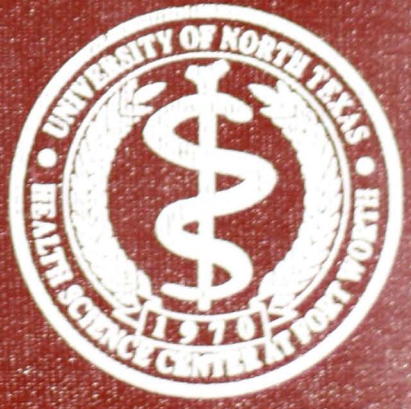
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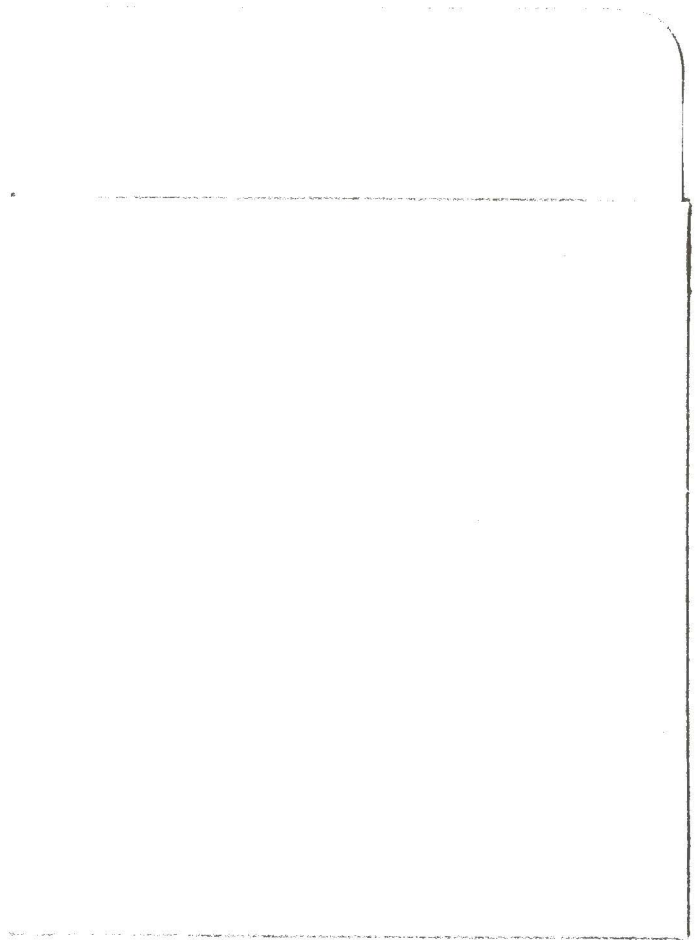

Das, Paromita, Elucidation of mechanism and molecular determinants important in picrotoxin action in the 5-hydroxytryptamine type 3 receptor. Doctor of Philosophy (Pharmacology and Neuroscience), September 2003, pp. 192, 3 tables, 26 illustrations, 67 titles.

The 5-HT_3 receptor belongs to the superfamily of ligand-gated ion channels (LGIC), which mediate fast neurotransmission. Till date, only two subtypes of the receptor i.e. 5-HT_{3A} and 5-HT_{3B} have been investigated. The $GABA_A$ receptor antagonist picrotoxin inhibits other anion-selective members of the LGICs. Whether PTX inhibits the cation-selective 5-HT_3 receptors was previously unknown. Thus, the primary goal of this study was to elucidate the mechanism of action of PTX and identify the amino acids involved in the action of PTX in 5-HT_3 receptors. The overall hypothesis tested was that PTX inhibits the 5-HT_3 receptor by interacting in the ion channel.

PTX-mediated blockade of the 5-HT_{3A} receptors was non-competitive and use-facilitated similar to $GABA_A$ receptors suggesting a conserved site of action of these ligands.

The inhibitory effect of PTX was reduced drastically in heteromeric 5-$HT_{3A/3B}$ receptors, compared to homomeric 5-HT_{3A} receptors. Picrotoxin should prove to be a useful probe for determining the presence of homomeric vs. heteromeric 5-HT_3 receptors in native tissue and recombinant receptor preparations.

In anion-selective ion channels, the 2', 3' and 6' residues in cytoplasmic aspect of TM2 are known to modulate PTX sensitivity. While mutation of 2' and 3' residues in 5-

dramatic loss of sensitivity to PTX in 5-HT_{3A} receptors. A converse mutation at 6' residue in the 5-HT_{3B} subunit caused gain of sensitivity to PTX, suggesting that 6' is a key determinant of PTX sensitivity. A novel finding was the involvement of 7' residue in increasing PTX sensitivity in 5-HT_{3A} but not the 5-HT_{3B} subunit. The lack of specific binding by radioligand [^{3}H]EBOB in 5-HT_{3A} receptors suggested that the site of action of convulsants may be different from that in the anion-selective receptors. The overall results suggest that PTX interacts in the ion channel in the 5-HT_3 receptors but also underscores the complexity of its interaction with LGICs.

ELUCIDATION OF MECHANISM AND MOLECULAR DETERMINANTS IMPORTANT IN PICROTOXIN ACTION IN THE 5-HYDROXYTRYPTAMINE TYPE 3 RECEPTOR

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DISSERTATION

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for the Degree of

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TABLE OF CONTENTS

LIST OF TABLES

TABLE **PAGE**

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

[^{3}H]DHP	[^{3}H]-dihydropicrotoxinin
[^{3}H]EBOB	[^{3}H]-ethynylbicycloorthobenzoate
5-HT_{3A}	5-Hydroxytryptamine type 3A receptor
5-HT_{3B}	5-Hydroxytryptamine type 3B receptor
m-CPBG	meta-chloro-phenylbiguanide
MTSET	2-trimethylammonioethylmethane thiosulfonate
MTSEA	2-aminoethylmethane thiosulfonate
PTX	Picrotoxin
SCAM	Substituted cysteine accessibility method
TBPS	tert-butylbicyclophosphorothionate
TBOB	tert-butylbicycloorthobenzoate
[^{35}S]TBPS	[^{35}S] tert-butylbicyclophosphorothionate
[^{3}H]TBOB	[^{3}H]tert-butylbicycloorthobenzoate

CHAPTER I

INTRODUCTION

The Serotonin 5-HT_3 Receptor - General History

Serotonin, a monoamine neurotransmitter in the central and peripheral nervous systems, was discovered more than fifty years ago and is known to be involved in the modulation of numerous physiological functions (Rapport et al., 1947). The multiple actions of 5-HT like feeding, learning, sexual behavior, sleep, nociception (Gingrich, 2002; Kroeze et al., 2002), are mediated through a variety of serotonin receptors (termed 5-HT_1-5-HT_7), most of which are G-protein coupled receptors (Hoyer and Martin, 1997; Hoyer et al., 2002). In contrast to the other serotonin receptors which mediate slow responses via second messenger signaling systems, the 5-HT_3 receptor, initially called the M receptor (due to blockade by morphine) (Gaddum and Picarelli, 1957), is the only serotonin receptor which is a ligand-gated ion channel (LGIC) and mediates fast synaptic transmission (Derkach et al., 1989). LGICs are complex receptor proteins to which neurotransmitters bind and result in opening of an integral ion channel. Other members of the LGIC superfamily include the $GABA_A$, glycine, the invertebrate glutamate-gated chloride channel (Glu-R) and the nicotinic acetylcholine receptor (nAChR) (Karlin, 2002). While the $GABA_A$ and glycine receptors are largely permeable to anions (specifically chloride), the 5-HT_3 receptor like the nAChR is a cation-selective

ion channel. The receptor is relatively non-selective with respect to monovalent ions as Na^+ and K^+ have equivalent permeability through the channel (Yakel et al., 1990). Activation of the receptor due to agonist binding results in membrane depolarization and subsequent desensitization (Barnes and Sharp, 1999; Jackson and Yakel, 1995).

The 5-HT_{3A} receptor was first cloned in 1991 from NCB-20 cells, which is a hybrid cell line of mouse neuroblastoma and chinese hamster brain cells (Maricq et al., 1991). The 5-HT_{3A} receptor shares notable sequence homology and structural similarity with other members of the LGICs. Like others members of the LGIC superfamily the receptor is believed to be formed from the assembly of five subunits, which align pseudosymmetrically around the pore (Boess et al., 1995, Fig.1, page 27). Each subunit consists of a large N-terminal region and four putative transmembrane domains designated TM1-TM4 (review by Reeves and Lummis, 2002, Fig.2, page 29). Experimental data obtained from studies in different members of the LGIC superfamily suggest that the agonist-recognition site is located in the N-terminal domain (Ortells and Lunt, 1995; Corringer et al., 2000; Yan et al., 1999) while the TM2 region lines the ion channel pore (Akabas et al., 1994; Panicker et al., 2001; Galzi et al., 1992; Wilson and Karlin, 2001; Yakel et al., 1993). Binding of the agonist to the N-terminal domain results in a conformational change, which is transducted to the pore domain, and results in opening of the ion channel, a process termed "gating".

In the LGIC superfamily, multiple subunits usually exist for each type of receptor, which contribute to functional diversity. Early pharmacological and functional data obtained from experiments in native tissues had suggested the existence of multiple

5-HT_3 receptor subtypes within a single species (Bonhaus et al., 1993; Peters et al., 1992). In addition, there appeared to be pharmacological heterogeneity of 5-HT_3 receptors amongst species (Newberry et al., 1991; Wong et al., 1993). Although the cloned 5-HT_{3A} subunit can form functional homomers in heterologous systems, many of the biophysical properties exhibited by this receptor appear to be distinct from those observed in native tissue (Hussy et al., 1994). For instance, single-channel recordings attempted in heterologously expressed 5-HT_{3A} receptor and those found in neuroblastoma cells, show negligible conductance (sub-picosiemens) in contrast to resolvable recordings obtained from neuronal 5-HT_3 receptors (9-17 picosiemens) (Lambert et al., 1989; Yang, 1990; Yang et al., 1992; Hussy et al., 1994). In contrast to neuronal 5-HT_3 receptors, which exhibit very modest inward rectification (Yang et al., 1992; Hussy et al., 1994), 5-HT_{3A} receptors expressed in recombinant systems exhibit a pronounced inward rectification (Maricq et al., 1991; Davies et al., 1999). Moreover, recombinant 5-HT_{3A} receptors are permeable to divalent cations like Ca^{+2} (Hargreaves et al., 1994) and Mg^{+2} (Maricq et al., 1991; Brown et al., 1998) whereas some neuronal 5-HT_3 receptors (those expressed in rodent ganglia) display very little permeability to Ca^{+2} (Robertson and Bevan, 1991; Yang et al., 1992). Thus, the unexplained differences in functional properties observed in native and recombinant systems were thought to be due to the existence of subtypes of the 5-HT_3 receptor (Hargreaves et al., 1994; review by Fletcher and Barnes, 1998). Unlike other members of the LGICs, the cloning of other subunits did not follow cloning of the 5-HT_{3A} receptor. However, splice variants of the mouse, rat and guinea pig 5-HT_{3A} receptor were subsequently cloned (lacking six, five and six amino

acids respectively in the putative TM3-TM4. loop) (Hope et al., 1993; Miquel et al., 1995; Lankiewicz et al., 1998) but no significant functional and pharmacological differences were observed in these variants (Downie et al., 1994; Werner at al., 1994).

Nearly a decade later, a second 5-HT_3 receptor subunit type known as the 5-HT_{3B} subunit, was cloned from human fetal kidney (Davies et al., 1999). This new class of receptor subunit was found to be non-functional in recombinant systems when expressed alone but could efficiently co-assemble with the 5-HT_{3A} subunit and form functional channels. Interestingly, co-expression of the two subunits produced receptors, which mimicked some of the properties of the neuronal 5-HT_3 receptors. For instance, heteromeric receptors exhibited a robust single-channel conductance (~16 picosiemens), negligible permeability to Ca^{+2} and a linear current-voltage relationship (lack of voltage dependence) similar to that observed in native tissue (Davies et al., 1999; Dubin et al., 1999). Heteromeric receptors also exhibited distinct pharmacological differences compared to the homomeric 5-HT_{3A} receptors. For example, recordings from *Xenopus* oocytes injected with 5-HT_{3A} or 5-HT_{3A} and 5-HT_{3B} cRNA showed that the apparent affinity for agonists like 5-HT and 2-methyl 5-HT were reduced in heteromeric receptors as compared to homomeric receptors (Davies et al., 1999). In contrast, the agonists meta-chlorophenylbiguanide (m-CPBG) and 1-Phenylbiguanide exhibited a higher affinity for 5-$HT_{3A/3B}$ heteromeric receptors as compared to 5-HT_{3A} homomers (Dubin et al., 1999). 5-HT_3 receptor selective antagonists like ondansetron and tropisetron did not display differential sensitivities in either receptor configuration. However, the nAChR antagonist, tubocurarine which is also a potent antagonist of the 5-HT_{3A} receptor was found to be less

potent (~2-5 fold) in human and rat heteromeric receptors (Davies et al., 1999; Hanna et al., 2000). The 5-HT_{3B} subunit thus confers distinct functional and pharmacological properties upon co-expression with the 5-HT_{3A} receptor. It was suggested that the discrepancies observed in recordings from neuronal 5-HT_3 receptors and recombinant systems could perhaps be explained in part due to the presence of homomeric and heteromeric receptors in the same neuron. Indeed, in rat dorsal root ganglia, transcripts of both 5-HT_{3A} and 5-$HT_{3A/3B}$ receptors were seen (Morales et al., 2001). Also, electrophysiological recordings from neuronal cells have shown the presence of both low and high conductance 5-HT_3 receptors suggesting a heterogeneous population of receptors (Hussy et al., 1994; Yang et al., 1992). Whereas transcripts of the 5-HT_{3B} subunit have been observed in cell lines like the N1E-115, NCB-20 and NG108-15 (Hanna et al., 2000) and from different brain regions (Davies et al., 1999; Dubin et al., 1999), recent studies indicate the 5-HT_{3B} subunit may not be expressed in central nervous system (van Hooft and Yakel, 2003). Morales and Wang (2002) did not find transcripts of the 5-HT_{3B} subunit in rat CNS but instead found that this subunit was exclusively present in neurons of the PNS. This subunit was also not detectable in rat hippocampal and neocortical interneurons (Ferezou et al., 2002; Sudweeks et al., 2002). Moreover, the presence of transcripts of 5-HT_{3B} may not necessarily be an indicator for the presence of functional heteromeric receptor. Stewart et al, (2003) demonstrated that although the neuroblastoma cell line NB41A3 had detectable levels of 5-HT_{3B} mRNA, the functional properties exhibited were like the 5-HT_{3A} receptors and upon transfection of the 5-HT_{3B} cDNA into these cells, the properties displayed, resembled those of heteromeric

receptors. Thus the molecular makeup of 5-HT_3 receptors in the CNS is currently unknown. The distinct properties of native 5-HT_3 receptors cannot be entirely accounted for by the known 5-HT_3 receptors and it is very likely that other 5-HT_3 receptor subunits exist. In fact, a human 5-HT_{3C} receptor subunit, which shares 39% sequence identity with the 5-HT_{3A} subunit, has recently been patented (review by Reeves and Lummis, 2002). Another group has recently reported the identification and cloning of novel 5-HT_3 receptor-like subunits termed 5-HT_{3D} and 5-HT_{3E} (Niesler et al., 2003). The functional characteristics of these receptors are not known at the present time.

It is evident that the 5-HT_3 receptor system is complex and there is a lack of information regarding the composition of this receptor in the CNS. Moreover, insight into some of the very fundamental questions like the mechanism of agonist recognition, the process of transduction of signal and gating, the molecular determinants of ion selectivity and permeation, the structure of the transmembrane domains etc. for this receptor is limited.

Distribution of 5-HT_3 receptors in the body

5-HT_3 receptors are found in neurons of the central and peripheral nervous system as well as in neuronally derived cell lines like NCB-20, N1E-115 and NG108-15 (Bradley et al., 1986). Expression of the 5-HT_{3A} receptor is ubiquitous in the central nervous system although the levels are low. In the forebrain, the receptors are expressed in the cerebral cortex, hippocampus and amygdala (Barnes et al., 1990; Kilpatrick et al., 1987; Tecott et al., 1993). Although no mRNA expression was detected in areas of the hindbrain i.e in area postrema, nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve, radioligand binding indicated the presence of 5-HT_3 receptors (Waeber et al., 1988; Kilpatrick et al., 1989; Parker et al., 1996(a)). Transcripts can also be located in the medulla oblongata, spinal cord, nucleus accumbens, striatum and substantia nigra (Tecott et al., 1993; Fonseca et al., 2001). The 5-HT_{3A} receptors are also detectable in many regions of the peripheral nervous system, which includes the sympathetic and parasympathetic ganglia and the myenteric plexus of the enteric nervous system (Johnson and Heinemann, 1995(a); Johnson and Heinemann, 1995(b)). Transcripts of the 5-HT_{3A} receptor have been found in the non-neuronal tissue like heart and retina (Maricq et al., 1991). Transcripts of the 5-HT_{3B} receptor have been found in the cerebral cortex, amygdala, hippocampus and testis. (Dubin et al., 1999) and in rabbit retina (Pootanakit and Brunken, 2001). Recently, expression analysis of the novel 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} genes, showed that expression of 5-HT_{3D} and 5-HT_{3E} was restricted to a few tissues (5-HT_{3D} expressed in adult kidney, colon and liver; 5-HT_{3E}

expressed in adult colon and intestine), whereas that of 5-HT_{3C} was more widespread (in adult brain, colon, intestine, lung, muscle and stomach) (Niesler et al., 2003).

Primary, Secondary, Tertiary and Quaternary Structure of the 5-HT_3 Receptor

Primary Structure: The 5-HT_{3A} subunit protein is made up of 487 amino acids and when compared to other members of the ligand-gated ion channels shows sequence similarity and shares the highest degree of homology (~30 %) with the $\alpha 7$ subunit of the neuronal nicotinic acetylcholine receptor. Hydrophobicity analysis indicated that the protein has four transmembrane domains (TM1 through TM4), a large N-terminal extracellular region containing a 23 amino acid long signal sequence and the characteristic cys-cys residues that are spaced 14 residues apart, a long cytoplasmic loop between TM3 and TM4 and an extracellular C-terminal region (Maricq et al.,1991). Experimental data derived from studies in other LGICs, suggested that the neurotransmitter-binding site was contained in the extracellular N-terminal region. Evidence that this was true for the 5-HT_3 receptor, came from the construction of a chimeric protein consisting of N-terminal region of the $\alpha 7$ nicotinic acetylcholine receptor linked to the C-terminal of the 5-HT_{3A} receptor. The resulting chimeric protein could bind nicotinic ligands but had channel properties of the 5-HT_{3A} receptor (Eiselle et al., 1993). Biochemical and site-directed mutagenesis studies in the nAChR have suggested that the ligand-binding site is located in a cleft at the interface between two subunits. A series of loops (termed loops A, B and C) from one of these subunits, forms the principle binding-site (Galzi et al., 1990; Dennis et al., 1988; Middleton and Cohen,

1988) and the loops D, E and F from the adjacent subunit, forms the complementary binding site (Corringer et al., 1995; Sine et al., 1995; Czajkowski et al., 1993) (Fig. 3, page 31). Mutational studies from other members of the LGICs, including the 5-HT_{3A} receptor, also suggest that the amino acid residues, which contribute to formation of the ligand-binding pocket, are largely aromatic amino acids like tyrosine and tryptophan (Schmieden et al., 1993; Vandenberg et al., 1992; Boess et al., 1997; Spier and Lummis, 2000). In particular, Trp183 in the 5-HT_{3A} receptor is critical residue involved in ligand binding as evidenced by the interaction of aromatic core of this residue with primary amine of the agonist serotonin (Beene et al., 2002). The recent discovery of a crystal structure of an acetylcholine binding protein (AChBP) has enabled the study of the ligand-binding domain in greater detail. AChBP, a glial protein found in the synaptic cleft of a snail, is a homopentamer lacking the transmembrane and intracellular domains of LGICs but is homologous to the ligand-binding domain of nAChR (Brejc et al., 2001). The crystal structure confirms the critical role of aromatic residues, including Trp143 (which is in a homologous position to Trp183 in 5-HT_{3A} receptor) in formation of the binding pocket.

The gene coding for the 5-HT_{3B} subunit resides in the q-arm of chromosome 11, has nine exons which code for 441 amino acids. The mouse and rat 5-HT_{3B} subunits (Hanna et al., 2000) exhibit ~40% sequence identity with rodent 5-HT_{3A} receptor, ~ 73% amino acid identity with the human 5-HT_{3B} receptor and 95% sequence identity with each other. Hydropathy analysis of the human 5-HT_{3B} receptor predicted the presence of four transmembrane domains and the cys-sys loop in the N-terminal region of the protein.

Even though the 5-HT_{3B} subunit possesses some of the conserved sequences that are involved in ligand binding, the protein itself is not targeted to the membrane (Dubin et al., 1999). Instead, it is retained in the endoplasmic reticulum and does so due to its inability to oligomerize (Boyd et al., 2002; Boyd et al., 2003). Thus, 5-HT_{3B} subunit by itself cannot form functional channels when expressed in heterologous systems but can form a heteromeric functional channel in conjunction with the 5-HT_{3A} subunit. The genes encoding the recently cloned putative 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} subunits are located on the long arm of chromosome 27 and share important features with the 5-HT_{3A} receptor namely four transmembrane domains and a large intracellular loop. While the putative 5-HT_{3C} and 5-HT_{3E} subunits have a large N-terminal region containing the cys-cys loop, the 5-HT_{3D} subunit lacks the signal sequence and the N-terminal agonist recognition site (Niesler et al., 2003).

