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Molecular determinants of picrotoxin inhibition of 5-hydroxytryptamine type 3 receptors

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Running title: Picrotoxin blocks 5-HT_3 receptors at transmembrane domain 2

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Abbreviations: DMSO, dimethysulfoxide; EGTA, ethylene glycol-bis (β-aminoethyl ether); [^{3}H]EBOB, [^{3}H]-ethynylbicycloorthobenzoate; GABA, γ-aminobutyric acid; $GABA_A$, type A GABA receptor; $GABA_C$, type C GABA receptor; Glu-Cl, glutamate-gated Cl^- channel; HEPES, N-2-hydroxyethylpiperazine-N-2-etanesulfonicacid N,N,N',N'-tetra acetic acid; 5-HT, 5-hydroxytryptamine; LGIC, ligand-gated ion channels; m-CPBG, meta-chloro-phenylbiguanide; nAChR, nicotinic acetylcholine receptor; PTX, picrotoxin; 5-HT_{3A}, serotonin type 3A; 5-HT_{3B}, serotonin type 3B; TM2, transmembrane domain 2; [^{35}S]TBPS, [^{35}S] tert-butylbicyclophosphorothionate

Abstract

Previously we reported that the $GABA_A$ receptor antagonist picrotoxin also antagonizes 5-HT_3 receptors, and that its effects are subunit-dependent. Here, we sought to identify amino acids involved in picrotoxin inhibition of 5-HT_3 receptors. Mutation of serine to alanine at the TM2 2' position did not affect PTX sensitivity in murine 5-HT_{3A} receptors. However, mutation of the 6' TM2 threonine to phenylalanine dramatically reduced PTX sensitivity. Mutation of 6' asparagine to threonine in the 5-HT_{3B} subunit enhanced PTX sensitivity in heteromeric 5-$HT_{3A/3B}$ receptors. Introduction of serine (native to the human 3B subunit) at the 6' position also increased PTX sensitivity, suggesting a species-specific effect. Mutation of the 7' leucine to threonine in 5-HT_{3A} receptors increased PTX sensitivity roughly ten-fold, comparable to that observed in $GABA_A$ receptors, and also conferred distinct gating kinetics. The equivalent mutation in the 3B subunit (i.e., 7' valine to threonine) had no impact on PTX sensitivity in 5-$HT_{3A/3B}$ receptors. Interestingly, [^{3}H]EBOB, a high affinity ligand to the convulsant site in $GABA_A$ receptors, did not exhibit specific binding in 5-HT_{3A} receptors. The structurally related compound, TBPS, which potently inhibits $GABA_A$ receptors, did not inhibit 5-HT_3 currents. Our results indicate the TM2 6' residue is a common determinant of PTX inhibition of both 5-HT_3 and $GABA_A$ receptors, and demonstrate a role of the 7' residue in PTX inhibition. However, lack of effects of EBOB and TBPS in 5-HT_{3A} receptors suggests the functional domains in the two receptors are not equivalent, and underscores the complexity of PTX modulation of LGICs.

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The 5-hydroxytryptamine type 3 (5-HT_3) receptor is a member of the cys-loop superfamily of ligand-gated ion channels (LGICs) that includes nicotinic acetylcholine receptors (nAChRs), $GABA_A$, $GABA_c$ and glycine receptors, and invertebrate glutamate-gated chloride channels (Glu-Cl) (Reeves et al., 2001; Karlin, 2002). 5-HT_3 receptor antagonists are useful as antiemetics in chemotherapy-induced emesis and in irritable bowel syndrome. They also may have utility in treatment of neuropsychiatric diseases such as anxiety and drug dependence and in management of pain (Costall and Naylor, 2004).

The 5-HT_{3A} subunit was identified initially, and shown to form functional homomeric receptors (Maricq et al., 1991). A second subunit (5-HT_{3B}) was subsequently cloned and characterized (Davies et al., 1999). The 5-HT_{3B} subunit is incapable of forming functional homomeric cell surface receptors as it is retained in the endoplasmic reticulum in the absence of the 3A subunit (Boyd et al., 2002). However, co-expression of 5-HT_{3B} with the 5-HT_{3A} subunit results in a heteromeric receptor with distinct biophysical properties (Davies et al., 1999). Recently, three additional putative 5-HT_3 subunits, 5-HT_{3C}, $_{3D}$ and $_{3E}$ have been cloned (Niesler et al., 2003). The pharmacological and functional properties of these novel 5-HT_3 –like subunits are currently unknown.

Like other members of this superfamily, the 5-HT_3 receptor is pentameric in nature, with the five subunits surrounding a central ion channel (Boess et al., 1995). The receptor shares structural features common to LGICs, such as a large extracellular N-terminal region, four transmembrane domains (TM1-TM4) and an intracellular loop between TM3 and TM4. Evidence suggests that the agonist-binding site is located in the N-terminal region, while TM2 forms the pore (Akabas et al., 1994; Brejc et al., 2001; Ortells and Lunt, 1995).

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The plant derived alkaloid picrotoxin was originally shown to be a non-competitive antagonist of $GABA_A$ receptors (Takeuchi and Takeuchi, 1969). It was subsequently demonstrated that picrotoxin inhibits other anion-selective ligand-gated anion channels, including glycine and $GABA_C$ receptors (Pribilla et al., 1992; Wang et al., 1995) and glutamate-gated Cl^- channels (Etter et al., 1999). Recently, we have shown that PTX also inhibits the cation-selective 5-HT_{3A} receptors in a non-competitive, use-facilitated manner (Das et al., 2003). Additionally, the interaction of PTX with the 5-HT_3 receptor is subunit-dependent. Co-expression of the 5-HT_{3B} subunit with the 5-HT_{3A} subunit results in a substantial decrease in PTX sensitivity, making PTX the only antagonist that displays notable selectivity between homomeric and heteromeric 5-HT_3 receptors (Das and Dillon, 2003).

In the present report, we sought to identify the molecular determinants of PTX inhibition of 5-HT_3 receptors. While the precise location of PTX binding in anion-selective channels is unknown, evidence indicates it acts at the cytoplasmic aspect of the highly conserved TM2 domain (Fig. 1) (ffrench-Constant et al., 1993; Gurley et al., 1995; Buhr et al., 2001; Shan et al., 2001; Dibas et al., 2002). Because the mechanism of block by PTX appears comparable in anion-selective and 5-HT_3 receptors, and because the TM2 domain of the 5-HT_3 receptor shares notable homology with the $GABA_A$ receptor (Fig. 1), we focused on this region as the probable site of action of PTX in 5-HT_3 receptors. In the present study, we demonstrate that the 6' and 7' positions of TM2 are major determinants of PTX sensitivity in the 5-HT_3 receptor.

