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## From mouse to man: the 5-HT<sub>3</sub> receptor modulates physical dependence on opioid narcotics

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### Abstract

**Objectives**—Addiction to opioid narcotics represents a major public health challenge. Animal models of one component of addiction, physical dependence, show this trait to be highly heritable. The analysis of opioid dependence using contemporary in-silico techniques offers an approach to discover novel treatments for dependence and addiction.

**Methods:** In these experiments, opioid withdrawal behavior in 18 inbred strains of mice was assessed. Mice were treated for 4 days with escalating doses of morphine before the administration of naloxone allowing the quantification of opioid dependence. After haplotypic analysis, experiments were designed to evaluate the top gene candidate as a modulator of physical dependence. Behavioral studies as well as measurements of gene expression on the mRNA and protein levels were completed. Finally, a human model of opioid dependence was used to quantify the effects of the 5-HT<sub>3</sub> antagonist ondansetron on signs and symptoms of withdrawal.

**Results**—The *Htr3a* gene corresponding to the 5-HT<sub>3</sub> receptor emerged as the leading candidate. Pharmacological studies using the selective 5-HT<sub>3</sub> antagonist ondansetron supported the link in mice. Morphine strongly regulated the expression of the *Htr3a* gene in various central nervous system regions including the amygdala, dorsal raphe, and periaqueductal gray nuclei, which have been linked to opioid dependence in previous studies. Using an acute morphine administration model, the role of 5-HT<sub>3</sub> in controlling the objective signs of withdrawal in humans was confirmed.

**Conclusion:** These studies show the power of in-silico genetic mapping, and reveal a novel target for treating an important component of opioid addiction.

### Keywords

addiction; hyperalgesia; morphine; ondansetron; opioid; serotonin

### Introduction

Addiction to illicit and prescription opioid narcotic drugs (heroin, morphine, codeine, oxycodone and related agents) is a significant public health issue because of the number of people it affects and its cost to society. Each month in the United States, 4.9% of persons aged

12 years or older (>11 million) use prescription pain relievers for nonmedical purposes [1]. Young adults (age 18–25 years) are particularly hard hit by this problem, they have the highest rate of abuse of prescription pain relievers [1]. Opioid addiction has significant adverse consequences for personal health and society [2–4]. As many as 90% of patients in chronic pain management settings receive opioid pain relievers, and the prevalence of drug abuse is 9–41% among these patients [4]. Anxiety, increased pain sensitivity, poor concentration, tachycardia and flu-like symptoms develop during opioid withdrawal, a syndrome reflecting physical dependence on these drugs [5]. The severity of the dependence and resulting withdrawal symptoms is a major contributor to the addictive potential of opioid narcotics. Current strategies for treatment of opioid withdrawal are suboptimal; they rely on the administration of controlled substances (methadone and buprenorphine), or medications with significant hemodynamic side effects (clonidine). As it can lead to new approaches for prevention or treatment of addiction, identification of novel genetic factors affecting dependence on opioids is of great public health significance.

Susceptibility to opioid addiction is heritable in humans [6]. One dimension of addiction is physical dependence, which can be modeled in rodents. The jumping behavior displayed by morphine-dependent mice after administration of naloxone, a potent opioid receptor antagonist, is a commonly used measure of physical dependence. Naloxone-precipitated jumping is a highly heritable trait amongst inbred mouse strains [7], and the interstrain differences are largely independent of differences in the method of drug administration or the duration of treatment [8,9]. Furthermore, naloxone-precipitated withdrawal has been used to quantify opioid dependence in human volunteers [10]. Despite these facts, no specific genes linked to physical dependence have been identified. We, however, recently used a computational genetic mapping method [11,12] to successfully identify several genetic factors underlying the variability in morphine-induced alterations in pain sensitivity (hyperalgesia) and responsiveness to analgesic medications in mice [13–15]. We therefore investigated whether haplotype-based computational genetic mapping could identify genes affecting susceptibility to opioid dependence in mice, and whether a pharmacological agent targeting the human homolog of the computationally identified murine gene could alleviate the signs and symptoms of withdrawal in humans.

## Methods

### Animals

All animal experiments were conducted using protocols approved by our Institutional Animal Care and Use Committee. Protocols complied with the Guide for the Care and Use of Laboratory Animals available through the National Academy of Sciences. Male mice from the inbred mouse strains (129/SvImJ, A/HeJ, A/J, AKR/J, B10.D2-H2/oSNJ, BALB/cByJ, BALB/cJ, BUB/BnJ, C3H/HeJ, C57BL/6J, DBA/2J, FVB/NJ, LP/J, LG/J, MRL/MpJ, NZB/BinJ, NZW/LaCJ, SM/J) were obtained from Jackson Labs (Bar Harbor, Maine, USA) at 7–8 weeks of age, and housed for 7–10 days in our animal care facility for acclimation before use in experiments. The mice were kept under pathogen-free conditions and were provided food and water ad libitum with a 12 : 12 h light:dark cycle.

### Behavioral assays

**Morphine treatment**—After baseline nociceptive testing, morphine (Sigma Chemical, St. Louis, Missouri, USA) was administered to mice subcutaneously (s.c.) 10 mg/kg twice per day on day 1, 20 mg/kg on days 2–3, and 40 mg/kg twice per day on day 4 in 50–100  $\mu$ l volumes of 0.9% NaCl similar to our previous protocols for generating opioid-induced hyperalgesia (OIH), tolerance and dependence [9,13,14].

**Precipitated withdrawal**—For dependence determinations, mice were assessed 18 h after the final dose of morphine when spontaneous dependence-related hyperalgesia was maximal [16]. Naloxone (Sigma Chemical) 10 mg/kg was injected s.c. in 50  $\mu$ l of NaCl as described previously by our laboratory and others [8,9]. After naloxone administration, mice were placed in clear plastic cylinders (10 cm in diameter and 40 cm in height), and the number of jumps during the following 15 min was counted. Naloxone-precipitated jumping behavior is the most robust response reflecting physical dependence on opioids observed across strains of inbred mice [17].

In some experiments, the selective 5-HT<sub>3</sub> antagonist ondansetron (Sigma Chemical) was administered. For systemic administration, ondansetron was injected s.c. in 100  $\mu$ l volumes of 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 paradigm, or given once over a range of doses 30 min before dependence or nociceptive testing. For intracerebroventricular (i.c.v.) administration, mice were briefly anesthetized with inhaled isoflurane. A 30-gauge half-inch needle was used to pierce the skull and enter the ventricles using an approach described previously [18]. Once inserted, 5  $\mu$ l of injectate was slowly administered using a microsyringe, and the animals were used within 20 min after the injection.

**Opioid dependence-related hyperalgesia**—Mechanical allodynia was assayed using nylon von Frey filaments according to the ‘up-down’ algorithm described by Chaplan *et al.* [19] as previously described [14,20]. 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, pressed upward to cause a slight bend in the fiber and left in place for 5 seconds. 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 allowing the estimation of the mechanical withdrawal threshold [21]. This data-fitting algorithm allowed the use of parametric statistics for analysis. This assay is sufficiently sensitive to detect mechanical thresholds as low as 0.02 g [14].