Secondary Structure: Direct secondary structure measurement of 5-HT_3 receptor is lacking. Alanine scanning mutagenesis of the N-terminal region predicts that the structure of the protein in this domain is predominantly a β sheet structure (Yan et al., 1999). Cysteine substituted mutagenesis suggests that the TM2, which forms the pore of the channel, may be largely α helical although the helical structure is not conserved throughout the TM2 (Panicker et al., 2002; Reeves et al., 2002). The secondary structures of the other TMs are not unequivocally deciphered. The deduced overall secondary structure of 5-HT_{3A} receptor from circular dichroism studies indicates a larger α helical content and a lesser contribution by the β-strand (Hovius et al., 1998).

Tertiary and Quaternary Structure: Electron microscopy analysis of homomeric 5-HT_{3A} receptor particles revealed a pentameric structure suggesting that the quaternary structure of the protein may be similar to other members of the LGICs (Boess et al., 1995). Detailed high-resolution structural analysis of the protein is limited due to the unavailability of a crystal structure. The quaternary structures of heteromeric receptors and other putative 5-HT_3 subunits are unknown at this time.

5-HT_3 Receptor Selective Agonists and Antagonists

Drugs like 2-methyl 5-HT, meta-chloro phenyl biguanide (mCPBG) and phenyl biguanide (PBG) are selective agonists of the 5-HT_3 receptor and interact at the ligand-binding site (Boess et al., 1992; Mochizuki et al., 1999; Niemeyer and Lummis, 1998). Selective antagonists of the 5-HT_{3A} receptor include ondansetron, granisetron, tropisetron, ramosetron (Endo et al., 1999). The agonists also interact at the heteromeric receptors with equal efficacy although the potency is moderately altered .The antagonists however, do not appear to select between the homomeric and heteromeric receptors (Dubin et al., 1999).

Allosteric Modulators of 5-HT_3 Receptor

A diverse array of pharmaceutical agents is known to modulate 5-HT_3 receptor function. Aside from their primary action, they possess either agonist or antagonist action on the receptor. In addition, the receptor is also modulated by divalent cations. The inhibitory role of calcium and to a lesser extent magnesium is well documented. While some studies in neuronal cell lines (mouse N18 neuroblastoma) have shown Ca^{+2} permeability in 5-HT_3 receptors (Yang, 1990; Yang et al., 1992), others indicate that the 5-HT_3 receptor (in mouse N1E-115 neuroblastoma) has negligible permeability to Ca^{+2} (Peters et al., 1988). However, regardless of this, extracellular Ca^{+2} has been reported to inhibit the receptor in all cases (Peters et al., 1988; Yang 1990; Yakel et al., 1993). Though the exact mechanism of inhibitory action is not known, it is thought that Ca^{+2} may interact at multiple sites in the receptor, which include the pore

and the extracellular ligand-binding domain (Brown et al., 1998; Niemeyer and Lummis, 2001). Other divalent cations like Mg^{+2}, Cd^{+2} and Cu^{+2} also inhibit the receptor (Lovinger, 1991; Gill et al., 1995, Niemeyer and Lummis, 2001).

A number of research groups have demonstrated the enhancement of 5-HT_3 receptor function by ethanol and related alcohols (Barann et al., 1995; Lovinger et al.,2000; Machu and Harris, 1994; Parker et al., 1996). At pharmacologically relevant concentrations, ethanol and lower alcohols potentiate 5-HT-mediated currents while larger alcohols inhibit the receptor. The site(s) of alcohol action in the receptor is not defined. Local and general anesthetics also modulate the 5-HT_{3A} receptor. Barann et al. (2000) demonstrated that 5-HT_{3A} receptor from mouse neuroblastoma cells (N1E-115) was a sensitive target for the general anesthetic propofol. Pentobarbital also inhibited 5-HT induced currents in N1E-115 cells (Barann et al., 1997). Volatile anesthetics like halothane and isoflurane can potentiate 5-HT_3 receptor-mediated currents (Machu and Harris, 1994; Zhang et al., 1997) while local anesthetics like procaine, cocaine and lidocaine inhibit the receptor (Barann et al., 1993; Fan et al., 1994).

A variety of other compounds modulating the 5-HT_3 receptor function include the phenothiazine, chlorpromazine, which presumably inhibits the receptor by binding to the ligand-binding site (Sepulveda et al., 1994; Lummis and Baker, 1997), gonadal steroids like 17-β estradiol and progesterone which are non-competitive antagonists (Wetzel et al., 1998) and neuromuscular blockers like d-tubocurarine, atracurium, mivacurium, vecuronium, which inhibit the receptor at low micromolar concentrations (Peters et al.,1990; Min et al., 2000).

5-HT_3 Receptors- Therapeutic Aspects

The first successful therapeutic application of 5-HT_3 receptor selective antagonists was the use of ondansetron in prevention of chemotherapy-induced nausea and vomiting (Currow et al., 1997). The severity of emesis as a result of chemotherapy in cancer patients can lead to discontinuation of therapy. Thus, 5-HT_3 antagonists like granisetron, zatosetron, ramosetron, tropisetron have found a place as effective anti-emetics. The mechanism of action of these drugs may be due to blockade of 5-HT_3 receptors located in gastrointestinal tract and the chemoreceptor trigger zone in area postrema of brain (Costall and Naylor, 1992; Tyers and Freeman, 1992). 5-HT_3 antagonists are also useful in treating irritable bowel syndrome (IBS), a complex disorder, characterized by recurrent abdominal pain and abnormal bowel activity (Berrada et al., 2003). Alosetron, in particular, was found to be effective in treating IBS in women (Camilleri et al., 1999; Jones et al., 1999) but was recently removed from market due to adverse side effects (reviews by Callahan, 2002; Spiller, 2002). Although 5-HT_3 receptor antagonists are predicted to be useful in numerous clinical conditions, currently they are approved only for use as anti-emetic agents. Studies in animal models and human subjects have suggested that 5-HT_3 antagonists may be potentially useful in treatment of alcohol and drug dependence, as analgesics, in neuropsychiatric disorders like anxiety, depression and psychoses (Greenshaw and Silverstone, 1997). Recent preliminary clinical data in patients exhibiting generalized anxiety disorder showed that zatosetron improved anxiety symptoms as compared to placebo although statistical significance was not observed (Smith et al., 1999). Treatment with ondansetron, of a

small number of schizophrenic patients, showed an improvement in positive and negative symptoms and also reduction in severity of tardive dyskinesia, a symptom associated with neuroleptic therapy. These effects were statistically significant (Sirota et al., 2000). Randomized placebo-controlled studies in alcohol-dependent males treated with ondansetron showed a clinically meaningful reduction in drinking (Sellers et al., 1994). 5-HT_3 antagonists may be also useful as analgesics in management of pain in migraine and fibromyalgia (review by Spath, 2002; Wolf, 2000). In patients suffering from primary fibromyalgia, short-term treatment with tropisetron showed a significant reduction in pain score compared to placebo group (Farber et al., 2001). Thus a growing body of evidence suggests a promising role for antagonists of 5-HT_3 receptor in management and treatment of various clinical disease states.

As described earlier, the 5-HT_3 receptor is a member of the diverse LGIC superfamily. Much of the information on LGICs known today has been obtained from studies in the nAChR receptors. A significant contribution to this field has also been made from studies in other members of the LGICs, particularly the $GABA_A$ receptors. These receptors are the major inhibitory neurotransmitter receptors in the mammalian brain, ubiquitous in distribution, and are important in regulating neuronal excitability (review by Sivilotti and Nistri, 1991). Moreover, they are specific targets for several psychoactive drugs including the anxiolytic benzodiazepines and barbiturates (review by Hevers and Luddens, 1998).

Anion-Selective LGICs-Interaction with Convulsant Drugs

A diverse array of compounds modulates $GABA_A$ receptors. Drugs like benzodiazepines and barbiturates that enhance $GABA_A$ receptor function and hence inhibit neuronal excitability, are used for sedation, to treat anxiety disorders (MacDonald and Barker, 1978; review by Sieghart, 1995; Olsen et al., 1981) and epilepsy (Bradford, 1995; Frazer, 1996). Agents that inhibit $GABA_A$ receptors are epileptogenic and include the allosteric antagonists picrotoxin, TBPT (t-butylbicyclophosphorothionate, also known as TBPS), TBOB (t-butylbicycloortho-benzoate (also called bicyclophosphate-like cage convulsants), pentylenetetrazole (PTZ) and the insecticides dieldrin and lindane (review by Hever and Luddens, 1998 and references cited therein). Despite the advent of advanced molecular biology techniques, some of these agents like picrotoxin, which inhibit all members of the anionic LGICs, have been useful as tools to investigate regions in the protein important for gating and function. Picrotoxin was initially isolated from a poisonous plant belonging to the family *Menispermaceae* (Porter, 1967). It inhibits chloride flux in the GABA (Takeuchi and Takeuchi, 1969; Constanti, 1978; Barker et al., 1983), glycine (Schmieden et al. 1989) and the glutamate-gated chloride channels (Etter et al., 1999). The pharmacology of PTX is complex and despite decades of research, the exact site where PTX binds in these receptors is not known. PTX and related depressant compounds are believed to bind to the same site or overlapping sites within the chloride channel complex.

Radioligand Binding Studies

In the early 1980's radioligand binding studies were used extensively to characterize the convulsant site(s). Although PTX was demonstrated to be a GABA antagonist in electrophysiological studies, binding studies with [^{3}H]GABA indicated that PTX did not interact with the GABA recognition site (Enna et al., 1977; Olsen et al., 1978). A radiolabeled analog of PTX known as [^{3}H]dihydropicrotoxinin ([^{3}H]DHP) was then synthesized to characterize the PTX binding-site (Ticku et al., 1978). Due to the low specific binding of this compound, other radioligands like [^{35}S]TBPS (Squires et al., 1983; Lawrence and Casida, 1983) and [^{3}H]TBOB (Lawrence et al., 1985) were used to characterize this site. A large number of structurally unrelated compounds like PTZ, PTX-like convulsants, bicyclophosphate-like cage convulsants, barbiturates, non-benzodiazepines depressants like etazolate were found to displace specific [^{3}S]TBPS binding. While the convulsant-site was thought to be distinct from the GABA recognition-site and the benzodiazepine-site (Ticku, 1983; Ticku and Ramanjaneyulu, 1984), from a number of studies it appeared that the PTX-site in the $GABA_A$ receptor complex was a modulatory site to which various components of the receptor were allosterically coupled. For example, pentobarbital and etazolate which inhibited [^{3}H]DHP and [^{35}S]TBPS binding (Ramanjaneyulu and Ticku, 1984) were found to enhance [^{3}H]benzodiazepine and [^{3}H]GABA binding (Olsen, 1981). This enhancement could be blocked by drugs binding to the [^{3}H]DHP/ [^{35}S]TBPS-site like PTX and related convulsants and by bicuculline, which acts at the GABA recognition-site. These studies

supported the notion that ligands, which bind to different sites on the GABA receptor complex, modulated the binding of ligands to other sites.

A disadvantage of [^{35}S]TBPS is the short half-life of these ligands and hence more recently, high-affinity radiolabeled drugs like [^{3}H] TBOB and its structurally related compound [^{3}H]4'-ethynyl-4-n-[2,3-$^{3}H_2$] propylbicycloorthobenzoate ([^{3}H]EBOB) have been used (Casida, 1993; Huang and Casida, 1996). Homogenate-binding studies with [^{3}H]EBOB have indicated that this ligand has identical binding sites as [^{35}S]TBPS. However autoradiographic studies revealed a discrepancy in the regional distribution of [^{3}H] EBOB binding as compared to [^{35}S]TBPS binding and it was suggested that bicyclophosphates like EBOB/TBOB interacted at a distinct site from TBPS binding site. By extension, it was hypothesized that multiple binding sites for PTX existed in the receptor (Edgar and Schwartz, 1990; Kume and Albin, 1994).

Electrophysiological Studies

Early electrophysiological studies had determined the type of inhibition produced by PTX to be non-competitive (Takeuchi and Takeuchi, 1969; Simmonds, 1982). Non-competitive inhibition can be produced as a result of different mechanisms like simple open-channel block, complex channel block or enhancement of desensitization. Inoue and Akaike (1988) suggested that PTX interacts within the ion channel and acts as an open-channel blocker. However, in a study by Newland and Cull-Candy (1992), whole-cell GABA current-voltage relationships indicated that PTX did not produce a voltage-dependent block thus arguing against an open-channel blocking

mechanism (open-channel blockers are typically voltage dependent). But the block was also "use-dependent' i.e. onset of block and recovery was faster in presence of GABA, which is typically a characteristic of open channel blockers. The authors proposed that such a block could be due to a complex channel blocking mechanism wherein PTX stabilized an agonist-bound closed state of the receptor. Single channel studies of blockade produced by PTX conducted in invertebrate tissue, also have suggested that PTX may inhibit the receptor non-competitively by stabilizing the shut-state of the receptor (Franke et al., 1986; Adelsberger et al., 1998). Yoon et al (1993) also argued against an open-channel blocking mechanism and instead proposed two different mechanisms of block *i)* an use-dependent mechanism wherein the block produced was due to binding at a use-dependent site which is outside the ion-channel but closely coupled to the GABA recognition-site *ii)* an use-independent site which is distant from the GABA recognition-site. Thus, the mechanism of block produced by PTX is still unequivocal.

With the advent of advanced molecular biology techniques it became possible to study ligand-receptor interactions at the molecular level. In particular, the TM2 region, which is largely conserved amongst the members of LGIC superfamily and believed to form the channel pore, became the focus of several studies. Several lines of evidence indicated that the TM2 is an important region for inhibitory action of PTX. Pribilla et al (1992) found that only glycine receptor α subunits were sensitive to PTX whereas incorporation of a β subunit rendered the receptors resistant to PTX. Chimeric

studies suggested that the TM2 region of the β subunit was responsible for this effect. Based on their data, the authors suggested that the binding site for PTX could be within the ion-channel. The most convincing evidence came from the studies of Reddy et al, (1993) who demonstrated that insertion of a synthetic peptide into lipid bilayer with a sequence similar to TM2 of a subunit of glycine receptor led to the formation of channels. These channels which were spontaneously open, exhibited properties similar to native glycine receptors and could be blocked by PTX.

Site-Directed Mutagenesis Studies

Site-directed mutagenesis studies have enabled the identification of amino acid residues, which may be involved in PTX interaction with the receptor. Fig. 4, (page 33), shows the map of TM2 domain in different members of the LGICs (the Miller numbering method (Miller, 1989) is a generally accepted method of numbering residues in the TM2 domain, which assigns 1' to the most intracellular residue and 20' to the most extracellular residue in the TM2). Most notably, mutation of amino acid residues at the 2' and 6' positions in the cytoplasmic aspect of the ion channel have been found to have dramatic effects in neurotoxin interaction. ffrench-Constant et al., (1993)(b) conducted studies on mutated *Drosophila* $GABA_A$ receptors, which had 2' alanine, substituted for by serine. These natural mutants, which were resistant to the insecticide dieldrin, (Rdl mutants-resistance to dieldrin) were also resistant to picrotoxin. The authors proposed that the 2' residue formed at least part of the binding site for PTX. Studies in the $GABA_C$ receptor, which is predominantly found in mammalian retina, has also shown that the 2'

residue is important for PTX sensitivity. $GABA_C$ receptors are formed from either *ρ1* or *ρ2* subunits and receptors formed from *ρ2* subunits are more sensitive to PTX than those formed from *ρ1* subunits. When the 2' proline from *ρ1* was substituted with serine (the corresponding residue in *ρ2*), it enhanced the PTX sensitivity of the mutant *ρ1* receptors to match that of *ρ2* receptors. The authors proposed that this single amino acid was a critical determinant of PTX sensitivity. Moreover, mutation of the 2' proline to valine, alanine, serine or glycine (the corresponding residues found in various subunits of $GABA_A$ and glycine receptor) increased PTX sensitivity (Wang et al., 1995). Based on the premise that PTX binds in the ion channel, Xu et al (1995) used substituted cysteine accessibility method (SCAM) to determine channel-lining residues that would be protected from modification with sulfhydryl reagents by PTX. They found that mutation of valine to cysteine in the rat α1 (valine aligns with the Rdl equivalent site on the mutant Drosophila $GABA_A$ receptors) and subsequent treatment with sulfhydryl reagents resulted in PTX protection of the 2' residue suggesting its role in PTX interaction. Studies in invertebrate glutamate-gated chloride channel (GluR) also show similar results. GluR receptors are either homomers of *α* subunits or *β* subunits or heteromers of *αβ* subunits. The heteromeric GluR*αβ* are several thousand-fold less sensitive to PTX than homomeric receptors. Mutation of the 2' alanine (which exists in *β* subunits) to threonine (which exists in *α* subunits) in GluR*β* receptors conferred ~ 10,000-fold resistance to PTX (Etter et al., 1999).

Another residue that appears to play an important role in the PTX inhibition of the $GABA_A$ receptor is the 6' residue. The critical role of 6' residue in the TMII is

evident from the studies of Gurley et al, (1995) who showed that resistance to PTX is conferred if the wildtype 6' threonine residue is mutated to phenylalanine in the β subunit of the $\alpha 1\beta 2\gamma 2$ $GABA_A$ receptors. In the $GABA_C$ receptors, co expression of $\rho 1$ and $\rho 2$ subunits produce receptors insensitive to PTX. Substituting the 6' methionine residue in $\rho 2$ subunit with threonine (the corresponding residue in $\rho 1$) produces heteromeric receptors which gain sensitivity to PTX (Zhang et al., 1995). A recent study by Shan et al, (2001) in glycine $\alpha\beta$ heteromeric receptors also showed that the 6' phenylalanine to threonine mutation in β subunit conferred increased PTX sensitivity to the PTX-resistant heteromeric receptors. Based on their results and comparison with data obtained from other groups, they proposed that a ring of threonine residues at the 6' position was essential and a common requirement for PTX sensitivity amongst members of the LGICs. Moreover, they suggested that if the site of action of PTX is identical in all members of the LGICs, then it is likely to be formed by the threonine residues in the 6' position. From molecular modeling studies, Zhorov et al, (2000) hypothesized that threonine at the 6' position contributed to hydrogen bonding with the hydrophillic domain of PTX (Fig.5, page 36). Thus, incorporation of phenylalanine, which disrupts hydrogen bond interaction with PTX, would prevent it from docking.

While some of the above studies are suggestive of a binding site for PTX in the cytoplasmic aspect of the ion-channel, there are certain discrepancies to account for. For instance, while the 2' engineered cysteine residue in $GABA_A$ receptors was protected from modification by sulfhydryl reagents, the 6' engineered cysteine was not (Xu et al., 1995). The authors in this study proposed that PTX could enter far enough into the

cytoplasmic aspect of the channel and bind to the 2' residue without causing obstruction for sulfhydryl reagents to access the 6' residue. The authors favored the hypothesis of PTX binding-site being inside the channel lumen rather than an allosteric effect. A similar finding was also reported by Perret et al, (1999) who used chemically reactive probes derived from non-competitive blockers to study the interaction of PTX and TBPS with the $GABA_A$ receptors. The authors discovered that the chemical probes blocked the wildtype and the 6' engineered cysteine mutant but the effect was reversible. In contrast, these probes irreversibly blocked the 2' engineered cysteine residue and the blockade could be protected by PTX. The authors hypothesized that PTX could enter the channel, travel through the channel (which is presumably wide) and reach deep into the cytoplasmic aspect i.e. the 2' position and act as a "plug at this position". In addition to this finding, an engineered cysteine residue at a more extracellular location i.e. 17' position was also protected from irreversible blockade by PTX. The authors speculated on the existence of another recognition-site for PTX in the extracellular aspect of the TM2. Indeed, another study by Dibas et al, (2001) demonstrated that mutation of the 15' serine residue to asparagine or glutamine (corresponding residues in the $GABA_A$ and Glu-R receptors) conferred a "use-facilitated" block by PTX in glycine receptors which otherwise do not exhibit use-dependence. These findings corroborate the possibility of multiple binding sites for PTX as suggested from early radioligand binding and electrophysiological studies and underscore the complexity of interaction of this and related ligands with members of the LGICs.

Objectives of Dissertation

PTX is known to inhibit all anion-selective members of the LGIC superfamily. While numerous groups have investigated the site and mechanism of neurotoxin interaction with the anion-selective members of the LGICs, direct interaction of neurotoxins with the cation-selective 5-HT_3 receptor has not been documented previously. As described earlier, the cation-selective 5-HT_3 receptor shares many structural features with other members of the LGIC superfamily. Furthermore, the TM2 domain (which is largely conserved across the LGICs) of the 5-HT_3 receptor shares notable homology with the anion-selective $GABA_A$ receptors. Thus, it is intuitive to expect that the 5-HT_3 receptor would be a target for neurotoxins.

The overall hypothesis tested in the study was that like anion-selective members of LGIC, PTX inhibits the cation-selective 5-HT_3 receptor by interacting in the ion channel. The specific aims of the study were the following: Specific Aim 1. Test the hypothesis that the mechanism of action of neurotoxins in the cation-selective 5-HT_3 receptor is similar to that in the anion-selective receptors. Specific Aim 2. Test the hypothesis that the interaction of PTX in the cation-selective 5-HT_3 receptor is subunit-dependent. Specific Aim 3. Test the hypothesis that the domain of interaction of PTX in the cation-selective 5-HT_3 receptors is similar to the anion-selective $GABA_A$ receptors.

