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RESEARCH PAPER

The 5-HT3B subunit affects high-potency inhibition of 5-HT_3 receptors by morphine

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BACKGROUND AND PURPOSE
Morphine is an antagonist at 5-HT_3A receptors. 5-HT_3 and opioid receptors are expressed in many of the same neuronal pathways where they modulate gut motility, pain and reinforcement. There is increasing interest in the 5-HT3B subunit, which confers altered pharmacology to 5-HT_3 receptors. We investigated the mechanisms of inhibition by morphine of 5-HT_3 receptors and the influence of the 5-HT3B subunit.

EXPERIMENTAL APPROACH
5-HT-evoked currents were recorded from voltage-clamped HEK293 cells expressing human 5-HT3A subunits alone or in combination with 5-HT3B subunits. The affinity of morphine for the orthosteric site of 5-HT_3A or 5-HT_3AB receptors was assessed using radioligand binding with the antagonist [^{3}H]GR65630.

KEY RESULTS
When pre-applied, morphine potently inhibited 5-HT-evoked currents mediated by 5-HT_3A receptors. The 5-HT3B subunit reduced the potency of morphine fourfold and increased the rates of inhibition and recovery. Inhibition by pre-applied morphine was insurmountable by 5-HT, was voltage-independent and occurred through a site outside the second membrane-spanning domain. When applied simultaneously with 5-HT, morphine caused a lower potency, surmountable inhibition of 5-HT_3A and 5-HT_3AB receptors. Morphine also fully displaced [^{3}H]GR65630 from 5-HT_3A and 5-HT_3AB receptors with similar potency.

CONCLUSIONS AND IMPLICATIONS
These findings suggest that morphine has two sites of action, a low-affinity, competitive site and a high-affinity, non-competitive site that is not available when the channel is activated. The affinity of morphine for the latter is reduced by the 5-HT3B subunit. Our results reveal that morphine causes a high-affinity, insurmountable and subunit-dependent inhibition of human 5-HT_3 receptors.

Introduction

The 5-HT type 3 (5-HT_3) receptor is a member of the cysteine (Cys)-loop pentameric ligand-gated ion channel family [receptor nomenclature follows Alexander *et al.*, (2011)]. 5-HT_3 receptors, with binding sites for 5-HT and several allosteric modulators, mediate rapid 5-hydroxytryptaminergic excitatory synaptic transmission (Sugita, 1992). The Cys-loop family of pentameric receptors also includes the nicotinic ACh (nACh), $GABA_A$ and glycine receptors and Zn^{2+}-activated ion channels (Barnes *et al.*, 2009). 5-HT3A subunits form homomeric receptors when expressed alone and can also combine with 5-HT3B subunits into heteromeric receptors (Davies *et al.*, 1999). The 5-HT3B subunit confers several unique properties. These include a higher conductance, faster desensitization kinetics, linear current–voltage relationship and reduced sensitivity to 5-HT and some non-competitive antagonists (Davies *et al.*, 1999; Peters *et al.*, 2005). Genes encoding 5-HT3C, 5-HT3D and 5-HT3E subunits have also been cloned but their functional significance is poorly understood (Niesler *et al*., 2003).

Drugs that affect 5-HT_3 receptor function include competitive antagonists such as the 'setrons' (including ondansetron), the nicotinic drugs curare (Peters *et al*., 1990), epibatidine and mecamylamine (Drisdel *et al*., 2008), metoclopramide (Walkembach *et al*., 2005), cannabinoids (Barann *et al*., 2002), as well as opioid alkaloids such as methadone (Deeb *et al*., 2009) and morphine (Fan, 1995; Wittmann *et al*., 2006). Setron 5-HT_3 receptor antagonists are typically used to treat nausea and vomiting and, to a lesser extent, irritable bowel syndrome (Galligan, 2002). 5-HT_3 receptors also participate in drug reinforcement and reward (Carboni *et al*., 1988; 1989; Allan *et al*., 2001). Ondansetron is effective in the treatment of early onset alcoholism (Kranzler *et al*., 2003) and appears to aid detoxification of heroin-dependent individuals (Ye *et al*., 2001).

Morphine is the primary active metabolite of heroin and is widely used as an analgesic to treat severe pain. While its analgesic and hedonic effects are mediated by μ-opioid receptors, morphine-induced hyperalgesia has been observed in mice lacking μ-, δ- and κ-opioid receptors, suggesting that some actions of the drug are mediated through additional targets (Juni *et al*., 2007). Morphine has potent inhibitory effects on 5-HT receptor subtypes in the guinea pig ileum (Bradley *et al*., 1986). 5-HTM receptors, originally named for their sensitivity for morphine (Gaddum and Picarelli, 1957), were later renamed 5-HT_3 receptors (Bradley *et al*., 1986). Morphine competitively inhibits $5\text{-HT}_3\text{A}$ receptors when applied simultaneously with 5-HT (Fan, 1995; Wittmann *et al*., 2006). Recent studies suggest that 5-HT_3 receptors influence morphine-induced hyperalgesia and tolerance (Liang *et al*., 2011). An association between polymorphisms in human 5-HT3A and 5-HT3B subunit genes and heroin addiction (Levran *et al*., 2008; 2009) further implicates these proteins in morphine dependence. We examined the influence of the 5-HT3B subunit on the modulation of 5-HT_3 receptors by morphine. We found that the presence of the 5-HT3B subunit reduced the potency of morphine as an insurmountable antagonist but did not affect the affinity of morphine for the orthosteric binding site.

Methods

Cell culture and transfection

HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum, 50 μg mL^{-1} streptomycin and 50 U·mL^{-1} penicillin in a humid atmosphere of 5% CO_2. Cells were transfected by either calcium phosphate precipitation (for electrophysiological experiments) or Lipofectamine (Invitrogen, Carlsbad, CA) reagent (for radioligand binding assays) with cDNA encoding the human 5-HT3A subunit either alone or in combination with either the human 5-HT3B subunit cDNA or the 5-HT3ABA construct (in a 1:1 ratio) in the pCDM8 vector as described previously (Davies *et al*., 1999). A fivefold lesser amount of cDNA encoding green fluorescent protein was included for electrophysiology experiments to enable identification of successfully transfected cells by fluorescence microscopy. Cells were used 48–96 h after transfection for electrophysiological experiments.