**Conditioned place preference**—To assess the dependence liability of morphine and subsequent effects of ondansetron on such dependence, a counter-balanced conditioned place preference (CPP) paradigm was used [22,23]. The CPP experiments were carried out using Place Preference System (MED Associates Inc., St. Albans, Vermont, USA), which consists of three compartments; two outer compartments for active association and a middle neutral compartment. One association compartment is constructed of white opaque plastic walls with a floor made of metal rods, whereas the other compartment is made of black opaque plastic walls with a metal mesh floor. The smaller middle neutral compartment is made of gray opaque plastic walls and floor. Experiments were conducted in a dimly lit room with homogenous lighting (approximately 20 lux). The place preference apparatus is equipped with motion photosensors with data recorded through a computer running MED PC software (MED Associates Inc.). C57BL/6J mice (8–12 weeks old) were used in all experiments, which were carried out during the second half of the light phase between 13 : 00 and 18 : 00 h. On Day 0, the mice were acclimated to the test room and the time spent in each compartment was recorded (preconditioning report). Any mouse that spent more than 75% of the time in either of the association compartments was excluded. On the next day, each mouse was randomized to a control or drug group and assigned to either the white or black association compartments in a counter-balanced manner ( $n = 10–15$  per group). On Days 1, 3, and 5 the drug group received intraperitoneal injections of the drug(s): vehicle (0.9% saline), morphine 5 mg/kg or ondansetron 1 mg/kg plus morphine 5 mg/kg (separate injections). Twenty-five minutes after injection(s), each mouse was placed into its assigned association compartment for 25 min

without access to the other compartments. On Days 2, 4, and 6, the Mice received saline injections and were placed in the alternative compartment after 25 min. On Day 7, mice were placed in the middle neutral compartment of the place preference apparatus with full access to the other two compartments and assessed for the length of time spent in each compartment (postconditioning report). The percentage of time spent in each association compartment was calculated relative to the total time spent in both association compartments.

### Computational haplotype-based genetic mapping

Haplotype-based computational genetic analysis of the phenotypic data was performed as previously described [11,14,24,25]. This technique has been used recently to identify genes associated with a number of different murine phenotypic traits including opioid narcotic drug responses [9,13-15]. In brief, allelic data from multiple inbred strains were analyzed and a haplotype block map of the mouse genome was constructed. Only a limited number of haplotypes – typically 2, 3, or 4 – are present within a haplotype block. This analysis identifies haplotype blocks in which the haplotypic strain grouping within a block correlates with the distribution of phenotypic data among the inbred strains analyzed. To do this, a *P* value that assesses the likelihood that genetic variation within each block could underlie the observed distribution of phenotypes among the inbred strains is calculated as described using analysis of variance [11,12]. The phenotypic data were evaluated using the average value for each strain, obtained by assessing eight mice per strain. The haplotype blocks are then ranked based upon the calculated *P* value. When this computational analysis was performed, the haplotype map had 5694 haplotype blocks generated from 215 155 single nucleotide polymorphisms (SNPs) characterized across 19 inbred strains covering 2609 genes. From this analysis, the candidate haplotype blocks empirically selected for further analysis had the best *P* values.

### Gene expression analysis

Mice were killed at specific time points by CO<sub>2</sub> asphyxiation. Whole brains were dissected in block from the skull, and spinal cords were harvested by extrusion. Using low-power binocular magnification, tissues were dissected on a chilled surface. Brain tissue was separated into cortex, cerebellum, and brainstem. Dissected tissue was then quick frozen in liquid nitrogen and stored at –80°C until use. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions, its purity and concentration were determined spectrophotometrically as described previously for brain and spinal cord samples [26]. cDNA was synthesized from total RNA using random hexamer priming and a First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, California, USA). Briefly, 1 µg of total RNA was mixed with 4 µl of 10x reverse transcription buffer, 8 µl of 25 mmol/l MgCl<sub>2</sub>, 4 µl 0.1mol/l dithiothreitol, 1 µl RNasin, 2 µl SSII (50 u/µl), 5 µl hexamers 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 of 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, California, USA). Briefly, 2 µl of a mixture of 2x Sybr Green and primers were loaded with 2 µl diluted cDNA template in each well. Following this, 8 µl of mineral oil was loaded in each well to prevent loss of solution. Using an ABI prism 7900HT system (Applied Biosystems, Foster City, California, USA), PCR was carried out using the parameters 52°C, 5 min→95°C, 10 min then (95°C, 30s→60°C, 60s) for 40 cycles. Samples were analyzed in triplicate. Melting curves were performed to document single product formation, and agarose electrophoresis confirmed appropriate product size. For internal control 18s RNA was used. The 18s primers were purchased from Ambion (Austin, Texas, USA). The expression of *Htr3a* in morphine-treated versus control samples was analyzed using the  $\Delta\Delta C_t$  method [27].

We used our previously described technique for laser capture microdissection (LCM) of central nervous system (CNS) tissue [26]. Mice underwent intracardiac perfusion with 10 cm<sup>3</sup> ice-cold 0.9% NaCl after CO<sub>2</sub> asphyxiation followed by brain harvest. Brainstems were rapidly dissected, embedded in optimal cutting temperature medium, and stored at -80°C until sectioning. Later, 15- $\mu$ mol/l sections were cut using a cryostat, placed on slides and rapidly dehydrated through ethanol and xylene. Each slide contained about six sections. The slides were then brought directly to the PixCell LCM instrument (Arcturus, Mountain View, California, USA), with a 15- $\mu$ m laser spot diameter, power of 40 mW and 500- $\mu$ s pulse duration to transfer tissue to the CapSure matrix (Arcturus). Each cap was used until 80–90% of the surface contained transferred tissue. Tissue was harvested from the amygdala, dorsal raphe, and periaqueductal gray nuclei using standard brain atlases to guide the process. The RNA was extracted using the PicoPure (Arcturus) RNA spin column purification kit according to manufacturer's directions followed by mRNA amplification. It has been shown by our laboratory and others that amplification of laser-captured RNA clarifies rather than distorts differences between samples [28]. This process was performed using the RiboAmp RNA amplification kit (Arcturus) according to the manufacturer's directions for a single round of amplification, and the amount of material was quantified by absorbance spectrophotometry and then subjected to reverse transcription using random hexamer primers as described above. Amplified mRNA was then used for real-time PCR-based quantification as described.

Western blotting for 5-HT<sub>3</sub> receptor protein was performed as described [29]. Briefly, tissues from specific CNS regions were homogenized in protease containing buffer and separated on acrylamide gels. After transfer, membranes were probed using rabbit polyclonal anti-5-HT<sub>3</sub> antibody (Abcam, Cambridge, Massachusetts, USA) at 1 : 500 dilution. Membranes were then stripped and reprobed for actin abundance thus allowing normalization.

### Human volunteers, study design, and procedures

Eight healthy male volunteers underwent an acute precipitated narcotic drug withdrawal protocol as previously described [30], with or without ondansetron pretreatment using a randomized double-blinded placebo-controlled crossover study design on two separate occasions. Test sessions for each individual volunteer were separated by at least 7 days. Female volunteers were excluded from the study because of modulation of opioid response by menstruation cycles [31]. The human experimental protocol was approved by the Institutional Review Board (Stanford University, USA). Each volunteer provided written informed consent before study enrolment, and the study was registered in the clinicaltrials.gov database (identifier NCT00661674).

All study sessions were conducted by a blinded research assistant (N.D.) and supervised by an unblinded physician (L.C.) who administered the study medication and monitored heart rate, blood pressure, and arterial oxygenation throughout the study. The Subjective Opioid Withdrawal scale (SOWS) and Objective Opioid Withdrawal scale (OOWS) were completed after establishing intravenous (i.v.) access and before administration of study medications, as originally described by Handelsman *et al.* [5]. All OOWS measurements were obtained by the same blinded research assistant (N.D.). Ondansetron (Bedford Laboratories, Bedford, Ohio, USA) 8 mg or placebo (0.9% saline solution, Hospira Inc., Lake Forest, Illinois, USA) was administered in a double-blinded manner as an i.v. bolus. Thirty minutes later, morphine (Baxter Healthcare Corporation, Deerfield, Illinois, USA) 10 mg/70 kg was administered over 10 min. Patients remained in the lab under observation for 105 min and were offered music or video entertainment and caffeine-free meals or snacks ad lib during this period of time. After 105 min, the patient's vital signs and OOWS and SOWS were reassessed. One hundred and twenty minutes after i.v. morphine administration, 10 mg/70 kg naloxone (Hospira Inc.) was

administered to the volunteer as an i.v. bolus. Vital signs, SOWS and OOWS were administered 5 and 15 min after the naloxone administration.

### Statistical analysis

All data are displayed as the means  $\pm$  SEM unless otherwise noted. Animal behavioral data were analyzed using one- or two-way analysis of variance with post-hoc Tukey testing or, for CPP data with *t*-testing. The outcome measures of interest between the placebo-treated and ondansetron-treated groups were compared using paired *t*-test and the Wilcoxon's signed-rank test where appropriate. Normal distribution was determined using QQ plots and the Kolmogorov–Smirnov test. Analyses were performed with SAS 9.1 statistical package (Cary, Illinois, USA) with *P* value of less than 0.05 considered statistically significant.

