviolet spectrophotometer; 1 \( \mu \)g of RNA/reaction was used in all of the reactions. Using Quantace SensiMix One-Step mix, reverse transcription was performed for 30 min at 42°C with an enzyme activation step for 10 min at 95°C. The PCR protocol consisted of 40 cycles of denaturation (15 s at 95°C), annealing (30 s at 60°C), and extension (30 s at 72°C). The cycle threshold value, \( C_t \), count, for each sample was measured by normalizing it from the \( C_t \) value of the reference gene 18S to establish \( \Delta C_t \) (\( \Delta C_t = C_t - 18S \) Ct) for each sample. The fold change was calculated as \( 2^{-\Delta C_t} \). All primers were designed using the Vector NTI software (Invitrogen), and the sequences in this study used were as follows: 5'-GCCTGTGTTCGGCTAATGAC-3’ (forward) and 5'-TCCAGAATACTAACAGCAG-3’ (reverse) for Na\(_v\)1.7(Scr9a, NM_018852); 5'-GTGTCGATCCCGAAGCTGAT-3’ (forward) and 5'-CAAACCTTCTGCGATATCT-3’ (reverse) for Na\(_v\)1.8 (Scr10a, NM_009134); 5'-CGACTTCTGGTCGTTAGA-3’ (forward) and 5'-AGAGCTTAGTTACCTCTGAGG-3’ (reverse) for Na\(_v\)1.9 (Scr11a, NM_011887); and 5'-TCAAGAAGCGAAAGTGGGAGG-3’ (forward) and 5'-GGACTCTTAAGGCGATCAC-3’ (reverse) for 18S (X00686).

RESULTS

Chronic Morphine-Induced Hypernociception

To assess the antinociceptive and hypernociceptive effects of morphine, we used the acetic acid-induced nociceptive assay in mice, a model of abdominal pain (50). The number of stretches after acetic acid administration in placebo were 49 \( \pm \) 3 for a 20-min period on day 1 and remained unchanged for 7 days [36 \( \pm \) 5 (day 3), 36 \( \pm \) 4 (day 7), \( n = 6 \) in each group]. On the other hand, in morphine-pelleted mice, we observed morphine-induced antinociceptive effects up to 3 days [4 \( \pm \) 1 (day 1), 4 \( \pm \) 1 (day 3)] while hypernociception was evident on day 7 following morphine pellet implantation. Each group represent means \( \pm \) SE from five to six mice (Fig. 1).

Excitability of DRGs After Chronic Morphine Administration

The effect of chronic morphine on the excitability of DRGs from the lumbosacral region L3 to S1 was determined by analyzing the AP parameters under current clamp. Small-sized DRG neurons with the mean diameter of 26 \( \pm \) 1 \( \mu \)m (placebo) (\( n = 20 \)) and 26 \( \pm \) 2 \( \mu \)m (morphine pellet) (\( n = 13 \)) were examined. Classifying the DRGs based on AP waveforms and other distinctions as reported in rat DRGs (12, 62) was not possible in this study, since none of our AP recordings showed any “shoulder” in the falling phase. Therefore, we followed the report by Lawson et al. (43) that demonstrated the properties associated with nociceptors in vivo, including small cell body diameter. Figure 2 shows typical records of AP firing in DRGs from both placebo- and morphine-pelleted mouse at day 7 (Fig. 2A) and day 3 (Fig. 2B). The rheobase (minimum pulse strength required to initiate AP) in placebo-pelleted mice at day 3 was 116 \( \pm \) 17 pA (\( n = 4 \)). This was not significantly different from morphine-pelleted mice (87 \( \pm \) 16 pA, \( n = 11 \)) at day 3. However, at day 7, the rheobase of the DRG from morphine-pelleted mice was significantly lower (38 \( \pm \) 7 pA, \( n = 13 \)) compared with placebo (100 \( \pm \) 15 pA, \( n = 20 \)) or the rheobase obtained at day 3 (Fig. 2, A-C). The lower rheobase suggests hyperexcitability of sensory neurons following chronic morphine treatment. Chronic morphine did not significantly affect the resting membrane potentials, as shown in Table 1. Other AP parameters such as the overshoot, peak-to-peak magnitude, duration measured at the half-maximal amplitude, and maximum rise slope were also not significantly different in DRGs between placebo- and morphine-pelleted groups (Table 1). Chronic morphine did not significantly affect the membrane input resistance of DRGs as measured from the electrotonic potential induced by a hyperpolarizing current step (Table 1). Therefore, the primary effect of chronic morphine administration was to significantly lower the required pulse strength to generate an AP without affecting other AP parameters. Furthermore, as shown in Fig. 2, A and C, right, the mean number of APs at double-strength rheobase (2\( \times \) rheobase) is more than doubled after chronic morphine (4.4 \( \pm \) 0.8) compared with placebo (2 \( \pm \) 0.5) on day 7 (\( P = 0.01, n = 12-20 \)), which is also an indication of enhanced excitability of DRGs induced by chronic morphine.