Materials and Methods

Materials. 5-HT, m-CPBG and PTX were purchased from Sigma (St. Louis, MO). [^{3}H]GR65630 (85.5 Ci/mmol) and [^{3}H]EBOB (38 Ci/mmol) were purchased from NEN (Boston, MA). 5-HT and m-CPBG stocks were made in ultrapure H_2O. PTX was made in DMSO and diluted in extracellular saline solution so that the final DMSO concentration (v/v) was < 0.3%. Primers for mutations were synthesized at IDT, Inc. (Coralville, IA).

Cell culture, site-directed mutagenesis and expression of 5-HT$_3$ receptor cDNA. Mouse 5-HT$_{3A}$ (Genbank accession number S41757) and 5-HT$_{3B}$ subunits (AAF73284, kindly provided by Dr. D. Julius, USCF, San Francisco, CA and Dr. E. Kirkness, Institute of Genomic Research, Maryland, respectively) were subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA) for expression in HEK-293 cells. HEK-293 cells were maintained in medium containing minimum essential media (MEM), 10% fetal bovine serum (FBS), L-glutamine (200 mM), penicillin and streptomycin (10,000 U/ml). Site-directed mutagenesis in the 5-HT$_{3A}$ (A-S2'A, 2' serine to alanine; A-T6'F, 6'threonine to phenylalanine; and A-L7'T, 7' leucine to threonine) and 5-HT$_{3B}$ (B-N6'S, 6' asparagine to serine; B-N6'T, 6' asparagine to threonine and B-V7'T, 7' valine to threonine) subunits was performed using the QuickChange^{TM} mutagenesis kit (Stratagene, La Jolla, CA). For the double mutation in 5-HT$_{3B}$ subunit (B-N6'T,V7'T), mutagenic primers were designed using B-N6'T as the template DNA. All mutations were confirmed by DNA sequencing (Texas Tech University, Lubbock). Wild-type and mutant receptors were transfected in HEK-293 cells using the modified calcium phosphate transfection method (Chen and Okayama, 1987). For each transfection approximately 4-10 μg of DNA was used. For co-transfection of receptor subunits, a 1:1 ratio of respective cDNA was used (unless otherwise stated). Cells were washed twice and were used for recording 12-48 hours after transfection.

Radioligand binding assays. Saturation experiments were carried out using membranes harvested from a stable cell line expressing the 5-HT_{3A} receptor. Briefly, HEK-293 cells stably expressing the 5-HT_{3A} receptor were grown as described previously (Das et al., 2003) and harvested using the method described by Yagle et al (2003). Membranes were suspended in sodium phosphate buffer (200 mM NaCl, 50 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4) such that the final concentration was approximately 2 mg/ml and stored at -80°C. Initial saturation experiments using tissue harvested from the stable cell line suggested high B_{max} values. Thus, relatively low amount of protein was used for subsequent assays. For experiments using [^{3}H]GR65630, tissue was thawed and diluted in sodium phosphate buffer and a fixed concentration of protein (5-12 μg) was added to varying concentrations of the radioligand (0.8 nM-20 nM). Non-specific binding was defined using 10 μM m-CPBG. Final volumes for all assays were 250 μl. Samples were incubated in borosilicate glass tubes for 30 minutes at 37°C and reaction was terminated by addition of ice-cold sodium phosphate buffer and bound ligand was separated from free using a Millipore 12 well manifold vacuum filtration system. Saturation experiments using [^{3}H]EBOB were carried out using a similar protocol as described above. In this case, non-specific binding was defined using 100 μM PTX and samples were incubated at room temperature for 90 minutes. All assays were performed in triplicate and a minimum of 3 experiments was carried out for each saturation assay. Radioactivity was measured using liquid scintillation (Packard). Data analysis was performed using Origin 5.0 (Yagle et al., 2003).

Electrophysiological recordings. Whole cell recordings were performed as described previously (Bell-Horner et al., 2000). Patch pipettes were pulled from thin-walled borosilicate glass using a horizontal micropipette puller (P-87/PC, Sutter Instrument Co., Navato, CA), and had a resistance of 1-3 MΩ when filled with the following internal pipette solution (in mM):

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CsCl, 140; EGTA, 10; Mg^{2+} - ATP, 4; HEPES-Na, 10; pH 7.2. Coverslips containing the cultured cells were transferred to a small chamber (1 ml) on the stage of an inverted microscope (Olympus IMT-2; Olympus, Tokyo, Japan) and superfused continuously with the following external solution (in mM): NaCl, 125; KCl, 5.5; $CaCl_2$, 1.5; $MgCl_2$, 0.8; HEPES-Na, 20; glucose, 10; pH 7.3. 5-HT-induced currents were obtained with an Axopatch 200A amplifier (Axon instruments, Foster City, CA) equipped with a CV-4 headstage. Currents were low-pass filtered at 5 kHz, monitored simultaneously on a storage oscilloscope and a thermal head pen recorder (Gould TA240; Gould, Cleveland, OH), and stored on a computer using an online data acquisition system (pClamp 6.0, Axon Instruments). If a change in access resistance was observed during the recording period, the patch was aborted and the data were not included in the analysis. All recordings were made at room temperature and all cells were clamped at -60 mV.

Experimental protocol. 5-HT (with or without PTX or TBPS) was dissolved in the external solution (above) and applied to the target cell through a Y tube positioned adjacent to the cell. With this system, solution exchange (measured as the 10-90% rise time of the junction potential at the open tip) averages 30 msec (Huang and Dillon, 1999). An equipotent concentration of 5-HT was used to gate the channel in most experiments evaluating the effects of antagonist. Once a stable 5-HT-gated current was established, the antagonist at varying concentrations was co-applied with 5-HT to the target cell.

Data analysis. Both PTX (Das et al., 2003; Dillon et al., 1995) and TBPS (Van Renterghem et al., 1987; Dillon et al., 1995) are use-dependent blockers. Thus, for all experiments the extent of inhibition by each agent was calculated as the current remaining at the end of the ligand application period (10 – 30 s), compared to the current amplitude at the same timepoint in the

control (5-HT alone) recording. Inhibition-response relationships for PTX were fitted with the equation : $I / I_{max} = 1/\{1 + (IC_{50}/[PTX])^n\}$, where I is the current amplitude normalized to control, IC_{50} is the half-maximal blocking concentration and n is the Hill coefficient. For inhibition curves, all Hill coefficients are understood to be negative. A minimum of four individual experiments was conducted for each paradigm. All data are presented as mean ± S.E.M.