Electrophysiological recording

The whole-cell configuration of the patch-clamp technique was used to record currents from HEK293 cells expressing $5\text{-HT}_3\text{A}$ or $5\text{-HT}_3\text{AB}$ receptors. Recording electrodes were fabricated from borosilicate glass capillaries and when filled with electrode solution had resistances of 1.3–2.5 MΩ. The electrode solution contained (in mM): 140 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA and 10 HEPES (pH 7.4 with KOH). The extracellular solution contained (in mM): 140 NaCl, 2.8 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES and 10 glucose (pH 7.4 with NaOH). Unless otherwise stated, cells were voltage-clamped at an electrode potential of −60 mV. 5-HT evoked currents at −60 and +60 mV were routinely compared before each experiment. A near unitary ratio of current amplitudes recorded at these potentials indicated the successful incorporation of the 5-HT3B subunit (Davies *et al*., 1999). In experiments investigating the voltage dependence of inhibition by morphine, the voltage was adjusted between −60 and +60 mV (in 20 mV increments). Permeability ratios for Na^+ and Ca^{2+} with respect to Cs^+ (P_{Na+}/P_{Cs+} and P_{Ca2+}/P_{Cs+}) were determined as described previously (Livesey *et al*., 2008). For determining P_{Na+}/P_{Cs+}, the extracellular solution was modified such that additional NaCl replaced KCl. $CaCl_2$ and $MgCl_2$ were both reduced to 0.1 mM to minimize the effects of divalent cations on the reversal potential. For determining P_{Ca2+}/P_{Cs+}, the extracellular solution contained (in mM): $CaCl_2$ 100, L-histidine 5 and glucose 10 (pH 7.2). A Cs^+ containing intracellular solution was used, which contained (in mM): CsCl 140, $CaCl_2$ 0.1, EGTA 1.1, HEPES 10 (pH 7.2) Currents were elicited using 1 μM 5-HT. At the plateau of the 5-HT induced current, a voltage ramp protocol was applied by stepping from −60 mV to −100 mV for 100 ms and ramped to +60 mV within 1 s. Current in the absence of 5-HT was subtracted from current in the presence of 5-HT to obtain the current–voltage relationship attributable to 5-HT. The reversal potential ($E_{5\text{-HT}}$) was used to determine the permeability ratios. No correction was made for the compensation for liquid junction potential except in the determination of $E_{5\text{-HT}}$ for Na^+ and Ca^{2+}. Liquid junction potentials arising at the tip of the electrode were determined empirically as described previously (Fenwick *et al*., 1982) and corrected *post hoc*. Currents were recorded using an Axopatch 200B amplifier, low-pass filtered at 2 KHz, digitized at 10 KHz using a Digidata 1320A interface and acquired using pCLAMP8 software (all from Molecular Devices, CA) on to the hard drive of a personal computer for off-line analysis. All experiments were performed at room temperature.

Drug application

5-HT_3 receptors were activated either by pressure (10 psi) ejection (Picospritzer II, General Valve Corp., Fairfield, NJ) of 5-HT from a micropipette placed close to the cell, or by the three-pipe Perfusion Fast-Step (SF-77B) solution exchange system (Warner Instruments, CA). Solution flow (0.3 mL·min^{-1}) through the pipes was controlled by a syringe pump (Cole-Parmer, Vernon Hills, IL). The voltage-clamped cell was lifted from the base of the recording chamber and placed in front of the stream of control solution. Perfusion pipes were moved rapidly, exposing cells to morphine or 5-HT, either alone or in combination. With an open electrode tip, the 10–90% rise time for junction currents generated by moving between adjacent perfusion pipes containing osmotically dissimilar solutions was approximately 1 ms. The 10–90% rise time for currents evoked by rapidly applying 5-HT (100 μM) to HEK293 cells expressing 5-HT_3 receptors was approximately 12 ms. Therefore, the solution 10–90% exchange time around the whole cell was 1–12 ms. The 5-HT_3A receptor concentration–response relationships determined by measuring peak current amplitudes generated by 5-HT, applied either rapidly or by pressure application, were similar (data not shown). The recording chamber was continuously perfused with extracellular solution (5 mL·min^{-1}).

Morphine sulphate was diluted from frozen stocks (10 mM) into the extracellular solution on the day of recording except in the case of the 1 mM morphine solution used to test agonist actions. In this case, morphine was dissolved directly into the recording solution. Morphine was either applied alone, co-applied with 5-HT or pre-applied prior to exposure to 5-HT. An interval of at least 60 s was allowed between each 5-HT application to enable the receptors to recover from desensitization.

Data analysis

The peak amplitudes of agonist-activated currents were measured using pCLAMP8 software. Systematic effects of 5-HT-evoked current rundown were corrected using linear regression analysis, normalizing current amplitudes to that evoked by 100 μM 5-HT. 5-HT concentration–response relationships were fitted with the following logistic function:

$$I = 100/(1 + (EC_{50}/C)^{n_H}$$

where I is the 5-HT activated current amplitude (as % of the current activated by 100 μM 5-HT), EC_{50} is the 5-HT concentration eliciting a half maximal response, C is the agonist concentration and n_H is the Hill coefficient. Schild analysis was performed by plotting the pEC_{50} values for 5-HT against morphine concentration. A slope of unity indicated that the inhibition was competitive. IC_{50} values for pre-applied morphine were determined using a modified version of this equation as described previously (Adodra and Hales, 1995).

Determining permeability ratios

Permeability ratios were calculated from measurements of $E_{5\text{-HT}}$ and calculated ion activities as described previously (Livesey *et al.*, 2008). P_{Na+}/P_{Cs+} ratios were calculated from the Goldman–Hodgkin–Katz equation:

$$E_{5\text{-HT}} = \frac{RT}{F} \ln \frac{(P_{Na}/P_{Cs})[Na^+]_0}{[Cs^+]_i}$$

where R, T and F have their standard meanings, $[Na^+]_o$ and $[Cs^+]_i$ are the calculated activities of extracellular Na^+ and internal Cs^+ respectively. For the calculation of P_{Ca2+}/P_{Cs+}, a modified version of the Goldman–Hodgkin–Katz equation was used (Brown *et al.*, 1998; Livesey *et al.*, 2008):

$$\exp^{E_{5\text{-HT}}F/RT} = 4\frac{P_{Ca}[Ca^{2+}]_0}{P_{Cs}(1+\exp^{E_{5\text{-HT}}F/RT})[Cs^+]_i}$$

Where $[Ca^{2+}]_o$ is the calculated ion activity of external Ca^{2+}.

Statistics

All data are presented as mean ± SEM. Statistical significance ($P < 0.05$) was established using either Student's *t*-test or one-way ANOVA with the *post hoc* Tukey's test.

Radioligand binding assay

HEK293 cells transiently expressing 5-HT_3A or 5-HT_3AB receptors were washed and harvested in HBSS and prepared for radioligand binding as described previously (Wu *et al.*, 2010). Cells were centrifuged at 3000× *g* for 5 min, supernatant was removed and pellets were either used immediately for [^{3}H]GR65630 (specific activity of 77 Ci $mmol^{-1}$) binding or stored at –80°C for later use. A crude membrane fraction of thawed cells was obtained by suspension in 10 mL ice-cold 50 mM HEPES buffer (pH 7.5) and centrifugation for 30 min at 40 000× *g*. Membranes were then resuspended in 50 mM HEPES buffer by ultrasonication, and the total protein concentration was measured using a Bradford assay. For competition binding studies, 100 μg of protein was incubated with [^{3}H]GR65630 (150 pM) for 60 min at 25°C with morphine (10 nM to 1 mM) in a final volume of 1 mL. The reaction was halted by rapid vacuum filtration using a Brandel cell harvester (Brandel, Gaithersburg, MD) with Whatman GF/B filter papers pre-soaked in 0.5 M polyethyleneamine in 50 mM HEPES buffer. Filters were allowed to air dry for 15 min and then assayed for radioactivity by liquid scintillation counting. For saturation binding studies, 100 μg of protein was incubated with [^{3}H]GR65630 ranging from 10 pM to 10 nM. Non-specific binding for both types of assay was determined in the presence of 1 μM ondansetron, and all experiments were performed in duplicate. Values of morphine binding affinity (K_i) were calculated using the Cheng–Prusoff equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/((1 + (L/K_d))$$

where, IC_{50} is the concentration of morphine or 5-HT required to displace 50% of bound [^{3}H]GR65630, L is the concentration of [^{3}H]GR65630 in competition binding experiments and K_d is the dissociation constant for [^{3}H]GR65630 determined from saturation binding.