In Chapter II, the first evidence for PTX and related neurotoxins interaction with the cation-selective recombinant mouse 5-HT_3 receptors is reported. The mechanism of block (e.g. competitive vs. non-competitive, use-dependence) is explored.

PTX is known to have differential effects in the anionic $GABA_A$, glycine invertebrate Glu-R and $GABA_C$ receptors. In chapter III, the selective effects of PTX in the 5-HT_{3A} receptor as compared to the heteromeric 5-$HT_{3A/3B}$ receptors is reported.

The molecular determinants important for PTX action have been defined in the anionic receptors to some extent. Some of the critical residues in the $GABA_A$ receptors and glycine receptors, which confer PTX sensitivity, are also present in the 5-HT_{3A} receptors. For example, the 5-HT_{3A} has a conserved threonine residue in the 6' position. In chapter IV, the effects of mutations of 2', 6' and additionally 7' residues in the 5-HT_{3A} receptor has been defined. PTX's effect on $GABA_A$, glycine, $GABA_C$ and Glu-R are subunit dependent. As described earlier, heteromeric assemblies of glycine$\alpha\beta$, $GABA_C\rho1\rho2$ and Glu-R$\alpha\beta$ receptors show diminished or no sensitivity to PTX. The molecular determinants, which contribute to decreased sensitivity of PTX upon co expression of the 5-HT_{3B} subunit with the 5-HT_{3A} subunit, are also evaluated. Finally, the results of radioligand binding studies in the 5-HT_{3A} receptor using [^{3}H]EBOB and [^{3}H] GR65630 are also presented.

In chapter IV, additional studies, which include substituted cysteine accessibility method (SCAM) on the 2' and 6' engineered cysteine residues in the 5-HT_{3A} receptor are also presented. Thus, a combination of whole-cell patch clamp technique, site-directed mutagenesis, radioligand binding, substituted cysteine accessibility method has been used in this study in an attempt to elucidate the mechanism and molecular determinants important for PTX interaction with the 5-HT_3 receptors.

This study was undertaken with the long-term goal of being able to identify a novel therapeutic target within the 5-HT_3 receptor. Although 5-HT_3 antagonists have been suggested to be useful in treatment and management of various clinical disorders, the actual use of these drugs have been restricted. Moreover, from the therapeutic point of view only antagonists that bind to the agonist-binding site are available for clinical use. Therapeutically useful drugs that inhibit this receptor by interacting at another site in the receptor are lacking. Thus, mapping the site through which neurotoxins act in the 5-HT_3 receptor will likely identify a hitherto undefined therapeutic target. Additionally, study of the molecular mechanism and determinants, which lead to subunit specific action of PTX, would help in development of drugs that display selectivity for homomeric or heteromeric receptors.

FIGURE I-1 Pentameric Structure of LGICs.

LGICs are multimeric proteins formed by the assembly of five subunits, which surround a central ion channel. The transmembrane domain 2 lines the channel pore.

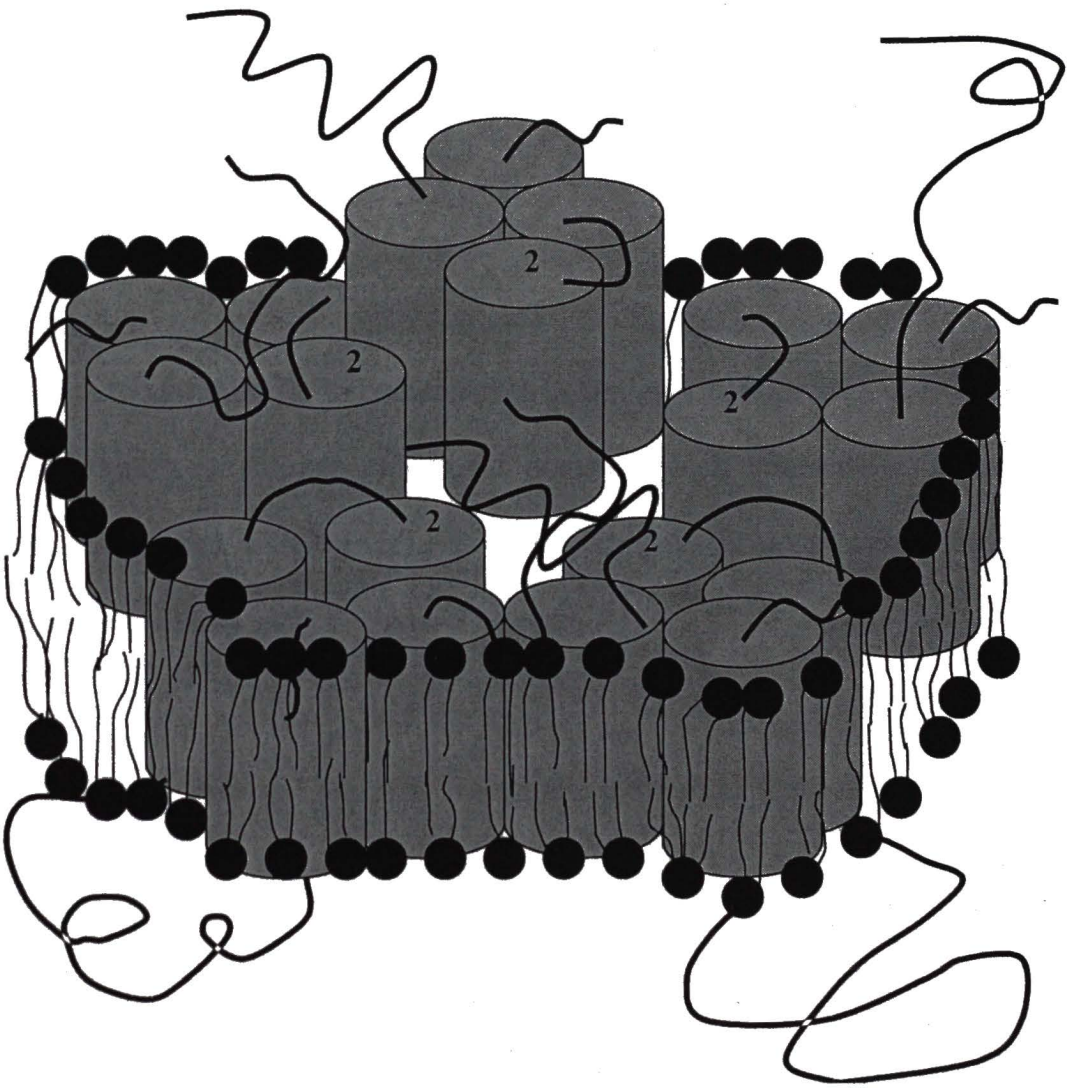

2
2
2
2
2

FIGURE I-2 Diagrammatic Representation of General Topography of LGICs.

Each subunit consists of a large N-terminal domain, which binds the agonist, four transmembrane domains (TM1-TM4), a large intracellular loop with consensus phosphorylation sites and a short C-terminal region.

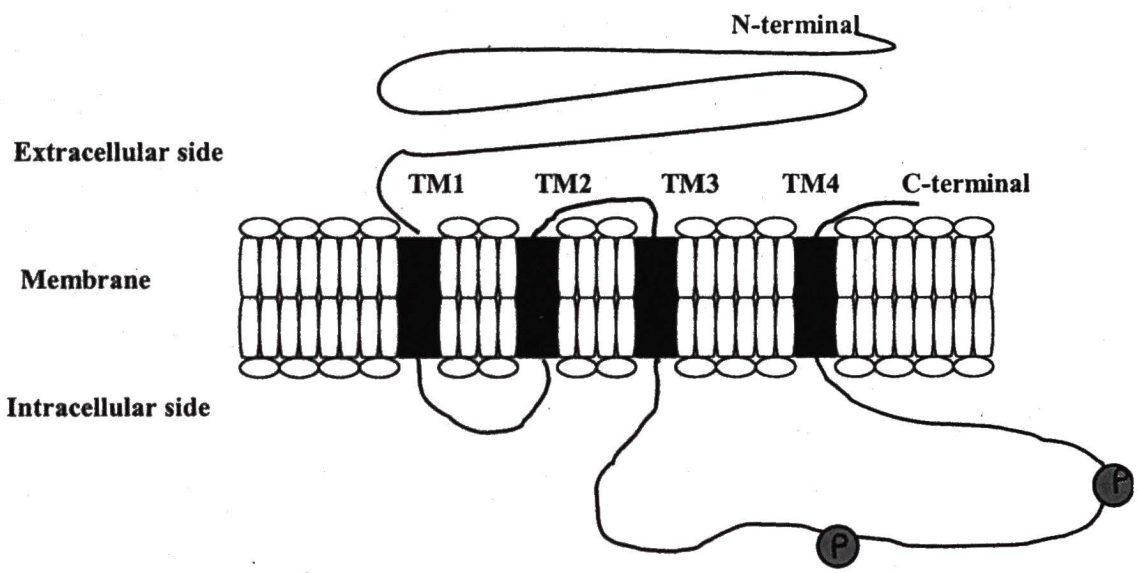

N-terminal
Extracellular side
TM1
TM2
TM3
TM4
C-terminal
Membrane
Intracellular side
P
P

FIGURE I-3 Model Depicting the Approximate Location of Loops A, B, C, D, E and F in the N-terminal Ligand-Binding Domain of a Subunit (from Reeves and Lummis, 2002). Loops A, B and C contribute to the principle binding-site and loops D, E and F form the complementary binding-site.

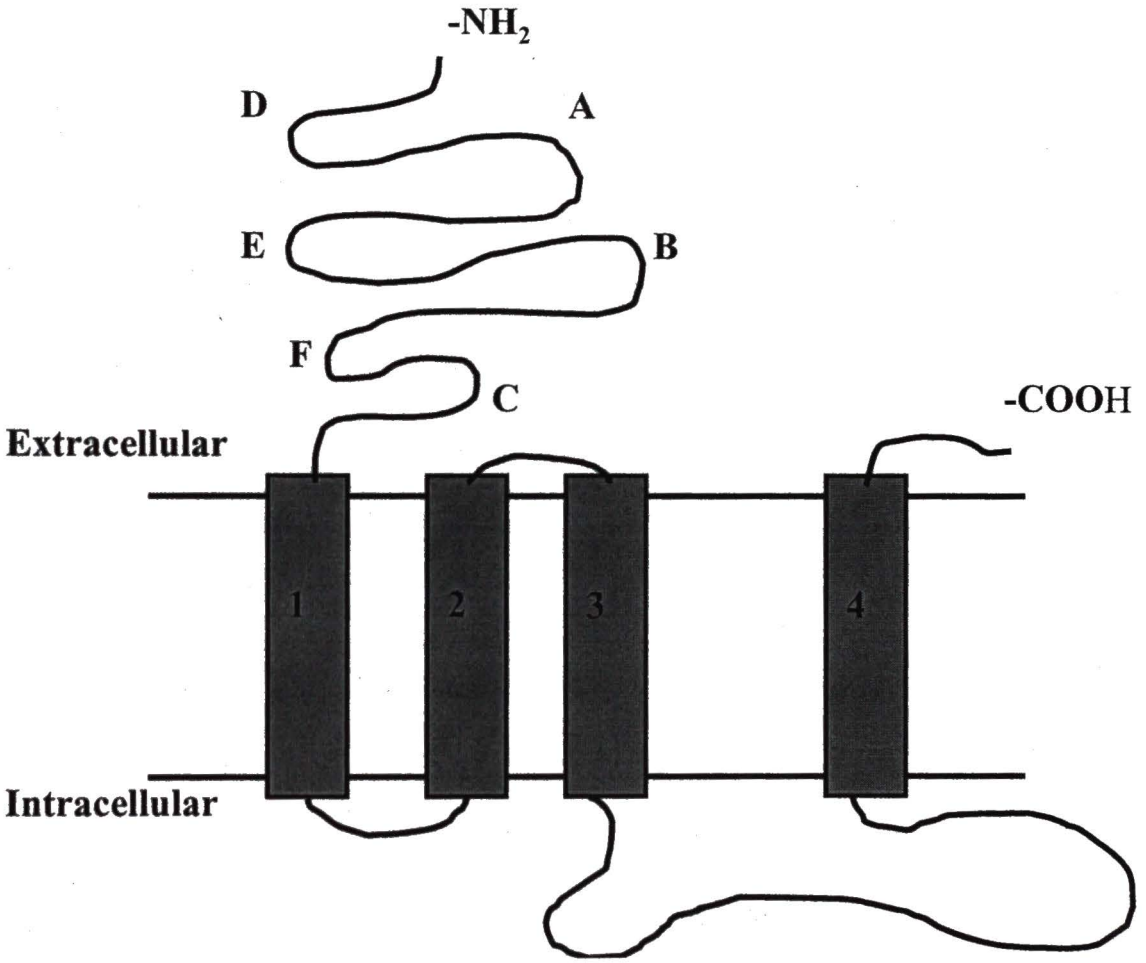
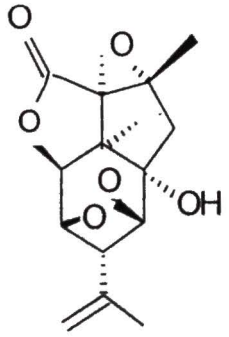

-NH2
D
A
E
B
F
C
-COOH
Extracellular
1
2
3
4
Intracellular

FIGURE I-4 Map of Transmembrane Domain 2 (TM2) of LGICs. The amino acid residues have been numbered according to the Miller numbering method (Miller, 1989) which assigns 1' to the most intracellular residue and 20' to the most extracellular residue in the TM2. m, r and Dro indicate mouse, rat and *Drosophila* subunits respectively.

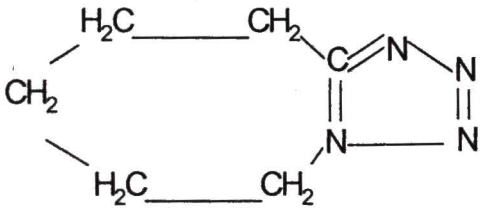
Receptor	0'	1'	2'	3'	4'	5'	6'	7'	8'	**9'**	10'	11'	12'	13'	14'	15'	16'	17'	18'	19'	20'
rGABA$\alpha 1$	R	T	V	F	G	V	T	T	V	**L**	T	M	T	T	L	S	I	S	A	R	N
rGABA$\beta 2$	R	V	A	L	G	I	T	T	V	**L**	T	M	T	T	I	N	T	H	L	R	E
rGABA$\gamma 2$	R	T	S	L	G	I	T	T	V	**L**	T	M	T	T	L	S	T	I	A	R	K
DroGABA	R	V	A	L	G	V	T	T	V	**L**	T	M	T	T	L	M	S	S	T	N	A
mAChRα7	K	I	S	L	G	I	T	V	L	**L**	S	L	T	V	F	M	L	L	V	A	E
m5HT$_{3A}$	R	V	S	F	K	I	T	L	L	**L**	G	Y	S	V	F	L	I	I	V	S	D
m5HT$_{3B}$	R	I	V	F	K	T	N	V	L	**V**	G	Y	T	V	F	R	V	N	M	S	D

FIGURE I-5 Structures of the Prototypical Convulsant Drug PTX and PTX-site ligands. The illustrated convulsant drugs, which presumably act through the PTX-site, each have a hydrophillic and hydrophobic domain.

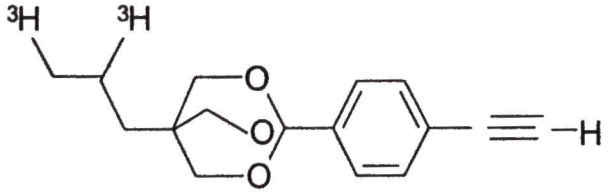
STRUCTURES OF CONVULSANT COMPOUNDS



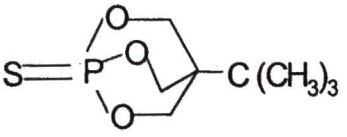
PICROTOXIN



PENTYLENETETRAZOLE



[^{3}H] EBOB



TBPS

REFERENCES

Adelsberger, H., Brunswieck, S., Dudel, J., 1998. Block by picrotoxin of a GABAergic chloride channel expressed on crayfish muscle after axotomy. European Journal of Neuroscience 10(1), 179-187.

Akabas, M.H., Kaufmann, C., Archdeacon, P., and Karlin, A., 1994. Identification of acetylcholine receptor channel lining residues in the entire M2 segment of alpha subunit. Neuron 13, 919-927.

Barann, M., Gothert, M., Fink, K., and Bonisch, H., 1993. Inhibition by anesthetics of 14C-guanidinium flux through the voltage-gated sodium channel and the cation channel of the 5-HT_3 receptor in N1E-115 neuroblsatoma cells. Naunyn Schmiedebergs Archives of Pharmacology 347, 125-132.

Barann, M., Ruppert, K., Gothert, M., and Bonisch, H., 1995. Increasing effect of ethanol on 5-HT_3 receptor-mediated 14C-guanidium influx in N1E-115 neuroblastoma cells. Naunyn Schmiedebergs Archives of Pharmacology 352, 149-156.

Barann, M., Gothert, M., Bonisch, H., Dybek, A., and Urban, B.W., 1997. 5-HT_3 receptors in outside-out patches of N1E-115 neuroblastoma cells: Basic properties and effects of pentobarbital. Neuropharmacology 36, 655-664.

Barann, M., Dilger, J.P., Bonisch, H., Gothert, M., Dybek, A., and Urban, B.W., 2000. Inhibition of 5-HT_3 receptors by propofol: equilibrium and kinetic measurements. Neuropharmacology 39, 1064-1074.

Barnes, J.M., Barnes, N.M., Champaneria, S., Costall, B., and Naylor, R.J., 1990. Characterization and autoradiographic localization of 5-HT_3 receptor recognition sites identified with [^{3}H]S-zacopride in the forebrain of the rat. Neuropharmacology 29(11), 1037-1045.

Barker, J.L., McBurney, R.N., and Mathers, D.A., 1983. Convulsant-induced depression of amino acid responses in cultured mouse spinal neurones studied under voltage clamp. British Journal of Pharmacology 80, 619-629.

Beene, D.L., Brandt, G.S., Zhong, W., Zacharias, N.M., Lester, H.A., Dougherty, D.A., 2002. Cation-pi interactions in ligand recognition by serotonergic (5-HT_{3A}) and nicotinic acetylcholine receptors: the anomalous binding properties of nicotine. Biochemistry 41(32), 10262-10269.

Berrada, D., Canenguez, K., Lembo, T., 2003. New approaches to the medical treatment of irritable bowel syndrome. Current Gastroenterology Report 5(4),337-342.

Boess, F.G., Sepulveda, M.I, Lummis, S.C., and Martin, I.L., 1992. 5-HT_3 receptors in NG108-15 neuroblastoma x glioma cells: effect of the novel agonist 1-(m-chlorophenyl)-biguanide. Neuropharmacology 31(6), 561-564.

Boess, F.G., Beroukhim, R., and Martin, I.L., 1995. Ultrastructure of the 5-hydroxytryptamine$_3$ receptor. Journal of Neurochemistry 64, 1401-1405.

Boess, F.G., Steward, L.J., Steele, J.A., Liu, D., Reid, J., Glencorse, T.A., and Martin, I.L., 1997. Analysis of the ligand-binding site of the 5-HT_3 receptor using site directed mutagenesis: Importance of glutamate 106. Neuropharmacology 36, 637-647.

Bonhaus, D.W., Wong, E.H., Stafanich, E., Kunysz, E.A., and Eglen, R.M., 1993. Pharmacological characterization of 5-hydroxytryptamine 3 receptors in murine brain and ileum using the novel radioligand [^{3}H]RS-42358-197: evidence for receptor heterogeneity. Journal of Neurochemistry 61(5), 1927-1932.

Boyd, G.W, Low, P., Dunlop, J.I, Robertson, L.A, Vardy, A., Lambert, J.J, Peters, J.A, Connolly, C.N., 2002. Assembly and cell surface expression of homomeric and heteromeric 5-HT_3 receptors: the role of oligomerization and chaperone proteins. Molecular and Cellular Neuroscience 21(1), 38-50.

Boyd, G.W, Doward, A.I, Kirkness, E.F, Millar, N.S, and Connolly, C.N., 2003. Cell surface expression of 5-HT_3 receptors is controlled by an endoplasmic reticulum retention signal. Journal of Biological Chemistry 278(30), 27681-27687.

Bradford, H.F., 1995. Glutamate, GABA and Epilepsy. Progress in Neurobiology 47(6), 477-511.

Bradley, P.B, Engel, G., Feniuk, W., Fozard, J.R., Humphrey, P.P., Middlemiss, D.N, Mylecharane, E.J, Richardson, B.P., Saxena, P.R., 1986. Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. Neuropharmacology 25(6), 563-576.

Brejc, K., van Dijk, W.J, Klaassen, R.V., Schuurmans, M., van Der Oost, J., Smit, A.B., and Sixma, T.K., 2001. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature 411(6835), 269-276.

Brown, A.M., Hope, A.G., Lambert, J.J., Peters JA., 1998. Ion permeation and conduction in a human recombinant 5-HT_3 receptor subunit (h5-HT_{3A}). Journal of Physiology 507 (Pt 3),653-65.

Callahan, MJ., 2002. Irritable bowel syndrome neuropharmacology. A review of approved and investigational compounds. Journal of Clinical Gastroenterology 35(1 Suppl), S58-67.

Casida, J.E., 1993. Insecticide action at the GABA-gated chloride channel: recognition, progress, and prospects. Archives in Insect Biochemical Physiology 22(1-2), 13-23.

