5-Hydroxytryptamine Type 3 Receptor Modulates Opioid-induced Hyperalgesia and Tolerance in Mice

De-Yong Liang, Ph.D.,* XiangQi Li, M.D.,† J. David Clark, M.D., Ph.D.‡

ABSTRACT

Background: Opioid-induced hyperalgesia (OIH) and tolerance are challenging maladaptations associated with opioids in managing pain. Recent genetic studies and the existing literature suggest the 5-hydroxytryptamine type 3 (5-HT3) receptor participates in these phenomena. The location of the relevant receptor populations and the interactions between the 5-HT3 system and other systems controlling OIH and tolerance have not been explored, however. We hypothesized that 5-HT3 receptors modulate OIH and tolerance, and that this modulation involves the control of expression of multiple neurotransmitter and receptor systems.

Methods: C57BL/6 mice were exposed to a standardized 4-day morphine administration protocol. The 5-HT3 antagonist ondansetron was administered either during or after the conclusion of morphine administration. Mechanical testing was used to quantify OIH, and thermal tail-flick responses were used to measure morphine tolerance. In other experiments spinal cord and dorsal root ganglion tissues were harvested for analysis of messenger RNA concentrations by real-time polymerase chain reaction or immunohistochemistry analysis.

Results: The results showed that (1) systemic or intrathecal injection of ondansetron significantly prevented and reversed OIH, but not local intraplantar injection; (2) systemic or intrathecal injection of ondansetron prevented and reversed tolerance; and (3) ondansetron blocked morphine-induced increases of multiple genes relevant to OIH and tolerance in dorsal root ganglion and spinal cord.

Conclusions: Morphine acts via a 5-HT3-dependent mechanism to support multiple maladaptations to the chronic administration of morphine. Furthermore, the use of 5-HT3 receptor antagonists may provide a new avenue to prevent or reverse OIH and tolerance associated with chronic opioid use.

What We Already Know About This Topic

• Opioid therapy results in tolerance and hyperalgesia, which reflects in part activation of 5-hydroxytryptamine type 3 (5-HT3) receptors

What This Article Tells Us That Is New

• In mice, systemic or intrathecal injection of the 5-HT3 receptor antagonist, ondansetron, prevented or reversed opioid-induced tolerance, hyperalgesia, and genetic changes in sensory neurons

OPIOIDS are a mainstay of treatment for acute and chronic pain. However, repeated or chronic administration of these medications is accompanied by various maladaptations. Tolerance (the reduction of opioid analgesic potency), hyperalgesia (opioid-induced hyperalgesia [OIH], the sensitization to noxious or painful stimuli), and physical dependence (the requirement to continue opioid administration to avoid a withdrawal state) all are challenging conditions associated with the utilization of opiates in managing pain. However, the relationships between and mechanisms of these maladaptations are complex and not fully understood.

Genetic strategies provide a novel approach to understanding the molecular basis underlying these phenomena.1-5 For example, our group used a murine haplotypic mapping approach to identify several target genes associated with specific maladaptations: the β2-adrenergic receptor with mechanical OIH,6 the P-glycoprotein drug transporter (Abcb1b) with thermal OIH,7 and the 5-hydroxytryptamine receptor subunit type 3A (5-HT3A) with physical dependence.7 Furthermore, correlative genetic analysis of the strain-specific data and limited pharmacologic analyses done in the course of these studies suggested that these three principal opioid maladaptations may share common mechanistic underpinnings.8,9 Not well established, however, is the location of the relevant populations of receptors such as 5-HT3 controlling OIH and tolerance.

The 5-HT3 receptor, the focus of the current studies, is a pentameric ligand-gated ion channel consisting of five...
monomers that form a structure centrally permeable to cat-
ions.10–12 The receptor subunits are expressed in brain, spi-
nal cord, and dorsal root ganglia (DRG) tissue.13–18 The 5-HT3 receptor has multiple functions including those in-
volved in nausea and vomiting, pain processing, the drug
reward system, and anxiety. A few studies concluded that
5-HT3 receptor antagonists can reduce various opioid mal-
adaptations.7,19–23 However, these studies involved limited
behavioral assessments, and efforts to determine site of action
as well as effects on gene expression or other mechanisms of
chronic adaptation are largely lacking.