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Results

Figure 2 shows the concentration-response curves for 5-HT in wild-type and all mutant 5-HT_{3A} receptors. With exception of the 5-HT_{3A} T6'F mutation (designated A-T6'F), only modest changes in EC_{50} values were seen (Table 1), indicating the mutations were well-tolerated and did not cause any gross alterations in channel structure. No detectable currents were recorded when the 5-HT_{3A} A-T6'F mutant was expressed alone. However, receptors incorporating this mutant were "rescued" when it was co-expressed with the wild-type 5-HT_{3A} subunit, and the resulting receptors had EC_{50} and Hill-coefficient values similar to wild-type (Fig. 2).

Lack of effect of TM2 2' serine on PTX sensitivity in homomeric 5-HT_{3A} receptors - As shown in Figure 3, PTX inhibited wild-type 5-HT_{3A} receptors with an IC_{50} of 41.2 ± 5.6 μM. This IC_{50} is comparable to the value we originally reported for PTX-mediated inhibition of these receptors (Das et al., 2003), and is 10-fold higher than picrotoxin's IC_{50} in $GABA_A$ receptors (Bell-Horner et al., 2000). The magnitude of inhibition was not different with cells recorded at -30 mV (current decreased to 38.5 ± 4.2 % of control with 100 μM PTX, n = 4) and +30 mV (current reduced to 42.4 ± 7.3 % of control with 100 μM PTX, n = 4), with no change in reversal potential, indicating picrotoxin's block is voltage-independent.

GABA receptors from a strain of *Drosophila* resistant to picrotoxin express a serine at the TM2 2' position instead of alanine, which is found in the picrotoxin-sensitive wild-type receptor (ffrench-Constant et al., 1993). The 2' position has also been shown to be involved in PTX sensitivity in mammalian $GABA_A$ receptors (Buhr et al., 2001). We considered the possibility that the presence of S instead of A at the 2' position may account for the lower sensitivity of 5-HT_{3A} receptors to PTX. The A-S2'A mutation did not significant alter the PTX sensitivity of 5-

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HT_{3A} receptors (Table 1), indicating the presence of a different residue at the 2' position in the 5-HT_{3A} receptor, compared to the $GABA_A$ receptor, does not account for lower PTX sensitivity.

The TM2 6' threonine influences PTX sensitivity in 5-HT_{3A} receptors- In $GABA_A$ and glycine receptors, mutation of the conserved 6' threonine to phenylalanine confers resistance to PTX (Gurley et al., 1995; Shan et al., 2001). We thus evaluated the role of this residue in conferring resistance to PTX in the 5-HT_{3A} receptor. Because we could not detect functional receptors when the A-T6'F mutant was expressed alone (no response to up to 100 μM 5-HT), we co-expressed it with wild-type cDNA in varying ratios. A 3:1 or greater ratio of A-T6'F to wild-type cDNA did not produce functional receptors. Co-transfection of these subunit cDNAs in 1:1 or 2:1 ratio produced functional receptors with comparable properties (i.e., EC_{50} values and PTX sensitivity). The data presented here were obtained using a 2:1 ratio of A-T6'F: wild-type cDNA. The effects of PTX on the 5-HT_{3A} + A-T6'F receptor are shown in Fig. 3C. The PTX sensitivity was markedly reduced in receptors incorporating the A-T6'F mutation, compared to wild-type receptors (Fig. 3D, Table 1). Although PTX sensitivity was not completely abolished by the mutation, the nearly 50-fold shift in sensitivity to PTX strongly suggests that the 6' residue is a critical determinant of PTX sensitivity in the 5-HT_{3A} receptor.

The TM2 7' residue modulates PTX sensitivity in 5-HT_{3A} receptors-The present and our previous results (Das et al., 2003) show that the affinity of picrotoxin and related ligands for the 5-HT_{3A} receptor is roughly 10-fold less than that observed in $GABA_A$ receptors. In an attempt to understand the molecular basis of this difference, we examined the possibility that other residues in the 2'- 6' vicinity of TM2 may influence PTX sensitivity.

Evidence from SCAM studies in both anionic $GABA_A$ and cationic 5-HT_{3A} receptors suggests that the amino acid residue at the 7' position may be exposed to the channel lumen

(Reeves et al., 2001; Xu et al., 1995). In most $GABA_A$ receptor subunits, a threonine exists at the 7' position. In contrast, a leucine residue is present at this position in the 5-HT_{3A} subunit. To evaluate the potential involvement of this residue in PTX-mediated inhibition, we mutated the 5-HT_{3A} TM2 7' leucine to threonine (A-L7'T). Whereas the A-L7'T mutation resulted in a very modest decrease in 5-HT sensitivity (Fig. 2), this mutation conferred a 10-fold increase in PTX sensitivity, compared to wild-type receptors (Fig. 4, Table 1). Interestingly, the sensitivity to PTX in A-L7'T receptors is comparable to that seen in $GABA_A$ receptors (Bell-Horner et al., 2000; Xu et al., 1995). Thus, the TM2 7' position appears to play a role in determining picrotoxin sensitivity in homomeric 5-HT_{3A} receptors.

The TM2 7' residue modulates gating kinetics in 5-HT_{3A} receptors-In addition to altering PTX sensitivity, the A-L7'T mutation dramatically altered channel kinetics. Activation, desensitization and deactivation were all enhanced compared to wild-type 5-HT_{3A} receptors. Figure 5 illustrates the changes in activation and deactivation conferred by this mutation. The 10-90% rise time in the presence of an EC_{50} 5-HT concentration was 3.5 ± 0.76 and 0.37 ± 0.08 s in wild-type and mutant receptors, respectively ($p < 0.01$). Deactivation time was similarly enhanced in the mutant (12.1 ± 0.91 s in wild-type compared to 2.28 ± 0.54 s in the mutant A-L7'T receptors, $p < 0.01$). Also, in contrast to wild-type 5-HT_{3A} receptors which showed little or no desensitization when gated with an EC_{50} concentration of 5-HT (current amplitude decreased only 1.7 ± 0.8 % at end of 5-HT application period), the A-L7'T receptors exhibited significant desensitization (current amplitude decreased 13.5 ± 2.9 % at end of 5-HT application period, $p < 0.01$ compared to wild type receptors). Thus, mutation of 7'L to T in the 5-HT_{3A} receptor also profoundly altered gating kinetics.

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The 3B subunit TM2 6' position is responsible for reduced PTX sensitivity in heteromeric receptors - We recently reported that heteromeric receptors formed by murine 5-HT_{3A} and 5-HT_{3B} subunits are notably less sensitive to PTX than homomeric 5-HT_{3A} receptors (Das and Dillon, 2003). Following our observation that the 6'T residue in the 5-HT_{3A} subunit was critical in PTX-mediated inhibition, we sought to examine if the 6' residue in the 5-HT_{3B} subunit (asparagine (N) instead of threonine, Fig. 1) was responsible for the reduced sensitivity to PTX in the heteromeric receptors. Serotonin sensitivity in heteromeric receptors expressing the B-N6'T mutation was modestly increased compared to that observed in wild-type receptors (Fig. 6, Table 1). Consistent with our previous report, sensitivity to PTX was significantly decreased in heteromeric receptors (Fig. 7). As expected, PTX sensitivity was significantly enhanced in 5-HT_{3A} + B-(N6'T) receptors (Fig. 7), confirming that the 6'N in the 5-HT_{3B} subunit is in part responsible for the reduced sensitivity of heteromeric receptors to PTX. However, PTX sensitivity was not fully converted to that seen in homomeric receptors, suggesting other residues contribute to the reduced PTX sensitivity in these receptors.