## Results

### Genetic variation within *Htr3a* affects morphine dependence

Eighteen inbred strains were made physically dependent on morphine, an archetypical opioid narcotic, by administration over a 4-day period. Then, the jumping behavior precipitated by the administration of an opioid receptor antagonist (naloxone) to the dependent mice was measured. There were very large interstrain differences in the withdrawal-induced jumping behavior; SM/J mice averaged only one jump in the 15-min period following naloxone administration, whereas BUB/BnJ mice jumped over 100 times under the same conditions (Fig. 1). Other investigators [8] evaluated eight of these strains by a similar method, and a comparative Pearson's correlation analysis of the data for the eight common strains indicated that two studies yielded similar results (coefficient of 0.87,  $P = 0.05$ ).

Haplotype-based computational genetic mapping was used to analyze data from this study [11,12]. The distribution of the naloxone-induced withdrawal behavior was compared with the pattern of genetic variation across the 18 strains analyzed. Interestingly, the two most highly correlated haplotype blocks ( $P$  value  $< 5 \times 10^{-6}$ ) were in close proximity on chromosome 9 and corresponded to the 5' and 3' regions of the *Htr3a* gene (Figs 1 and 2). There are seven haplotype blocks covering this gene though the dependence-associated blocks account for the majority of SNPs. The remaining small noncorrelated blocks are located between the second and forth exons. None of the SNPs in the associated blocks alter the predicted amino acid sequence of the protein. This gene encodes the 5-HT<sub>3a</sub> receptor, which has well-established roles in modulating nausea, anxiety, and pain [32]. Although more speculative, it has also been postulated that this receptor could also affect opioid tolerance and dependence [33].

To determine whether 5-HT<sub>3</sub> receptor function affects opioid withdrawal, the effect of a selective 5-HT<sub>3</sub> receptor antagonist (ondansetron) on withdrawal-associated jumping was assessed. Administration of ondansetron before measurement of naloxone-induced jumping significantly reduced this response in morphine-dependent C57BL/6J mice in a dose-dependent manner (Fig. 3a). In addition, simultaneous administration of ondansetron with each morphine dose during the 4-day protocol for establishing dependence diminished the naloxone-precipitated withdrawal response (Fig. 3b). The latter effect was unlikely to be because of the presence of residual ondansetron at the time of the dependence measurement as a 1-mg/kg dose was not able to effectively inhibit withdrawal when given acutely, and approximately 5 half-lives of the drug had passed in the time between the last dose of ondansetron and the naloxone-precipitated withdrawal procedure. We next investigated whether 5-HT<sub>3</sub> receptors expressed within the CNS were capable of altering the severity of withdrawal. In these experiments, ondansetron (or saline) was injected i.c.v. before naloxone was administered to morphine-dependent C57BL/6J mice. The i.c.v. administration of ondansetron profoundly blocked the naloxone-precipitated jumping behavior in a dose-dependent manner (Fig. 3c).

The spontaneous hyperalgesia observed in humans and mice after chronic exposure to opioids, which is referred to as OIH [34], is another measure of physical dependence [35-37]. In our experiments, chronic morphine treatment caused hyperalgesia in C57BL/6J mice. We chose to measure sensitization to a mechanical stimulus as this provides a robust degree of sensitization [14]. Ondansetron administration had no effect on pain sensitivity in control mice that were not treated with morphine, but reversed the hyperalgesia that developed during morphine abstinence (Fig. 4). Taken together, these experiments show that a 5-HT<sub>3</sub> receptor antagonist markedly reduced the severity of two distinct measures of physical dependence on morphine.

On account of the positive findings with respect to 5-HT<sub>3</sub> blockade and reduction in signs of physical dependence, we extended our testing to a paradigm probing the reinforcing properties of opioid administration by using CPP. The group of mice that were administered morphine 5 mg/kg and placed in the conditioning chamber 25 min after injection had a mean difference between pre and postconditioning percentage of time spent in the compartment associated with drug of 21.29 percent (95% confidence interval: 16.82–25.75;  $P < 0.001$ ). The group of mice that received ondansetron 1 mg/kg in addition to morphine 5 mg/kg displayed a mean difference of 0.40 percent (95% confidence interval: –9.666–10.47;  $P = 0.93$ ) (Fig. 5). Thus, ondansetron completely blocked the place preference associated with morphine administration.

### Chronic morphine exposure reduces *Htr3a* mRNA expression within key brainstem nuclei

The i.c.v. injection experiments showed that ondansetron modulated naloxone-induced jumping, by acting within the CNS. Furthermore, none of the SNPs within either *Htr3a*-associated haplotype blocks altered its predicted amino acid sequence. Many SNPs were located within 3' and 5' regulatory regions, suggesting that they may alter mRNA transcription or stability. Thus, differences in CNS *Htr3a* mRNA expression could be responsible for interstrain differences in the severity of naloxone-precipitated withdrawal. To investigate this possibility, we evaluated *Htr3a* mRNA expression in several brain regions in the presence or absence of chronic morphine treatment. Measurements were made in strains with high (C57BL/6J) or low (129/SvImJ) morphine dependence, which possess different *Htr3a* haplotypes. Chronic morphine treatment induced strain and brain-region-specific changes in *Htr3a* expression (Fig. 6); the C57BL/6J strain exhibited a more drastic decrease in *Htr3a* mRNA in cortical and brainstem tissue, whereas the 129/SvImJ strain exhibited a larger decrease in the cerebellum. An approximately equal change in the spinal cord *Htr3a* expression was observed in both strains.

We also investigated whether chronic morphine exposure would alter *Htr3a* mRNA levels within specific brainstem nuclei that were previously associated with physical dependence on opioids. The amygdala [38,39], dorsal raphe [38,40], and periaqueductal gray [41] are brainstem nuclei and are known to modulate signs of opioid dependence and withdrawal. Tissue from these three brainstem regions was harvested from dependence-developing C57BL/6J mice by LCM. *Htr3a* mRNA expression in all three of these brainstem nuclei was markedly reduced (two-fold to five-fold) after chronic morphine treatment (Fig. 6). Consistent with the computational prediction, therefore, there are strain-specific differences in the effect of chronic morphine exposure on CNS *Htr3a* mRNA expression, and chronic morphine exposure reduced *Htr3a* mRNA in three brainstem nuclei associated with opioid dependence.

The expression levels of several additional genes were measured as a specificity control in C57BL/6J and 129/SvImJ mice. There were no interstrain differences after chronic morphine treatment in the brain levels of expression of *Kcnj6*, *C3ar1*, *C5ar1* or *Htr3b* mRNA between these two strains (Fig. 7). Importantly, the *Htr3b* gene is located adjacent to *Htr3a* on chromosome 9, and encodes a protein that forms heteromultimeric 5-HT<sub>3</sub> receptors, with biophysical properties that are distinct from *Htr3a* homomultimers [42].

### Chronic morphine exposure reduces 5-HT3 protein expression

Western blot analysis was conducted to determine whether the changes in 5-HT3 protein followed the observed changes in *Htr3a* mRNA. This analysis showed that protein preparations from the brainstems of C57BL/6J mice had statistically significant decreases in 5-HT3 content after morphine treatment, whereas no such changes were observed in preparations made from 129/SvImJ mice (Fig. 8).