Materials

Morphine sulphate and 5-HT hydrochloride were purchased from Sigma-Aldrich (St Louis, MO and Poole, UK respectively). [^{3}H]GR65630 was purchased from Perkin Elmer (Waltham, MA). All tissue culture reagents were from Invitrogen. All other reagents used in experimental solutions were of the highest laboratory grade purchased from Sigma (Poole, UK) or Fisher Scientific (Loughborough, UK).

Results

High-potency insurmountable inhibition of 5-HT$_3$ receptors by pre-applied morphine

In addition to its orthodox interaction with the μ-opioid receptor, morphine directly inhibits 5-HT-activated currents mediated by 5-HT$_3$A receptors (Fan, 1995; Wittmann *et al.*, 2006). We used the whole-cell patch-clamp technique to record currents from voltage-clamped HEK293 cells transiently expressing human 5-HT$_3$A or 5-HT$_3$AB receptors. Morphine (0.03–10 μM) applied to the recording chamber ≥120 s prior to 5-HT caused a concentration-dependent inhibition of currents evoked by 5-HT (30 μM) applied locally to HEK293 cells expressing recombinant 5-HT$_3$A or 5-HT$_3$AB receptors (Figure 1A). 5-HT was unable to overcome inhibition of 5-HT$_3$A receptors by morphine (10 μM) even when applied at high concentrations (Figure 1B). These data demonstrate that the pre-application of morphine caused insurmountable inhibition of 5-HT$_3$ receptors.

The IC$_{50}$ values for morphine were 0.33 ± 0.07 μM for the 5-HT$_3$A receptor and 1.2 ± 0.1 μM for the 5-HT$_3$AB receptor. Therefore, the incorporation of the 5-HT3B subunit caused an approximate fourfold reduction in the potency of pre-applied morphine as an antagonist of the 5-HT$_3$ receptor.

Morphine caused a slowing of receptor activation (Figure 1B inset). In the absence of morphine, the 10–90% rise time of 5-HT-evoked current was 12 ± 2 ms (n = 6) and 12 ± 1 ms (n = 5) for 5-HT$_3$A and 5-HT$_3$AB receptors respectively. In the presence of pre-applied morphine (10 μM), the 10–90% rise times were 48 ± 8 ms (n = 6) and 35 ± 6 ms (n = 5) for 5-HT$_3$A and 5-HT$_3$AB receptors respectively. The slowing in 10–90% rise time in the presence of morphine was statistically significant for both homomeric (P < 0.005, paired *t*-test) and heteromeric receptors (P = 0.01, paired *t*-test).

Morphine is not a 5-HT$_3$ receptor agonist

A previous study demonstrated that the alkaloid apomorphine acts as a weak partial agonist at 5-HT$_3$ receptors (van Hooft and Vijverberg, 1998). If morphine acts as a partial agonist, its pre-application could induce desensitization. Under these circumstances, subsequently applied 5-HT would be unable to maximally activate receptors. Such a phenomenon could produce an insurmountable inhibition by pre-applied morphine. We examined whether morphine (1 mM) activated 5-HT$_3$ receptors when applied rapidly to cells expressing 5-HT$_3$A or 5-HT$_3$AB receptors. Cells, expressing either receptor subtype, that responded robustly to 5-HT (30 μM), failed to exhibit currents in response to morphine (n ≥ 3, data not shown). This suggests that morphine lacks efficacy as an agonist of 5-HT$_3$ receptors and, taken together with the morphine pre-application findings, suggests that the high-potency insurmountable inhibition occurs through non-competitive antagonism and not through desensitization.

Inhibition of 5-HT$_3$ receptors by simultaneously applied morphine

Previous studies in which morphine and 5-HT were applied simultaneously suggest that its antagonism is competitive (Fan, 1995; Wittmann *et al.*, 2006). We investigated the effect

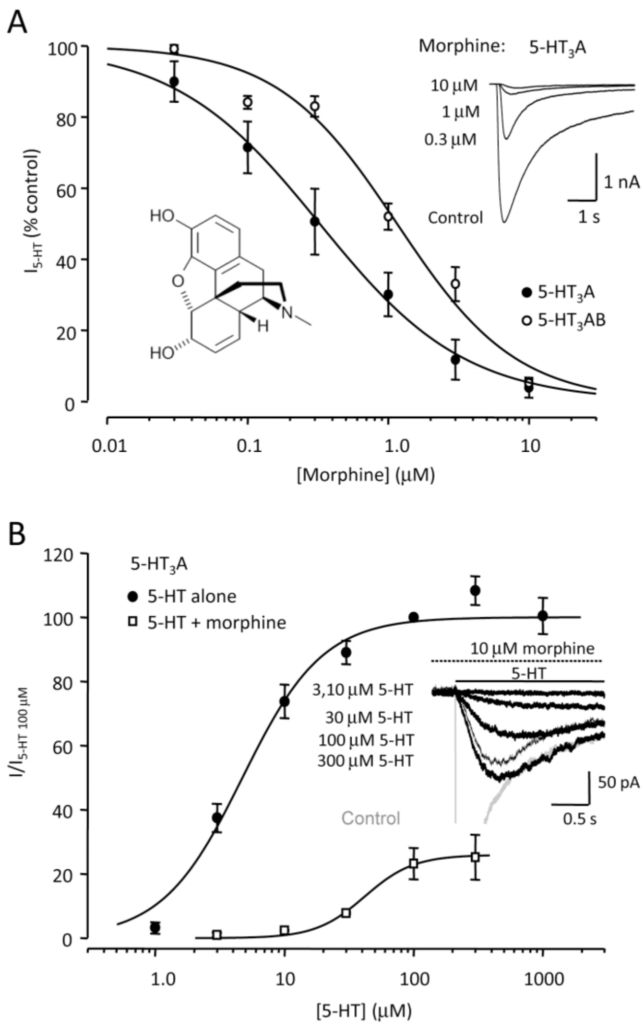

Figure 1

High-potency insurmountable antagonism of 5-HT$_3$A and 5-HT$_3$AB receptors by morphine. (A) Concentration–response relationships of pre-applied morphine on 5-HT$_3$A and 5-HT$_3$AB receptors. Each point represents the mean ± SEM of at least four recordings from different cells. Data are normalized to the current at 30 μM 5-HT in the absence of morphine. The logistic fit to the concentration–response curve yielded an IC$_{50}$ of 0.33 ± 0.07 μM for 5-HT$_3$A receptors and 1.15 ± 0.11 μM for 5-HT$_3$AB receptors. Morphine was either bath applied or pre-applied for 2 s using a rapid application system (see Methods). Inset shows representative, superimposed traces of currents recorded from 5-HT$_3$A receptor expressing HEK293 cells in the absence or presence of 0.3, 1 and 10 μM morphine pre-applied prior to 5-HT (30 μM). (B) Graph of the 5-HT concentration–response relationship in the absence and presence of pre-applied morphine (10 μM). Current amplitudes are normalized to the amplitude of control currents activated by 100 μM 5-HT. When morphine was pre-applied, the inhibition of the 5-HT-induced current was insurmountable even by maximally efficacious concentrations of 5-HT. Inset shows superimposed traces of 5-HT (3–300 μM)-induced currents in the presence of pre-applied morphine (10 μM). The grey trace represents part of a control current induced by 5-HT (100 μM) provided for comparison.