Differential PTX sensitivity in homomeric vs. heteromeric 5-HT_3 receptors is likely species-specific - Whereas the murine 5-HT_{3B} subunit has N at the 6' position, we noted that the human 5-HT_{3B} subunit has a serine (S) residue at this position (Davies et al., 1999). Based on our findings that the presence of T at the 6' position confers enhanced PTX sensitivity, we speculated that the presence of S would also result in receptors sensitive to PTX. As shown in Fig. 7C, PTX sensitivity was similarly enhanced in heteromeric receptors incorporating the B-N6'S mutation. Thus, unlike in murine 5-HT_3 receptors, the degree of differential PTX sensitivity in homomeric versus heteromeric human 5-HT_3 receptors may be minimal.

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The 7' residue in the 3B subunit is a minimal determinant of PTX sensitivity in heteromeric receptors – Based on our above finding that the 7' position of the 3A subunit affects sensitivity to PTX in homomeric receptors, we assessed whether this position in the 3B subunit could similarly alter PTX-mediated inhibition in heteromeric receptors. Receptors expressing the wild-type 3A subunit along with the 3B subunit 7' valine to threonine (B-V7'T) mutant had slightly enhanced 5-HT sensitivity compared to wild-type heteromeric receptors (Fig. 6). Fig. 8 (A, C) illustrates that PTX sensitivity in these receptors was not significantly increased. To further evaluate a possible contribution of the 3B subunit 7' position to PTX sensitivity, we generated a 5-HT_{3B} double mutant (B-N6'T, V7'T). As shown in Figs. 8B and C, heteromeric receptors expressing the double mutant did not display any additional sensitivity to PTX than receptors expressing only the 6' mutation. Thus, in heteromeric receptors, the 7' position of the 5-HT_{3B} subunit does not appear to play a significant role in modulating the effects of PTX.

The caged convulsants EBOB and TBPS do not interact with 5-HT_{3A} receptor- [^{3}H]EBOB binds with high affinity to the convulsant site in $GABA_A$ receptors (Yagle et al., 2003; Huang and Casida, 1996). Although the exact binding site for [^{3}H]EBOB has not been defined, it is believed to overlap the binding site for PTX, since PTX and related convulsant drugs competitively displace [^{3}H]EBOB (Cole et al., 1995). Based on our above findings, we hypothesized that [^{3}H]EBOB would also bind to 5-HT_{3A} receptors. To test this possibility, we performed [^{3}H]EBOB saturation binding studies using membranes harvested from a stable cell line expressing the 5-HT_{3A} receptor. As expected, [^{3}H]GR65630, a ligand for the 5-HT_3 receptor agonist binding site, bound to these receptors with high affinity (K_d = 8 ± 1.7 nM, B_{max} = 23.8 ± 1.6 pmol/mg protein). In contrast, we detected no interaction of [^{3}H]EBOB with 5-HT_{3A} receptors, as exposure of the membranes to up to 180 nM of [^{3}H]EBOB did not yield significant

specific binding (Fig. 9A). We subsequently tested whether TBPS, which is structurally related to EBOB and also presumably binds to the PTX site in $GABA_A$ receptors, (Dillon et al., 1995; Van Renterghem et al., 1987) could interact with 5-HT_{3A} receptors. As shown in Fig. 9B, application of up to 100 μM TBPS had no significant effect on 5-HT currents. In contrast, a concentration of TBPS ten-fold lower completely abolished steady-state GABA-activated currents (Fig. 9C). Thus, whereas the interaction of PTX with 5-HT_{3A} receptors is similar to that observed in $GABA_A$ receptors, the caged convulsants EBOB and TBPS do not similarly interact with the two receptors.

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Discussion

For decades, picrotoxin was considered to be a fairly selective antagonist of $GABA_A$ receptors. The discovery that PTX also inhibits glycine receptors and other anion-selective ligand-gated ion channels is more recent (Pribilla et al., 1992; Etter et al., 1999; Shan et al., 2001). Following our report that PTX also inhibits the cation-selective 5-HT_3 receptor (Das et al., 2003), and that it does so in a subunit-dependent manner (Das and Dillon, 2003), the focus of the present investigation was to determine the molecular basis for PTX inhibition of 5-HT_3 receptors.

Minimal role of the TM2 2' residue in PTX sensitivity of 5-HT_3 receptors - In the homomeric *Drosophila* GABA receptor, a naturally-occuring mutation at the 2' position (A2'S) confers resistance to PTX (ffrench-Constant et al., 1993). Mutations at this position also modify PTX sensitivity in mammalian $GABA_A$ receptors (Xu et al., 1995), $GABA_C$ receptors (Wang et al., 1995), and glutamate-gated Cl^- channels (Etter et al., 1999). Because the presence of serine or threonine instead of alanine at the 2' position in the *Drosophila* GABA receptor and glutamate-gated Cl^- channels, respectively, causes PTX resistance, we hypothesized that the reverse mutation in the homomeric 5-HT_{3A} receptor (S2'A) might lead to an increase in sensitivity to PTX. However, the picrotoxin sensitivity in 5-HT_{3A} receptors expressing the S2'A mutant was not significantly different from wild-type 5-HT_{3A} receptors. Our results are analogous to those of Shan et al. (2001), who found that substitution of the native 2' residue in glycine α1 or β subunits (glycine and proline, respectively) with alanine did not increase PTX sensitivity in homomeric α1 or heteromeric α1β receptors. Thus, the role of the 2' position on picrotoxin sensitivity is receptor-dependent; in 5-HT_3 receptors, like in glycine receptors (Shan et al., 2001), it appears to play a minimal role.

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The TM2 6' residue is a critical determinant of PTX sensitivity in both 5-HT_{3A} and 5-$HT_{3A/3B}$ receptors - The presence of phenylalanine at the TM2 6' position has been shown to confer PTX resistance in $GABA_A$ (Gurley et al., 1995), $GABA_C$ (Zhang et al., 1995) and glycine receptors (Shan et al., 2001). Our finding that 5-HT_{3A} receptors that express a T6'F mutation are significantly less sensitive to PTX demonstrates the 6' position is also a critical determinant of PTX sensitivity in cation-selective LGICs. In addition, our ability to convert picrotoxin-resistant heteromeric 5-$HT_{3A/3B}$ receptors to picrotoxin-sensitive by mutation of the native 3B 6' residue (asparagine) confirms its involvement in PTX-mediated inhibition of 5-HT_3 receptors.