Camilleri, M., Mayer, E.A., Drossman, D.A., Heath, A., Dukes, G.E., McSorley, D., Kong, S., Mangel, A.W., Northcutt, A.R., 1999. Improvement in pain and bowel function in female irritable bowel patients with alosetron, a 5-HT_3 receptor antagonist. Aliment Pharmacology Therapy 13(9),1149-1159.

Constanti, A., 1978. The mixed effect of picrotoxin on the GABA dose/conductance relation recorded in lobster muscle. Neuropharmacology 17, 159-167.

Corringer, P.J., Galzi, J.L., Eisele, J.L., Bertrand, S., Changeux, J.P., Bertrand, D., 1995. Identification of a new component of the agonist binding site of the nicotinic alpha 7 homooligomeric receptor. Journal of Biological Chemistry 270(20), 11749-11752.

Corringer, P.J., Le Novere, N., and Changeux, J.P., 2000. Nicotinic receptors at the amino acid level. Annual Reviews in Pharmacology and Toxicology 40, 431-438.

Costall, B., and Naylor., R.J., 1992. Anxiolytic potential of 5-HT_3 receptor antagonists. Pharmacology and Toxicology 70(3),157-162.

Currow, D.C, Coughlan, M., Fardell, B., and Cooney, N.J., 1997. Use of ondansetron in palliative medicine. Journal of Pain Symptom Management 13(5), 302-307.

Czajkowski, C., Kaufmann, C., and Karlin, A., 1993. Negatively charged amino acid residues in the nicotinic receptor delta that contribute to the binding of acetylcholine. Proceedings of the National Academy of Sciences. U.S.A. 90, 6285-6289.

Davies, P.A., Pistis, M., Hanna, M.C., Peters, J.A., Lambert, J.J., Hales, T.G., and Kirkness, E.F., 1999. The 5-HT_{3B} subunit is a major determinant of serotonin receptor function. Nature 397, 359-363.

Derkach, V., Suprenant, A., and North, R.A., 1989. 5-HT_3 receptors are membrane ion channels. Nature 339, 700-709.

Dibas, M.I., Gonzales, E.B., Das, P., Bell-Horner, C.L., and Dillon, G.H., 2002. Identification of a key residue within the second transmembrane domain that confers use-facilitated block by picrotoxin in glycine α1 receptors Journal of Biological Chemistry 277, 9112-9117.

Dennis, M., Giraudat, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J.Y., Lazure, C., Chretien, M., Changeux, J.P., 1988. Amino acids of the Torpedo marmorata acetylcholine receptor alpha subunit labeled by a photoaffinity ligand for the acetylcholine binding site. Biochemistry 27(7),2346-2357.

Downie, D.L., Hope, A.G., Lambert, J.J., Peters, J.A., Blackburn, T.P., and Jones, B.J., 1994. Pharmacological characterization of the apparent splice variants of the murine 5-HT_3R-A subunit expressed in *Xenopus laevis* oocytes. Neuropharmacology 33, 473-482.

Dubin, A.E., Huvar, R., Andrea, M.R., Pyati, J., Zhu, J.Y., Joy, K.C., Wilson S.J., Galindo, J.E., Glass, C.A., Luo, L., Jackson, M.R., Lovenberg, T.W., and Erlander, M.G., 1999. The pharmacological and functional characteristics of the serotonin 5-HT_{3A} receptor are specifically modified by the 5-HT_{3B} receptor subunit. Journal of Biological Chemistry 274(43), 30799-30810.

Edgar, P.P, Schwartz, R.D., 1990. Localization and characterization of ^{35}S-t-butylbicyclophosphorothionate binding in rat brain: an autoradiographic study. Journal of Neuroscience 10(2), 603-612.

Eiselle, J.L., Bertrand, S., Galzi, J.L., Devillers-Thiery, A., Changeux, J.P., and Bertrand, D., 1993. Chimeric nicotinic-serotonergic receptor combines distinct ligand binding and channel specificities. Nature 366, 479-483.

Endo, T., Minami, M., Kitamura, N., Teramoto, Y., Ogawa, T., Nemoto, M., Hamaue, N., Hirafuji, M., Yasuda, E., Blower, P.R., 1999. Effects of various 5-HT_3 receptor antagonists, granisetron, ondansetron, ramosetron and azasetron on serotonin (5-HT) release from the ferret isolated ileum. Research Communications in Molecular Pathology and Pharmacology 104(2), 145-155.

Enna, S.J., Bennett, J.P, Bylund, D.B., Creese, I., Burt, D.R., Charness, M.E., Yamamura, H.I., Simantov, R., and Snyder, S.H., 1977. Neurotransmitter receptor binding: regional distribution in human brain. Journal of Neurochemistry 28(1), 233-306.

Etter, A., Cully, D.F., Liu, K.K., Reiss, B., Vassilatis, D.B., Schaeffer, J.M., and Arena J.P., 1999.Picrotoxin blockade of invertebrate glutamate gated chloride channels: subunit dependence and evidence for binding within the pore. Journal of Neurochemistry 72, 318-326.

Farber, L., Stratz, T.H., Bruckle, W., Spath, M., Pongratz, D., Lautenschlager, J., Kotter, I., Zoller, B., Peter, H.H,, Neeck, G., Welzel, D., Muller, W., 2001. German Fibromyalgia Study Group. Short-term treatment of primary fibromyalgia with the 5-HT_3-receptor antagonist tropisetron. Results of a randomized, double-blind, placebo-controlled multicenter trial in 418 patients. International Journal of Clinical Pharmacology Research 21(1),1-13.

Fan, P., Visentin, S., and Weight, F.F.,1994. Effects of cocaine on the serotonin-induced inward current in rat nodose ganglion neurons. Journal of Pharmacology Experimental Therapeutic 271, 262-266.

Ferezou, I., Cauli, B., Hill, E.L., Rossier,J., Hammel, E., and Lambolez, B., 2002. 5-HT_3 receptors mediate serotonergic fast excitation of neocortical vasoactive intestinal peptide/cholecystokinin interneurons. Journal of Neuroscience 22(17), 7389-7397.

ffrench-Constant, R.H., Rocheleau, T.A., Steichen, J.C., and Chalmers, A.E., 1993. A point mutation in a Drosophila GABA receptor confers insecticide resistance. Nature 363, 449-451.

Fletcher, S., and Barnes, N.M., 1998. Desperately seeking subunits: are native 5-HT_3 receptors really homopentameric complexes. Trends in Pharmaceutical Sciences 19(6), 212-215.

Fonseca, M.I, Ni, Y.G., Dunning, D.D., and Miledi, R., 2001. Distribution of serotonin 2A, 2C and 3 receptor mRNA in spinal cord and medulla oblongata. Brain Research Molecular Brain Research 89(1-2), 11-19.

Franke, C.H., Hatt, H., and Dudel, J., 1986. The inhibitory chloride channel activated by glutamate γ-aminobytyric acid (GABA). Journal of Comparitive Physiology 159, 591-609.

Fraser, A.D., 1996. New drugs for the treatment of epilepsy. Clinical Biochemistry 29(2),97-110.

Gaddum, J.H., and Picarelli, Z.P., 1957. Two kinds of tryptamine receptor. British Journal of Pharmacology. 12,323-328.

Galzi, J.L., Revah, F., Black, D., Goeldner, M., Hirth, C., Changeux, J.P., 1990 Identification of a novel amino acid alpha-tyrosine 93 within the cholinergic ligands-binding sites of the acetylcholine receptor by photoaffinity labeling. Additional evidence for a three-loop model of the cholinergic ligands-binding sites. Journal of Biological Chemistry 265(18), 10430-10437.

Gingrich, J.A., 2002. Mutational analysis using the serotonergic system: Recent findings using knockout mice. Current Drug Target CNS Neurological Disorders 1(5), 449-465.

Gill, CH., Peters, J.A, Lambert, J.J., 1995. An electrophysiological investigation of the properties of a murine recombinant 5-HT_3 receptor stably expressed in HEK 293 cells. British Journal of Pharmacology 114(6), 1211-1221.

Greenshaw, A.J., and Silverstone, P.H., 1997. The non-antiemetic uses of serotonin 5-HT_3 receptor antagonists. Clinical pharmacology and therapeutic applications Drugs 53(1), 20-39.

Gurley, D.J., Amin, P.C., Ross, D.S., Weiss, D.S., and White, W., 1995. Point mutations in the M2 region of the α, β, or γ subunit of the $GABA_A$ channel that abolish block by picrotoxin. Receptors Channels 3, 12-30.

Hanna, M.C., Davies, P.A., Hales, T.G., and Kirkness, E.F., 2000. Evidence for expression of heteromeric serotonin 5-HT_3 receptors in rodents. Journal of Neurochemistry 75(1), 240-247.

Hargreaves, A.C., Lummis, S.C., and Taylor, C.W., 1994. Ca+2 permeability of cloned and native 5-hydroxytryptamine type 3 receptors. Molecular Pharmacology 46, 1120-1128.

Hevers, W., and Luddens, H., 1998. The diversity of $GABA_A$ receptors: Pharmacological and electrophysiological properties of $GABA_A$ channel subtypes. Molecular Neurobiology 18(1), 35-86.

Hope, A.G., Downie, D.L., Sutherland, L., Lambert, J.J., Peters, A., and Burchell, B., 1993. Cloning and functional expression of an apparent splice variant of the murine 5-HT_3 receptor A subunit. European Journal of Pharmacology 245, 187-192.

Hovius, R., Tairi, A.P., Blasey, H., Bernard, A., Lundstrom, K., Vogel, H., 1998. Characterization of a mouse serotonin 5-HT_3 receptor purified from mammalian cells. Journal of Neurochemisty 70(2), 824-834.

Hoyer, D., and Martin, G., 1997.5-HT receptor classification and nomenclature: towards a harmonization with the human genome. Neuropharmacology 36, 419-428.

Hoyer, D., Hannon, J.P., and Martin, G.R., 2002. Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacology, Biochemistry and Behavior 71(4), 533-554.

Huang, J., and Casida, J.E., 1996. Characterization of [^{3}H]ethynylbicycloorthobenzoate ([^{3}H]EBOB) binding and the action of insecticides on the gamma-aminobutyric acid-gated chloride channel in cultured cerebellar granule neurons. Journal of Pharmacology and Experimental Therapeutics 279(3), 1191-1196.

Hussy, N., Lucas, W., and Jones, K.A., 1994. Functional properties of a cloned 5-hydroxytryptamine ionotropic receptor subunit: comparison with native mouse receptors. Journal of Physiology 481, 311-323.

Inoue, M., and Akaike, N., 1988. Blockade of gamma-aminobutyric acid-gated chloride current in frog sensory neurons by picrotoxin. Neuroscience Research 5, 380-394.

Jackson, M.B., and Yakel, J.L., 1995. The 5-HT_3 receptor channel. Annual Reviews in Physiology. 57, 447-468.

Johnson, D.S., and Heinemann, S.F., 1995 (a). Detection of 5-HT_3R-A, a 5-HT_3 receptor subunit, in submucosal and myenteric ganglia of rat small intestine using in situ hybridization. Neuroscience Letters 184(1), 67-70.

Johnson, D.S., Heinemann, S.F., 1995 (b). Embryonic expression of the 5-HT_3 receptor subunit, 5-HT_3R-A, in the rat: an in situ hybridization study. Molecular and Cellular Neuroscience 6(2), 122-138.

Jones, R.H., Holtmann, G., Rodrigo, L., Ehsanullah, R.S., Crompton, P.M., Jacques, L.A., Mills, J.G., 1999. Alosetron relieves pain and improves bowel function compared with mebeverine in female nonconstipated irritable bowel syndrome patients. Aliment Pharmacology and Therapy 13(11),1419-1427.

Karlin, A., 2002. Emerging structure of the nicotinic acetylcholine receptors. Nature Reviews Neuroscience 3(2):102-114

Kilpatrick, G.J, Jones, B.J, Tyers, M.B., 1987. Identification and distribution of 5-HT_3 receptors in rat brain using radioligand binding. Nature 330(6150),746-748.

Kilpatrick, G.J., Jones, B.J., Tyers, M.B., 1989. Binding of the 5-HT_3 ligand, [^{3}H]GR65630, to rat area postrema, vagus nerve and the brains of several species. European Journal of Pharmacology 159(2), 157-164.

Kroeze, W.K., Kristiansen, K., and Roth, B.L., 2002. Molecular biology of the serotonin receptors structure and function at molecular level. Current Topics in Medicinal Chemistry. 2(6), 507-528.

Kume, A., and Albin, R.L., 1994. Quantitative autoradiography of 4'-ethynyl-4-n-[2,3-3H2]propylbicycloorthobenzoate binding to the $GABA_A$ receptor complex. European Journal of Pharmacology 263(1-2),163-173.

Lambert, J.J., Peters, J.A., Hales, T.G., and Dempster, J., 1989. The properties of 5-HT_3 receptors in clonal cell lines studied by patch clamp techniques. British Journal of Pharmacology 97(1), 27-40.

Lankiewicz, S., Lobitz, N., Wetzel, C.H., Rupprecht, R., Gisselmann, G., and Hatt, H., 1998. Molecular cloning, functional expression, and pharmacological characterization of 5-hydroxytryptamine3 receptor cDNA and its splice variants from guinea pig. Molecular Pharmacology 53, 202-212.

Lawrence, L.J., and Casida, J.E., 1983. Stereospecific action of pyrethroid insecticides on the gamma-aminobutyric acid receptor-ionophore complex. Science 221(4618), 1399-1401.

Lawrence, L.J., Gee, K.W., Yamamura, H.I., 1985. Interactions of pyrethroid insecticides with chloride ionophore-associated binding sites. Neurotoxicology 6(2),87-98.

Lovinger, D.M.,1991. Inhibition of 5-HT_3 receptor-mediated ion current by divalent metal cations in NCB-20 neuroblastoma cells. Journal of Neurophysiology 66(4), 1329-1337.

Lovinger, D.L., Sung ,K.W., and Zhou, Q., 2000. Ethanol and trichloroethanol alter gating of the 5-HT_3 receptor channels in NCB-20 neuroblastoma cells. Neuropharmacology 39, 561-570.

Lummis, S.C.R. and Baker, J., 1997. Radioligand binding and photoaffinity labelling studies show a direct interaction of phenothiazines at 5-HT_3 receptors. Neuropharmacology 36, 665-670.

Machu, T.K., and Harris, R.A., 1994. Alcohols and anesthetics enhance the function of 5-hydroxytryptamine $_3$ receptors expressed in Xenopus laevis oocytes. Journal of. Pharmacology and Experimental Therapeutics 271(2), 898-905.

Macdonald, R.L, Barker, J.L., 1978. Different actions of anticonvulsant and anesthetic barbiturates revealed by use of cultured mammalian neurons. Science 200(4343), 775-777.

Maricq, A.V., Peterson, A.S., Brake, A.J., Myers, R.M., and Julius, D., 1991. Primary structure and functional expression of the 5-HT_3 receptor, a serotonin-gated ion channel. Science 254, 432-437.

Middleton, R.R., and Cohen, J.B., 1991. Mapping of the acetylcholine binding site of the nicotinic acetylcholine receptor: [^{3}H] nicotine as an agonist photoaffinity label. Biochemistry 30, 6987-6997.

Miller, C., 1989. Genetic manipulation of ion channels: a new approach to structure and mechanism. Neuron 2(3),1195-1205.

Min, K.T., Wu, C.L., and Yang, J., 2000. Nondepolarizing neuromuscular blockers inhibit the serotonin type$_{3A}$ receptor expressed in Xenopus oocytes. Anesthesia Analgesia 90, 476-481.

Miquel, M.C., Emerit, M.B., Gingrich, J.A., Nosjean, A., Hamon, M., and el Mestikawy, S., 1995. Developmental changes in differential expression of two serotonin 5-HT_3 receptor splice variants in the rat. Journal of Neurochemistry 65(2), 257-261.

Mochizuki, S., Miyake, A., Furuichi, K., 1999. Ion permeation properties of a cloned human 5-HT_3 receptor transiently expressed in HEK 293 cells. Amino Acids 17(3), 243-255.

Morales, M., McCollum, N., and Kirkness, E., 2001. 5-HT_3 receptor A and B subunits are co-expressed in neurons of dorsal root ganglion. Journal of Comparative Neurology 438(2), 163-172.

Morales, M., and Wang, S.D., 2002. Differential composition of 5-hydroxytryptamine 3 receptors synthesized in the rat CNS and peripheral nervous system. Journal of Neuroscience 22(15), 6732-6741.

Newberry, N.R., Cheshire, S.H., and Glibert, M.J. 1991. Evidence that the 5-HT_3 receptors of rat, mouse and guinea pig superior cervical ganglion may be different. British Journal of Pharmacology 102(3), 615-620.

Newland, C.F., and Cull-Candy, S.G., 1992. On the mechansim of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurons of the rat. Journal of Physiology 447,191-231.

Niemeyer, M.I., and Lummis, S.C., 1998. Different efficacy of specific agonists at 5-HT_3 receptor splice variants: the role of the extra six amino acid segment. British Journal of Pharmacology 123(4), 661-666.

Niemeyer, M.I, Lummis, S.C., 2001. The role of the agonist binding site in Ca (2+) inhibition of the recombinant 5-HT_{3A}receptor. European Journal of Pharmacology 428(2),153-161.

Niesler, B., Frank, B., Kapeller, J., and Rappold, G.A., 2003. Cloning, physical mapping and expression analysis of the human 5-HT_3 serotonin receptor like genes HTR3C, HTR3D and HTR3E. Gene 310, 101-111.

Ortells, M.O., and Lunt, C.G., 1995. Evolutionary history of the ligand-gated ion channel superfamily of receptors. Trends in Neuroscience 18(3), 121-127.

Olsen, R.W., Ticku, M.K., Miller, T., 1978. Dihydropicrotoxinin binding to crayfish muscle sites possibly related to gamma-aminobutyric acid receptor-ionophores. Molecular Pharmacology 14(3),381-390.

Olsen, R.W.,1981. GABA-benzodiazepine-barbiturate receptor interactions. Journal of Neurochemistry 37(1), 1-13.

Panicker, S., Cruz, H., Arrabit, C., Slesinger P.A., 2002. Evidence for a centrally located gate in the pore of a serotonin-gated ion channel. Journal of Neuroscience 22(5),1629-1639.

Parker, R.M, Barnes, J.M, Ge, J, Barber, P.C, and Barnes, N.M., 1996 (a). Autoradiographic distribution of [^{3}H]-(S)-zacopride-labelled 5-HT_3 receptors in human brain. Journal of Neurological Sciences 144(1-2), 119-127.

Parker, R.M, Bentley, K.R, and Barnes, N.M., 1996 (b). Allosteric modulation of 5-HT_3 receptors: focus on alcohols and anaesthetic agents. Trends in Pharmacological Sciences 17(3), 95-9.

Perret, P., Sarda, X., Wolff, M., Wu, T.-T., Bushey, D., and Goeldner, M., 1999. Interaction of non-competitive blockers within the γ-aminobutyric acid type A chloride channel using chemically reactive probes as chemical sensors for cysteine mutants. Journal of Biological Chemistry 274, 25350-25354.

Peters, J.A., Hales, T.G., and Lambert, J.J., 1988. Divalent cations modulate 5-HT_3 receptor-induced currents in N1E-115 neuroblastoma cells. European Journal of Pharmacology. 151, 491-495.

Peters, J.A., Malone, H.M., and Lambert, J.J., 1992. Recent advances in electrophysiological characterization of 5-HT_3 receptors. Trends in Pharmacological Sciences 13, 391-397.

Porter, L.A., 1967. Picrotoxinin and related substances. Chemistry Reviews 67(4), 441-464.

Pootanakit, K., and Brunken, W.J., 2001. Identification of 5-HT_{3A} and 5-HT_{3B} receptor subunits in mammalian retinae: potential pre-synaptic modulators of photoreceptors. Brain Research 896(1-2),77-85.

Pribilla, I., Takagi, T., Langosch, D., Bormann, J., and Betz, H., 1992. The atypical M2 segment of the beta subunit confers picrotoxinin resistance to inhibitory glycine receptor channels. EMBO Journal 12, 4305-4311.

Ramanjaneyulu, R., and Ticku, M.K., 1984. Binding characteristics and interactions of depressant drugs with [^{35}S]t-butylbicyclophosphorothionate, a ligand that binds to the picrotoxinin site. Journal of Neurochemistry 42, 221-229.

Rapport, M.M., Green, A.A., and Page, I.H., 1947. Purification of the substance which is responsible for vasoconstrictor activity of serum. Federal Proceedings 6, 184.

Reddy, G.L., Iwamoto, T., Tomich, J.M., and Montal, M., 1993. Synthetic peptides and four helix bundle proteins as model systems for the pore forming structure of channel proteins. Transmembrane segment M2 of the brain glycine receptor is a plausible candidate for the pore-lining structure. Journal of Biological Chemistry 298, 14608-14615.

Reeves, D.C., and Lummis, S.C.R., 2002. The molecular basis of the structure and function of the 5-HT_3 receptor: a model ligand-gated ion channel. Molecular Membrane Biology 19, 11-26.

Robertson, B., and Bevan, S., 1991. Properties of 5-hydroxytryptamine receptor-gated currents in adult rat dorsal root ganglions. British Journal of Pharmacology 102(1), 272-276.

Schmieden, V., Grenningloh, G., Schofield, P.R., and Betz, H., 1989. Functional expression in *Xenopus* oocytes of the strychnine binding 48 kd subunit of the glycine receptor. EMBO Journal 8, 695-700.