Voltage Dependence and Activation/Inactivation Kinetics of TTX-R Na\(^+\) Channels

DRG express TTX-R Na\(^+\) channels, which are important to generate an AP (15) and have been shown to be altered in other
pathological states that affected the cellular excitability. Therefore, we studied the biophysical characteristics of TTX-R Na⁺ channels to determine the changes, if any, caused by chronic morphine. Under voltage clamp, TTX-R Na⁺ currents were recorded in the presence of 1 μM TTX to block TTX-sensitive (TTX-S) Na⁺ currents, internal NMDG, external TEA⁺, and Cs⁺ to block K⁺ channels and 0.03 μM Cd²⁺ to block Ca²⁺ channels, from a holding potential of −80 mV with depolarizing test pulses (300 ms) stepped at a 10-mV increment up to +60 mV. The mean capacitance in these DRGs was similar. In the present study, both TTX-S and TTX-R Na⁺ currents were studied. The TTX-S Na⁺ currents were calculated by subtracting TTX-R Na⁺ current series from the series of total Na⁺ currents in the absence of TTX. While the current-voltage relationship of TTX-S Na⁺ currents (Fig. 3A, bottom) did not change significantly, the TTX-R currents (Fig. 3B, bottom) showed relatively higher magnitude of peak currents normalized to cell capacitance, with a hyperpolarized shift in the voltage dependence of activation in DRGs from the chronic morphine group. Voltage-dependent activation and steadystate inactivation of Na⁺ channels contributes to membrane excitability (5). The voltage dependence of activation and inactivation in both placebo- and morphine-pelleted groups was measured as described in MATERIALS AND METHODS and fit by the Boltzmann function to determine the voltage (V₅₀) for half-maximal activation or inactivation and the corresponding slope factors (Fig. 3D). The values of V₅₀ of activation of TTX-R Na⁺ channels were −9 mV more negative after chronic morphine treatment [−28 ± 1 mV, k = 5 ± 1 (placebo), −37 ± 1 mV, k = 2 ± 1 (morphine), n = 10] while the values of V₅₀ of inactivation were −33 ± 1 mV, k = 5 ± 1 in placebo and −40 ± 1 mV, k = 5 ± 1 in the morphine groups (n = 4). The corresponding slope factors (k) of activation and inactivation of TTX-R Na⁺ channels in placebo- and morphine-pelleted groups showed a steeper slope for activation after morphine. Moreover, the time-dependent properties of TTX-R Na⁺ currents (TTX-R Iₕa) were altered by chronic morphine. The kinetics of TTX-R Iₕa were analyzed by monoeponential fitting to the descending phase of the current traces for activation and the rising phase for inactivation, and the corresponding averaged rate constants of activation (tₐₐₚₜ) and inactivation (tᵢₐₜ) at pulses from −20 to +10 mV are displayed in Fig. 3C. At respective test potentials of −20, −10, 0, and +10 mV, the tₐₐₚₜ for placebo were 6 ± 2, 5.9 ± 2, 4.3 ± 1, and 2.1 ± 0.5 ms (n = 5) and for the morphine group were 5.1 ± 1, 2.2 ± 0.6, 1 ± 0.2, and 0.5 ± 0.1 ms (n = 6) while the tᵢₐₜ for placebo were 5.6 ± 1, 4.6 ± 0.3, 3.4 ± 0.4, and 2.7 ± 0.3 ms (n = 5) and for the morphine group were 5.9 ± 1.2, 4.4 ± 0.7, 3.5 ± 0.6, and 2.9 ± 0.5 ms (n = 6). Chronic morphine resulted in significantly faster activation kinetics of TTX-R Na⁺ channels compared with the placebo group without affecting the inactivation kinetics.