The precise role the 6' residue plays in PTX sensitivity is not clear. Based on molecular modeling, Zhorov and Bregestovski (2000) hypothesized that picrotoxin's electronegative hydrophilic domain is stabilized in the channel of $GABA_A$ and glycine receptors by hydrogen bonding with the native 6' threonine residues; the inability of phenylalanine to hydrogen bond destabilizes PTX binding. The experimental results of Shan et al. (2001) support this hypothesis. However, our results demonstrate that asparagine, which can act as a hydrogen bond donor, confers resistance when present at the 6' position of the 5-HT_{3B} subunit. Thus, either aparagine's NH_2 group is not properly oriented to act as a hydrogen bond donor for the picrotoxin molecule, or other factors at this 6' position are also critical in conferring sensitivity to PTX.

The TM2 7' position differentially affects PTX sensitivity in 5-HT_{3A} and 5-$HT_{3A/3B}$ receptors - All α, β and γ subunits of the $GABA_A$ receptor have a threonine at the 7' position of TM2 (Tyndale et al., 1995), whereas a leucine exists at this position in the 5-HT_{3A} subunit. Based on the fact that the 7' residue is exposed to the lumen of the channel in both $GABA_A$ (Xu et al., 1995) and 5-HT_{3A} receptors (Reeves et al., 2001), and the fact that it is adjacent to the 6'

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residue, we hypothesized that the 7' position may be responsible for the 5-10 lower PTX sensitivity of 5-HT_{3A} receptors than $GABA_A$ receptors (Bell-Horner et al., 2000). Mutation of the TM2 7' leucine to threonine resulted in a 10-fold increase in sensitivity to PTX in the 5-HT_{3A} receptor. This is the first report of a role of the 7' residue in PTX inhibition of a member of the LGIC superfamily. Interestingly, this residue in the 3B subunit does not appear to play in PTX sensitivity in 5-$HT_{3A/3B}$ receptors, as mutation of this residue to T did not enhance sensitivity to PTX when expressed alone or in combination with the 6' threonine mutation (i.e., B-N6'T, V7'T). The basis for the differential effect of the 7' mutation in the two subunits is not known. Although the 7' residue is known to project toward the channel lumen in homomeric 5-HT_{3A} receptors (Reeves et al., 2001), the orientation of the 7' position of the 3B subunit in heteromeric 5-$HT_{3A/3B}$ receptors has not been determined. Subunit-dependent effects of equivalent TM2 residues near the 7' position have been reported in both $GABA_A$ (Chang and Weiss, 1999) and glycine receptors (Shan et al., 2001). However, the similarity of effect of 6' mutations in the two subunits would seem to indicate the orientation of residues at this position is not dramatically different. Thus, differences at the level of 7' would likely be subtle.

Because it has been postulated that picrotoxin acts by stabilizing a desensitized state in other receptors (Dillon et al., 1995; Newland and Cull-Candy, 1992), we considered the possibility that the enhanced effect of PTX in A-L7'T receptors may be secondary to the altered kinetics present in these receptors. This does not appear to be the case however, as we have recently made the reverse mutation (T7'L) in homomeric glycine α1 receptors. As expected, this mutation in glycine receptors caused opposite effects on kinetics than the L7'T mutation caused in 5-HT_{3A} receptors. However, PTX sensitivity was in fact slightly enhanced, not decreased as would be expected if its effects were secondary to alterations in kinetics of the channel

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(unpublished observations). Thus, the effects of the 7' residue on PTX sensitivity do not appear to be due to alterations in channel kinetics.

Is the binding site for PTX the same in 5-HT$_3$ receptors and GABA$_A$ receptors? The present report demonstrates that the 6' TM2 residue, like in the GABA$_A$ receptor, is a critical determinant of PTX action in 5-HT$_3$ receptors. However, the differential contribution in the two receptors of the 2' residue to PTX sensitivity indicates the binding domains are not equivalent. Our finding that the picrotoxin-site ligands [^{3}H]EBOB and TBPS had insignificant effects on the 5-HT$_3$ receptor demonstrates an additional difference of the convulsant site in the two receptors. The lack of effect of these ligands on 5-HT$_3$ receptors was initially somewhat unexpected. However, given that [^{3}H]EBOB binding is strongly influenced by the 2' residue (Cole et al., 1995), its lack of affinity for the 5-HT$_3$ receptors is not unreasonable. With regard to TBPS, the 3' leucine of the GABA$_A$ receptor β subunit may contribute to a high affinity binding-site for this convulsant (Jursky et al., 2000). Thus in the GABA$_A$ receptor, radioligand-binding studies, site-directed mutagenesis and SCAM studies are suggestive of a PTX binding-site at or near the cytoplasmic aspect of the ion-channel, encompassing the 2'-3' vicinity. [^{3}H]EBOB, which displaces PTX binding in the GABA$_A$ receptors, did not specifically bind to the 5-HT$_{3A}$ receptors. In addition, TBPS, a GABA$_A$ receptor antagonist which presumably binds at the 3' region in GABA$_A$ receptors (Jursky et al., 2000), did not inhibit whole-cell currents induced by 5-HT in the 5-HT$_{3A}$ receptor. Taken together, our data demonstrate the 6'-7' region is the more likely site of PTX action than the 2'-3' region in 5-HT$_3$ receptors. An allosteric effect of the mutations we have made on PTX sensitivity cannot be ruled out, however. It is possible, for instance, that mutations at the 6' -7' region are inducing conformational changes in receptor structure at a distant site that are influencing the ability of PTX to bind at that site. Indeed, there

is evidence in anion-selective receptors that picrotoxin may interact at the extracellular aspect (15'-17') of TM2 (Dibas et al., 2002; Perret et al., 1999).

The TM2 7' position is a determinant of channel gating kinetics – In addition to influencing PTX sensitivity, substitution of the native 7' leucine with threonine also notably altered kinetics in 5-HT_{3A} receptors. Activation, desensitization and deactivation kinetics were all increased significantly compared to wild-type receptors. The TM2 7' residue is likely a determinant of gating kinetics in the LGIC superfamily in general, because, as noted above, the reverse mutation in glycine α1 receptors (T7'L) had opposite effects on channel kinetics (unpublished observations). The involvement of the 7' position in gating kinetics is not surprising given that neighboring residues, including the 9' (Chang and Weiss, 1999) and 6' positions (Xu et al., 1995; Shan et al., 2001), are known to influence channel gating. A better understanding of the physicochemical traits of 7' residues necessary for rapid channel kinetics will require additional investigation.