In light of the confirmed genetic finding of 5-HT3 recep-
tor regulation of physical dependence and existing evidence
supporting the hypothesis that 5-HT3 receptor might medi-
ate opioid tolerance and OIH, we conducted a series of ex-
periments to define the role of this receptor in opioid toler-
ance and OIH through pharmacology and molecular
analysis. In an attempt to define the mechanism of this mod-
ulation we evaluated the location of the relevant 5-HT3 re-
ceptor expression and the ability of the 5-HT3 receptor to
control the expression of other genes established to partici-
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control the expression of other genes established to partici-

Materials and Methods

Animals

All animal experiments were done after approval of protocols
by the Veterans Affairs Palo Alto Health Care System Institu-
tional Animal Care and Use Committee (Palo Alto, Cali-
rifornia) and complied with the Guide for the Care and Use of
Laboratory Animals available through the National Academy
of Sciences. Male C57BL/6j mice were obtained from Jack-
son Laboratory (JAX, Bar Harbor, ME) at 7–8 weeks of age.
Mice were kept an additional 7–10 days from the date of
arrival in our animal care facility before use to allow for
acclimation. Mice were housed four to six per cage under
pathogen-free conditions with soft bedding and were pro-
vided food and water ad libitum with a 12:12 light/dark
cycle.

Chronic Morphine Administration

After baseline nociceptive testing, morphine (Sigma Chem-
ical, St. Louis, MO) was subcutaneously administered to
mice 10 mg/kg twice daily on day 1, 20 mg/kg twice daily on
days 2 and 3, and 40 mg/kg twice daily on day 4 in 50- to
100-μl volumes of 0.9% NaCl similar to our previous pro-
tocols for OIH and tolerance.3,6–8

Ondansetron Administration

Ondansetron (Sigma Chemical) was administered acutely
and chronically via systemic application (subcutaneous and
intrathecal injection) or local hind paw site application. For

systemic administration, ondansetron was injected subcuta-
neously in a 100-μl volume in 0.9% NaCl to some groups of
mice. The drug was either given at a dose of 1 mg/kg along
with each dose of morphine during the chronic dosing par-
adigm, or given once at a dose of 2 mg/kg 30 min before
tolerance or nociceptive testing. The procedure for intra-
thecal drug administration was based on the technique de-
scribed by Hylden and Wilcox.24 Briefly, the intervertebral
space between L5 and L6 was punctured directly using a
28-gauge needle attached to a microsyringe. Mice were
lightly anesthetized with isoflurane during these procedures.
A tail flick was used as an indication that the needle had
penetrated the dura. Once inserted, 5 μl injectate was slowly
administered with use of a microsyringe, and the animals
were used within 20 min of the injection.

Behavioral Measurement

Opioid-induced Hyperalgesia. Mechanical allodynia was
assessed using nylon von Frey filaments according to the “up-
down” algorithm described by Chaplan et al.25 as previously
described.6,26 In these experiments, mice were placed on wire
mesh platforms in clear cylindrical plastic cylinders. After 15
min of acclimation, fibers of sequentially increasing stiffness
were applied to the plantar surface of one hind paw, and left
in place 5 s. Withdrawal of the hind paw from the fiber was
scored as a response. When no response was obtained, the
next stiffest fiber in the series was applied to the same paw; if
a response was obtained a less stiff fiber was applied. Testing
proceeded in this manner until four fibers had been applied
after the first one, causing a withdrawal response and allow-
ing the estimation of the mechanical withdrawal threshold.27
This data-fitting algorithm allowed the use of parametric
statistics for analysis.

Morphine Dose-Response. Cumulative morphine dose-
response curves were constructed using the tail-flick assay
and methods similar to those we described previously.8,9 For
these measurements mice were gently restrained within a
cone-shaped tube made of cotton toweling. Using a tail-flick
analgesic apparatus (Columbus Instruments, Columbus,
OH), tail-flick latency was measured with 0.1-s precision. A
10-s cutoff time was used to prevent permanent tissue dam-
age. Two measurements were made per mouse with the light
beam focused on two different points 1 cm apart on the tail.
The lamp intensity was the same for all animals, which re-
sulted in baseline tail-flick measurements of 3–4 s. For the
assessment of tolerance, these dose–response experiments
followed 18 h after the final dose of morphine given as part of
the chronic morphine administration protocol. The cumu-
lat"et al.
Messenger RNA Expression.