### Pretreatment with ondansetron reduces signs of opioid withdrawal in humans

On the basis of the murine results, the efficacy of the 5-HT3 receptor antagonist ondansetron was tested in human volunteers using an experimental protocol for inducing opioid withdrawal [30]. Eight healthy male volunteers were pretreated with placebo or ondansetron (8 mg) before i.v. administration of morphine and subsequent naloxone-precipitated withdrawal. The effect of the pretreatment drug was assessed using well-established objective (OOWS, primary outcome) and subjective (SOWS) measures of opioid withdrawal [5]. Ondansetron pretreatment caused a substantial ( $76.4\% \pm 22.6$ ) and statistically significant ( $P=0.0313$ ) decrease in mean OOWS score (Fig. 9). Seven of the eight volunteers developed objective signs of opioid withdrawal, and ondansetron pretreatment reduced these signs in all seven affected individuals. The OOWS score is a composite measure of 13 physically observable signs. The volunteers manifested 12 of the 13 measured signs, and ondansetron pretreatment decreased all 12 of these individual signs indicating a broad-spectrum effect (Fig. 9). In contrast, there was only a very small mean decrease in the subjective symptoms (SOWS score) that did not reach statistical significance ( $4.1\% \pm 62.5$ ,  $P>0.05$ , Fig. 10).

## Discussion

Computational haplotype-based genetic analysis indicated that genetic variation within the *Htr3a* gene could affect interstrain differences in the degree of physical dependence developing after chronic morphine exposure. This computational prediction was confirmed in pharmacological experiments; a 5-HT3 receptor antagonist reduced two distinct manifestations of opioid withdrawal in mice. Similarly, the CPP paradigm commonly used to estimate the addictive properties of drugs showed that blockade of the 5-HT3 receptor completely abolished the reinforcing properties of morphine. Furthermore, *Htr3a* mRNA expression within three brainstem nuclei associated with opioid dependence was strongly down-regulated after chronic morphine exposure. Alterations in brainstem 5-HT3 protein followed the same pattern. The strain-dependent and brain-region-specific differences in *Htr3a* mRNA and 5-HT3 protein expression within the CNS provide a potential mechanism that could partially explain how genetic differences within *Htr3a* affect susceptibility to opioid dependence. On the basis of these results, a widely used antiemetic drug (ondansetron) was tested in human volunteers, and these 5-HT3 receptor antagonists were shown to significantly reduce the objective signs of morphine withdrawal in humans. Therefore, this report provides the first demonstration that a 5-HT3 antagonist, which is a readily available medication, can reduce the severity of narcotic drug withdrawal symptoms in humans.

The molecular biology, genetics, expression pattern, and function of the 5-HT3 receptor have been extensively characterized. 5-HT3 receptors are transmitter-gated, cation selective ion channels that mediate neuronal depolarization and neurotransmitter release in both the CNS and peripheral nervous system. This receptor is targeted by antiemetic drugs during chemotherapy and anesthesia, and is also considered a potential target for treatment of gastrointestinal disorders, anxiety, and pain [32]. Although the data are far less clear and compelling, 5-HT3 receptor antagonists have also been speculatively considered for treatment of addiction [43,44]. 5-HT3 receptors modulate the activity of the mesolimbic system, which are heavily implicated in addiction to multiple substances [45]. Animal studies provide data

indicating that 5-HT<sub>3</sub> receptors could be involved in addiction and withdrawal. Mice deficient in the *Htr3a* gene display diminished sensitization to cocaine, a commonly used laboratory model for cocaine addiction [46]. Ondansetron reduced the CPP induced by morphine in rodents, which is a measure of the use-reinforcing properties of a drug [47], and reduced place aversion, a measure of unpleasantness during naloxone-precipitated opioid withdrawal [48]. Ondansetron reduced naloxone-precipitated withdrawal behaviors in mice and rats in some [33,49,50] but not in all studies [48]. However, three analyses [51-53] of place conditioning and related models, found that 5-HT<sub>3</sub>-blocking agents were ineffective. In one of these studies, ondansetron was not effective at lower doses in rats, and was only variably effective at higher doses when the drug itself had an effect on place conditioning. In addition, this antagonist had no effect on heroin self-administration [54]. In humans, one-time low-dose oral ondansetron was not effective in reducing opioid cravings in addicts exposed to video materials containing drug-related cues [55]. On the basis of the results of the mouse genetic model, we designed a completely different type of clinical study in human volunteers. The results presented here represent the first indication that 5-HT<sub>3</sub> antagonists can effectively ameliorate narcotic withdrawal symptoms in humans.

The role of the amygdala in modulating opioid withdrawal is of particular interest; this was one of the nuclei showing prominent opioid modulation of *Htr3a* expression in our studies. The expression of *Htr3a* is particularly high in the amygdala [56]. It has long been recognized that this structure is involved in various physiological aspects of the opioid withdrawal syndrome in rodents. For example, microinjection of naloxone and its derivatives into the amygdala precipitates jumping behavior in morphine-dependent rats [57,58]. Additional experiments have identified the periaqueductal gray matter as a key center mediating the signs of opioid withdrawal [57]. This was the area showing the greatest degree of *Htr3a* modulation of opioids in our experiments. Moreover, the amygdala is one of the structures at the center of the neuroadaptive changes supporting the larger syndrome of addiction to opioids and other substances of abuse [59]. Although not tested for opioids specifically, ondansetron microinjection into the mouse amygdala (and dorsal raphe) blocked an aversive behavior (light/dark exploration) exhibited by rodents during withdrawal from nicotine, cocaine, benzodiazepines, and ethanol [38].

Painful symptoms are also part of the opioid withdrawal syndrome, and increased pain sensitivity can be measured in chronic pain and addict populations when the serum drug concentrations are near their nadirs [35,60]. Rodents display nociceptive sensitization after chronic morphine administration, even without antagonist-precipitated withdrawal. This sensitization is used as a measure of opioid dependence [36,37]. In addition to blocking naloxone-induced withdrawal behaviors, ondansetron effectively reversed the spontaneous hyperalgesia in our morphine-dependent mice. Other investigators have reported that spinal 5-HT<sub>3</sub> receptors may modulate OIH as well [61]. These findings emphasize that the 5-HT<sub>3</sub> is involved in at least two objectively quantifiable manifestations of opioid dependence.

Although the murine *Htr3a* gene was the focus of the present studies, many additional studies indicate important differences between naturally occurring human *Htr3a* and *Htr3b* variants. For example, five naturally occurring human *Htr3a* variants cause changes in the cell surface expression of 5-HT<sub>3</sub> receptors [62]. More recently four naturally occurring variants of the human *Htr3b* gene were found to alter the expression and function of heteromeric 5-HT<sub>3A/B</sub> receptors [63]. Many gene association studies linking variants of these genes with depression, bipolar affective disorder, nausea, and other phenotypes are available. One functional variant of the *Htr3b* gene has particularly strong linkage to depression in women and nausea after antidepressant administration [64,65].

Opioid addiction is a complex syndrome involving a maladaptive pattern of drug use that is characterized by tolerance, physical dependence (withdrawal during abstinence), continued drug use despite acknowledged harm from the drug, excessive preoccupation with use of the drug, and social isolation. A medication that reduces the physiological manifestations could be an important part of addiction prevention and treatment, but would not address all of the complex neuroplastic events that underlie the overall symptom complex [66,67]. In contrast, the increased use of opioid narcotics for control of chronic pain has focused attention on the physical dependence associated with chronic opioid use. Although a patient receiving chronic opioid medications may not develop addiction, the physical dependence, tolerance and hyperalgesia that can develop may limit the value of chronic narcotic therapy and can complicate ongoing patient management [34,68]. Therefore, treatment with 5-HT<sub>3</sub> antagonists may provide part of the solution to significant public health problems associated with opioid use.