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Footnotes

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Figure Legends

Figure 1. **Map of the second transmembrane domain of members of the ligand-gated ion channel superfamily.** Sequences for the $GABA_A$ α1 subunit, the glycine α1 subunit, and 5-HT_{3A} and 5-HT_{3B} subunits are shown. The 0' position represents the most cytoplasmic aspect of the channel, while the 19' position represents the extracellular aspect of the channel. The conserved 9' leucine is believed to form the channel gate. Residues at the 2' and 6' position are strongly involved in picrotoxin-mediated inhibition, although other residues may also play a role (see text).

Figure 2. **5-HT Sensitivity in wild-type and mutant 5-HT_{3A} receptors.** Mutations in 2' and 7' positions in the 5-HT_{3A} receptor did not appreciably affect 5-HT sensitivity, indicating that the gross structure of the mutant receptors was unaltered. The EC_{50} values and Hill coefficients are reported in Table 1. Values for the wild-type receptor are comparable to what we have reported previously (Das et al., 2003). Mutation of the 6' residue led to non-functional receptors which could be "rescued" upon co-expression with the wild-type 5-HT_{3A} receptor. All data points are from a minimum of 4-6 cells.

Figure 3. **Effect of S2'A and T6'F mutations on picrotoxin sensitivity in 5-HT_{3A} receptors.** Typical traces showing effects of picrotoxin in wild-type (*A*), A-S2'A receptors (*B*), or A-T6'F 5-HT_{3A} receptors (*C*). Receptors were gated with an EC_{50} 5-HT concentration. The PTX IC_{50} was not significantly shifted in receptors with the S2'A mutation as compared to the wild-type receptor, but was drastically shifted in receptors expressing the T6'F mutation (Table 1). *D,*

Mean PTX inhibition curves for wild-type and mutant receptor configurations. n = 4-6 for each data point.

Figure 4. Effect of L7'T mutation in the 5-HT$_{3A}$ receptor. In A-L7'T mutant receptors, PTX sensitivity was increased 10-fold compared to wild-type receptor (Table 1). *B,* Traces in *A* are replotted from Fig. 3 for comparison. This mutation also conferred to the receptor distinct gating kinetics (see Figure 5 below). Traces in *A* are replotted from Figure 3 for comparison. *C,* Mean PTX concentration-inhibition curve for the A-L7'T receptor compared to the wild-type receptor. n = 4-6 for each data point.

Figure 5. Effect of A-L7'T mutation on gating kinetics. *A,* Mean activation kinetics in wild-type (solid line) and A-L7'T receptors (dotted line) in response to an EC_{50} 5-HT concentration. Activation is reported as the 10-90% rise time. Mean results are given in *Ab*. *B,* Deactivation was also enhanced in the A-L7'T receptors (dotted line) compared to wild type receptors (solid line). Deactivation is reported as the 10-90% decay time. Mean deactivation times for the two receptors are given in *Bb*. Both activation and deactivation kinetics were significantly different in mutant compared to wild-type receptors (* denotes $p < 0.01$, Student's t-test).

Figure 6. 5-HT Sensitivity in wild-type and mutant 5-HT$_{3A/3B}$ receptors. Agonist sensitivity was increased approximately two-fold on average in heteromeric receptors with mutations in the 5-HT$_{3B}$ subunit (Table 1). Values for the wild-type receptor are similar to what we have reported previously (Das and Dillon, 2003).

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Figure 7. PTX sensitivity is partially restored in heteromeric receptors with a converse mutation at the 6' position. *A,* Wild-type heteromeric 5-$HT_{3A/3B}$ receptors show reduced sensitivity to PTX, as reported previously (Das and Dillon, 2003). *B,* Heteromeric receptors in which the B subunit 6' asparagine has been mutated to threonine (B-N6'T) displayed an increased sensitivity to PTX, although this was not fully converted to the sensitivity observed in homomeric 5-HT_{3A} receptors. *C,* Mutation of the 6' asparagine to serine, which is present in the human 5-HT_{3B} subunit, also conferred enhanced sensitivity to PTX. *D,* Mean PTX concentration-inhibition curves in wild-type and mutant heteromeric 5-HT_3 receptors. PTX IC_{50} and Hill coefficient values for receptors expressing these mutations are reported in Table 1. Effects of PTX on homomeric receptors (solid squares) is replotted here and in Fig. 8 for comparison.

Figure 8. Effect of the TM2 7' position in the 5-HT_{3B} subunit on PTX sensitivity in heteromeric 5-$HT_{3A/3B}$ receptors. *A,* Effect of PTX in cells expressing 3A + B-(V7'T) subunits. No significant increase in PTX sensitivity was observed in these receptors compared to wild-type 5-$HT_{3A/3B}$ receptors. *B,* Typical recordings showing effects of PTX in cells expressing the B-N6'T, V7'T double mutant along with the wild-type 3A subunit. No additional gain in sensitivity was observed in these receptors when compared to heteromeric receptors expressing the B-N6'T mutation alone. *C,* Mean PTX concentration-inhibition curves in cells expressing wild-type and mutant 5-$HT_{3A/3B}$ heteromeric receptors.

Figure 9. The noncompetitive $GABA_A$ receptor antagonists EBOB and TBPS do not interact with 5-HT_{3A} receptors. *A,* Saturation curve for analysis of [^{3}H]EBOB binding in 5-

HT_{3A} receptors. The plot shown is representative of one experiment performed in triplicate. Filled triangles represent total binding, open squares represent nonspecific binding, and open circles represent specific binding. A similar lack of specific binding of [^{3}H]EBOB to 5-HT_{3A} receptors was observed in three additional experiments. *B*, TBPS, a high affinity noncompetitive antagonist of $GABA_A$ receptors, was tested for its ability to block 5-HT_{3A} receptors. As shown, 100 μM TBPS had no significant effect of 5-HT-gated current. In contrast, steady-state GABA currents recorded from HEK293 cells expressing α1β2γ2 $GABA_A$ receptors were completely abolished by a 10-fold lower concentration of TBPS.

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Table 1. Functional properties of wild type and mutant 5-HT3 receptors. EC50 values for 5-HT and IC_{50} values for picrotoxin (PTX) are reported in μM. n = number of cells tested; nH = Hill coefficient. * = significantly different from the wild-type receptor ($p < 0.05$); ** = significantly different from the wild-type receptor ($p < 0.01$); + = significantly different from wild type $5\text{-}HT_{3A}$ receptor.