![Graphical representation of the data](http://ajpcell.physiology.org/)

Table 1. Comparison of passive and action potential parameters of DRG neurons from placebo- and morphine-pelleted mice on day 7

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo</th>
<th>Morphine</th>
</tr>
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<tbody>
<tr>
<td>Capacitance, pF</td>
<td>20 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Resting potential, mV</td>
<td>−37 ± 3</td>
<td>−40 ± 3</td>
</tr>
<tr>
<td>Input resistance, GΩ</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Overshoot, mV</td>
<td>43 ± 5</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>Peak to peak, mV</td>
<td>77 ± 5</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>Duration, ms</td>
<td>11 ± 2</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Maximum rise slope, mV/ms</td>
<td>12 ± 2</td>
<td>14 ± 2</td>
</tr>
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Values are means ± SE; n = 11–20 mice in each group. DRG, dorsal root ganglia. Data were analyzed by unpaired Student’s t-test.

Fig. 2. Effect of chronic morphine on action potential (AP) generation in dorsal root ganglia (DRG) neurons. Raw traces of typical AP elicited under current clamp at 2× rheobase in DRGs from placebo control (left) and morphine-pelleted (right) groups on day 7 (A) and day 3 (B) following pellet implantation. C: left, the difference in the mean rheobase (minimum pulse strength required to initiate AP) between placebo and morphine groups on day 7 but not day 3. The rheobase was significantly (P < 0.01) lower in the chronic morphine group (38 ± 7 pA, n = 13) than that of placebo (100 ± 15 pA, n = 20) on day 7 but not on days 1 to 3 [control (116 ± 17 pA, n = 4), morphine pellet (87 ± 16 pA, n = 11)]; right, increased mean number of APs at double rheobase (2×) pulse in the chronic morphine group on day 7 but not on day 3 compared with the placebo group can be seen. Data are expressed as means ± SE (n = 13–20, analyzed by unpaired Student’s t-test, *P < 0.05 and **P < 0.01 vs. placebo).
Recovery of TTX-R Na⁺ Channel from Inactivation

We next analyzed the recovery of TTX-R Na⁺ channels from inactivation using a paired-pulse protocol as illustrated in Fig. 4A: identical clamp pulses (P₁, P₂) were delivered from a holding potential of −80 mV to a step potential of −20 mV with a variable interpulse interval (1-ms increment, P₁–P₂) at 0.2 Hz. The current during P₂ (I₂) relative to the current during P₁ (I₁) was plotted as a function of the P₁–P₂ reactivation interval (t) as shown in Fig. 4B. The reactivation curves were fitted by a monoexponential function to assess the reactivation time constants. Chronic morphine did not affect the recovery rate (2.5 ± 0.3 ms) of TTX-R Na⁺ channels from inactivation compared with the placebo group (2 ± 0.3 ms) (n = 5). Therefore, the hyperexcitability or the generation of multiple APs at double rheobase is not due to any change in the recovery rate of TTX-R Na⁺ channels from inactivation states after chronic morphine administration.

Genetic Expression of Na⁺ Channel Subtypes

Hyperexcitability in neurons could be due to increased expression of Na⁺ channels. Various subtypes of voltage-dependent Na⁺ channels are expressed in the cell bodies of primary nociceptive neurons (7, 48) among which NaV1.8 and NaV1.9 are TTX-resistant (20). Therefore, using real-time PCR...
technique, we determined the changes in mRNA expression, if any, induced by chronic morphine administration. We detected and analyzed the transcripts of pertinent Na\(^+\) channel subtypes, including Na\(_{\text{v1.7}}\) (scn9A), Na\(_{\text{v1.8}}\) (scn10A), and Na\(_{\text{v1.9}}\) (scn11A), in DRGs from both placebo and morphine groups. As depicted in Fig. 4C, there were no significant fold changes in any of the Na\(^+\) channel subtypes, including the TTX-resistant subtypes, following chronic morphine administration. Therefore, the changes we observed in the excitability of neurons as well as the Na\(^+\) currents in the chronic morphine group were not related to changes in the gene expression of any Na\(^+\) channel isotype.