Table 1

Parameters of 5-HT concentration–response relationships in the presence and absence of simultaneously applied morphine

	5-HT$_3$A receptor EC$_{50}$ (μM)	I_{max} (%)	Hill slope	5-HT$_3$AB receptor EC$_{50}$ (μM)	I_{max} (%)	Hill slope
5-HT alone	5.1 ± 0.6	102 ± 3	1.3 ± 0.2	16 ± 2	110 ± 4	1.2 ± 0.1
5-HT + 3 μM morphine	8.2 ± 1.1	102 ± 4	2.0 ± 0.4	NT	NT	NT
5-HT + 30 μM morphine	22 ± 3	107 ± 4	1.9 ± 0.3	30 ± 8	108 ± 10	1.4 ± 0.3
5-HT + 100 μM morphine	41 ± 4*	105 ± 3	1.8 ± 0.2	51 ± 5*	104 ± 3	1.8 ± 0.2
5-HT + 300 μM morphine	175 ± 22*	101 ± 5	1.9 ± 0.3	221 ± 16*	109 ± 4	1.8 ± 0.2

5-HT concentration–response relationships in the presence and absence of simultaneously applied morphine at the concentrations indicated. Current amplitudes were normalised to those recorded from the same cell by 100 μM 5-HT alone. Concentration–response relationships were fitted with a logistic equation and are shown in Figure 2, yielding the tabulated values.
*Significantly different from corresponding value for 5-HT alone, $P < 0.05$, ANOVA, *post hoc* Dunnett's test.
NT, not tested.

of simultaneously applied morphine on the 5-HT concentration–response relationship (Figure 2A and B). Locally applied 5-HT (1–1000 μM) caused concentration-dependent activation of recombinant 5-HT$_3$A and 5-HT$_3$AB receptors expressed in HEK293 cells (Figure 2C and D, Table 1). Consistent with previous reports (Davies *et al.*, 1999; Stewart *et al.*, 2003), 5-HT had a lower potency as an agonist of heteromeric 5-HT$_3$AB receptors compared to homomeric 5-HT$_3$A receptors (Table 1). The simultaneous application of morphine (3–300 μM) reduced the amplitude of 5-HT$_3$A receptor-mediated currents evoked by low concentrations of 5-HT, but the inhibition was completely surmounted by high concentrations of 5-HT (Figure 2A and B, Table 1). The rightward shift in the 5-HT concentration–response relationship was dependent on morphine concentration. Simultaneous application of morphine (30–300 μM) also led to a surmountable inhibition of 5-HT (1–1000 μM)-evoked currents recorded from HEK293 cells expressing recombinant 5-HT$_3$AB receptors (Figure 2C and D, Table 1). Similar to its effect on the 5-HT$_3$A receptor, morphine caused rightward shifts of the 5-HT concentration–response relationship mediated by the 5-HT$_3$AB receptor (Figure 2D; Table 1).

Schild analysis of the shift in pEC$_{50}$ values in the absence and presence of morphine (as described in Methods) yielded slopes of 0.8 and 1.2 for 5-HT$_3$A and 5-HT$_3$AB receptors respectively (Figure 2B and D). These values did not significantly deviate from unity when compared with the 95% confidence intervals of the fits. These analyses indicate that the interaction between simultaneously applied morphine and 5-HT$_3$ receptors is competitive.

Comparison of the concentration–response relationships for morphine with pre- and simultaneous application reveals a marked reduction in potency for inhibition of both 5-HT$_3$A and 5-HT$_3$AB receptors using the latter protocol. IC$_{50}$ values for morphine were compared at 10 μM 5-HT for 5-HT$_3$A receptors and 30 μM for 5-HT$_3$AB receptors, to reflect differences in the potency of 5-HT at these receptor subtypes. The IC$_{50}$ values of simultaneously applied morphine for the 5-HT$_3$A and 5-HT$_3$AB receptors were 5.2 ± 1.5 and 39 ± 6 μM respectively (data not shown). These values are significantly higher than those obtained for pre-applied morphine ($P < 0.05$, t-test) and suggest that high-potency non-competitive inhibition by morphine requires its application prior to 5-HT. We also determined the apparent binding affinity of morphine to 5-HT$_3$A and 5-HT$_3$AB receptors from the Schild analysis described above. The K_d values for the competitive interaction of morphine with 5-HT$_3$A and 5-HT$_3$AB receptors were 18 and 55 μM respectively.

Morphine is a competitive inhibitor of [^{3}H]GR65630 binding

We used radioligand binding of [^{3}H]GR65630 to investigate further the competitive binding of morphine to 5-HT$_3$A and 5-HT$_3$AB receptors. GR65630 binds to the orthosteric site and is therefore a competitive antagonist. Saturation binding experiments revealed that [^{3}H]GR65630 bound to 5-HT$_3$A and 5-HT$_3$AB receptors with an affinity of 0.15 ± 0.07 and 0.17 ± 0.02 nM respectively (Figure S1). These values are consistent with published reports of [^{3}H]GR65630 affinity at 5-HT$_3$A and 5-HT$_3$AB receptors (Walstab *et al.*, 2010).

Morphine displaced [^{3}H]GR65630 (150 pM) from 5-HT$_3$A and 5-HT$_3$AB receptors with IC$_{50}$ values of 27 ± 4 μM ($n = 4$) and 19 ± 4 μM ($n = 4$) respectively (Figure 3). The K_i values for morphine calculated using the Cheng–Prusoff equation were 13 ± 2 μM for 5-HT$_3$A and 8 ± 1 μM for 5-HT$_3$AB receptors. The data suggest that morphine binds to the 5-HT/GR65630 binding site on 5-HT$_3$A and 5-HT$_3$AB receptors with a similar affinity.

The antagonism of 5-HT$_3$ receptors by morphine lacks voltage dependence and is not mediated through the channel pore

We previously demonstrated that the opioid methadone caused a non-competitive inhibition of 5-HT$_3$AB receptors through voltage-dependent channel blockade (Deeb *et al.*, 2009). We examined whether non-competitive inhibition by pre-applied morphine also exhibited voltage dependence by recording currents mediated by 5-HT$_3$A and 5-HT$_3$AB receptors at different holding potentials in the presence and absence of the opioid. Morphine (3 μM), bath applied to cells