## Acknowledgements

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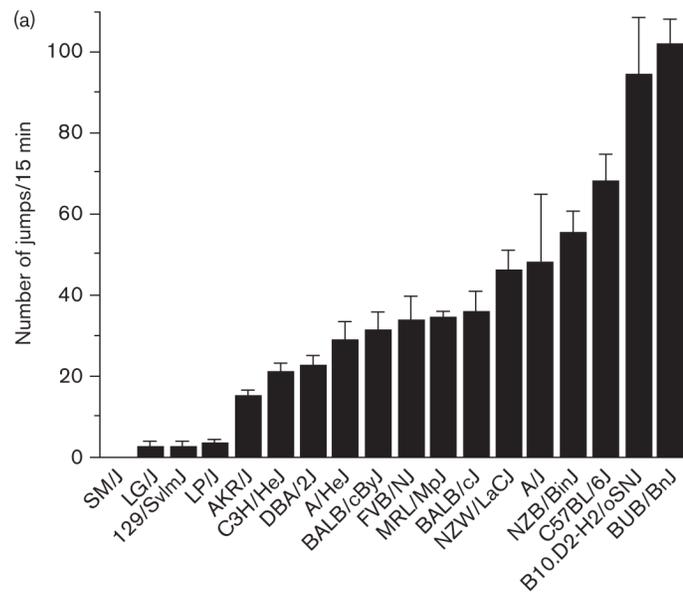
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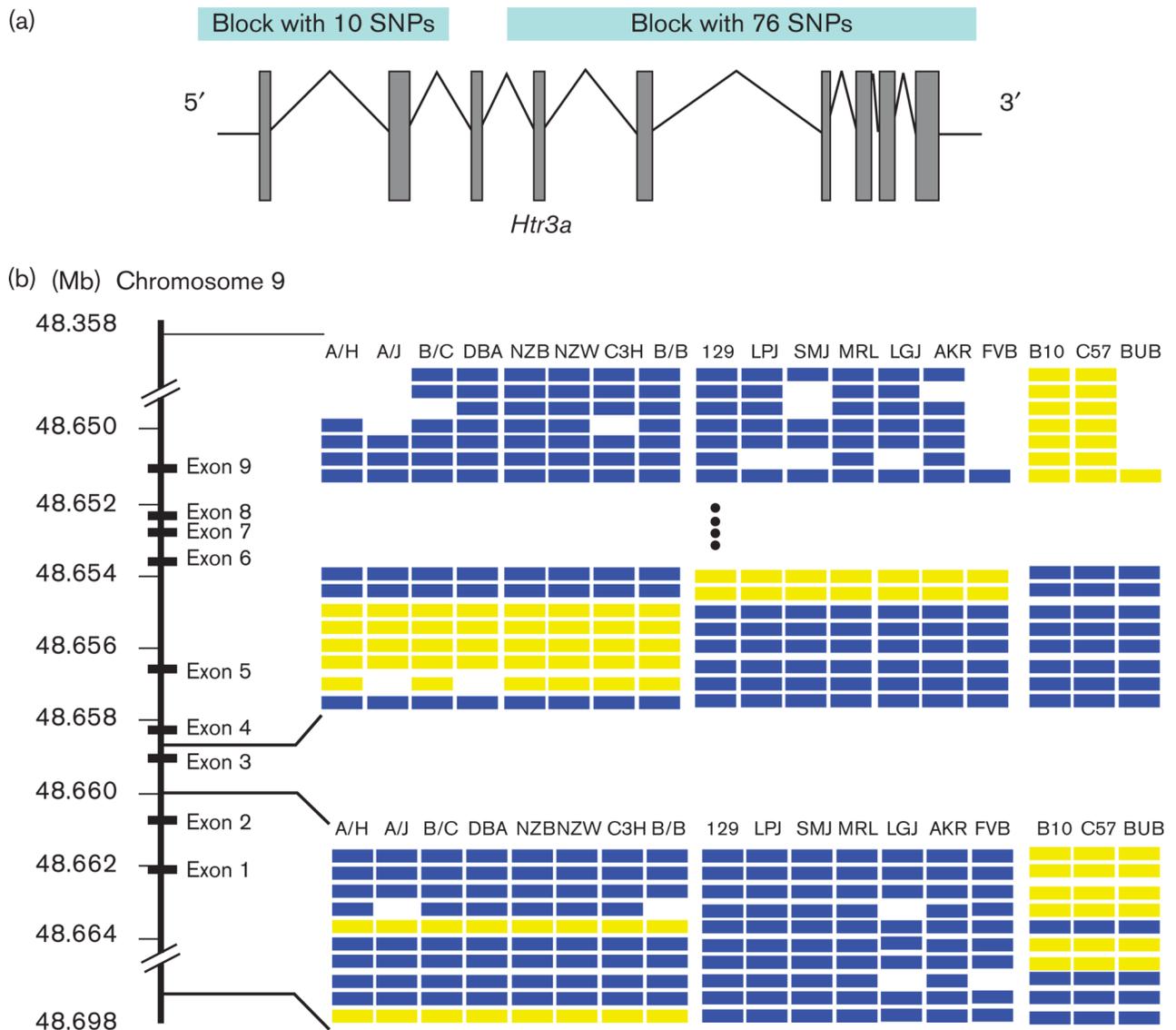
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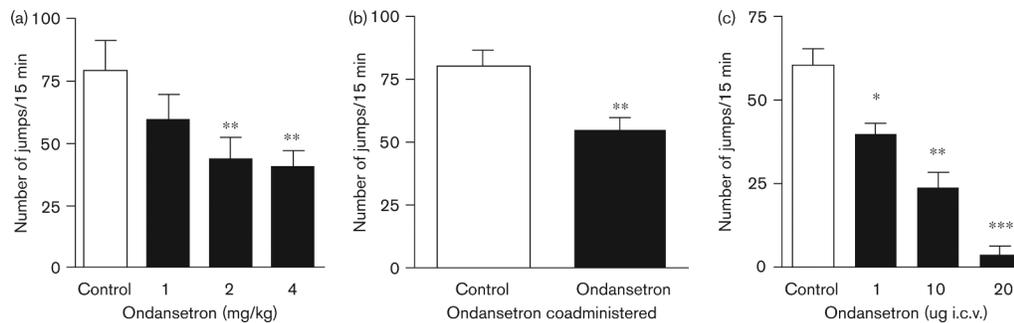
(b)

<i>P</i> value	Haplotype	Chr.	Position (Mb)	Number of SNPs	Symbol
4.60E-06		9	48.358~48.659	76	<i>Htr3a</i>
4.60E-06		9	48.660~48.697	10	<i>Htr3a</i>
4.00E-06		2	169.246~169.247	9	N/A
4.00E-05		6	108.305~108.316	7	<i>Itpr1</i>
7.10E-05		8	87.524~87.860	8	<i>Cacna1a</i>
0.00019		11	107.612~107.690	27	<i>Cacng5</i>
0.00023		14	53.395~53.570	63	<i>Mmp14</i>
0.00024		15	79.175~79.824	10	<i>Maff</i>
0.00029		1	5.110~5.113	9	<i>Atp6v1h</i>
0.00029		6	108.318~108.430	5	<i>Itpr1</i>

**Fig. 1.** Computational genetic analysis of interstrain differences in physical dependence on morphine. (a) Eighteen strains (eight mice per strain) were treated for 4 days with morphine to establish physical dependence. On the fifth day, the number of jumps made during the 15-min period after naloxone injection was measured as an indicator of the degree of opioid dependence. The data represent the mean number of jumps for each indicated strain  $\pm$  SEM. (b) The morphine physical dependence data (mean number of jumps for each strain) was analyzed by computational genetic mapping. The 10 most strongly correlated haplotype blocks are shown. For each block, the chromosomal location (chr.), number of single nucleotide polymorphisms (SNPs) within a block and its gene symbol are listed. For each gene, the haplotypes are represented by a colored block, and the blocks are presented in the same rank order as the phenotypic data. Strains sharing the same haplotype have the same colored block. The calculated *P* value measures the probability that the strain groupings within a block would have the same degree of association with the phenotypic data by random chance. The genetic effect indicates the fraction of the interstrain variance that is potentially attributable to the haplotype. The letter ‘E’ is appropriate as an indicator of ‘exponent’.