**Chronic Morphine Enhances Capsaicin-Induced TRPV1 Currents**

The TRPV1 receptor, which belongs to the family of transient receptor potential (TRP) channels, is considered as a molecular transducer of noxious stimuli such as vanilloids and acids (17, 19). Also, TRPV1 expression plays a critical role in the development of inflammation-induced hyperalgesia (17, 18, 25, 38). Because chronic morphine also induces hyperalgesia, we hypothesized that capsaicin-induced TRPV1 currents might be increased in DRGs under persistent morphine influence. Under whole cell voltage clamp, DRGs of similar cell size (below 30 pF capacitance) (Fig. 5C) from placebo- and morphine-pelleted groups were held at \(-50\) mV, and capsaicin (10 \(\mu\)M)-induced nonselective cationic inward currents were recorded and analyzed. Typical tracings of capsaicin-induced TRPV1 currents are displayed in Fig. 5, top. Chronic morphine significantly enhanced the magnitude of capsaicin-induced TRPV1 currents compared with the cells from the placebo group (Fig. 5A). As shown in Fig. 5B of averaged data, peak TRPV1 currents normalized to cell capacitance were significantly \((P = 0.008)\) enhanced by chronic morphine \((-64 \pm 11\) pA/pF; \(n = 9\)) relative to the placebo control \((-18 \pm 6\) pA/pF; \(n = 6\)).

**DISCUSSION**

Because chronic morphine has the potential to cause paradoxical hypernociception, we attempted to determine chronic morphine-induced changes at the cellular level on excitability as well as underlying remodeling of TTX-R Na\(^+\) and nonselective cationic TRPV1 channels in DRGs. The salient findings in this study are that chronic morphine (after 7 days) \(1\) leads to hypernociception, measured by acetic acid abdominal stretching assay, \(2\) induces hyperexcitability as demonstrated by lower rheobase for AP generation and increased multiple APs at double-strength rheobase, \(3\) shifts the voltage dependence of activation of TTX-R Na\(^+\) currents to more hyperpolarized potential, \(4\) changes the activation kinetics of TTX-R Na\(^+\) channels to a significantly faster time course but does not affect the inactivation kinetics, \(5\) does not affect the recovery rate of TTX-R Na\(^+\) channels from inactivation states, \(6\) does not change the gene expression of Na\(^+\) channels, and \(7\) enhances the magnitude of capsaicin-induced TRPV1 current in DRG neurons.