Expression Studies

Messenger RNA Expression. Mice were sacrificed at specific time points by carbon dioxide asphyxiation. Spinal cord lumbar segments were harvested by extrusion and rapid dissection on a prechilled surface. The DRGs were dissected using low-power binocular magnification. Tissue was then quick-frozen in liquid nitrogen and stored at −80°C until use. For synthesis of complementary DNA and real-time polymerase chain reaction (PCR), total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, and its purity and concentration were determined spectrophotometrically as described previously.28 Complementary DNA was synthesized from total RNA using random hexamer priming and a first strand synthesis system (Invitrogen, Carlsbad, CA). Briefly, 1 μg total RNA was mixed with 4 μl 10X reverse transcription buffer, 8 μl 25 mM MgCl2, 4 μl 0.1 M dithiothreitol, 1 μl RNasin, 2 μl Super Script II reverse transcriptase (50 U/μl), 5 μl hexomers, and RNase-free water to 40 μl. Incubation was then carried out at 42°C for 60 min followed by heat inactivation at 70°C. Finally, 1 μl RNase H was added to each reaction and incubated at 37°C for 20 min to degrade the RNA. For real-time quantitative PCR, reactions were conducted in a volume of 4 μl using the Sybr Green I master kit (PE Applied Biosystems, Foster City, CA). Briefly, 2 μl a mixture of 2× sybr green and target gene primers (see table 1) was loaded with 2 μl diluted complementary DNA template in each well. After this, 8 μl mineral oil was loaded in each well to prevent loss of solution. With use of an ABI prism 7900HT system (Applied Biosystems, Foster City, CA), PCR was carried out using the parameters 52°C, 5 min → 95°C, 10 min then [95°C, 30 s → 60°C, 60 s] for 40 cycles. Samples were analyzed in triplicate. Melting curves were performed to document single product formation. 18S RNA was used as an internal control. The 18S primers were purchased from Ambion (Ambion, Austin, TX). Quantification was accomplished according to the standard curve method as described previously.9 To achieve the same PCR efficiency for each analyte, serial dilution of complementary DNA was used to construct a standard curve for the target genes. The R2 values for the standard curves of the test genes approached 1.0, suggesting the same amplification efficiency in the PCRs under these conditions. The expression level of specific genes was normalized to the level of 18S expression in each sample.

Immunohistochemistry. The localization of expression of 5-HT3A and substance P in DRG was tested by using fluorescence confocal microscopy as previously described.29 Mice used in these experiments first underwent asphyxiation by carbon dioxide and perfusion by intracardiac injection of 10 ml 0.9% NaCl. This was followed by perfusion with 20 ml 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The DRG was then dissected under low-power magnification and fixed in 4% paraformaldehyde for 4 h at room temperature followed by overnight incubation in 30% sucrose at 4°C. The tissues were then embedded in optimal cutting temperature medium, and 8-μm sections made on a cryostat with subsequent processing on slides. Blocking took place overnight at 4°C in Tris-buffered saline containing 5% dry milk, followed by exposure to the primary antibodies: polyclonal anti-5-HT3A, 1:500 dilution (Abcam, Cambridge, MA); goat polyclonal anti-substance P antibody, 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA). The anti-5-HT3A antibody is against a synthetic peptide corresponding to a selective portion of the first extracellular loop of mouse 5-HT3A. This peptide corresponds to amino acids 40–140 of the mouse protein. For the specificity of 5-HT3A and substance P antibody, the preabsorption of the antibody with specific blocking peptide was conducted before adding to