Schmieden, V., Kuhse, J., Betz, H., 1993. Mutation of glycine receptor subunit creates beta-alanine receptor responsive to GABA. Science 262(5131), 256-258.

Sellers, E.M., Toneatto, T., Romach, M.K., Somer, G.R., Sobell, L.C., Sobell, M.B., 1994. Clinical efficacy of the 5-HT_3 antagonist ondansetron in alcohol abuse and dependence. Alcohol Clinical Experimental Research 18(4), 879-885.

Sepulveda, M.I., Baker, J., Lummis, S.C., 1994. Chlorpromazine and QX222 block 5-HT_3 receptors in N1E-115 neuroblastoma cells. Neuropharmacology 33(3-4), 493-499.

Shan, Q., Haddrill, J.L., and Lynch, J.W., 2001. A single β subunit M2 domain controls the picrotoxin sensitivity of αβ heteromeric glycine receptor chloride channels. Journal of Neurochemistry 76, 1109-1120.

Sieghart, W., 1995. Structure and pharmacology of gamma-aminobutyric acid A receptor subtypes. Pharmacology Reviews 47(2),181-234.

Simmonds, M.A., 1982. Classification of some GABA antagonists with regard to site of action and potency in slices of rat cuneate nucleus. European Journal of Pharmacology 80(4),347-358.

Sine, S.M., Kreienkamp, H.J., Bren, N., Maeda, R., and Taylor, P., 1995. Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of determinants of alpha-conotoxin M1 selectivity. Neuron 15, 205-211.

Sirota, P., Mosheva, T., Shabtay, H., Giladi, N., and Korczyn, A.D., 2000. Use of the selective serotonin $_3$ receptor antagonist ondansetron in the treatment of neuroleptic induced tardive dyskinesia. American Journal of Psychiatry 157, 287-289.

Sivilotti, L., and Nistri, A., 1991. GABA receptor mechanisms in the central nervous system. Progress in Neurobiology 36(1), 35-92.

Smith,W.T., Londberg, P.D., Blomgren, S.L., Tollefson, G.D., and Sayler, M.E., 1999.

Pilot study of zatosetron (LY277359) maleate, a 5-hydroxytryptamine3 antagonist, in the treatment of anxiety. Journal of Clinical Psychopharmacology 19(2), 125-131.

Spath, M., 2002. Current experience with 5-HT_3 receptor antagonists in fibromyalgia Rheumatology Disorder Clinic North America 28(2), 319-328.

Spier, A.D., and Lummis, S.C.R., 2000. The role of tryptophan residues in the 5-hydroxytryptamine$_3$ receptor ligand binding domain. Journal of Biological Chemistry 275, 5620-5625.

Spiller, R., 2002. Serotonergic modulating drugs for functional gastrointestinal diseases. British Journal of Clinical Pharmacology 54(1),11-20.

Squires, R.F., Casida, J.E., Richardson, M., Saederup, E., 1983. [^{35}S]t-butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to gamma-aminobutyric acid-A and ion recognition sites. Molecular Pharmacology 23(2),326-336.

Stewart, A., Davies, P.A., Kirkness, E.F., Safa, P., Hales, T.G., 2003. Introduction of the 5-HT_{3B} subunit alters the properties of 5-HT_3 receeptors native to neuroblastoma cells. Neuropharmacology 44(2), 214-223.

Sudweeks, S.N., Hooft, J.A., and Yakel, J.L., 2002. Serotonin 5-HT_3 receptors in CA1 hippocampal interneurons: functional and molecular characterization. Journal of Neuroscience 544, 715-726.

Takeuchi, A., and Takeuchi, N., 1969. A study of the action of picrotoxin on the inhibitory neuromuscular junction of the crayfish. Journal of Physiology 205, 377-391.

Tecott, L.H, Maricq, A.V, Julius, D., 1993. Nervous system distribution of the serotonin 5-HT_3 receptor mRNA. Proceedings of the National Academy of Sciences U.S.A. 90(4), 1430-1434.

Ticku, M.K., Ban, M., Olsen, RW., 1978. Binding of [^{3}H]alpha-dihydropicrotoxinin, a gamma-aminobutyric acid synaptic antagonist, to rat brain membranes. Molecular Pharmacology 14(3), 391-402.

Ticku, M.K., 1983. Benzodiazepine-GABA receptor-ionophore complex:Current concepts. Neuropharmacology 22(12B), 1459-1470.

Ticku, M.K., and Ramanjaneyulu, R., 1984. Differential interactions of GABA agonists, depressant and convulsant drugs with [^{35}S]-t-butylbicyclophosphorothionate binding sites in cortex and cerebellum. Pharmacology Biochemistry and Behavior 21(1), 151-158.

Tyers, M.B., and Freeman, A.J., 1992. Mechanism of the anti-emetic activity of 5-HT_3 receptor antagonists. Oncology 49(4), 263-268.

Vandenberg, R.J., Handford, C.A., Schofield, P.R., 1992. Distinct agonist- and antagonist-binding sites on the glycine receptor. Neuron 9(3), 491-496.

Van Hooft, J.A., and Yakel, J.L., 2003. 5-HT_3 receptors in the CNS; 3B or not 3B. Trends in Pharmacological Sciences 24(4), 157-160.

Waeber, C., Dixon, K., Hoyer, D., Palacios, J.M., 1988. Localisation by autoradiography of neuronal 5-HT_3 receptors in the mouse CNS. European Journal of Pharmacology 151(2), 351-352.

Wang, T.L., Hackam, A.S., Guggino, W.B., and Cutting, G.R., 1995. A single amino acid in gamma-aminobutyric acid rho 1 receptors affects competitive and noncompetitive components of picrotoxin inhibition. Proceedings of the National Academy of Sciences U.S.A. 92(25), 11751-11755.

Werner, P., Kawashima, E., Reid, J., Hussy, N., Lundstrom, K., Buell, G., Humbert, Y., and Jones, K.A., 1994. Organization of the mouse 5-HT_3 receptor gene and functional expression of two splice variants. Molecular Brain Research 26, 233-241.

Wetzel, C.H, Hermann, B., Behl, C., Pestel, E., Rammes, G., Zieglgansberger, W., Holsboer, F., Rupprecht, R., 1998. Functional antagonism of gonadal steroids at the 5-hydroxytryptamine type 3 receptor. Molecular Endocrinology 12(9),1441-1451.

Wolf, H., 2000. Preclinical and clinical pharmacology of the 5-HT_3 receptor antagonists. Scandinavian Journal of Rheumatology Supplements 113, 37-45.

Wong, E.H., Bonhaus, D.W., Lee, J.A., Wu, I., Loury, D.N., and Eglen, R.M., 1993. Different densities of 5-HT3 receptors are labeled by [^{3}H]quizapine, [^{3}H]GR65630 and [^{3}H]granisetron. Neuropharmacology 32(9), 869-875.

Xu, M., Covey, D.F., and Akabas, M.H., 1995. Interaction of picrotoxin with $GABA_A$ receptor channel-lining residues probed in cysteine mutants. Biophysics Journal 69, 1858-1867.

Yan, D., Schulte, M.K., Bloom, K.E., and White, M.M., 1999. Structural features of the ligand-binding domain of the serotonin 5-HT_3 receptor. Journal of Biological Chemistry 274, 5537-5541.

Yang, J., 1990. Ion permeation through 5-hydroxytryptamine-gated channels in neuroblastoma N18 cells. Journal of General Physiology 96, 1177-1198.

Yang, J., Mathie, A., and Hille, B., 1992. 5-HT_3 receptor channels in dissociated rat superior cervical ganglion neurons. Journal of Physiology 448, 237-256.

Yakel, J.L., Shao, X.M., and Jackson, M.B., 1990. The selectivity of channel coupled to the 5-HT_3 receptor. Brain Research 533(1), 46-52.

Yakel, J.L., Lagrutta, A., Adelman, J.P., and North, R.A., 1993. Single amino acid substitution affects desensitization of the 5-hydroxytryptamine type $_3$ receptor expressed in *Xenopus* oocytes. Proceedings of the National Academy of Sciences U.S.A. 90, 5030-5033.

Yoon, K.W., Covey, D.F., and Rothman, S.M., 1993. Multiple mechanisms of picrotoxin block of GABA induced currents in rat hippocampal neurons. Journal of Physiology 464, 423-439.

Zhang, H.G., ffrench-Constant, R.H., Brideau, A.D., and Lipton, S.A., 1995. Cloning of a γ-aminobutyric acid type $_C$ receptor subunit in rat retina with a methionine residue critical for picrotoxinin channel block. Proceedings of National Academy Sciences 92, 11756-11760.

Zhang, L., Oz, M., Stewart, R.R., Peoples, R.W., and Weight, F.F., 1997.Volatile general anaesthetic actions on recombinant nAChα7, 5-HT_3 and chimeric nAChα7-5-HT_3

receptors expressed in Xenopus oocytes. British Journal of Pharmacology 120, 353-355.

Zhorov, B., and Bregestovski, P., 2000. Chloride channels of glycine and GABA receptors with blockers: Monte Carlo minimization and structure-activity relationships. Biophysics Journal 78, 1786-1803.

THE $GABA_A$ RECEPTOR ANTAGONIST PICROTOXIN INHIBITS 5-HYDROXYTRYPTAMINE TYPE $_{3A}$ RECEPTORS

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Running title:

5-HT_{3A} receptor inhibition by picrotoxin

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Abbreviations:

5-HT, 5-hydroxytryptamine; 5-HT_{3A}, serotonin type 3A; DMSO, dimethysulfoxide; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetra acetic acid; 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; LGIC, ligand gated ion channels; nAChR, nicotinic acetylcholine receptor; PTX, picrotoxin; PTZ, pentylenetetrazole; U-93631, [4-Dimethyl-3-t-butylcarboxyl-4,5-dihydro (1,5-a) quinoxaline].

Das et al., 2003. Neuropharmacology 44(4), 431-438.

CHAPTER II

THE $GABA_A$ RECEPTOR ANTAGONIST PICROTOXIN INHIBITS 5-HYDROXYTRYPTAMINE TYPE 3A RECEPTORS

SUMMARY

For a number of years it has been known that the CNS convulsant picrotoxin inhibits the $GABA_A$ receptor, an anion-selective member of the ligand-gated ion channel (LGIC) superfamily. PTX also inhibits other anion-selective LGIC members, such as $GABA_C$, glycine and glutamate-gated Cl^- channels. In the present report, we tested the ability of picrotoxin to inhibit cation-selective 5-HT_{3A} receptors. Murine 5-HT_{3A} receptors were expressed in HEK293 cells, and functionally evaluated using whole-cell patch clamp recording. Picrotoxin inhibited 5-HT-gated currents in a concentration-dependent manner, with an IC_{50} of approximately 30 μM. Moreover, the blockade by PTX was non-competitive and use-facilitated. Pentylenetetrazole and U-93631, ligands that act at a domain similar to that of picrotoxin in $GABA_A$ receptors, also inhibited the 5-HT_{3A} receptor. For each ligand tested, its potency was 5-10 fold lower than typically observed in $GABA_A$ receptors. Our results demonstrate that, in addition to being a relatively non-selective inhibitor of anionic LGICs, picrotoxin also inhibits the cation-selective 5-HT_{3A} receptor. Moreover, the fact that both PTZ and U-93631 similarly

inhibit the 5-HT_{3A} receptor is consistent with the suggestion that the site of picrotoxin action in this receptor may be comparable to that in anion-selective LGICs.

INTRODUCTION

The 5-hydroxytryptamine type 3 (5-HT_3) receptor is a member of the superfamily of ligand-gated ion channels (LGICs) that includes nicotinic acetylcholine receptors (nAChRs), $GABA_A$, $GABA_c$ and glycine receptors, and glutamate-gated chloride channels (Glu-Cl) (Karlin, 2002). Two 5-HT_3 receptor subunits, designated 5-HT_{3A} and 5-HT_{3B}, have been identified to date (Maricq et al., 1991; Davies et al., 1999). Heteromeric assemblies of 5-HT_{3A} and 5-HT_{3B} receptor subunits exhibit distinct biophysical properties as compared to homomeric 5-HT_{3A} receptors (Davies et al., 1999; Dubin et al., 1999).

Both cation-selective (nAChR and 5-HT_3) and anion-selective ($GABA_A$, $GABA_c$, glycine and Glu-Cl) receptors make up the LGIC superfamily. All members of the superfamily have a large extracellular N-terminal region where agonist binds, and are composed of five subunits that surround a central ion channel. Each subunit has 4 transmembrane domains (TM1-TM4), and TM2 is believed to line the ion channel (Karlin, 2002). Despite the difference in ion selectivity, there is notable homology within the ion-conducting TM2 domain of anion-selective and cation-selective LGICs. For instance, approximately 30% of residues in the cytoplasmic aspect of the channel are identical or highly conserved among subunits of nicotinic, 5-HT_{3A}, $GABA_A$ and glycine receptors.

It has been known for decades that the convulsant picrotoxin (PTX) is an antagonist of central nervous system GABA (later identified as $GABA_A$) receptors (Takeuchi and Takeuchi, 1969; Constanti, 1978; Barker et al., 1983). In more recent years, PTX has also been found to inhibit Cl^- flux in glycine (Schmieden et al. 1989), $GABA_c$ (Zhang et al., 1995), and Glu-Cl receptors (Etter et al., 1999). Although the exact binding site of picrotoxin is not known, there is strong evidence indicating that PTX acts at the cytoplasmic aspect of the TM2 domain. Thus, while picrotoxin appears to be an effective antagonist in all anion-selective members of the LGIC family, its ability to block cation-selective 5-HT_3 channels is untested.

In the present study, we report that picrotoxin inhibits 5-HT_{3A} receptors in a non-competitive fashion. Moreover, PTZ and U-93631, ligands that act at a domain similar to picrotoxin in anion-selective channels, also inhibited this receptor. The mechanism of inhibition by picrotoxin in 5-HT_{3A} receptors is similar to that observed in $GABA_A$ receptors. Thus, the domain of action of PTX in 5-HT_{3A} receptors is likely similar to that in anion-selective channels. Our results further demonstrate the lack of specificity of picrotoxin, and extend this lack of specificity to a cation-selective member of the ligand-gated ion channel superfamily. Part of this work has been presented in abstract form (Das et al., 2000).

Electrophysiological recordings. The conventional whole cell configuration of the patch clamp technique was used to study 5-HT-activated currents. 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): 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). To monitor the possibility that access resistance changed over time or during experimental conditions, at the initiation of each recording we measured and stored on our digital oscilloscope the current response to a 5 mV voltage pulse. This stored trace was continually referenced throughout the recording. 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, PTZ or U-93631) was dissolved in the external solution (above) and applied to the target cell through a Y tube

positioned within 100 μm of the cell. An EC_{20} concentration of 5-HT was chosen to gate the channel in most experiments evaluating the effects of antagonist, because it activated a stable current without inducing receptor desensitization. Once a stable 5-HT gated current was established, the antagonist at varying concentrations was co-applied with 5-HT to the target cell. Recovery from PTX was generally achieved (except at high PTX concentrations, see Results). In experiments where the effects of pre-incubation of PTX were assessed, a stable 5-HT current was established and then cells were bathed in a solution containing PTX (100 μM) for 3 minutes. PTX and 5-HT were then co-applied to the target cell.

Chemicals. 5-HT, PTX and PTZ were obtained from Sigma (St. Louis, MO). U-93631 was a generous gift from Pharmacia (Kalamazoo, MI). 5-HT stocks were made in ultrapure H_2O. PTX, PTZ and U-93631 were made in DMSO and diluted in the above extracellular saline solution so that the final DMSO concentration (v/v) was < 0.3%.

Data analysis. Inhibition-response relationships for PTX, PTZ and U-93631 were fitted with the equation: $I / I_{max} = 1/\{1 + (IC_{50}/[DRUG])^n\}$, where I is the current amplitude normalized to control, DRUG is the test drug, 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 (typically 4-8) was conducted for each paradigm.

To analyze the pharmacologic mechanism of PTX-mediated inhibition of $5\text{-}HT_{3A}$ receptors, 5-HT concentration-response profiles in the presence and absence of PTX were constructed. At low concentrations of 5-HT, PTX had comparable effects on peak and

steady-state currents. At high concentrations of 5-HT, PTX had minimal effects on peak current amplitude but substantially enhanced current decay. In all cases, 5-HT current amplitudes with and without PTX were quantified at the end of the 5-HT application for concentration-response profiles. We refer to these curves as semi-equilibrium concentration-response profiles. Whole-cell currents were fitted to the following equation: $I/I_{max}= 1/\{1 + (EC_{50}/[5\text{-HT}])^n\}$, where I and I_{max} represent the normalized 5-HT-induced current at a given concentration and the maximum current induced by a saturating [5-HT], EC_{50} is the half-maximal effective 5-HT concentration, and n is the Hill coefficient. In some experiments, the time constant (τ) for PTX-induced current decay was obtained by fitting a mono-exponential function to time course-current profiles with the aid of a computer software program (Origin 5.0, Microcal Software). All data were presented as mean ± S.E.M. Statistical significance was tested using paired or unpaired Student's t-test where $p<0.05$ represented significance.

RESULTS

All studies were conducted on 5-HT_{3A} receptors expressed in HEK293 cells. To compare the effects of PTX, PTZ and U-93631 on the 5-HT_{3A} receptor (below), it was necessary to activate the receptors with equipotent concentrations of 5-HT. Thus, a 5-HT concentration-response curve (0.3 to 30 μM) was initially generated. 5-HT-activated currents could be blocked by MDL 72222, a selective 5-HT_3 receptor antagonist. The EC_{50} for 5-HT was 1.2 ± 0.06 μM, and the Hill-coefficient was 1.9 ± 0.19 (n=4). These values are consistent with those previously reported for recombinant 5-HT_{3A} receptors expressed in HEK293 cells (Hanna et al., 2000).

PTX-induced inhibition of 5-HT-activated currents in HEK 293 cells expressing the murine 5-HT_{3A} receptor. The effect of picrotoxin on recombinant 5-HT_{3A} receptors is shown in Fig. 1. When co-applied with 0.6 μM 5-HT (EC_{20} concentration), PTX inhibited 5-HT currents in a concentration-dependent manner (Fig. 1A). In all concentration-response profiles, application of PTX was not cumulative. At lower concentrations, the effects of PTX were easily washed out and thus current amplitude could be fully restored to control levels for subsequent testing of a different PTX concentration. At higher concentrations of PTX (>30 μM), washout was typically slow and thus full recovery of control current amplitude was not always achieved. When recovery was not achieved, the patch was aborted and a new whole-cell recording was obtained. Differences in current amplitude between cells were normalized to the control

5-HT response. The IC_{50} for PTX-mediated inhibition of 5-HT-gated currents was 30 ± 3.1 μM (Fig. 1B).

To gain additional insight into the rate at which PTX blocks $5\text{-}HT_{3A}$ receptors, 5-HT (0.6 μM) was bath-applied to induce a stable current, and then PTX (30 or 100 μM) and 5-HT were co-applied to the agonist-bound receptors. Application of 30 μM PTX caused 5-HT currents to decay mono-exponentially, with a mean time constant τ of 2.8 ± 0.5 s (Fig 1C). As expected, the PTX-induced current decay rate was significantly enhanced ($\tau = 1.4 \pm 0.2$ s, $p < 0.05$) in response to application of 100 μM PTX. Thus, PTX-induced current decay is concentration-dependent and equilibrates within seconds.

Non-competitive blockade produced by picrotoxin in the $5\text{-}HT_{3A}$ receptor. In $GABA_A$ receptors, the mechanism of PTX inhibition is generally defined as noncompetitive (Yakushiji et al., 1987; Yoon et al., 1993), although a competitive component of block may also be present (Krishek et al., 1996). To elucidate the mechanism of block by PTX in $5\text{-}HT_{3A}$ receptors, semi-equilibrium 5-HT concentration-response curves were created in the absence and presence of PTX. Fig. 2A shows typical responses to application of 1, 3 and 10 μM 5-HT in the absence and in the presence of 300 μM PTX. In the presence of 100 and 300 μM PTX, there was a depression of the maximal response without any significant change in the apparent affinity of 5HT for the receptor (Fig. 2B). These data demonstrate that picrotoxin-mediated inhibition of $5\text{-}HT_{3A}$ receptors is non-competitive.

Use-facilitated blockade by picrotoxin of the $5\text{-}HT_{3A}$ receptor. PTX-mediated inhibition of $GABA_A$ and Glu-Cl receptors is largely dependent on channel opening

(Newland and Cull-Candy, 1992; Dillon et al., 1995; Etter et al., 1999), a phenomenon referred to as use-dependence or use-facilitation. In contrast, no significant use-facilitation of PTX inhibition is observed in glycine α1 receptors (Lynch et al., 1995; Dibas et al., 2002). We used two different methods to analyze whether PTX-mediated inhibition of 5-HT_{3A} receptors is use-facilitated. First, receptors were incubated with PTX following establishment of a control 5-HT current. If PTX can gain access to its binding site in the closed state, then peak 5-HT currents following this incubation period should be greatly decreased in amplitude compared to control currents. Because of the slow activation kinetics of 5-HT_{3A} receptors, we used 3 μM 5-HT to conduct these experiments. When 100 μM PTX was co-applied with 5-HT, peak current amplitude was similar (91 ± 3% of control) to that observed with 5-HT alone. When receptors were incubated with 100 μM PTX (3 min), the amplitude of the subsequent response to 3 μM 5-HT was not significantly different (83 ± 4% of control) from that observed with no PTX incubation (Fig. 3). Moreover, the effects of PTX on steady-state current amplitudes were comparable with or without PTX incubation (Fig. 3B). From these experiments, it is evident that PTX binds poorly in the absence of agonist and that the binding site for PTX becomes accessible upon the binding of 5-HT to the receptor. Thus, similar to the $GABA_A$ receptor, PTX blockade of the 5-HT_{3A} receptor is use-facilitated.