Opiates are potent analgesics, clinically used in the therapeutic management of moderate to severe pain. Sustained opiate treatment is required to treat conditions like cancer pain. Notably, patients under chronic opiate administration have reported enhanced pain in body parts even other than the original pain area for which opiates were administered, a
potential problem in the therapeutic management of chronic pain. Unexpected hyperalgesia after opioid administration has been reported in several clinical studies (24, 28). We confirmed this paradoxical hypernociceptive phenomenon in this study in mice. The implantation of morphine pellets produced initial potent analgesic effect while by day 7 induced significantly enhanced pain response, as determined by an increase in acetic acid-induced abdominal stretches. The observed hypernociception does not seem to be due to tolerance development to morphine, since the manifestation of pain in the morphine-pelleted group is significantly above the placebo group. If it is due to morphine tolerance, the pain response should have been similar to the placebo group. The time course of development of chronic morphine-induced hypernociception observed in this study is in agreement with the report on a rat model of gastric ulcer (11), neuropathic pain (54), and local inflammation (59) that cause hypernociception are accompanied by hyperexcitability of DRGs, and an upregulated function or expression of TTX-R Na$^+$ channels (34) and DRG (53). Also, the enhanced overlap between activation and inactivation curves in the chronic morphine-pellet groups, i.e., the window current, could increase the amplitude of the slow depolarizing potential and accelerate a long train of APs (34). These data suggest that chronic morphine adapts the TTX-R Na$^+$ channels to activation at a more negative membrane potential. Previous reports show comparable shifts toward hyperpolarizing potential in the voltage-dependent activation of TTX-R $I_{Na}$, seemingly due to the activation of G proteins (30, 31). However, it is not presently clear whether the hyperpolarizing shift observed in this study is due to chronic G protein activation by morphine. In addition to changes in voltage dependence of activation, chronic morphine also alters the time-dependent parameter of activation. The activation time course analyzed by monoexponential fitting of the descending phase of TTX-R $I_{Na}$ currents normalized to cell capacitance were not affected by chronic morphine while some of the biophysical characteristics were altered. Analysis of the current density-voltage relationships demonstrated that current density at $-30$ mV was much larger after chronic morphine relative to the placebo group. This was due to shift in the voltage dependence of activation. Voltage-dependent activation and steady-state inactivation analyzed by Boltzmann fits evinced a $V_{1/2}$ of activation to a much hyperpolarized potential ($\sim 9$ mV more negative) in the chronic morphine group along with an $\sim 7$-mV shift in the voltage dependence of inactivation. Unlike in the morphine group, the half-activation and half-inactivation voltages observed in the placebo group were almost similar to previously reported values for TTX-R Na$^+$ channels in trigeminal ganglion neurons (34) and DRG (53). Also, the enhanced overlap between activation and inactivation curves in the chronic morphine pellet groups, i.e., the window current, could increase the amplitude of the slow depolarizing potential and accelerate a long train of APs (34). These data suggest that chronic morphine adapts the TTX-R Na$^+$ channels to activation at a more negative membrane potential. Previous reports show comparable shifts toward hyperpolarizing potential in the voltage-dependent activation of TTX-R $I_{Na}$, seemingly due to the activation of G proteins (30, 31). However, it is not presently clear whether the hyperpolarizing shift observed in this study is due to chronic G protein activation by morphine. In addition to changes in voltage dependence of activation, chronic morphine also alters the time-dependent parameter of activation. The activation time course analyzed by monoexponential fitting of the descending phase of TTX-R Na$^+$ currents was significantly faster in the chronic morphine-pelleted group relative to the
placebo without altering the inactivation time constants. The faster time course of activation of TTX-R Na\(^+\) currents after chronic morphine could be due to a change in single channel open times and latent times of the TTX-R Na\(^+\) channels (34) being shorter and less dispersed than those in the placebo group. However, the contribution of the faster activation kinetics to the AP is not clear, since the maximum rise slope of the AP was not altered by chronic morphine. The recovery of TTX-R Na\(^+\) channels from inactivation states tends to show no difference after chronic morphine, which probably may not contribute to the altered firing of sensory neurons. It is also noteworthy that in either group the recovery was not complete even after a 200-ms interval, which could probably be due to a small fraction of TTX-R Na\(^+\) channels having entered inactivated states (slow inactivation) from which recovery was slower (13).

The increased excitability upon chronic morphine exposure is unlikely to be due to changes in expression of Na\(_{\text{v}1.7}\), Na\(_{\text{v}1.8}\), or Na\(_{\text{v}1.9}\), since the gene expression of the Na\(_{\text{v}}\) channel subtype \(\alpha\)-subunits was not significantly altered. However, auxiliary subunits have been shown to influence the voltage dependence, time course, and gating processes of activation and inactivation of TTX-R Na\(^+\) channels (45). The influence of chronic morphine over the expression of auxiliary subunits needs further study. Also, it has to be noted that the real-time PCR studies in this manuscript are performed with all DRG neurons combined. Possible single-cell PCR studies would be more informative to delineate between different types of DRGs with respect to the change in Na\(_{\text{v}}\) channel expression, if any, and its corresponding function. Taken together, chronic morphine shifts the voltage dependence of activation of TTX-R Na\(^+\) channels to a more hyperpolarized potential, causing a larger window of channel availability, and speeds the activation kinetics, without affecting the channel expression. It is highly possible that these changes in the biophysical properties of Na\(^+\) channels could be the underlying mechanisms in chronic opioid hyperexcitability.