Table 1. Primer Sequences for Target Genes

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward (5′→3′)</th>
<th>Reverse (5′→3′)</th>
<th>Product Size (bp)</th>
<th>Genbank Accession</th>
</tr>
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<tbody>
<tr>
<td>α-CGRP</td>
<td>tgacaggagctaaagctgaagtgcc</td>
<td>aacctcaacaggtaggtagttatg</td>
<td>142</td>
<td>NM_001033954</td>
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<tr>
<td>β2-AR</td>
<td>caactctgcttcctcaactttta</td>
<td>ctgtagttgcctgctccataagctt</td>
<td>117</td>
<td>NM_007420</td>
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<td>5HT3A</td>
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<td>NM_008712</td>
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<td>gatacctcaatgtagcctcctct</td>
<td>177</td>
<td>NM_008169</td>
</tr>
<tr>
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<td>actgcactaagagcacacgc</td>
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<td>AF026537</td>
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<tr>
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<td>109</td>
<td>NM_001001445</td>
</tr>
<tr>
<td>18S</td>
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<td>tccgctaatcctttgattttca</td>
<td>160</td>
<td>NR_003278</td>
</tr>
</tbody>
</table>

The information of primer sequences of target genes tested, including gene access number in GenBank, polymerase chain reaction product size (bp).

αCGRP = α-calcitonin gene-related peptide; β2-AR = β2-adrenergic receptor; 5-HT3A = 5-hydroxytryptamine receptor subunit type 3A; NOS1 = nitric oxide synthase-1; NR1 = N-methyl-D-aspartate receptor-1; PDP = prodyorphin; PPT-A = preprotachykinin-A; TRPV1 = transient receptor potential vanilloid-1; 18S = 18S ribosomal RNA gene.

The percent maximal possible effect (%) was determined according to the following formula:

\[
\text{The percent maximal possible effect } (\%) = \left( \frac{\text{measured latency} - \text{baseline latency}}{\text{cutoff latency} - \text{baseline latency}} \right) \times 100
\]

Expression Studies

Messenger RNA Expression. Mice were sacrificed at specific time points by carbon dioxide asphyxiation. Spinal cord lumbar segments were harvested by extrusion and rapid dissection on a prechilled surface. The DRGs were dissected using low-power binocular magnification. Tissue was then quick-frozen in liquid nitrogen and stored at −80°C until use. For synthesis of complementary DNA and real-time polymerase chain reaction (PCR), total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, and its purity and concentration were determined spectrophotometrically as described previously.28 Complementary DNA was synthesized from total RNA using random hexamer priming and a first strand synthesis system (Invitrogen, Carlsbad, CA). Briefly, 1 μg total RNA was mixed with 4 μl 10X reverse transcription buffer, 8 μl 25 mM MgCl2, 4 μl 0.1 M dithiothreitol, 1 μl RNasin, 2 μl Super Script II reverse transcriptase (50 U/μl), 5 μl hexomers, and RNase-free water to 40 μl. Incubation was then carried out at 42°C for 60 min followed by heat inactivation at 70°C. Finally, 1 μl RNase H was added to each reaction and incubated at 37°C for 20 min to degrade the RNA. For real-time quantitative PCR, reactions were conducted in a volume of 4 μl using the Sybr Green I master kit (PE Applied Biosystems, Foster City, CA). Briefly, 2 μl a mixture of 2× sybr green and target gene primers (see table 1) was loaded with 2 μl diluted complementary DNA template in each well. After this, 8 μl mineral oil was loaded in each well to prevent loss of solution. With use of an ABI prism 7900HT system (Applied Biosystems, Foster City, CA), PCR was carried out using the parameters 52°C, 5 min → 95°C, 10 min then [95°C, 30 s → 60°C, 60 s] for 40 cycles. Samples were analyzed in triplicate. Melting curves were performed to document single product formation. 18S RNA was used as an internal control. The 18S primers were purchased from Ambion (Ambion, Austin, TX). Quantification was accomplished according to the standard curve method as described previously.9 To achieve the same PCR efficiency for each analyte, serial dilution of complementary DNA was used to construct a standard curve for the target genes. The R2 values for the standard curves of the test genes approached 1.0, suggesting the same amplification efficiency in the PCRs under these conditions. The expression level of specific genes was normalized to the level of 18S expression in each sample.

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the section. Sections were then rinsed and transferred to milk-Tris-buffered saline containing either CY3 conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) or Texas Red conjugated secondary antibody (Vector Labs, Burlington, CA) and incubated for 1 h. After washing, coverslips were applied. Confocal laser-scanning microscopy was carried out using a Zeiss LSM/510 META microscope (Thornwood, NY). Control experiments included incubation of slices in primary or secondary antibody-free solutions. Both conditions lead to low-intensity, nonspecific staining patterns in preliminary experiments.