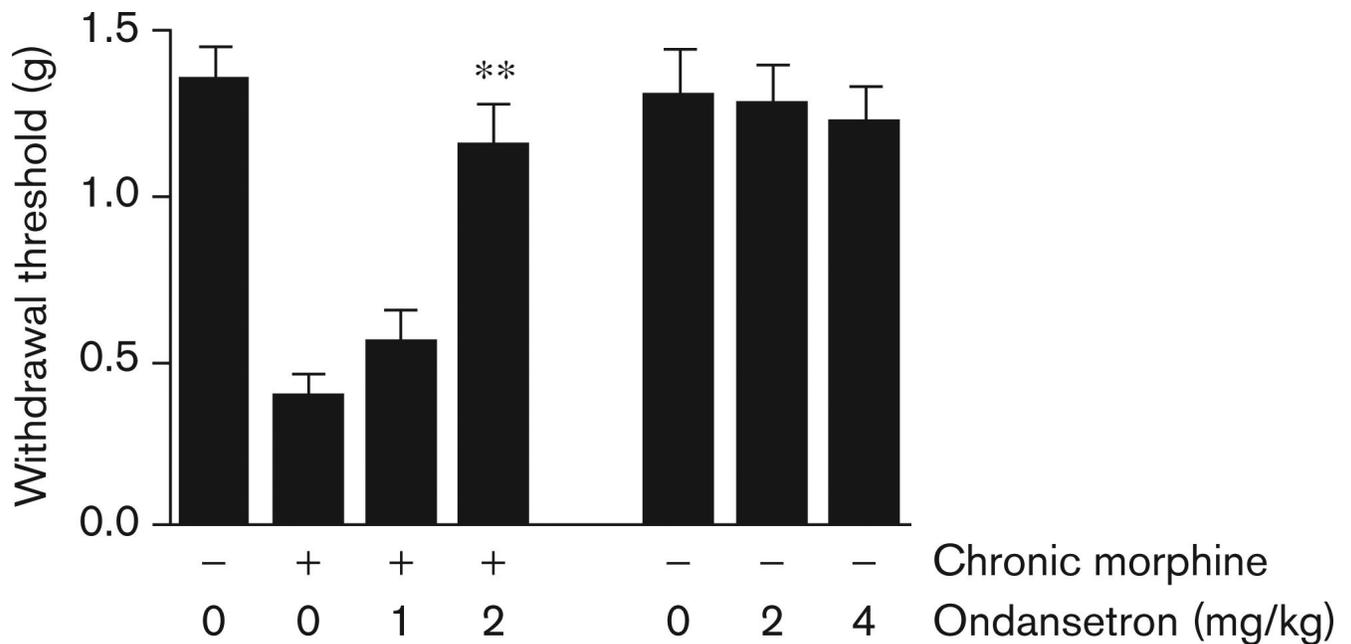


**Fig. 2.** Diagram of the *Htr3a* gene and associated haplotypic blocks. (a) The intron/exon structure of the *Htr3a* gene along with the relative positions of the two associated haplotypic blocks are displayed. The two haplotype blocks whose pattern of genetic correlation best correlated with the severity of the naloxone-precipitated jumping response are located at the 5' and 3' regions of the *Htr3a* gene. Each of these haplotype blocks has three different haplotypes, and none of the 86 single nucleotide polymorphisms (SNPs) within these two blocks alters the predicted amino acid sequence of the 5-HT<sub>3a</sub> receptor (<http://mouseSNP.roche.com>). (b) There are seven haplotype blocks across the *Htr3a* gene. The two major blocks are the dependence correlated 3' and 5' blocks, each with 76 and 10 SNPs, respectively, covering most of *Htr3a* gene. The other five small blocks (not shown in the figure) are between the 5' and 3' blocks (between exons 2 and 4) and contain total 15 SNPs. The 5' and 3' blocks, which exhibited the highest correlation with physical dependence on morphine, had the same pattern of genetic variation across the 18 strains of mice used in our studies.



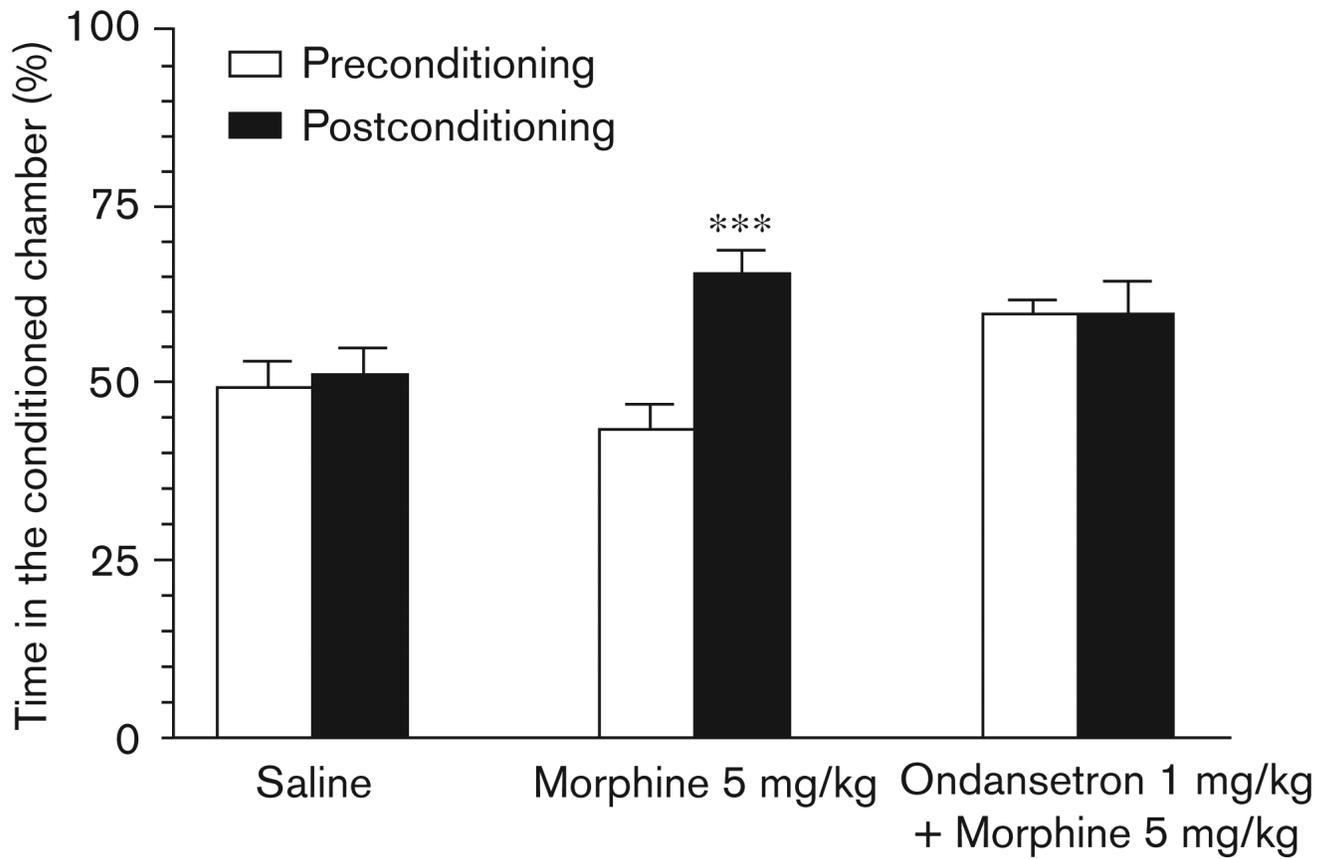
**Fig. 3.**

Administration of a selective 5-HT<sub>3</sub> receptor antagonist (ondansetron) decreases naloxone-precipitated withdrawal behavior. (a) Mice (C57BL/6J) were treated with morphine over a 4-day period, and then were treated with saline or the indicated dose of ondansetron on the fifth day before assessment of naloxone-precipitated jumping behavior. Ondansetron treatment induced a statistically significant and dose-dependent reduction in jumping behavior. (b) Mice (C57BL/6J) were treated with saline or ondansetron (1 mg/kg) at the time of each of the bi-daily morphine injections during the 4-day dependence building protocol. Eighteen hours after the final dose, naloxone-precipitated jumping behavior was assessed. (c) Mice (C57BL/6J) were treated with saline or the indicated dose of ondansetron intracerebroventricular (i.c.v.) before assessment of naloxone-precipitated jumping behavior. Ondansetron treatment induced a statistically significant and dose-dependent reduction in jumping behavior. For all experiments six to eight mice were used per group. Data represent mean values  $\pm$  SEM. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