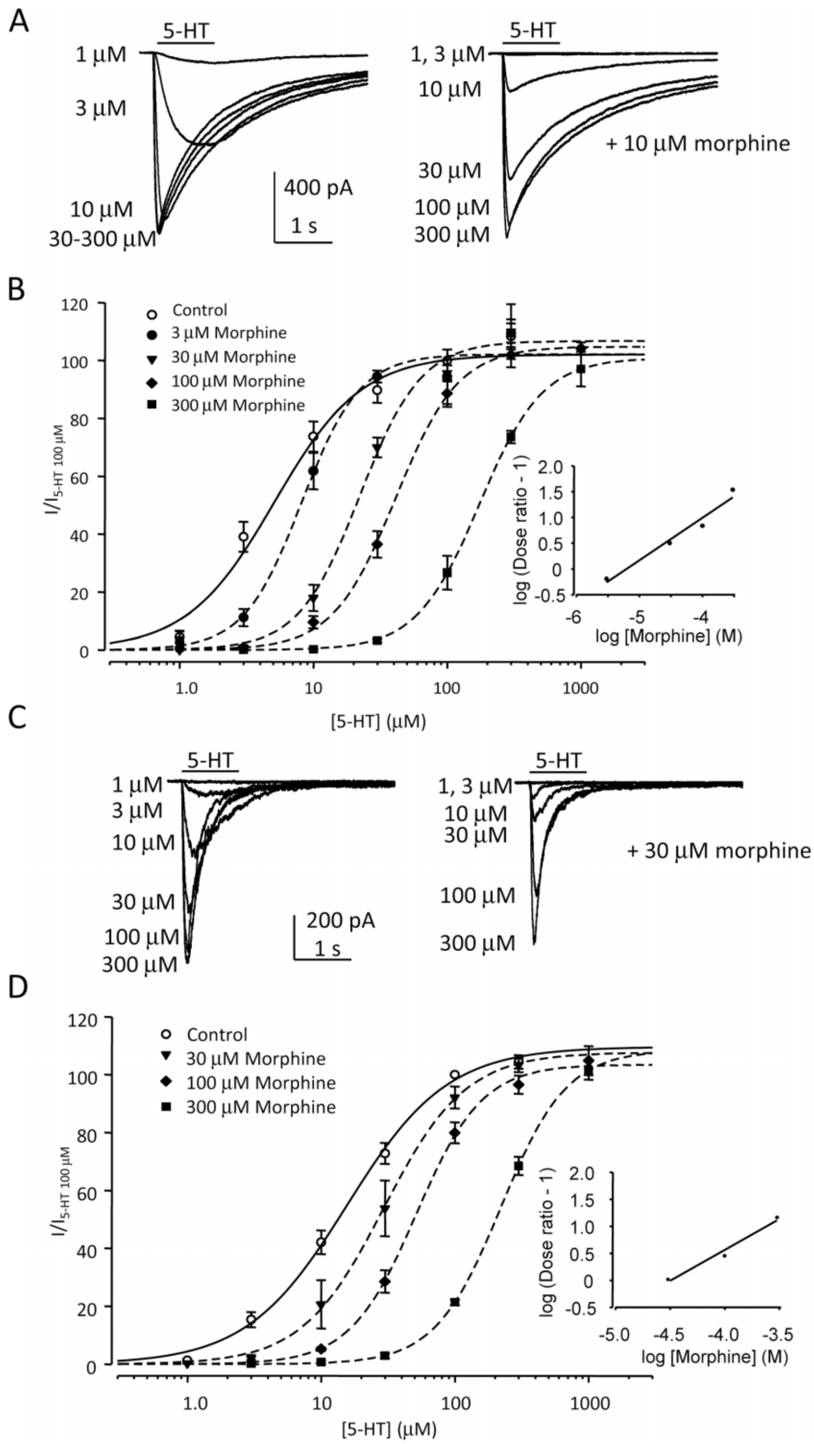

Figure 2

Competitive and surmountable antagonism of 5-HT_3A and 5-HT_3AB receptors by morphine simultaneously applied with 5-HT. (A) Superimposed traces of 5-HT (1–300 µM)-induced currents recorded from the same HEK293 cell expressing 5-HT_3A receptors in the absence and presence of simultaneously applied morphine (10 µM). (B) Graph of the concentration–response relationship for 5-HT in the presence of increasing concentrations (3–300 µM) of co-applied morphine on 5-HT_3A receptors. Inset, Schild plot fitted with a linear regression with a slope of 0.8. (C) Superimposed traces of 5-HT (1–300 µM)-induced currents recorded from the same HEK293 cell expressing 5-HT_3AB heteromeric receptors in the absence and presence of morphine (30 µM) applied simultaneously with 5-HT. (D) Graph of the 5-HT_3AB receptor concentration–response relationship for activation by 5-HT in the absence and presence of increasing concentrations of morphine. Inset, Schild plot fitted with a linear regression with a slope of 1.2. Current amplitudes in the concentration–response relationships are expressed as percentage of the mean current induced by 5-HT (100 µM) in control conditions. Each data point represents the mean ± SEM of at least four recordings from separate cells. EC_{50} values and Hill coefficients were determined from logistic fits to the concentration–response curves and are summarized in Table 1.

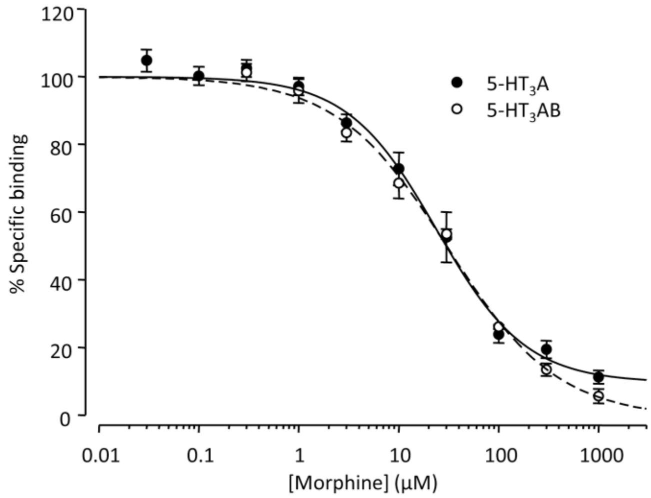

Figure 3

Morphine competes for the binding site of the competitive antagonist [^{3}H]GR65630 on 5-HT_3 receptors. Membranes containing 5-HT_3A receptors were incubated with 150 pM [^{3}H]GR65630. See Figure S1 for saturation binding of [^{3}H]GR65630. The graph in this figure shows the reduction of specific [^{3}H]GR65630 binding (expressed as percent control) with increasing concentrations of morphine. Each data point represents the mean ± SEM of at least three separate binding assays. Morphine inhibited [^{3}H]GR65630 binding to 5-HT_3A and 5-HT_3AB receptors with IC_{50} values of 27 and 19 μM respectively.

expressing either 5-HT_3A or 5-HT_3AB receptors, inhibited 5-HT (30 μM)-evoked currents at potentials between –60 and 60 mV. There was no change in the inhibition of the peak current amplitude by morphine at any potential (n = 3, data not shown). This is consistent with a lack of voltage-dependent open channel blockade by morphine.