Receptor	5-HT EC_{50}	nH	n	PTX IC_{50}	nH	n
3A	1.2 ± 0.07	2.7 ± 0.4	4	41.2 ± 5.6	0.9 ± 0.09	8
3A(S2'A)	1.0 ± 0.03*	2.5 ± 0.22	5	45.6 ± 5.2	1.0 ± 0.1	5
3A + 3A(T6'F)	0.9 ± 0.09*	2.3 ± 0.25	5	1900 ± 300**	1.04 ± 0.19	5
3A(L7'T)	2.0 ± 0.05**	2.3 ± 0.09*	6	4.0 ± 0.5**	1.1 ± 0.1	4
3A + 3B	1.0 ± 0.1	1.4 ± 0.26	4	1135 ± 131	1.3 ± 0.13	6
3A + 3B(N6'T)	0.5 ± 0.03**	1.8 ± 0.27	5	213 ± 24**,+	1.0 ± 0.1	5
3A + 3B(N6'S)	0.4 ± 0.01**	1.7 ± 0.04**	5	173 ± 28**,+	0.9 ± 0.12	5
3A + 3B(V7'T)	0.6 ± 0.09**	1.7 ± 0.29	4	866 ± 35	1.27 ± 0.1	4
3A + 3B(N6'T, V7'T)	0.4 ± 0.01**	1.2 ± 0.05**	4	343 ± 45**,+	1.3 ± 0.2	4

Receptor	0’	1’	2’	3’	4’	5’	6’	7’	8’	9’	10’	11'	12'	13'	14'	15’	16'	17'	18'	19'
$GABA_A$ α1	R	T	V	F	G	V	T	T	V	L	T	M	T	T	L	S	I	S	A	R
Glycine α1	R	V	G	L	G	I	T	T	V	L	T	M	T	T	Q	S	S	G	S	R
$5\text{-}HT_{3A}$	R	V	S	F	K	I	T	L	L	L	G	Y	S	V	F	L	I	I	V	S
$5\text{-}HT_{3B}$	R	I	V	F	K	T	N	V	L	V	G	Y	T	V	F	R	V	N	M	S

Figure 1

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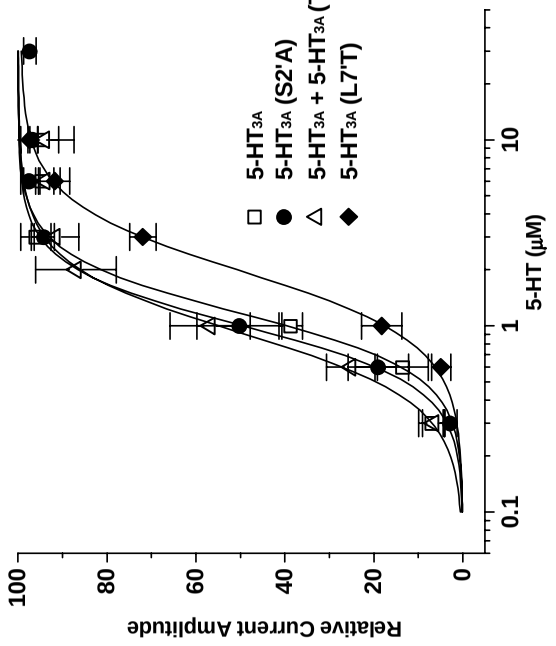

Figure 2

Figure 3

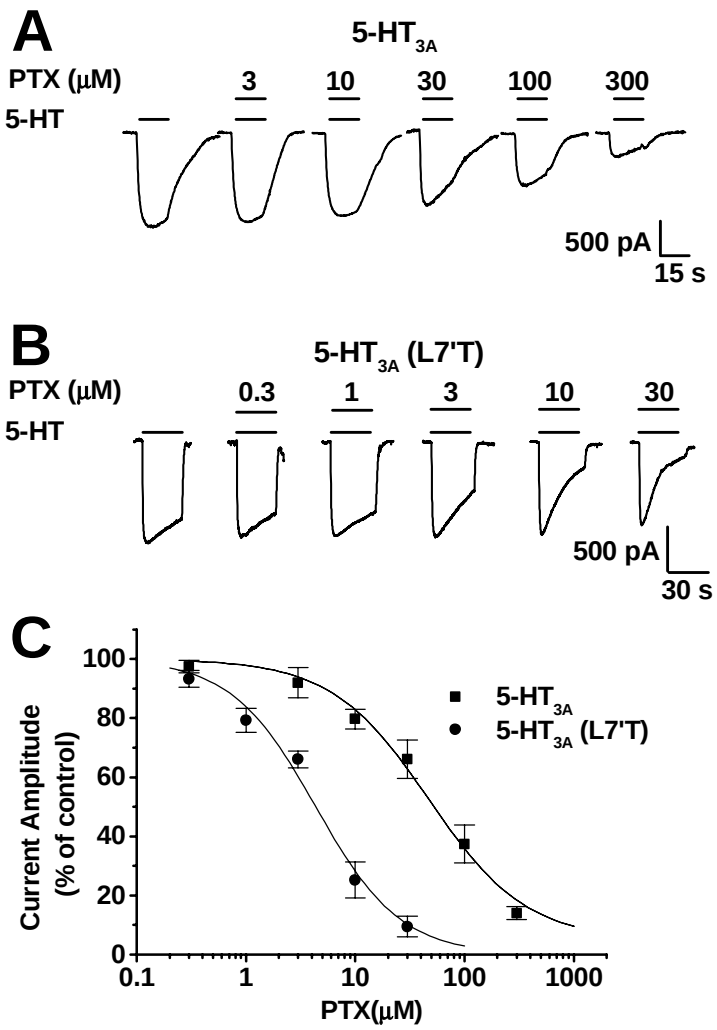

Figure 4

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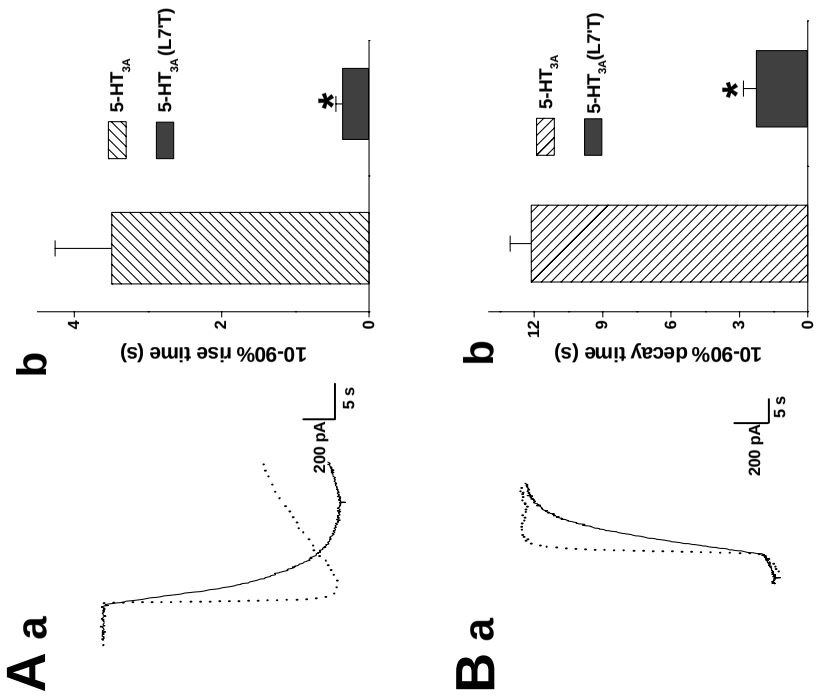