In a second approach to study use-facilitation in the 5-HT_{3A} receptor, we examined whether the kinetics for onset of block produced by PTX were dependent on 5-HT gating concentration. 5-HT (0.3 or 0.6 μM) was bath-applied until a steady-state current was obtained. Both concentrations of 5-HT elicited stable currents that did not desensitize

during the bath application period. 5-HT and PTX were then co-applied to the agonist-bound receptor, and the rate of block of these currents by PTX was determined. A typical experiment is illustrated in Fig. 4. When 100 μM PTX was co-applied with 0.3 μM 5-HT to the 0.3 μM 5-HT-bound receptor, currents decayed mono-exponentially until an equilibrium block was reached. Analysis of the decaying currents yielded a time constant for PTX block of 2.7 ± 0.6 s. When receptors were first equilibrated with 0.6 μM 5-HT, which significantly enhances channel open probability, the rate of block produced by 100 μM PTX was enhanced significantly (to 1.4 ± 0.2 s, $p < 0.05$). A similar 5-HT concentration-dependent enhancement of PTX-induced decay was observed when 30 μM PTX was assessed (data not shown). These data further illustrate that PTX inhibition of the 5-HT_{3A} receptor is use-facilitated.

Inhibition of the 5-HT_{3A} receptor by the picrotoxin-site ligands pentylenetetrazole and U-93631. To gain some insight into the site of picrotoxin action in 5-HT_{3A} receptors, we evaluated the effects of two other ligands, pentylenetetrazole and U-93631, that are known to interact at a domain comparable to that of picrotoxin in $GABA_A$ receptors (Ramanjaneyulu and Ticku, 1984; Dillon et al., 1995; Bell-Horner et al., 2000; Huang et al., 2001). As shown in Fig. 5, both PTZ and U-93631 also inhibited the 5-HT-gated currents in HEK293 cells expressing 5-HT_{3A} receptors. The PTZ IC_{50} was 3.7 ± 0.6 mM when tested against an EC_{20} concentration of 5-HT (Fig. 5C). Due to both a small supply of U-93631 and solubility concerns, we did not construct a full concentration-response relationship for its actions on 5-HT_{3A} receptors. Assuming U-

93631 is also fully efficacious, its IC_{50} can be estimated to be approximately 50 μM. Thus, in addition to PTX, two other picrotoxin-site ligands also inhibit 5-HT_{3A} receptors.

DISCUSSION

The CNS convulsant picrotoxin is known to inhibit all anion-selective members of the ligand-gated ion channel superfamily. Our results demonstrate that the spectrum of channels that picrotoxin blocks can be extended to also include the 5-HT_{3A} receptor, a cation-selective member of the ligand-gated ion channel superfamily.

Following our initial observation that PTX blocks the 5-HT_{3A} receptor, a focus of subsequent studies was to more fully characterize the interaction of PTX action with these receptors. Our data demonstrate that the pharmacologic mechanism of PTX blockade of 5-HT_{3A} receptors is non-competitive inhibition[1]. In $GABA_A$ receptors, it is well established that PTX also blocks the channel non-competitively; however, a competitive component of PTX block exists in some preparations (Yakushiji et al., 1987; Yoon et al., 1993; Krishek et al., 1996). We observed no evidence of a competitive aspect of PTX inhibition of 5-HT_{3A} receptors. EC_{50} values for 5-HT were extremely consistent with 0, 100 μM and 300 μM PTX present, while current amplitude was decreased by PTX across the full 5-HT concentration-response curve. Our data are most consistent with the conclusion that PTX-mediated inhibition of 5-HT_{3A} receptors is likely occurring through a single site.

We also evaluated the possibility that channel state influences the actions of picrotoxin on 5-HT_{3A} receptors. In $GABA_A$ receptors, the association of PTX with its

binding site is minimal in the absence of channel opening (Newland and Cull-Candy, 1992; Dillon et al., 1995). This use-facilitation of PTX blockade is not observed in glycine receptors (Lynch et al., 1995; Dibas et al., 2002). Our data demonstrate that PTX-mediated inhibition of 5-HT_{3A} receptors is use-facilitated. This is based on the results from two studies. First, incubation of 5-HT_{3A} receptors with PTX had no effect on subsequent 5-HT currents. If PTX was accessing its binding site in the closed channel state, then its incubation with the receptors should have diminished the amplitude of the following 5-HT-activated currents. Second, the interaction of PTX with 5-HT_{3A} receptors was dependent on 5-HT gating concentration. Bath application of both 0.3 μM and 0.6 μM 5-HT elicited stable, non-desensitizing currents. The rate of block by PTX was two-fold more rapid at the higher gating concentration. This corresponds roughly to the increase in channel open probability observed with 0.6 μM compared to 0.3 μM 5-HT. Thus, mechanistically, the action of picrotoxin in 5-HT_{3A} receptors more closely resembles that observed in $GABA_A$ receptors than that seen in glycine receptors. This suggests the possibility of a conserved site of action for PTX in $GABA_A$ and 5-HT_{3A} receptors.

If the site of action of PTX in 5-HT_{3A} receptors were in fact comparable to its site in $GABA_A$ receptors, then other ligands that bind at the PTX site in $GABA_A$ receptors would presumably also inhibit 5-HT_{3A} receptors. Both the CNS convulsant PTZ and the novel molecule U-93631, which are known to interact at the picrotoxin site of the $GABA_A$ receptor (Dillon et al., 1995; Bell-Horner et al., 2000; Huang et al., 2001), also

[1] Preliminary data (Das et al., 2000) suggested a possible competitive inhibition by PTX. Subsequent

inhibited 5-HT_{3A} receptor currents. It is also worth noting that, for all three ligands, the estimated potency at 5-HT_{3A} receptors compared to $GABA_A$ receptors is reduced approximately five to ten-fold (Dillon et al., 1993; Bell-Horner et al., 2000; Huang et al., 2001). Taken together, the above findings are consistent with the suggestion that the site of action of picrotoxin is generally conserved in the two receptors.

In a recent abstract, Erkkila et al. (2001) reported that picrotoxin inhibits $\alpha 3\beta 4$ nicotinic acetylcholine receptors, which are also cation-selective. The reported IC_{50} was comparable (60 μM) to that we obtained in 5-HT_{3A} receptors, and the pharmacologic mechanism was also noncompetitive inhibition. The results of Erkkila et al. (2001) are consistent with ours, and suggest picrotoxin may inhibit other cation-selective members of the ligand-gated ion channel superfamily.

There is considerable evidence indicating that PTX acts in anion-selective LGICs at the TM2 domain of the receptor (ffrench-Constant et al., 1993; Gurley at al., 1995; Etter et al., 1999; Buhr et al., 2001; Shan et al., 2001; Dibas et al., 2002). Residues in the cytoplasmic aspect of TM2 have been shown to influence PTX sensitivity in several receptors (Gurley et al., 1995; Etter et al., 1999; Buhr et al., 2001; Shan et al., 2001), although residues toward the extracellular aspect of TM2 can also affect sensitivity to PTX (Perret et al., 1999; Dibas et al., 2002). Whether or not any of these candidate residues, some of which are conserved in 5-HT_{3A} receptors, are responsible for PTX-mediated inhibition of 5-HT_{3A} receptors awaits further investigation.

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detailed analysis reported here demonstrates the PTX-mediated inhibition is non-competitive.

In summary, the present results demonstrate that, in addition to blocking anion-selective channels of the ligand-gated ion channel superfamily, picrotoxin also inhibits the cation-selective 5-HT_{3A} receptor. The similarity in mechanism of block, sensitivity to other picrotoxin-site ligands, and the presence of amino acids known to be involved in picrotoxin action in anionic channels suggests its site of action may be conserved in the two classes of receptors.

FIGURE LEGENDS

FIGURE II-1 Picrotoxin-Mediated Inhibition of 5-HT_{3A} Receptors. *A,* Typical response of 5-HT-gated currents to the $GABA_A$ receptor antagonist picrotoxin. Picrotoxin inhibited 5-HT-activated currents in a concentration-dependent manner. Recovery from PTX was readily achieved at low PTX concentrations, but was relatively slow at PTX concentrations greater than 30 μM. ***B,*** Mean effect of picrotoxin in recombinant murine 5-HT_{3A} receptors (n = 4-6 at each data point). The IC_{50} for PTX was 30 ± 3.1 μM and the Hill-coefficient was 1.1 ± 0.12. The potency of picrotoxin is roughly 5-10 fold lower than that observed under similar conditions in $GABA_A$ receptors. ***Ca,*** To determine kinetic constants of interaction of PTX with the 5-HT_{3A} receptor, individual cells were activated by bath-application of 0.6 μM 5-HT. PTX at 30 and 100μM concentrations was then co applied to the cells along with 5-HT. Decaying currents were fitted with a mono-exponential time constant τ. The time course for onset of block produced by 100 μM PTX was significantly faster than that produced at 30 μM PTX ($p < 0.05$, n = 4). ***Cb,*** Graph showing the mean effect on 5-HT current decay rate obtained with 30 and 100 μM PTX.

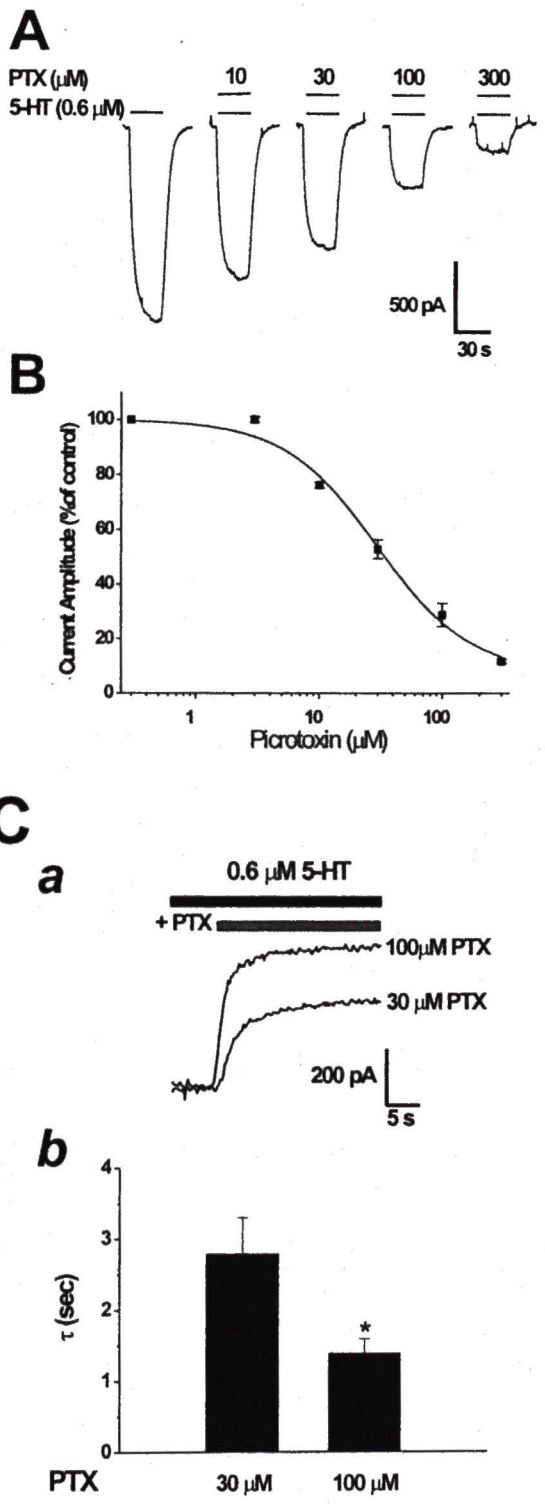

A
PTX (μM)
5-HT (0.6 μM)
10
30
100
300
500 pA
30 s
B
Current Amplitude (%of control)
100
80
60
40
20
0
1
10
100
Picrotoxin (μM)
C
a
0.6 μM 5-HT
+ PTX
100μM PTX
30 μM PTX
200 pA
5 s
b
τ (sec)
4
3
2
1
0
*
PTX
30 μM
100 μM

FIGURE II-2 Non-Competitive Inhibition by PTX of the 5-HT_{3A} Receptor. ***A,*** Typical recordings illustrating comparable inhibition by 300 μM PTX of 1, 3 and 10 μM 5-HT-gated currents. Whereas peak currents are relatively unaffected by PTX at high 5-HT concentrations, current amplitudes at the end of 5-HT application are comparably reduced regardless of 5-HT gating concentration. ***B,*** 5-HT concentration curves were obtained in absence and presence of 100 and 300 μM PTX (n = 4-6 at each data point). The graph was constructed by normalizing the current amplitude at the end of ligand application with or without PTX present. The EC_{50} value in absence of PTX was 0.73 ± 0.07 μM. In the presence of picrotoxin, there was a concentration-dependent depression of the maximum current amplitude with no significant change in the EC_{50} value (0.70 ± 0.07 and 0.74 ± 0.04 μM in 100 and 300 μM PTX, respectively).

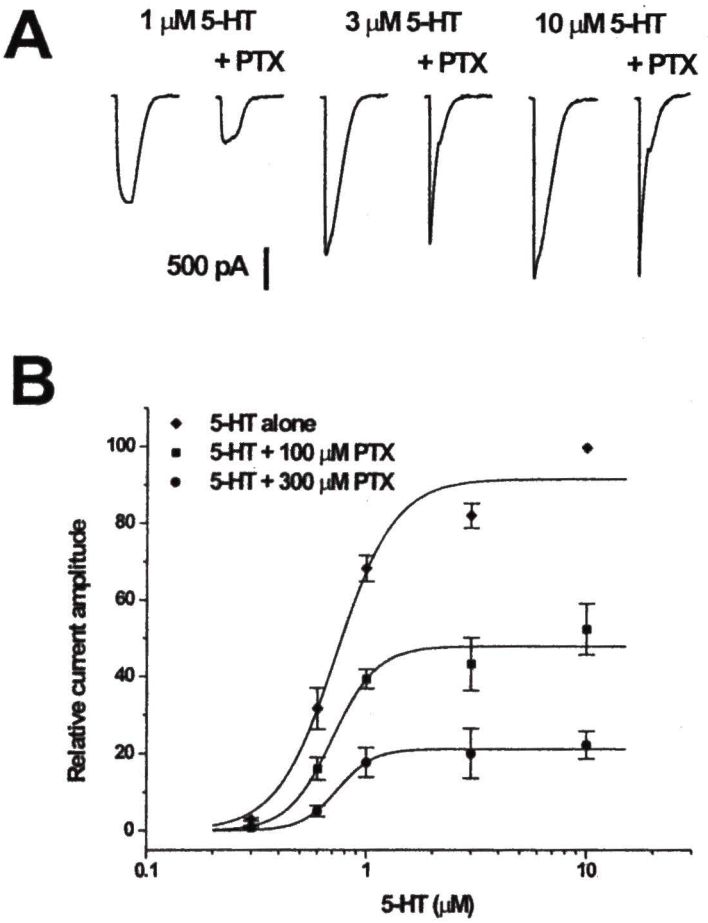

A
1 μM 5-HT
+ PTX
3 μM 5-HT
+ PTX
10 μM 5-HT
+ PTX
500 pA
B
5-HT alone
5-HT + 100 μM PTX
5-HT + 300 μM PTX
Relative current amplitude
100
80
60
40
20
0
0.1
1
10
5-HT (μM)

FIGURE II-3 Use-Facilitated Blockade by Picrotoxin of the $5HT_{3A}$ Receptor. A*a*, Example of typical responses obtained in absence of pre-incubation with PTX. When 100 μM PTX was co-applied with 3 μM 5-HT, it had minimal effect on peak current amplitude while greatly enhancing current decay. ***Ab***, When receptors were pre-incubated with PTX (3 min), peak amplitude of the subsequent 5-HT current was not significantly different from that obtained in absence of pre-incubation ($p = 0.14$, $n = 4$, unpaired Student's t-test). In addition, current amplitudes at the end of the ligand application period were not significantly different with or without pre-incubation of PTX ($p = 0.3$, $n = 4$, unpaired Student's t-test). ***B,*** Mean results for the experiments illustrated in ***A,*** where peak represents initial peak current amplitude and final represents current amplitude at the conclusion of the 5-HT + PTX application.

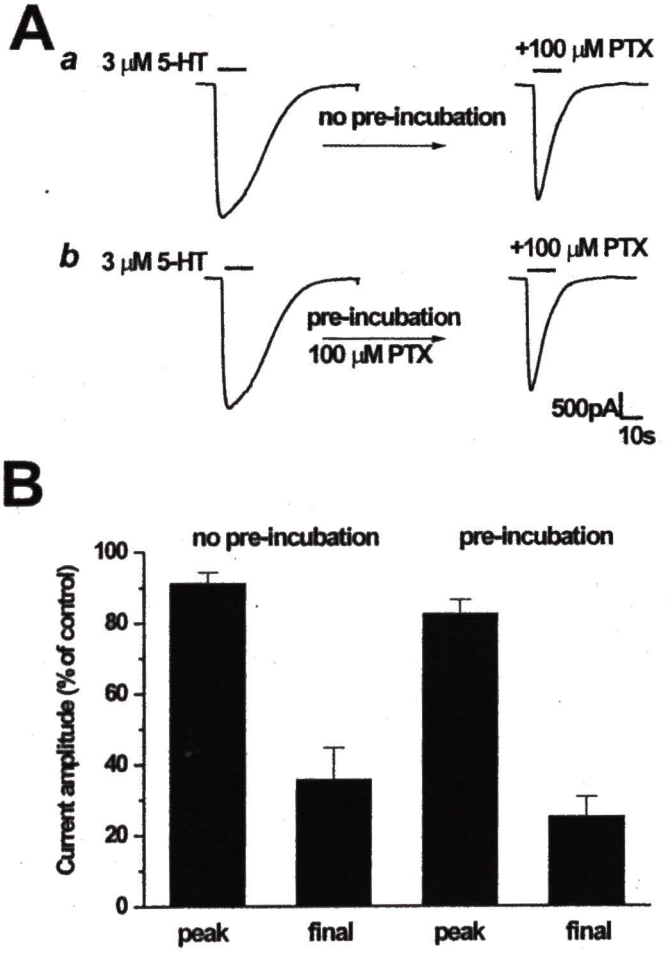

A
a
3 μM 5-HT
+100 μM PTX
no pre-incubation
b
3 μM 5-HT
+100 μM PTX
pre-incubation
100 μM PTX
500pA
10s
B
no pre-incubation
pre-incubation
Current amplitude (% of control)
100
80
60
40
20
0
peak
final
peak
final

FIGURE II-4 Dependence of Serotonin Concentration on the Rate of Block Produced by PTX. ***A and B,*** Example of records obtained with bath-application of two gating concentrations of 5-HT and then co-application of 100 μM PTX with 5-HT. **C,** The two traces from ***A*** and ***B*** have been normalized in scale and overlaid to better illustrate the differences in rate of block. Decaying currents were fitted to a mono-exponential time constant τ. The rate of block produced by PTX when channels were gated with 0.6 μM 5HT was significantly enhanced as compared to the block produced in presence of 0.3 μM 5HT. ***D,*** Mean results illustrating rate of current decay in response to 100 μM PTX when channels are gated with 0.3 μM (n = 5) or 0.6 μM (n = 4).