A lack of voltage-dependent inhibition does not necessarily rule out a channel blocking effect of morphine, which could occur through an interaction with pore residues beyond the sphere of influence of the membrane potential. The channel pore is lined by second transmembrane (M2) domains, one M2 provided by each of the five 5-HT3 subunits. We investigated whether the introduction of the M2 domain of the 5-HT3B subunit into the 5-HT3A subunit was sufficient to confer reduced sensitivity to insurmountable inhibition of 5-HT_3 receptors by morphine. The 5-HT3ABA construct (Figure 4A) was produced by replacing residues 269–298 of the 5-HT3A by those of the 5-HT3B subunit (Kelley *et al.*, 2003). As reported previously, the 5-HT3ABA construct (referred to from here onwards as ABA) failed to form functional receptors when expressed alone. However, when combined with the 5-HT3A subunit, the resulting 5-HT_3A + ABA heteromeric receptors were distinguishable from 5-HT_3A receptors by virtue of their low permeability to Ca^{2+} relative to Cs^+ (Figure 4B). The P_{Ca2+}/P_{Cs+} values for 5-HT_3A, 5-HT_3AB and 5-HT_3A + ABA receptors were 1.1 ± 0.1 (n = 4), 0.4 ± 0.1 (n = 4) and 0.5 ± 0.1 (n = 4) respectively. The permeability to Na^+ was similar for all three receptor types (Figure 4B). Having demonstrated that the ABA construct is functionally incorporated as 5-HT_3A + ABA heteromeric

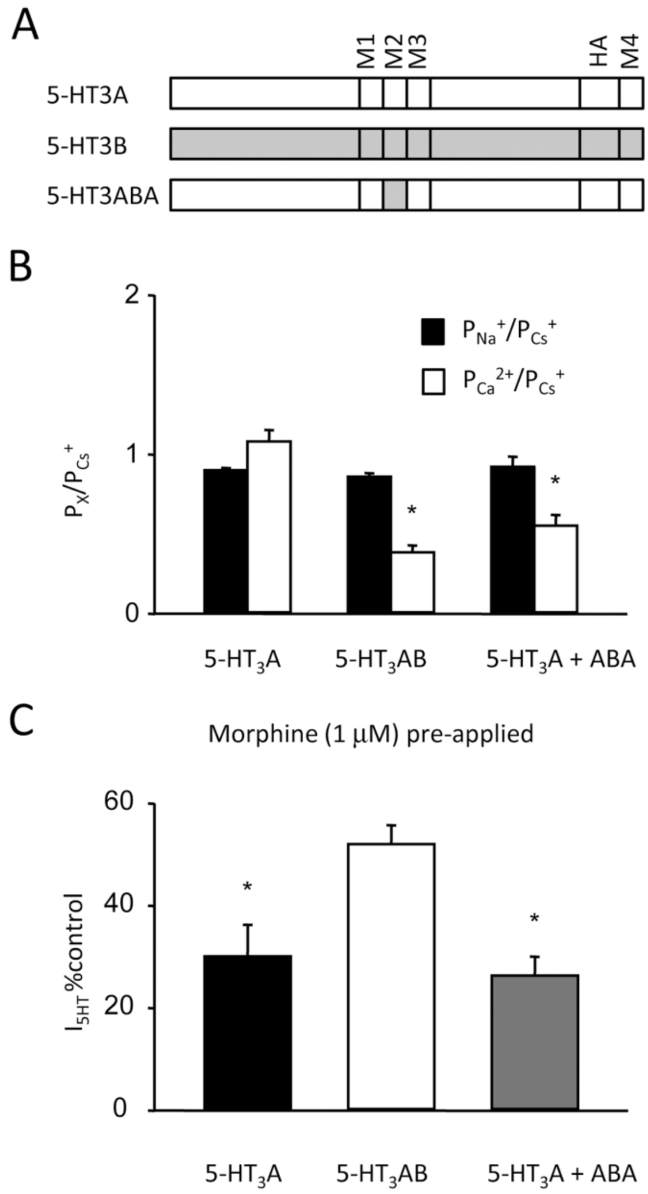

Figure 4

The M2 domain is not responsible for the insurmountable antagonism by pre-applied morphine. (A) Schematic diagram of the 5-HT3ABA construct, which is the 5-HT3A subunit containing the 5-HT3B M2 domain. (B) Na^+ and Ca^{2+} permeability ratios with respect to Cs^+ for 5-HT_3A, 5-HT_3AB and 5-HT_3A + ABA receptors. P_{Ca2+}/P_{Cs+} in 5-HT_3AB and 5-HT_3A + ABA receptors are significantly lower than that of 5-HT_3A receptor (P < 0.05, ANOVA, *post hoc* Tukey's test), thus confirming the incorporation of the ABA construct into heteromeric receptors. (C) Bar graph shows the current elicited by 5-HT (30 μM) following pre-application of morphine (1 μM) expressed as percent control current amplitude. The % control values for 5-HT_3A, 5-HT_3AB and 5-HT_3A + ABA receptors were 30 ± 6%, 52 ± 4% and 26 ± 4% respectively. The values for 5-HT_3A and 5-HT_3A +ABA receptors are significantly lower than that of 5-HT_3AB receptors (P < 0.05, one-way ANOVA, *post hoc* Tukey's test), indicating that the presence of the 5-HT3B M2 domain has no effect on the inhibition by pre-applied morphine.

receptors, we investigated whether the presence of the 5-HT3B M2 influenced inhibition by morphine. Pre-applied morphine (1 μM) caused a >70% inhibition of both the 5-HT$_3$A and 5-HT$_3$A + ABA receptors (Figure 4C). By contrast, morphine (1 μM) caused an approximately 50% inhibition of 5-HT$_3$AB receptors. The 5-HT3B M2 had no discernable influence on the inhibition by pre-applied morphine. Taken together, these data suggest that insurmountable inhibition by morphine occurs through a site outside the channel pore.

Activation of 5-HT$_3$ receptors prevents the non-competitive inhibition by morphine

Cells expressing 5-HT$_3$A receptors were activated by stepping rapidly into 5-HT (100 μM) either alone or in combination with morphine (10 μM) for 2 s. Currents recorded in the presence or absence of simultaneously applied morphine were indistinguishable (Figure S2). Desensitization kinetics of 5-HT-evoked currents were also unaffected by morphine. These data suggest that morphine binds more slowly than 5-HT, and, once activated, 5-HT$_3$ receptors are resistant to the high-potency insurmountable component of morphine inhibition.

Kinetics of high-potency insurmountable inhibition by morphine

We used rapid application to examine the time course for the onset and reversal of the insurmountable inhibition by morphine. We explored the morphine pre-application time required for the onset of the insurmountable inhibition of 5-HT-evoked currents by stepping into morphine (10 μM) for durations between 0 and 100 ms (in 10 ms increments) before stepping into 5-HT (100 μM) plus morphine (10 μM). Insurmountable inhibition in this context is defined as the failure of 5-HT (100 μM) to reverse the inhibitory effect of morphine (10 μM). Figure 5A shows a representative experiment. Morphine had no effect on the 5-HT-evoked current when applied for 10 ms prior to 5-HT$_3$A receptor activation. The appearance of insurmountable inhibition by morphine had a time constant (τ) of 35 ± 11 ms (n = 4; Figure 5C). Incorporation of the 5-HT3B subunit led to a reduction in the onset time constant for insurmountable inhibition to 17 ± 2 ms (n = 4). The difference between the onset time constants of homomeric and heteromeric receptors was statistically significant (P < 0.0001, t-test). These data suggest that morphine can reach its high-potency site faster when the 5-HT3B subunit is present.