Figure 5

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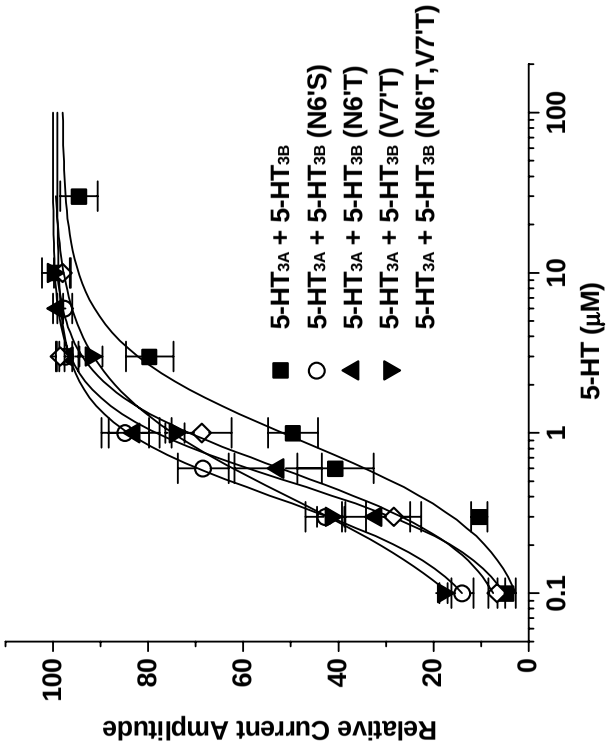

Figure 6

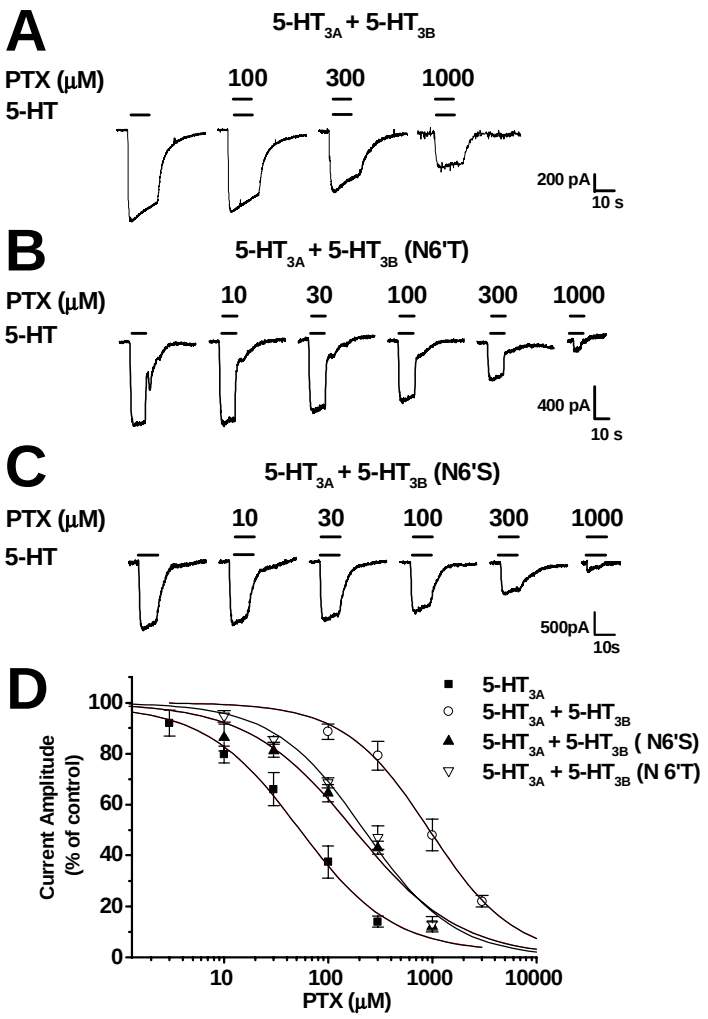

Figure 7

Figure 8

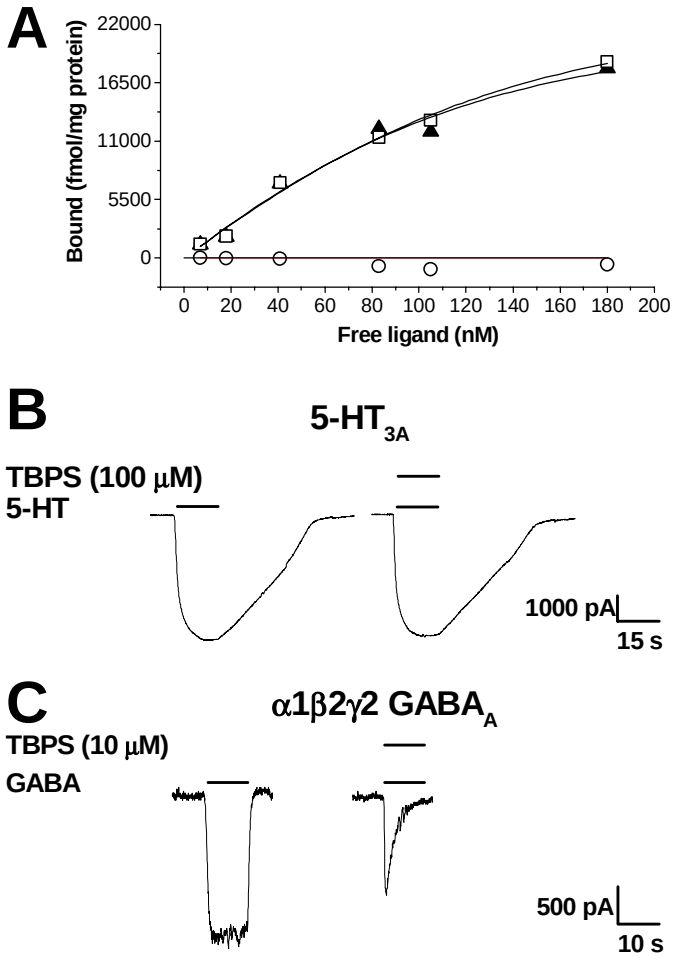

Figure 9