**Statistical Analysis**

All data are expressed as mean ± SEM unless otherwise noted. The data of mechanical sensitivity, tail-flick response, and gene expression in DRG and spinal cord were analyzed by two-way ANOVA followed by the Bonferroni post hoc test for multiple comparisons. Simple comparisons of two groups involved unpaired t-testing with two-tailed P values. Dose–response data were fitted using a sigmoidal function with variable slope as shown in a four-parameter logistic equation below and at the top of the curve set at 100% to determine 50% analgesic effective dose (ED50) values and 95% CI (Prism 5, GraphPad Software, La Jolla, CA). Pairs of dose–response curves were compared using the F test.

\[
Y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{(1 + 10^{(@\log EC_{50} - X)}) \times \text{hill slope}}
\]

**Results**

**The Effects of Systemic 5-HT3 Receptor Blockade on OIH**

We first hypothesized that if OIH was supported by the 5-HT3 receptor, the administration of a selective receptor antagonist should reduce sensitization after chronic opioid administration. Figure 1A shows data demonstrating that after 4 days of escalating-dose morphine administration, mice display alldynia to mechanical stimuli. In this setting of established OIH, administration of the selective 5-HT3 receptor antagonist ondansetron (2 mg/kg, subcutaneously) significantly reversed OIH over the first few hours in comparison with a control group of OIH mice treated with saline.

Although the acute systemic administration of ondansetron reversed OIH, we conducted a series of experiments to determine whether administration of ondansetron during the morphine treatment phase could prevent the development of OIH. To accomplish this, morphine and ondansetron were coinjected daily in a study group of mice. The data presented in figure 1B show that chronic administration of ondansetron fully prevented OIH development normally induced by chronic morphine treatment. Chronic ondansetron treatment alone did not induce any change of mechanical sensitivity in comparison with the control group.

**Localization of Ondansetron's Site of Action in Reversing OIH**

Next we determined whether ondansetron influenced OIH by acting on central 5-HT3 receptors versus those in the periphery. Intrathecal injection of ondansetron (1 μg/2 μl) dramatically reduced opioid-induced mechanical sensitivity as shown in figure 2A. Similar to systemic administration, the effect of ondansetron after intrathecal injection was maximal 2 h after administration. However, peripheral hind paw administration of ondansetron at the same dose and a 10-fold higher dose did not change the morphine-induced sensitization. These data are presented in figure 2B.

**The Effects of 5-HT3 Receptor Blockade on Morphine Antinociception in Morphine Tolerant Mice**

Next we treated the mice with morphine for 4 consecutive days in a chronic morphine dosing paradigm. Figure 3A shows the large rightward shift in the morphine dose–response relationship after this treatment protocol. Also provided are data for a separate group of tolerant mice in which ondansetron was administered immediately before dose–response testing (fig. 3B). These data demonstrate that the acute administration of ondansetron to morphine-tolerant mice completely reverses morphine
tolerance. Figure 3C demonstrates that if ondansetron was administered along with morphine during the chronic phase of treatment (see Methods). The assessment mechanical sensitivity at baseline was conducted before morphine treatment (baseline), after morphine treatment and after subsequent OND administration (arrow). (A) The effect of intrathecal injection of OND (1 μg) or saline in morphine-treated mice. (B) The effect of intraplantar injection of OND (1 and 10 μg) or saline in morphine-treated mice. Data are presented as mean ± SEM; **P < 0.01; ***P < 0.001. Six mice were used in each group.

The Effects of Spinal 5-HT3 Receptor Blockade on Tolerance
In the OIH experiments we had confirmed that intrathecal injection of a 5-HT3 receptor antagonist reversed morphine-induced nociceptive sensitization. Therefore, we hypothesized that blockade of spinal 5-HT3 receptors also would reverse tolerance. Figure 4 shows that intrathecal injection of ondansetron reverses opioid tolerance in mice previously treated with morphine, but does not affect the morphine dose–response relationship if the drugs are coadministered.