We also looked at the reversal time (offset time course) for the insurmountable inhibition by morphine. Cells expressing 5-HT$_3$A receptors were stepped rapidly into a maximally efficacious concentration of morphine (10 μM) for 2 s (Figure 5B). From our onset kinetics experiments (see above), we established that 100 ms was sufficient for maximal inhibition. Following this length of exposure to morphine, cells were rapidly stepped into a morphine-free solution for durations of between 0 and 1.3 s (in 100 ms increments), before activation by 100 μM 5-HT (Figure 5B). After a period of 300–400 ms, during which there was no recovery in the amplitude of 5-HT-induced current, currents recover with a mean time constant of 0.53 ± 0.04 s (n = 6) for 5-HT$_3$A receptors (Figure 5D). Incorporation of the 5-HT3B subunit reduced both lag time (100–200 ms) and recovery time constant (0.32 ± 0.07 s; n = 5). The difference in recovery time constants between 5-HT$_3$A and 5-HT$_3$AB receptors was significant as determined by the t-test (P = 0.02). These results suggest that morphine has a lower affinity when pre-applied to heteromeric receptors and therefore dissociates faster, consistent with the lower potency of inhibition of 5-HT$_3$AB compared with 5-HT$_3$A receptors (Figure 1A).

Discussion

There are two components to the inhibitory actions of morphine on 5-HT$_3$ receptors. A potent insurmountable inhibition occurred when morphine was applied prior to 5-HT$_3$ receptor activation. The presence of the 5-HT3B subunit caused an approximate fourfold reduction in the potency of this component of morphine's inhibition. By contrast, when applied simultaneously with 5-HT, morphine caused a lower-potency insurmountable inhibition of 5-HT$_3$ receptors. The affinity of morphine for the orthosteric binding site was not influenced by subunit composition.

Low-potency competitive inhibition of human 5-HT$_3$ receptors is a property shared by several heterocyclic alkaloids including the opioids methadone and fentanyl (Wittmann *et al.*, 2008; Deeb *et al.*, 2009). It is not surprising that this component of inhibition by morphine was unaffected by the 5-HT3B subunit because competitive inhibitors appear not to discriminate between homomeric 5-HT$_3$A and heteromeric 5-HT$_3$AB receptors (Brady *et al.*, 2001). A recent study suggests that the orthosteric binding site may be restricted to the interface between adjacent 5-HT3A subunits in both 5-HT$_3$A and 5-HT$_3$AB receptors (Lochner and Lummis, 2010). Our observation that the binding affinities of [^{3}H]GR65630 and 5-HT were unaffected by incorporation of the 5-HT3B subunit is consistent with this proposal. This implies that the lower potency of 5-HT as an agonist at human 5-HT$_3$AB compared with 5-HT$_3$A receptors (Davies *et al.*, 1999) is not caused by differing binding affinities but may instead reflect a lower efficacy of gating in the former (Colquhoun, 1998). Indeed, substitution of residues outside the 5-HT$_3$A receptor agonist binding site within the intracellular M3–M4 loop alter the potency of 5-HT (Livesey *et al.*, 2008).

In contrast to competitive antagonists, some non-competitive antagonists do discriminate between 5-HT$_3$A and 5-HT$_3$AB receptors. For example, the 5-HT3B subunit reduces the potency of inhibition by picrotoxin, a non-competitive use-dependent antagonist that interacts with residues in the channel pore within the M2 domains of 5-HT$_3$ receptors as well as other Cys-loop receptors (Das and Dillon, 2003). By contrast, in addition to its competitive antagonism of 5-HT$_3$ receptors, methadone also causes a non-competitive voltage-dependent blockade, which is dependent on the presence of the 5-HT3B subunit (Deeb *et al.*, 2009). The insurmountable inhibition of 5-HT$_3$A and 5-HT$_3$AB receptors by morphine is different from both of these examples in that it is neither use- nor voltage-dependent. This implies that morphine does not require access to the open channel. The use of a chimeric ABA construct, in which the 5-HT3A subunit's M2 is replaced by that of the 5-HT3B subunit, revealed that the actions of morphine were not mediated via the channel pore. The