It is not clear whether any other endogenous molecule plays a role in chronic morphine-induced hyperexcitability of DRGs. Inflammatory mediators like prostaglandins E\(_2\) and 5-hydroxytryptamine increase excitability in nociceptors through an increase in TTX-R Na\(^+\) current (16, 32). Morphine can activate spinal microglia (51, 52) and may cause hyperexcitability through inflammatory mediators released from microglia. Indeed, in a rat model of narcotic bowel syndrome, inhibition of microglia by minocycline reduced the morphine-induced visceral hyperalgesia (1). Therefore, the involvement of spinal glial cells on chronic morphine-induced hyperexcitability and altered TTX-R Na\(^+\) biophysics in DRGs is an attractive area for further studies. Another probable mechanism involved in chronic morphine-induced reduction of threshold of excitability and TTX-R Na\(^+\) currents may be due to posttranslational modification of channels by protein kinase A, protein kinase C (PKC), extracellular signal-regulated kinase, phosphatidylinositol 3-kinase, and phospholipase C (3, 8, 21). Our study also demonstrates that chronic morphine significantly enhances the capsaicin-induced TRPV1 currents in DRGs, which could lead to sensitization of primary afferent neurons. The enhanced TRPV1 current is hypothesized to be due to increased expression of TRPV1 channels and/or elevated phosphorylation by kinases like P38 mitogen-activated protein kinase (38), PKC (57), or Src kinases (22, 40). In this study, we did not find any significant change in the gene expression of TRPV1 channels measured by real-time PCR (data not shown). However, the status of the phosphorylation state of the TRPV1 channel could be altered by chronic morphine, resulting in enhanced capsaicin-induced cationic currents, which needs further study. The cation influx through TRPV1 channels can depolarize the membrane, in turn activating voltage-gated Na\(^+\) channels (10). Therefore, the chronic morphine-induced enhanced TRPV1 function can bring the voltage threshold required for activation of TTX-R Na\(^+\) channels, which is already shifted to a more hyperpolarized potential by chronic morphine, increasing the probability of hyperexcitability in sensory neurons. The role of TRPV1 channels in chronic opioid-induced hypernociception is supported by studies in TRPV1 knockout mice, where the analgesic effects of acute morphine were not affected by gene deletion of TRPV1 but eliminated the hyperalgesia induced by chronic morphine (56).

Whether the chronic morphine-induced changes in TTX-R Na\(^+\) channels and TRPV1 channels occur in the DRG neurons that express opioid receptors is not known. Previous immunohistochemistry studies have demonstrated co-localization of \(\mu\)-opioid receptors and TRPV1 channels in DRG neurons (29). Expression of \(\mu\)-opioid receptors is demonstrated in nociceptive small-diameter DRG neurons (58) that express TTX-R Na\(^+\) channels (32, 44). Therefore, we believe that it is highly likely that chronic morphine-induced adaptive changes in TTX-R Na\(^+\) channels and TRPV-1 channels occur in the DRG neurons that also express \(\mu\)-opioid receptors. However, further studies either functional or anatomical may be needed to determine the existence of any nociceptive DRG neurons without \(\mu\)-opioid receptor expression.

In conclusion, chronic morphine-induced hypernociception is associated with hyperexcitability of DRGs, in addition to altered biophysical properties of TTX-R Na\(^+\) channels. This includes a hyperpolarized shift in voltage dependence of activation and fast activation kinetics, without affecting the gene expression of any relevant Na\(^+\) channel subtype. In addition, enhanced TRPV1 cationic current in DRG neurons following chronic morphine could increase nociception. The altered biophysics of TTX-R Na\(^+\) channels is hypothesized to be the underlying mechanism of hyperexcitability of sensory neurons, leading to hypernociception observed after chronic morphine administration. Further identification of regulatory mechanisms that are involved in the regulation of TTX-R Na\(^+\) channels will be crucial for elucidation of the basal molecular mechanism involved in the regulation of neuronal excitability and conditions like chronic morphine-induced hyperexcitability.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS
REFERENCES