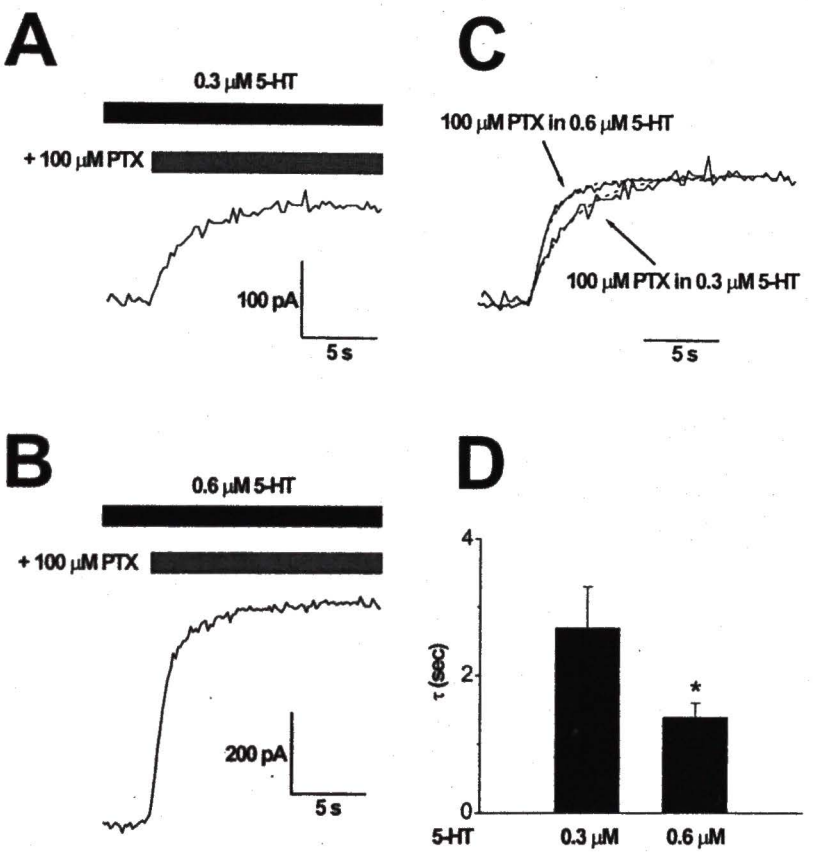
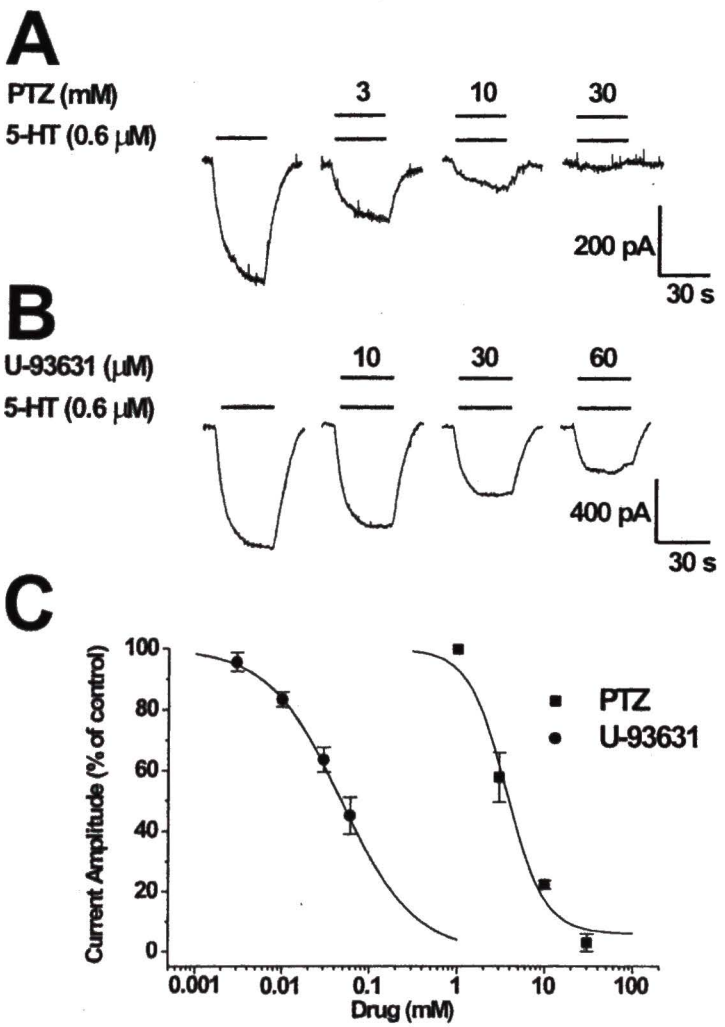

A
0.3 μM 5-HT
+ 100 μM PTX
100 pA
5 s
B
0.6 μM 5-HT
+ 100 μM PTX
200 pA
5 s
C
100 μM PTX in 0.6 μM 5-HT
100 μM PTX in 0.3 μM 5-HT
5 s
D
τ (sec)
4
2
0
*
5-HT
0.3 μM
0.6 μM

FIGURE II-5 Inhibition of the 5-HT_{3A} Receptor by the Picrotoxin-site Ligands PTZ and U-93631. ***A, B,*** Typical traces demonstrating inhibition of 5-HT currents by picrotoxin-site ligands PTZ and U-93631. Both drugs blocked the receptor in a concentration-dependent and reversible manner. ***C,*** Mean-concentration response profiles obtained for PTZ and U-93631 in HEK293/5-HT_{3A} cells (n = 4-7 cells at each concentration). The IC_{50} for PTZ mediated inhibition was 3.7 ± 0.6 mM and the Hill-coefficient was 1.8 ± 0.5 whereas the IC_{50} for U-93631 mediated was estimated to be approximately 50 μM.


A
PTZ (mM)
5-HT (0.6 μM)
3
10
30
200 pA
30 s
B
U-93631 (μM)
5-HT (0.6 μM)
10
30
60
400 pA
30 s
C
Current Amplitude (% of control)
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
Drug (mM)
PTZ
U-93631

REFERENCES

Barker, J.L., McBurney, R.N., and Mathers, D.A., 1983. Convulsant-induced depression of amino acid responses in cultured mouse spinal neurones studied under voltage clamp. British Journal of Pharmacology 80, 19-629.

Bell-Horner, C.L., Dibas, M.I., Huang, R.Q., Drewe, J.A., and Dillon, G.H., 2000. Influence of subunit configuration on the interaction of picrotoxin site ligands with recombinant $GABA_A$ receptors. Molecular Brain Research 76, 47-55.

Buhr, A., Wagner, C., Fuchs, K., Sieghart, W., and Siegel, E., 2001. Two novel residues in M2 of the γ-aminobutyric acid type A receptor affecting gating by GABA and picrotoxin affinity. Journal of Biological Chemistry 276, 7775-7781.

Chen, C., and Okayama, H., 1987. High efficiency transformation of mammalian cells by plasmid DNA. Molecular Cell Biology 7, 2745-2750.

Constanti, A., 1978. The mixed effect of picrotoxin on the GABA dose/conductance relation recorded in lobster muscle. Neuropharmacology 17, 159-167.

Das, P., Machu, T.K., and Dillon, G.H., 2000. Picrotoxin is a competitive antagonist of 5-HT_{3A} receptors. Society for Neuroscience Abstracts 26, 811.

Davies, P.A., Pistis, M., Hanna, M.C., Peters, J.A., Lambert, J.J., Hales, T.G., and Kirkness, E.F., 1999. The 5-HT_{3B} subunit is a major determinant of serotonin receptor function. Nature 397, 359-363.

Dibas, M.I., Gonzales, E.B., Das, P., Bell-Horner, C.L., and Dillon, G.H., 2002. Identification of a key residue within the second transmembrane domain that confers use-facilitated block by picrotoxin in glycine α1 receptors Journal of Biological Chemistry 277, 9112-9117.

Dillon G.H., Im, H.K., Hamilton, B.J., Carter, D.B., Gammill, R.B., and Im, W.B., 1993. U-93631 causes rapid decay of GABA-induced Cl^- current in recombinant rat $GABA_A$ receptors. Molecular Pharmacology 44, 860-865.

Dillon, G.H., Im, W.B., Carter, D.B., and McKinley, D.D., 1995. Enhancement by GABA of the association rate of picrotoxin and *tert*-butylbicyclophosphorothionate to the rat cloned α1ß2γ2 $GABA_A$ receptor subtype. British Journal of Pharmacology 115, 539-545.

Dubin, A.E., Huvar, R., Andrea, M.R., Pyati, J., Zhu, J.Y., Joy, K.C., Wilson, S.J., Galindo, J.E., Glass, C.A., Luo, L., Jackson, M.R., Lovenberg, T.W., and Erlander, M.G., 1999. The pharmacological and functional characteristics of the serotonin $5\text{-}HT_{3A}$ receptor are specifically modified by the $5\text{-}HT_{3B}$ receptor subunit. Journal of Biological Chemistry 274, 30799-30810.

Erkkila, B.E., Wotring, V.E., and Weiss, D.S., 2001. Picrotoxin inhibits α3β4 nicotinic acetylcholine receptors. Society for Neuroscience Abstracts, 27, 35.16.

Etter, A., Cully, D.F., Liu, K.K., Reiss, B., Vassilatis, D.B., Schaeffer, J.M., and Arena, J.P., 1999. Picrotoxin blockade of invertebrate glutamate gated chloride channels: subunit dependence and evidence for binding within the pore. Journal of Neurochemistry 72, 318-326.

ffrench-Constant, R.H., Rocheleau, T.A., Steichen, J.C., and Chalmers, A.E., 1993. A point mutation in a *Drosophila* GABA receptor confers insecticide resistance. Nature 363, 449-451.

Gurley, D.J., Amin, P.C., Ross, D.S., Weiss, D.S., and White, G.W., 1995. Point mutations in the M2 region of the α, β, or γ subunit of the $GABA_A$ channel that abolish block by picrotoxin. Receptors and Channels 3, 12-30.

Hanna, M.C., Davies, P.A., Hales, T.G., and Kirkness, E.F., 2000. Evidence for expression of heteromeric serotonin 5-HT_3 receptors in rodents. Journal of Neurochemistry 75, 240-247.

Huang, R.Q., Bell-Horner, C.L., Dibas, M.I., Covey, D.F., Drewe, J.A., and Dillon, G.H., 2001. Pntylenetetrazole induced inhibition of recombinant gamma-aminobutyric acid type A receptors: mechanism and site of action. Journal of Pharmacology and Experimental Therapeutics 298, 986-995.

Karlin, A., 2002. Emerging structure of the nicotinic acetylcholine receptors. Nature Reviews Neuroscience 3, 102-114.

Krishek, B.J., Moss, S.J., and Smart, T.G., 1996. A functional comparison of the antagonists bicuculline and picrotoxin at recombinant $GABA_A$ receptors. Neuropharmacology 35, 1289-1298.

Lynch, J.W., Rajendra, S., Bany, P.H., and Schofield, P.R., 1995. Mutations affecting the glycine receptor agonist transduction mechanism convert the competitive antagonist, picrotoxin, into an allosteric potentiator. Journal of Biological Chemistry 270, 13799-13806.

Maricq, A.V., Peterson, A.S., Brake, A.J., Myers, R.M., and Julius, D., 1991. Primary structure and functional expression of the 5-HT_3 receptor, a serotonin-gated ion channel. Science 254, 432-437.

Newland, C.F., and Cull-Candy, S.G., 1992. On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurons of the rat. Journal of Physiology 447, 191-231.

Perret, P., Sarda, X., Wolff, M., Wu, T.-T., Bushey, D., and Goeldner, M., 1999. Interaction of non-competitive blockers within the γ-aminobutyric acid type A chloride channel using chemically reactive probes as chemical sensors for cysteine mutants. Journal of Biological Chemistry 274, 25350-25354.

Ramanjaneyulu, R., and Ticku, M.K., 1984. Binding characteristics and interactions of depressant drugs with [^{35}S]t-butylbicyclophosphorothionate, a ligand that binds to the picrotoxinin site. Journal of Neurochemistry 42, 221-229.

Schmieden, V., Grenningloh, G., Schofield, P.R., and Betz, H., 1989. Functional expression in *Xenopus* oocytes of the strychnine binding 48 kd subunit of the glycine receptor. EMBO Journal 8, 695-700.

Shan, Q., Haddrill, J.L., and Lynch, J.W., 2001. A single β subunit M2 domain controls the picrotoxin sensitivity of αβ heteromeric glycine receptor chloride channels. Journal of Neurochemistry 76, 1109-1120.

Takeuchi, A., and Takeuchi, N., 1969. A study of the action of picrotoxin on the inhibitory neuromuscular junction of the crayfish. Journal of Physiology 205, 377-391.

Yakushiji, T., Tokutomi, N., Akaike, N., and Carpenter, D.O., 1987. Antagonists of GABA responses, studied using internally perfused frog dorsal root ganglion neurons. Neuroscience 22, 1123-1133.

Yoon, K.W., Covey, D.F., and Rothman, S.M., 1993. Multiple mechanisms of picrotoxin block of GABA induced currents in rat hippocampal neurons. Journal of Physiology 464, 423-439.

Zhang, H.G., ffrench-Constant, R.H., Brideau, A.D., and Lipton, S.A., 1995. Cloning of a γ-aminobutyric acid type $_C$ receptor subunit in rat retina with a methionine residue critical for picrotoxinin channel block. Proceedings of the National Academy of Sciences 92, 11756-11760.

In the previous chapter (chapter II), the mechanism of action of PTX in the homomeric 5-HT_{3A} receptors was explored. Similar to the anion-selective $GABA_A$ receptor, PTX was demonstrated to be a non-competitive inhibitor of the 5-HT_{3A} receptors. In addition, the block produced was also use-dependent. In the anionic receptors, PTX is known to exhibit subunit selective blockade. For instance, glycine$\alpha\beta$ heteromeric receptors are less sensitive to PTX than homomeric receptors formed by glycineα subunits alone (Pribilla et al., 1992). Whether, this is also true for the 5-HT_3 receptor is investigated in chapter III. In this chapter, the diminished effect of PTX upon co-expression of the 5-HT_{3A} and 5-HT_{3B} subunits is demonstrated.

(i) **THE 5-HT_{3B} SUBUNIT CONFERS REDUCED SENSITIVITY TO PICROTOXIN WHEN CO-EXPRESSED WITH THE 5-HT_{3A} RECEPTOR**

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(iv) Number of pages: 11
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Das, P., and Dillon, G.H., 2003. Molecular Brain Research 119(2), 207-212.

CHAPTER III

THE 5-HT_{3B} SUBUNIT CONFERS REDUCED SENSITIVITY TO PICROTOXIN WHEN CO-EXPRESSED WITH THE 5-HT_{3A} RECEPTOR

ABSTRACT

There are currently no known agents that display selectivity between homomeric 5-HT_{3A} and heteromeric 5-$HT_{3A/3B}$ receptors. In the present study, we show that the CNS convulsant picrotoxin selectively interacts with 5-HT_{3A} receptors. In whole-cell patch clamp recordings, the inhibitory effect of PTX was reduced one hundred-fold in heteromeric murine 5-$HT_{3A/3B}$ receptors, compared to homomeric 5-HT_{3A} receptors. Picrotoxin should prove to be a useful probe for determining the presence of homomeric vs. heteromeric 5-HT_3 receptors in both native tissue and recombinant receptor preparations.

Keywords: 5-HT_{3A}, 5-HT_{3B}, ligand-gated ion channel, picrotoxin

INTRODUCTION

The 5-hydroxytryptamine type 3 (5-HT_3) receptor is a member of the superfamily of ligand-gated ion channels (LGICs) that includes nicotinic acetylcholine receptors (nAChRs), $GABA_A$, $GABA_c$ and glycine receptors, and glutamate-gated chloride channels (Glu-Cl) [11]. Like other members of the LGIC superfamily, the 5-HT_3 receptor forms a pentameric assembly that surrounds a central ion channel. Functional 5-HT_3 receptors form as either homomeric receptors expressing 5-HT_{3A} subunits, or as heteromeric receptors incorporating both 5-HT_{3A} and 5-HT_{3B} subunits [5,12]. In comparison to homomeric 5-HT_{3A} receptors, heteromeric assemblies of 5-HT_{3A} and 5-HT_{3B} receptor subunits exhibit distinct biophysical properties, some of which mimic properties of neuronal 5-HT_3 channels [5,7].

Presence of the 5-HT_{3B} subunit also results in some changes in the pharmacology of 5-HT_3 receptors [5,7,8]. For instance, co-expression of the 5-HT_{3B} subunit with the 5-HT_{3A} subunit decreases sensitivity to the agonist 5-HT [5,7]. In addition, the antagonist tubocurarine has lower affinity in 5-$HT_{3A/3B}$ receptors compared to 5-HT_{3A} receptors [5,8]. In all reports to date, however, changes in ligand sensitivity caused by the presence of the 5-HT_{3B} subunit are modest (no greater than 5-fold difference in 3_A-expressing vs. $3_A/3_B$-expressing receptors). Indeed, Brady *et al.* [2] found no appreciable difference in the ability of thirteen ligands to displace [^{3}H]-granisetron in 5-HT_{3A} compared to 5-$HT_{3A/3B}$ receptors. Thus, no currently available ligands have been shown to display selectivity between the two receptor subtypes that is sufficient in magnitude to

be useful experimentally. Because the 5-HT_{3B} subunit transcripts (along with 5-HT_{3A} transcripts) are present in both central [5] and the peripheral nervous systems [14], an unambiguous way to pharmacologically identify heteromeric vs. homomeric receptors would be extremely advantageous.

Recently we have reported that the $GABA_A$ receptor antagonist picrotoxin inhibits the mouse 5-HT_{3A} receptor with μM affinity [4]. In the present study, we demonstrate that incorporation of the 5-HT_{3B} subunit drastically (approximately one hundred-fold) reduces sensitivity to picrotoxin-mediated inhibition. This dramatic decrease in sensitivity PTX in the 5-$HT_{3A/3B}$ receptors can be exploited to pharmacologically probe for the presence of homomeric versus heteromeric 5-HT_3 receptors in heterologous systems and neuronal cell lines or native tissue preparations.

RESULTS AND DISCUSSION

HEK293 cells were transiently transfected with the mouse 5-HT_{3A} cDNA (provided by Dr. D. Julius, University of California, San Francisco), or with mouse 5-HT_{3A} and 5-HT_{3B} (the 5-HT_{3B} subunit was a generous gift from Dr. E. Kirkness (Institute of Genomic Research, Maryland)) using the modified calcium phosphate method [3]. The cells were maintained and cultured as described previously [4]. The mouse 5-HT_{3A} and 5-HT_{3B} cDNA were subcloned into the vector pcDNA3.1. For co transfection of 5-HT_{3A} and 5-HT_{3B} subunits, a 1:1 ratio of respective cDNAs was used. Twenty-four hours after transfection, the cells were washed twice and used for recording subsequently. Whole-cell recordings were done as described previously [1]. Briefly, patch pipettes with resistance of 1-3 MΩ were filled with internal pipette solution containing (in mM): CsCl, 140; EGTA, 10; Mg^{2+}-ATP, 4; HEPES-Na, 10; pH 7.2. Recordings were made using an Axopatch 200A amplifier (Axon instruments, Foster City, CA) and data were stored on a computer using an online data acquisition system (pClamp 6.0, Axon Instruments). 5-HT (with or without PTX) was dissolved in external solution containing (in mM): NaCl, 125; KCl, 5.5; $CaCl_2$, 1.5; $MgCl_2$, 0.8; HEPES-Na, 20; glucose, 10; pH 7.3 and applied to the target cell through a Y tube positioned within 100 μm of the cell. We have shown previously that the 10 – 90% rise time of the junction potential averages 30 ms with this system [9]. All recordings were made at room temperature and all cells were clamped at –60 mV. Data analysis was performed as reported previously [6].

Initially, 5-HT concentration-response profiles were generated in HEK 293 cells expressing either mouse 5-HT_{3A} homomers or 5-$HT_{3A/3B}$ heteromers. Previous studies have reported roughly a two-fold decrease in affinity and loss of cooperativity for 5-HT in human [5] and rat heteromeric receptors [8]. We observed no appreciable difference in 5-HT sensitivity between the two receptor configurations in the present studies (EC_{50} = 1.2 ± 0.06 μM and 1.0 ± 0.07 μM for homomeric and heteromeric receptors, respectively). We did, however, observe a decrease in cooperativity in 5-$HT_{3A/3B}$ receptors (Hill coefficient = 1.06 ± 0.10) compared to 5-HT_{3A} receptors (Hill coefficient = 1.9 ± 0.19). In addition, the two receptor configurations showed striking differences in channel activation kinetics. A concentration of 0.6 μM 5-HT produced slowly activating currents in homomeric receptors (Fig. 1). The same 5-HT concentration elicited rapid activation kinetics in heteromeric 5-$HT_{3A/3B}$ receptors (Fig. 1A). This enhancement of activation kinetics by the presence of the 3B subunit has also been observed in rat and human 5-HT_3 receptors [5,7].

We have recently shown that picrotoxin, a classical $GABA_A$ receptor antagonist, inhibits the 5-HT_{3A} receptor [4]. We sought to examine if co-expression of the 5-HT_{3B} subunit would alter the PTX sensitivity of the 5-HT_{3A} receptor. Fig. 2 illustrates typical responses to PTX in 5-HT_{3A} (Fig. 2A) and 5-$HT_{3A/3B}$ receptors (Fig. 2B). Whereas 300 μM nearly completely abolished 5-HT_{3A} currents, the same concentration of PTX had negligible effects on 5-$HT_{3A/3B}$ currents. Fig. 2C shows the mean PTX inhibitory responses. The IC_{50} value for PTX in the 5-HT_{3A} receptor was 30 μM while that in the 5-

$HT_{3A/3B}$ receptor was greater than 3 mM. Thus, picrotoxin displays 100-fold selectivity for inhibition of 5-HT_{3A} receptors compared to 5-$HT_{3A/3B}$ receptors.

Picrotoxin-mediated inhibition of several channels, including the 5-HT_{3A} receptor, is facilitated when channels are gated with a higher concentration of the neurotransmitter [4, 6]. We thus assessed whether PTX inhibition of 5-$HT_{3A/3B}$ receptors might approach that seen in 5-HT_{3A} receptors if channels were activated with a near saturating concentration of 5-HT. As shown in Fig. 3, block of the heteromeric receptor by 100 μM PTX was modestly enhanced when channels were gated with 3 μM 5-HT, compared to 0.6 μM 5-HT; the degree of inhibition was still marginal compared to that observed in 5-HT_{3A} receptors (Fig. 2, also [4]). There was no significant difference in the degree of block induced by 1 mM PTX at the two different 5-HT gating concentrations (Fig. 3B). Thus, at both low and high gating concentrations of 5-HT, PTX-mediated inhibition of in 5-$HT_{3A/3B}$ receptors is minimal compared to that seen in 5-HT_{3A} receptors.

We considered the possibility that pre-incubating 5-$HT_{3A/3B}$ receptors with PTX may enhance the inhibitory effects of PTX. To test this, we pre-incubated receptors with PTX (1 mM, 3 min) before co-applying 5-HT and PTX. As illustrated in Fig. 4, 5-HT_3 currents were inhibited to a similar extent in cells pre-incubated with PTX compared to cells that were not initially exposed to PTX. Thus, as also seen in 5-HT_{3A} receptors [4], pre-incubation with PTX does not enhance its ability to inhibit 5-$HT_{3A/3B}$ receptors.