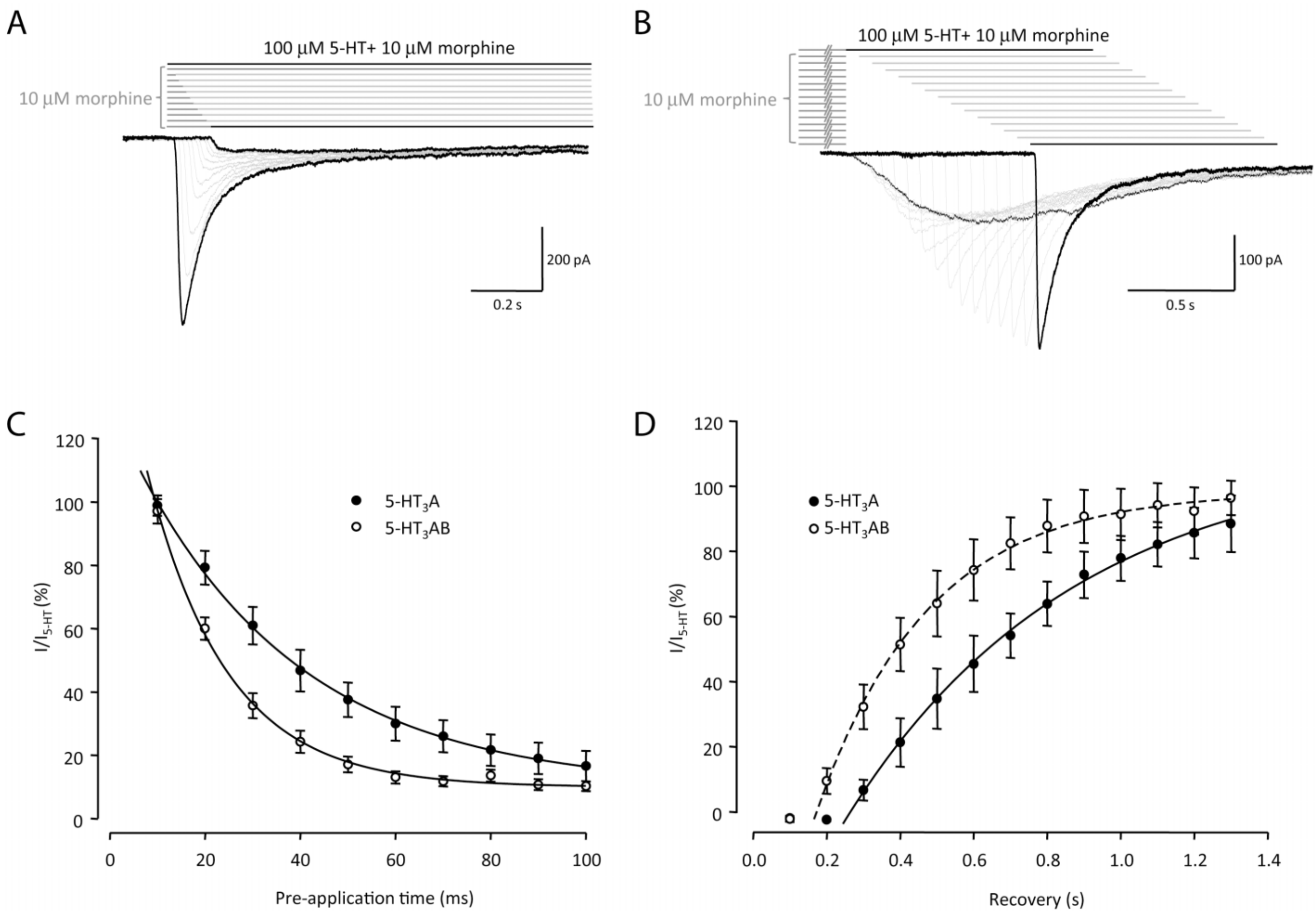

Figure 5

Onset and offset time course of inhibition by pre-applied morphine of 5-HT-induced currents mediated by 5-HT$_3$A and 5-HT$_3$AB receptors. (A) Typical traces of sequential 5-HT-induced currents recorded from an HEK293 cell expressing 5-HT$_3$A receptors. The cell was pre-exposed to morphine (10 µM) for progressively longer durations (10 ms increments) before the application of 5-HT (100 µM) plus morphine (10 µM). (B) currents recorded from a cell expressing 5-HT$_3$A receptors pre-exposed to morphine (10 µM) for 2 s followed by progressively increasing wash durations (100 ms increments) before the application of 5-HT (100 µM) plus morphine (10 µM). The first and last traces are shown in black, and the protocol for solution change is shown above. (C) Mean onset and (D) offset time courses for pre-applied morphine inhibition in 5-HT$_3$A and 5-HT$_3$AB receptors. Graphs show the mean peak amplitude [as % of control 5-HT (100 µM)-evoked current] with either increasing exposure time (for onset kinetics in C) or increasing wash time (for offset kinetics in D) for 5-HT$_3$A and 5-HT$_3$AB receptors. In experiments involving 5-HT$_3$AB receptors, a higher concentration of morphine was used (30 µM), reflecting the lower-potency inhibition of heteromeric receptors (Figure 1). Each data point represents the mean ± SEM of four to six experiments. A single exponential function was fitted to each set of data. The time constants for the onset of morphine inhibition were 35 ± 11 ms ($n = 4$) and 17 ± 2 ms ($n = 4$) for the 5-HT$_3$A receptor and 5-HT$_3$AB receptor respectively. The time constants for the offset of morphine inhibition were 0.53 ± 0.04 s ($n = 6$) and 0.32 ± 0.07 s ($n = 5$) for the 5-HT$_3$A receptor and 5-HT$_3$AB receptor respectively. There were significant differences between the onset and offset time constants ($P < 0.0001$ for onset, $P = 0.02$ for offset) for homomeric and heteromeric receptors.

potency of morphine was unaffected by the presence of the 5-HT3B M2.

Similar to its effect on inhibition by morphine, the 5-HT3B subunit reduces the potency of 5-HT$_3$ receptor inhibition by the heterocyclic alkaloid tubocurarine. Reduced tubocurarine potency conferred by the 5-HT3B subunit is relatively small compared with the remarkable shift between 5-HT$_3$A receptors of differing species (Mair *et al*., 1998; Davies *et al*., 1999). Rodent 5-HT$_3$A receptors are approximately 100-fold more sensitive to tubocurarine block than are human 5-HT$_3$A receptors and this effect has been traced to several residues within the agonist binding site (Hope *et al*., 1999; Zhang *et al*., 2007). As the 5-HT3B subunit appears not to affect binding to the agonist binding site, the modest reduction in the potency of tubocurarine inhibition conferred by the subunit is also likely to be caused by an interaction of the molecule with additional residues outside the site.

The observation that receptor activation prevents insurmountable inhibition by morphine suggests that the high affinity non-competitive site becomes unavailable. X-ray crystallographic structural models in the resting and agonist bound conformations of the acetylcholine binding protein (evolutionarily related to the Cys-loop receptors) reveal movement upon agonist binding within and around the orthosteric binding site (Hibbs *et al*., 2009). The binding site becomes capped by loop C, a conformation that in the 5-HT_3 receptor may be incompatible with high-affinity morphine binding. The structures of the bacterial pentameric ligand-gated ion channels isolated from *Erwinia chrysanthemi* and *Gloeobacter violaceus* in presumed closed and open conformations, respectively, reveal additional movement throughout the membrane spanning M1–M4 domains (Bocquet *et al*., 2009; Hilf and Dutzler, 2009). The slow onset rate of insurmountable inhibition by morphine, revealed by fast application, suggests that the binding site suffers from poor accessibility even in the resting receptor. The faster rate of onset upon incorporation of the 5-HT3B subunit suggests that accessibility to the site of high-potency inhibition by morphine improves and offers a strategy for additional future chimeric studies targeting its location.

The significance of 5-HT_3 receptor inhibition to the behavioural effects of morphine remains unclear. Interestingly, the 5-HT_3 receptor antagonist ondansetron aids detoxification from heroin (Ye *et al*., 2001). Furthermore, 5-HT_3 receptor antagonism decreases both the morphine-induced stimulation of dopamine release within the nucleus accumbens and reward associated with morphine administration (Carboni *et al*., 1988; 1989). Therefore, 5-HT_3 receptor inhibition by morphine may mitigate reward. Recent studies demonstrate that 5-HT_3 receptor antagonism may also reduce morphine-induced hyperalgesia and analgesic tolerance (Liang *et al*., 2011).

Neuronal μ-opioid receptors in the brain mediate the hedonic actions of morphine (Matthes *et al*., 1996). The morphine IC_{50} for 5-HT_3A receptor inhibition is close to its EC_{50} for activation of μ receptors (McPherson *et al*., 2010). Morphine is therefore likely to be present in the brain during heroin-induced euphoria, at a sufficient concentration to bind to the non-competitive site on inactive 5-HT_3A receptors, and impair their subsequent activation by 5-HT. However, the affinity of morphine for this site is reduced by the 5-HT3B subunit as revealed by both a higher IC_{50} value and a substantially accelerated recovery from inhibition, consistent with faster dissociation of morphine from heteromeric receptors. Furthermore, following i.v. administration to patients, morphine can reach a concentration of 50 nM in the CSF (Meineke *et al*., 2002). According to our concentration–response relationships, this concentration causes inhibition of 5-HT_3A receptors but has a negligible effect on 5-HT_3AB receptors. Therefore, although the difference in sensitivity of the two receptor subtypes to morphine is relatively modest, at clinically relevant concentrations, heteromeric 5-HT_3AB receptors may be spared from morphine inhibition.

5-HT3A and 5-HT3B subunit-specific antibodies reveal the presence of both subunits in human and rodent brain (Brady *et al*., 2007; Doucet *et al*., 2007). Furthermore, polymorphisms affecting the 5-HT3B subunit are associated with major depression and bipolar affective disorder (Frank *et al*., 2004; Yamada *et al*., 2006; Krzywkowski *et al*., 2007). Recent studies reveal that polymorphisms in genes encoding 5-HT3A and 5-HT3B subunits are also associated with heroin dependence (Levran *et al*., 2008; 2009). These polymorphisms lie outside the genes' open reading frames and are therefore most likely to affect subunit expression. It remains to be determined whether there are altered levels of homomeric versus heteromeric 5-HT_3 receptors in individuals harbouring these polymorphisms. However, our demonstration that the potency of insurmountable inhibition of 5-HT_3 receptors is reduced by the 5-HT3B subunit provides a mechanism by which altered expression of genes encoding subunits of the 5-HT_3 receptor may influence the effects of morphine.

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Conflicts of Interest

None to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Saturable binding of [^{3}H]GR65630 to 5-HT_3A and 5-HT_3AB receptors.
Figure S2 Simultaneous application of morphine does not alter 5-HT_3 receptor kinetics.

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