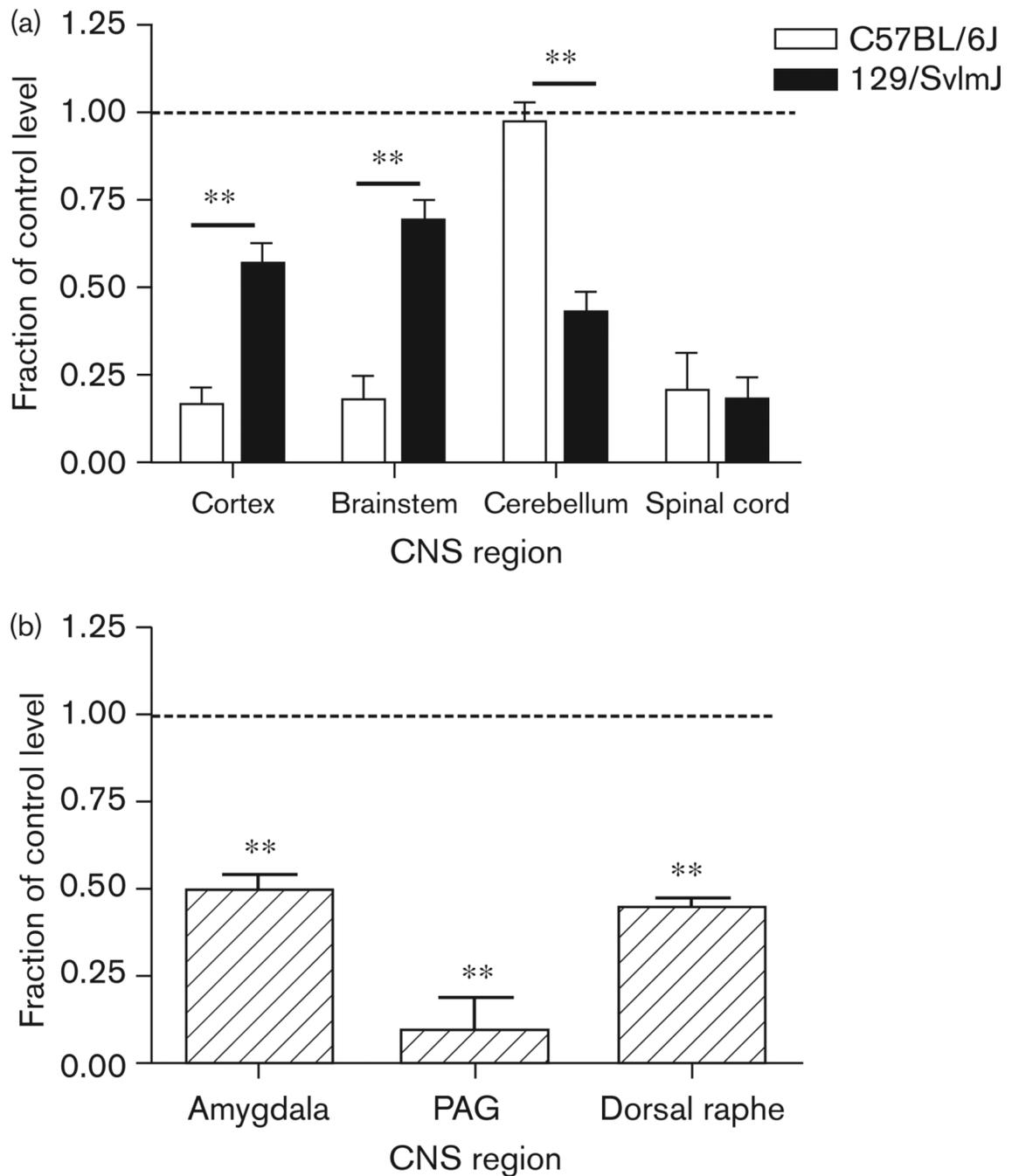


**Fig. 4.**

Ondansetron treatment reduces morphine dependence-related hyperalgesia. The effect of the selective 5-HT<sub>3</sub> antagonist ondansetron was evaluated in mice that were made dependent on morphine over a 4-day period. As a measure of physical dependence on morphine, mechanical nociceptive sensitization was measured 18 h after administration of the last dose of morphine, the point of maximal nociceptive sensitization. Mice were treated with saline (control) or the indicated dose of ondansetron 30 min before threshold assessment. Ondansetron dose dependently reversed the mechanical nociceptive sensitization caused by morphine withdrawal, but did not alter baseline nociceptive thresholds in control animals, even when doses of ondansetron higher than those effectively reversing hyperalgesia were administered. Six mice per group were used in these experiments, and the data represent mean values  $\pm$  SEM. \*\* $P < 0.01$ .

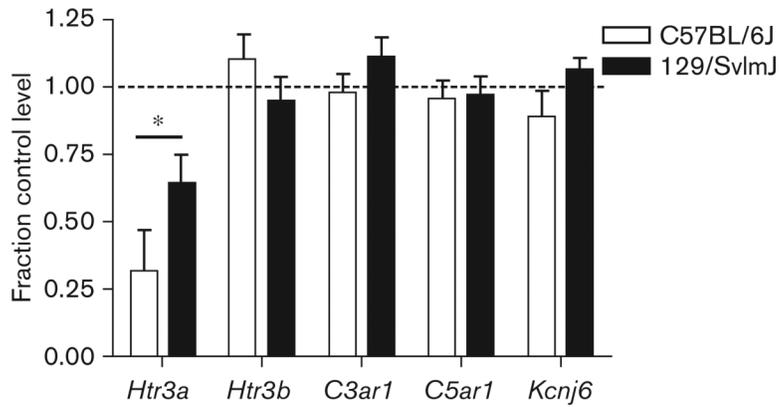


**Fig. 5.** Ondansetron blocks the conditioned place preference associated with morphine administration. In this figure the mean percent time spent in the assigned drug associated chamber in the conditioned place preference assessments before and after conditioning is displayed. Three groups of mice were used: vehicle ( $n = 15$ ); morphine 5 mg/kg ( $n = 14$ ); ondansetron 1 mg/kg and morphine 5 mg/kg ( $n = 10$ ). \*\*\* $P < 0.001$ .



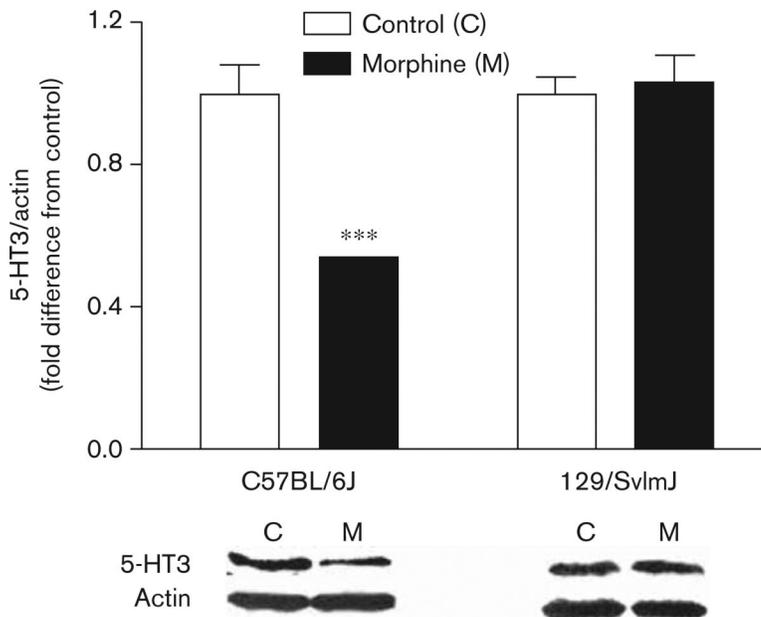
**Fig. 6.** Morphine treatment has a differential and brain region-specific effect on *Htr3a* mRNA expression. (a) Mice with a high (C57BL/6J) or low propensity (129/SvImJ) to develop morphine dependence were exposed to saline or morphine for 4 days. On the fifth day the mice were killed, the indicated brain regions were dissected, and level of *Htr3a* mRNA expression was measured using real-time quantitative PCR. Data represent mean values  $\pm$  SEM from duplicate measurements made on at least six mice per group. (b) Morphine induces changes in *Htr3a* mRNA expression in selected brainstem nuclei. Mice (C57BL/6J) were treated with saline or morphine as above. The mice were then killed, the indicated brainstem nuclei were isolated by laser capture microdissection, and *Htr3a* mRNA expression in the brainstem nuclei

was analyzed. The *Htr3a* mRNA levels in morphine-treated mice were normalized relative to those in saline-treated animals. The data are displayed as the mean normalized value for tissues from  $n = 6$  mice per group  $\pm$  SEM. CNS, central nervous system; PAG, periaqueductal gray. \*\* $P < 0.01$ .