The Effects of 5-HT3 Receptor Blockade on Pain and Tolerance-related Gene expression in DRG and Spinal Cord Tissue
The behavioral data demonstrate that 5-HT3 receptor blockade prevented the development of OIH and tolerance. Given the current results and the genetic association of the 5-HT3 with several opioid maladaptations (OIH, tolerance, and physical dependence), we hypothesized that the morphine-induced up-regulation of multiple pain and tolerance-related genes in the spinal cord and DRG tissue would be prevented by 5-HT3 receptor blockade during opioid treatment. The intrathecal administration of ondansetron could be hypothesized to act on either DRG or spinal cord tissue. For the analysis of DRG tissue we measured the expression of messenger RNA species coding for α-calcitonin gene-related peptide, the substance P precursor preprotachykinin-A, the N-methyl-D-aspartate receptor-1, the transient receptor potential vanilloid-1, the β2-adrenergic receptor, and 5-HT3A.

Fig. 2. The differential effects of ondansetron (OND) on opioid-induced hyperalgesia (OIH) when administered via intrathecal or intraplantar injection. Mice were used after chronic morphine treatment (see Methods). The assessment mechanical sensitivity at baseline was conducted before morphine treatment (baseline), after morphine treatment and after subsequent OND administration (arrow). (A) The effect of intrathecal injection of OND (1 μg) or saline in morphine-treated mice. (B) The effect of intraplantar injection of OND (1 and 10 μg) or saline in morphine-treated mice. Data are presented as mean ± SEM; **P < 0.01; ***P < 0.001. Six mice were used in each group.

Fig. 3. The effect of ondansetron (OND) on morphine tolerance. Morphine dose–response relationships were assessed using the tail-flick assay. MPE% = the percentage maximal possible effect. (A) The morphine dose–response relationships before (baseline) and after 4 days of morphine treatment. (B) The effects of OND (2 mg/kg, subcutaneous injection) on morphine analgesic tolerance when administered immediately before dose–response testing. (C) The effects of OND administration (1 mg/kg, subcutaneous injection) on morphine analgesic tolerance when administered at the time of each of the bidaily morphine injections during the 4-day morphine treatment period. Data are presented as mean ± SEM. Six mice were used in each group.
show that 5-HT3 blockade reduced the morphine-induced up-regulation of all members of this panel of genes.

To determine the role of the 5-HT3 receptor in controlling morphine-induced changes in spinal cord tissue, we chose to analyze the expression of nitric oxide synthase 1, the N-methyl-D-aspartate receptor-1, and prodynorphin, all of which, again, have been linked to OIH and/or tolerance.34,35 The data in figure 6 demonstrate that 5-HT3 receptor blockade suppressed the increases of these genes driven by chronic morphine treatment. In additional experiments we found that expression of 5-HT3A was not significantly enhanced by chronic morphine treatment.

**Protein Expression of 5-HT3 Receptor and Substance P in DRG**

Our results with messenger RNA indicated that the population of 5-HT3A most strongly linked to OIH and tolerance may be those expressed on sensory nerves. Figure 7 demonstrates that 5-HT3A is strongly expressed on DRG peptidergic neurons stained with substance P. The immunostaining signal was abolished when the sections were incubated with antibody preabsorbed with the block peptide of 5-HT3A or substance P. It demonstrates that such immunoactive signal is specific for 5-HT3A or substance P. The coexpression of these targets may not have been carefully studied before this time, although other reports have confirmed existence of the 5-HT3A on DRG neurons in the rat.18,36

**Discussion**

In this series of studies we addressed the ability of the 5-HT3 receptor to limit and reverse key opioid maladaptations ob-

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**Table 2. Effect of OND on Morphine Analgesic Potency**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Analgesic ED$_{50}$ (mg/kg)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>4.31</td>
<td>3.961–5.146</td>
</tr>
<tr>
<td>Chronic morphine</td>
<td>15.08*</td>
<td>13.56–16.78</td>
</tr>
<tr>
<td>Acute OND + chronic morphine</td>
<td>5.02</td>
<td>4.614–5.462</td>
</tr>
<tr>
<td>Chronic OND + chronic morphine</td>
<td>4.28</td>
<td>3.8000–4.817</td>
</tr>
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</table>

50% morphine analgesic effective dose (ED$_{50}$) values in animals treated with or without OND were determined by using the sigmoidal fitting-curve method (see Statistical Analysis).

*P < 0.05 in comparison of chronic morphine alone with other groups.