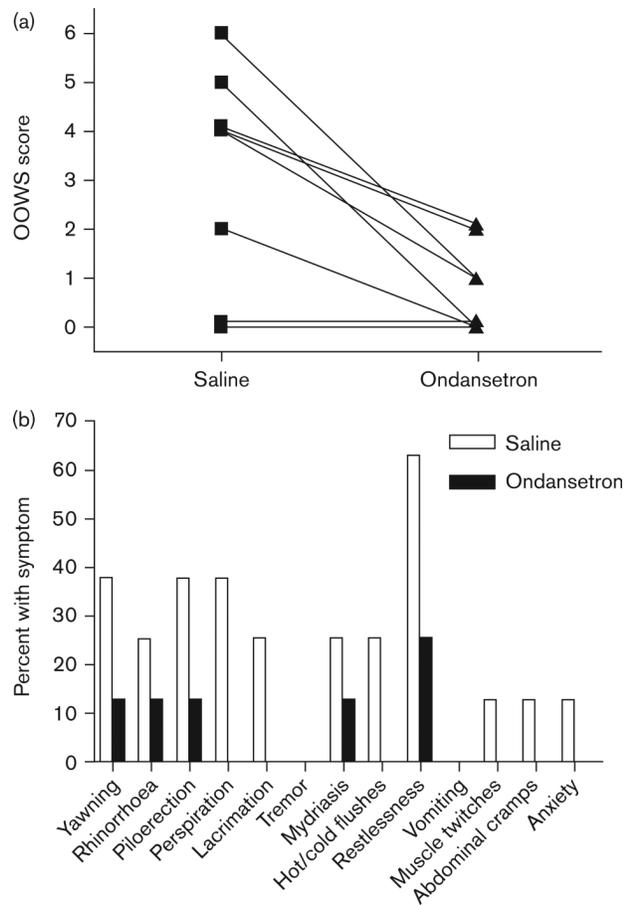


**Fig. 7.**

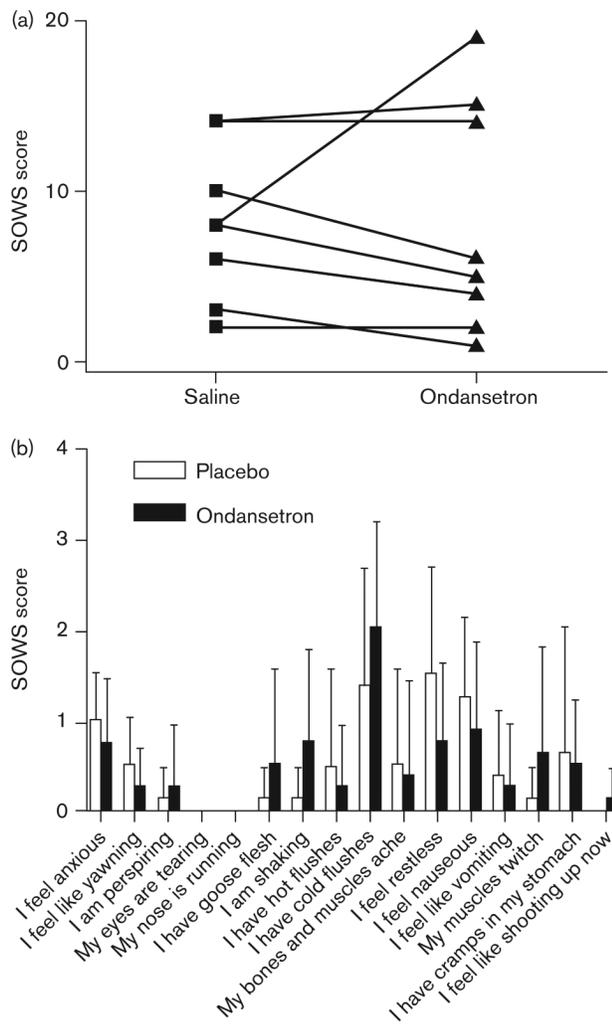
The regulation of central nervous system expression for several genes by morphine. Brain tissue was harvested after 4 days of saline versus morphine treatment for both high (C57BL/6J) and low (129/SvImJ) dependence developing strains. In this survey of genes only expression of the *Htr3a* gene coding for the 5-HT<sub>3</sub> serotonin receptor was opioid regulated ( $P < 0.05$ ). Importantly, expression of the adjacent *Htr3b* gene coding for an alternate form of the 5-HT<sub>3</sub> receptor was not altered by morphine treatment. Five mice per group were used in these experiments, and the displayed data represent mean values  $\pm$  SEM. \* $P < 0.05$  (difference between strains).



**Fig. 8.** The regulation of brainstem 5-HT3 protein levels by morphine. Brain tissue was harvested after 4 days of saline versus morphine treatment for both high (C57BL/6J) and low (129/SvImJ) dependence developing strains. Only in tissue from the C57BL/6J strain showed morphine-induced 5-HT3 changes. Five mice per group were used in these experiments, and the displayed data represent mean values±SEM. Average control mouse expression was set to 1. \*\*\* $P < 0.001$  (difference between control and morphine-treated groups).



**Fig. 9.** The effect of ondansetron pretreatment on the acute, naloxone-precipitated withdrawal response in human volunteers. Ondansetron 8 mg intravenous (i.v.) or placebo (normal saline) i.v. was administered 30 min before morphine (10 mg/70 kg) i.v. administration in eight volunteers. Naloxone-precipitated (10 mg/70 kg) withdrawal was then induced 120 min after morphine administration. (a) The composite OOWS scores for each volunteer after naloxone-precipitated opioid withdrawal after saline or ondansetron pretreatment are displayed. A  $P$  value=0.0313 was calculated based on signed-rank test of the difference in OOWS score after pretreatment with ondansetron and placebo. (b) The Objective Opioid Withdrawal scale (OOWS) subcategory responses to ondansetron pretreatment are displayed. The OOWS scale is composed of thirteen physically observable signs, which are rated as present (1) or absent (0) during the observation period. The percent of the volunteers who experienced each indicated naloxone-precipitated withdrawal sign after ondansetron or placebo pretreatment is shown.



**Fig. 10.** The effect of ondansetron pretreatment on the acute, naloxone-precipitated Subjective Opiate Withdrawal scale (SOWS) response in human volunteers. Ondansetron 8 mg intravenous (i.v.) or placebo (normal saline) i.v. was administered 30 min before morphine (10 mg/70 kg) i.v. administration in eight volunteers. Naloxone-precipitated (10 mg/70 kg) opioid withdrawal was then induced 120 min after morphine administration. (a) The composite SOWS scores for each volunteer after naloxone-precipitated opioid withdrawal for volunteers receiving saline or ondansetron pretreatment are displayed. A  $P$  value=0.5625 was calculated based on signed-rank test of the difference in SOWS score after pretreatment with ondansetron and placebo. (b) The SOWS subcategory responses to ondansetron pretreatment in humans are displayed. The SOWS score is composed of 16 subjective symptoms rated on a scale of 0–4 (0=not at all, 1=a little, 2=moderately, 3=quite a bit, 4=extremely) based on what volunteers were experiencing at the time of testing. The mean score for volunteers who experienced each indicated naloxone-precipitated withdrawal symptom after ondansetron or placebo pretreatment is shown.