OND = ondansetron.
served after chronic administration, namely OIH and tolerance. Such ability was suggested by previous pharmacologic studies and by genetic investigations. Lacking from the literature, however, was information from systematic studies on the location of relevant 5-HT3 receptor populations, and careful exploration of the prevention versus reversal of established adaptations. Moreover, little information before this time was available to address the mechanisms by which the 5-HT3 receptor might regulate opioid adaptations such as by controlling gene expression in specific tissues involved in nociception and analgesia. Using a well-characterized protocol for sustained morphine treatment in a strain of mice known to display robust adaptations to morphine exposure, we observed that (1) both systemic and intrathecal but not local administration of the selective 5-HT3 antagonist ondansetron could prevent and reverse OIH, and (2) systemic and intrathecal injection of ondansetron prevents and reverses tolerance to morphine, and (3) the concomitant treatment of mice with ondansetron prevents adaptive effects on gene expression in spinal cord and DRG tissues. Importantly, the genes studied were all ones for which functional roles in OIH and tolerance have been demonstrated.

Tolerance and hyperalgesia have recently been recognized as maladaptations complicating acute and chronic pain management. Studies using both methadone-maintained patients addicted to opioid and opioid-maintained patients with chronic pain have revealed enhanced pain sensitivity to various stimuli, including ice-water immersion of a limb and the injection of lidocaine. Studies addressing the topic universally demonstrate increased opioid requirements postoperatively in those patients with a history of chronic opioid consumption, and often increased pain scores as well. Difficulties in managing these patients have led to the publication of several reviews on the perioperative management of chronic opioid consumption in these patients. Even the acute administration of relatively high-dose opioids during surgery increases postoperative opioid requirements. Despite these observations, we currently lack proven techniques for limiting the development of OIH or tolerance in clinical populations. With the possible exception of postoperative infusion of ketamine, we also lack therapies that can reverse OIH or tolerance to a clinically useful degree once established.

One of our key observations was that intrathecal administration of ondansetron was effective in reversing tolerance and OIH, whereas injection of this selective antagonist at the same or 10-fold higher dose into the paw at the site of noxious stimulation failed to reduce OIH. Because intracereally administered drugs have access to spinal cord tissue and at

Fig. 7. Immunohistochemical analysis of 5-hydroxytryptamine receptor subunit type 3A (5-HT3A) and substance P expression in DRG. (A) 5-HT3A staining. (B) Substance P staining. (C) The double staining. The top section is preabsorbed primary antibody stained with specific block peptide as control. Scale bar is 50 μm.
least some portion of the DRG, the relevant 5-HT3 receptor populations may be expressed in either spinal neurons or afferent sensory fibers. Primary sensory neurons are known to express 5-HT3 receptors, and functional receptors capable of causing excitation and neurotransmitter release have been identified on both the sensory neuron cell bodies and on presynaptic terminals within the dorsal horn of the spinal cord. Many of these receptors are expressed on capsaicin-sensitive small afferent fibers consistent with our observations of 5-HT3 regulation of substance P, α-calcitonin gene-related peptide, and transient receptor potential vanilloid-1. Data that demonstrate the expression of 5-HT3 receptors on distal primary afferent nerve terminals are sparse. Although 5-HT3 is an important inflammatory mediator, and the injection of 5-HT in distal peripheral tissues causes hyperalgesia sensitive to selective 5-HT3 blocker, this is postulated to occur via an indirect action of serotonin on afferent neurons requiring local norepinephrine release and the activation of β2-adrenergic receptors. Although mechanisms of OIH involving peripheral nerve terminals and the enhanced production of inflammatory mediators in skin tissue have been demonstrated, we failed to find evidence for the participation of distally expressed 5-HT3 receptors in the current studies.

On the other hand, abundant evidence exists for the participation of 5-HT3 receptors in multiple aspects of nociceptive signal transmission in the spinal cord. Immunohistochemical studies have revealed the dense expression of 5-HT3 receptors in the superficial dorsal horn, an area that receives nociceptive input. Available reports indicate that 5-HT3 action can be either inhibitory to nociceptive signal transmission or facilitatory, with the latter action more consistent with OIH and tolerance. For example, the intrathecal injection of the selective 5-HT3 agonist 2-Me-5-HT has analgesic activity in some models. This activity may be the result of 5-HT3 mediated enhancement of γ-aminobutyric acid–mediated (GABAergic) inhibitory signaling. Other evidence, however, demonstrates a clear role for descending serotonergic facilitatory mechanisms in supporting hyperalgesia and facilitating nociceptive signal transmission. For example electrophysiologic, pharmacologic, and behavioral evidence supports roles for descending neurons from the rostral ventromedial medulla in facilitating nociceptive signaling in models of cancer-induced bone pain, inflammatory pain, and neuropathic pain. Importantly, facilitatory descending circuitry from the rostral ventromedial medulla to the dorsal horn of the spinal cord also supports both opioid tolerance and OIH, and it has been suggested that tolerance and OIH are essentially different manifestations of a common set of alterations in spinal cord neurophysiology. One report using single acute intrathecal ondansetron injections reported reversal of morphine tolerance and OIH in rats. In the current studies, we demonstrated the ability of ondansetron to prevent as well as reverse tolerance and OIH, and further explored the effects of systemic, intrathecal, and local peripheral administration of the 5-HT3 blocker.

Although our observations regarding the acute administration of ondansetron confirm and extend previous reports regarding the facilitatory activity of 5-HT3 receptors in pain and opioid adaptations, we also addressed the ability of 5-HT3 blockade to prevent opioid adaptations from occurring. The approach involved ondansetron-morphine coadministration paradigms in conjunction with behavioral and gene expression studies. For both the OIH and tolerance testing, nociceptive measurements were made at a point more than 6 half-lives after the final dose of ondansetron was delivered making ongoing ondansetron blockade unlikely. Our gene expression experiments provide a basis for explaining these observations. When we studied the morphine-induced expression of a group of genes in the DRG, which had been implicated in previous reports as being functionally involved in OIH and tolerance. These genes include α-calcitonin gene-related peptide, preprotachykinin A, the N-methyl-D-aspartate receptor-1, the transient receptor potential vanilloid-1, and the β2-adrenergic receptor. They are mainly synthesized in primary afferent neurons. Chronic morphine treatment induces increased messenger RNA synthesis, encoding these targets mostly in DRG as our group and other groups reported. Blockade of these targets with inhibitors or antagonists reduces opioid tolerance and OIH development. In the current investigation, we demonstrated that 5-HT3 receptor antagonist, ondansetron, prevented morphine-enhanced expression in all instances. Likewise, when a second group of genes expressed on spinal cord neurons with proven involvement in OIH and tolerance was studied (nitric oxide synthase-1, N-methyl-D-aspartate receptor-1, and prodynorphin), again all genes showed morphine-enhanced expression, which was completely prevented by the coadministration of ondansetron. The widespread prevention of gene expression adaptations to chronic morphine administration in DRG and spinal cord tissue provides a rationale for the ability of ondansetron to prevent OIH and tolerance.

Although the effects of 5-HT3 antagonists were not tested in clinical models of OIH or tolerance, it is notable that 5-HT3 antagonists have a remarkable record of safety even when used in high doses. Furthermore, in some clinical settings such as surgery and cancer care, 5-HT3 blockers are commonly used for their antiemetic effects along with opioids. The current findings might therefore be translated to clinical studies in which populations vulnerable to poor pain relief because of tolerance or OIH might be provided 5-HT3 antagonists to determine whether opioid use decreases or the quality of analgesia improves. In the United States, several selective 5-HT3 drugs, including ondansetron, granisetron, tropisetron, palonosetron, and dolasetron would be available for such trials.

In summary, the 5-HT3 system has been explored in humans and animals as controlling addiction, anxiety, de-
pression, irritable bowel syndrome, pain, and nausea/vomiting. Our studies combined with others suggest that the 5-HT3 system may participate in the acquisition and maintenance of two clinically key opioid maladaptations, OIH and tolerance. In this regard, it seems most likely that 5-HT3 receptors expressed on the soma or the central terminals of afferent neurons are most important. Acute 5-HT3 blockade may reduce tolerance and OIH by blocking descending serotonergic facilitation, whereas the blockade of changes in gene expression may be more central to the preventive effects of the antagonists. The long safety record associated with the use of these drugs and the availability of several well-characterized agents speaks to the potential translation of our rodent observations into human studies.

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