The ability of picrotoxin to inhibit murine 5-$HT_{3A/3B}$ receptors did not approach that seen in 5-HT_{3A} receptors under any of our experimental conditions. This is the first report of the existence of a ligand that displays selective interaction with homomeric 5-

HT_{3A} compared to heteromeric 5-$HT_{3A/3B}$ receptors (or vice versa). This ability of the 5-HT_{3B} subunit to confer resistance to picrotoxin is reminiscent of glycine receptor pharmacology. Like 5-HT_3 receptors, glycine receptors may form as either homomeric α-expressing receptors, or heteromeric $\alpha\beta$-expressing receptors. The IC_{50} for PTX in glycine receptors expressing only an α subunit is approximately 5-10 μM; incorporation of the glycine β subunit reduces by approximately 100-fold the sensitivity to picrotoxin [15]. This ability of PTX to selectively interact with homomeric vs. heteromeric glycine receptors has proven to be useful in assessing for expression of the two different glycine receptor configurations. For instance, the developmental switch from primarily glycine α homomeric to $\alpha\beta$ heteromeric receptors [13] can be detected by assessing PTX sensitivity [10,16].

There are conflicting reports regarding the presence of the 5-HT_{3B} subunit in the CNS. Initial reports detected 5-HT_{3B} transcripts in a number of CNS regions [5,7], while a recent study indicated 5-HT_{3B} subunits are present only in peripheral neurons [14]. In addition to lack of knowledge of regional expression of heteromeric 5-$HT_{3A/3B}$ receptors, possible developmental expression patterns of homomeric compared to heteromeric receptors have not been defined. Our data demonstrate that picrotoxin can be used as a pharmacological probe to detect expression of 5-HT_{3A} or 5-$HT_{3A/3B}$ serotonin receptors in native tissue and recombinant receptor preparations.

FIGURE LEGENDS

FIGURE III-1. The Mouse 5-HT_{3B} Subunit Modifies Activation Kinetics of the 5-HT_{3A} Receptor. A, Traces obtained upon application of 0.6 μM 5-HT in HEK 293 cells expressing either the 5-HT_{3A} subunit alone or both 5-HT_{3A} and 5-HT_{3B} subunits. Note the rapid activation kinetics in cells expressing the heteromeric receptors. **B,** Mean activation kinetics (measured as 10-90 % rise time of 5-HT-gated currents) in the 5-HT_{3A} and the 5-$HT_{3A/3B}$ receptors. The activation time was approximately 16-fold faster in the 5-$HT_{3A/3B}$ receptor as compared to the 5-HT_{3A} receptor. *** denotes $p < 0.001$ compared to 5-HT_{3A} receptors, Student's t-test, $n = 7$ for both receptor configurations. pA, current amplitude in picoamperes.

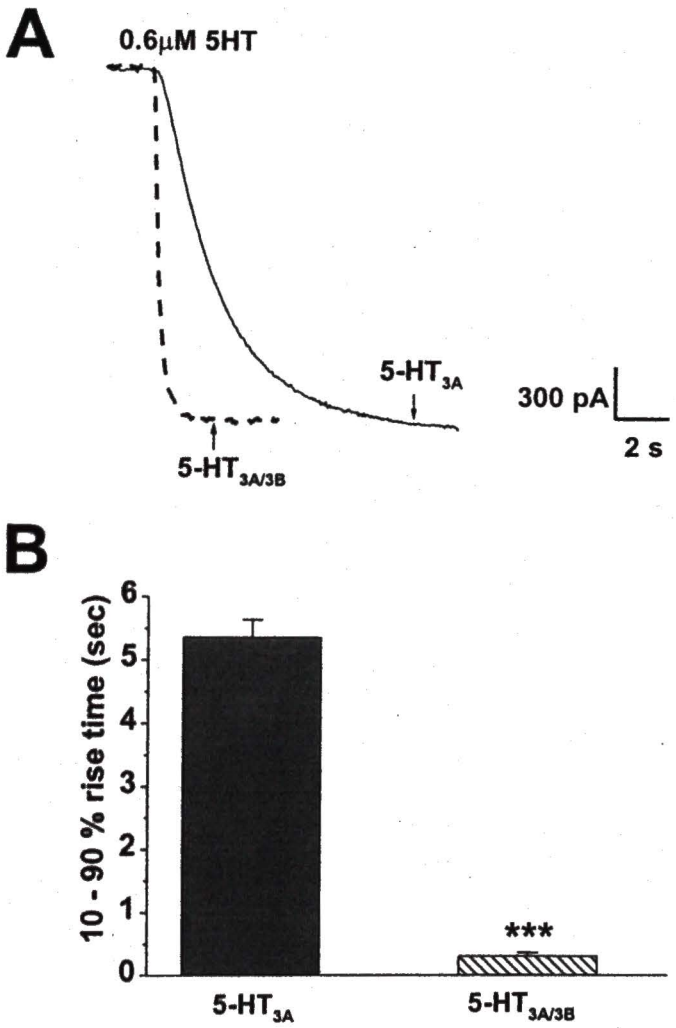

A
0.6μM 5HT
5-HT3A
300 pA
2 s
5-HT3A/3B
B
10 - 90 % rise time (sec)
6
5
4
3
2
1
0
***
5-HT3A
5-HT3A/3B

FIGURE III-2. Reduced Sensitivity to PTX in Heteromeric 5-$HT_{3A/3B}$ Receptors. **A**, Typical response of 5-HT-gated currents to picrotoxin in the 5-HT_{3A} receptor. Picrotoxin inhibited 5-HT-activated currents in a concentration-dependent manner. **B**, Traces obtained in the presence of PTX in the 5-$HT_{3A/3B}$ heteromeric receptors. The sensitivity to PTX is dramatically reduced in the 5-$HT_{3A/3B}$ receptor. **C**, Mean effect of picrotoxin in mouse 5-HT_{3A} receptor and 5-$HT_{3A/3B}$ receptor (n = 4-6 at each data point for each receptor configuration). The IC_{50} for PTX in 5-HT_{3A} receptors was 30 ± 3.1 μM and the Hill coefficient was 1.1 ± 0.12. Solubility concerns prevented evaluating the effects of PTX at concentrations greater than 3 mM. Nevertheless, assuming PTX is fully efficacious in 5-$HT_{3A/3B}$ receptors, the data points up to 3 mM PTX could be extrapolated to yield an IC_{50} for PTX in 5-$HT_{3A/3B}$ receptor of 3100 ± 300 μM, and a Hill coefficient of 0.9 ± 0.05.

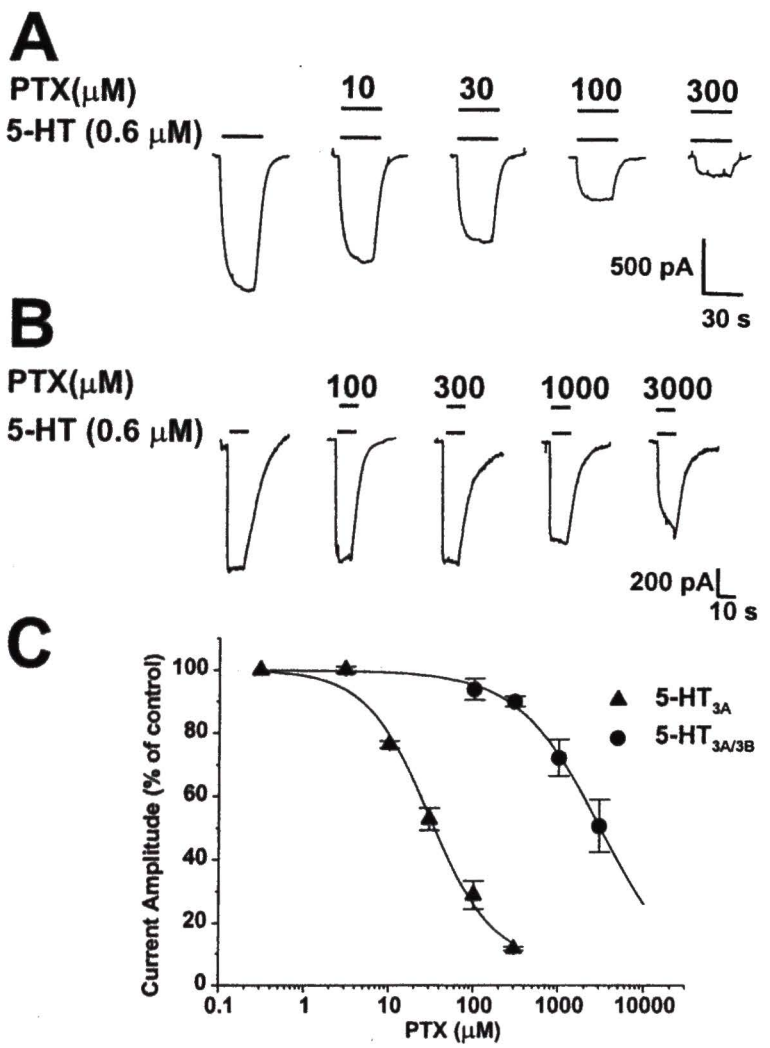

A
PTX(μM)
10
30
100
300
5-HT (0.6 μM)
500 pA
30 s
B
PTX(μM)
100
300
1000
3000
5-HT (0.6 μM)
200 pA
10 s
C
Current Amplitude (% of control)
100
80
60
40
20
0
0.1
1
10
100
1000
10000
PTX (μM)
5-HT3A
5-HT3A/3B

FIGURE III-3. Influence of 5-HT Gating Concentration on PTX Inhibition of 5-$HT_{3A/3B}$ Receptors. **A,** Typical response illustrating inhibitory effects of PTX on 5-$HT_{3A/3B}$ receptors when channels are gated with 0.6 μM 5-HT (A*a*) or 3 μM 5-HT (A*b*). **B,** Mean results from above experiments. The ability of 100 μM to block 5-$HT_{3A/3B}$ receptors was modestly enhanced when channels were gated with near saturating 5-HT (B*a*), whereas the response to 1 mM PTX was not significantly altered with the higher 5-HT gating concentration (B*b*). * denotes significantly different ($p < 0.05$, Student's t-test, n = 4-6 for each experimental condition) from value obtained with 3 μM 5-HT.

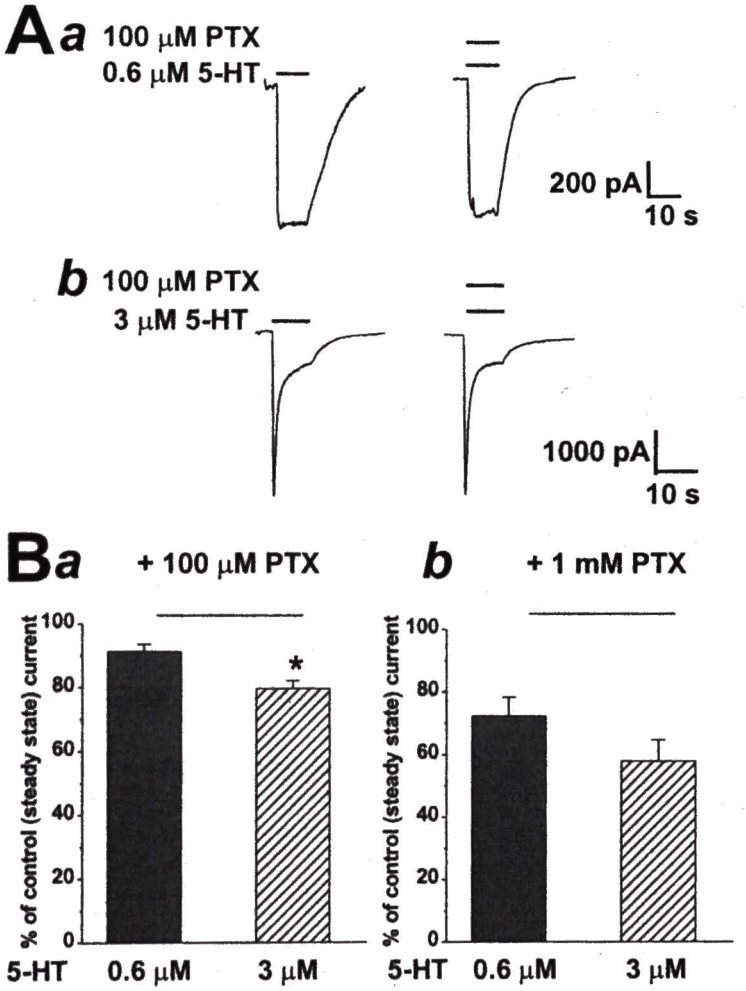
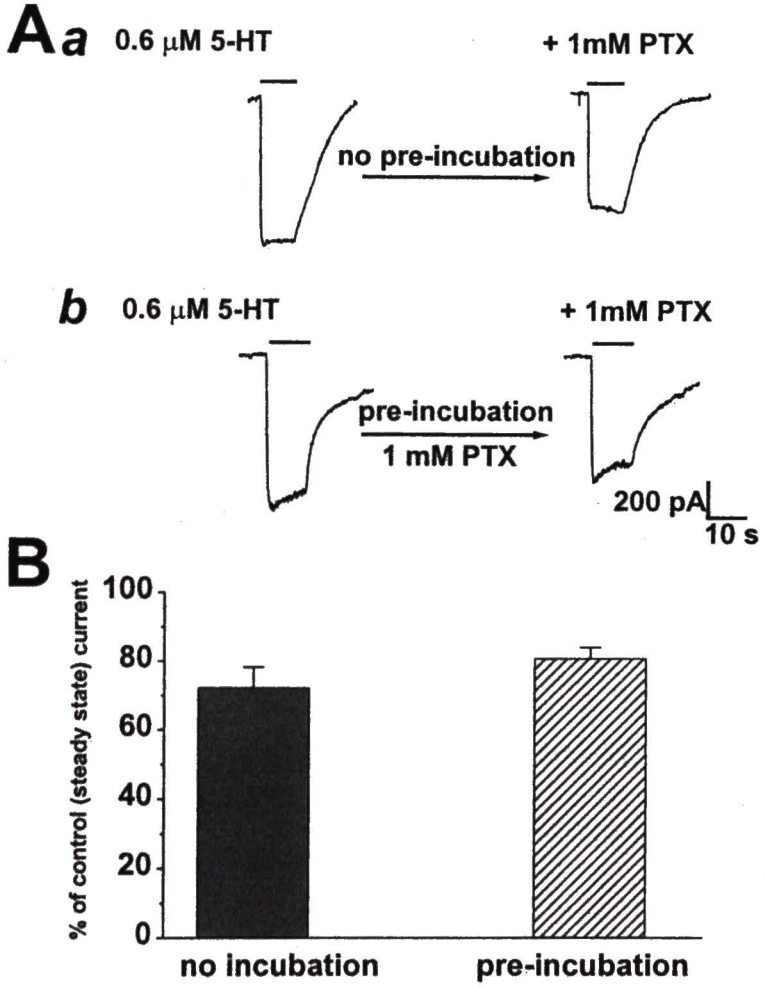

Aa
100 μM PTX
0.6 μM 5-HT
200 pA
10 s
b
100 μM PTX
3 μM 5-HT
1000 pA
10 s
Ba
+ 100 μM PTX
b
+ 1 mM PTX
% of control (steady state) current
100
80
60
40
20
0
*
5-HT
0.6 μM
3 μM
% of control (steady state) current
100
80
60
40
20
0
5-HT
0.6 μM
3 μM

FIGURE III-4. Effect of PTX Pre-exposure on PTX-mediated Inhibition of 5-$HT_{3A/3B}$ Receptors. **A**, Typical traces showing inhibitory effects of 1 mM PTX on 5-$HT_{3A/3B}$ receptors when cells were not pre-incubated with PTX (A*a*) compared to effects obtained when cells were pre-incubated with PTX (1 mM PTX, 3 min) prior to co-application of 5-HT and PTX. Calibration bars are for both sets of traces. **B**, Mean results of above experiments. The magnitude of inhibition seen with PTX was not different between those receptors pre-exposed to PTX and those not pre-exposed (n = 4 in both conditions, Student's t-test), and in neither case is the magnitude of inhibition near that seen in 5-HT_{3A} receptors (Fig. 2, also [4]).


A
a
0.6 μM 5-HT
+ 1mM PTX
no pre-incubation
b
0.6 μM 5-HT
+ 1mM PTX
pre-incubation
1 mM PTX
200 pA
10 s
B
% of control (steady state) current
100
80
60
40
20
0
no incubation
pre-incubation

REFERENCES

[1] C.L. Bell-Horner, M.I. Dibas, R.Q. Huang, Drewe J.A. and G.H. Dillon, Influence of subunit configuration on the interaction of picrotoxin site ligands with recombinant $GABA_A$ receptors, Mol. Brain Res. 76 (2000) 47-55.

[2] C.A. Brady, I.M. Stanford, I. Ali, L. Lin, J.M. Williams, A.E. Dubin, A.G. Hope and N.M. Barnes, Pharmacological comparison of human homomeric 5-HT_{3A} receptors versus heteromeric 5-$HT_{3A/3B}$ receptors, Neuropharmacology 41 (2001) 282-284.

[3] C. Chen and H. Okayama, High efficiency transformation of mammalian cells by plasmid DNA, Mol. Cell Biol. 7 (1987) 2745-2750.

[4] P. Das, C.L. Bell-Horner, T.K. Machu and G.H. Dillon, The $GABA_A$ receptor antagonist picrotoxin inhibits the 5-Hydroxytryptamine type 3A receptors, Neuropharmacology 44 (2003) 431-438.

[5] P.A. Davies, M. Pistis, M.C. Hanna, J.A. Peters, J.J. Lambert, T.G. Hales and E.F. Kirkness, The 5-HT_{3B} subunit is a major determinant of serotonin receptor function, Nature 397 (1999) 359-363.

[6] M.I. Dibas, E.B. Gonzales, P. Das, C.L. Bell-Horner and G.H. Dillon, Identification of a key residue within the second transmembrane domain that confers use-facilitated block by picrotoxin in glycine $\alpha 1$ receptors, J. Biol. Chem. 277 (2002) 9112-9117.

[7] A.E. Dubin, R. Huvar, M.R. Andrea, J. Pyati, J.Y. Zhu, K.C. Joy, S.J. Wilson, J.E. Galindo, C.A. Glass, L. Luo, M.R. Jackson, T.W. Lovenberg and M.G. Erlander,

The pharmacological and functional characteristics of the serotonin 5-HT_{3A} receptor are specifically modified by the 5-HT_{3B} receptor subunit, J. Biol. Chem. 274 (1999) 30799-30810.

[8] M.C. Hanna, P.A. Davies, T.G. Hales and E.F. Kirkness, Evidence for expression of heteromeric serotonin 5-HT_3 receptors in rodents, J. Neurochem. 75 (2000) 240-247.

[9] R.Q. Huang and G.H. Dillon, Effects of extracellular pH on GABA-activated current in rat recombinant receptors and thin hypothalamic slices. J. Neurophysiol. 82 (1999) 1233-1243.

[10] R.Q. Huang and G.H. Dillon, Direct inhibition of glycine receptors by genistein, a tyrosine kinase inhibitor, Neuropharmacology 39 (2000) 2195-2204.

[11] A. Karlin, Emerging structure of the nicotinic acetylcholine receptors, Nature Rev. Neurosci. 3 (2002) 102-114.

[12] A.V. Maricq, A.S. Peterson, A.J. Brake, R.M. Myers and D. Julius, Primary structure and functional expression of the 5-HT_3 receptor, a serotonin-gated ion channel, Science 254 (1991) 432-437.

[13] M.L. Malosio, B. Marqueze-Pouey, J. Kuhse and H. Betz, Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain, EMBO J. 10 (1991) 2401-2409.

[14] M. Morales and S.D. Wang, Differential composition of 5-hydroxytryptamine3 receptors synthesized in the rat CNS and peripheral nervous system, J. Neurosci. 22 (2002) 6732-6741.

[15] I. Pribilla, T. Takagi, D. Langosh, J. Bormann and H. Betz, The atypical M2 segment of β subunit confers picrotoxin resistance to inhibitory glycine receptor channel, EMBO J. 11 (1992) 4305-4311.

[16] J.C. Tapia and L.G. Aguayo, Changes in the properties of developing glycine receptors in cultured mouse spinal neurons, *Synapse* 28 (1998) 185-194.

In chapters II and III, the effect of PTX in homomeric 5-HT_{3A} and heteromeric 5-$HT_{3A/3B}$ have been illustrated respectively. The similarity in mechanism of action of PTX in the 5-HT_{3A} receptors when compared to $GABA_A$ receptors and subunit dependence as evidenced by the dramatic reduction in sensitivity to PTX in the heteromers was suggestive of a conserved site of action. Several amino acid residues in the TM2 domain of anionic receptors have been implicated in PTX recognition. In the next chapter (chapter IV), using site-directed mutagenesis, amino acid residues, which affect PTX interaction in the 5-HT_{3A} receptor, are identified. Furthermore, amino acid residues, which may contribute to decreased inhibitory effect of PTX in heteromers, are also ascertained.

IDENTIFICATION OF FUNCTIONAL DOMAIN OF PICROTOXIN INHIBITION IN THE 5-HYDROXYTRYPTAMINE TYPE 3 RECEPTOR

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Running title:

Interaction of PTX in TM2 domain of 5-HT_3 receptor

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CHAPTER IV

IDENTIFICATION OF FUNCTIONAL DOMAIN OF PICROTOXIN INHIBITION IN THE 5-HYDROXYTRYPTAMINE TYPE 3 RECEPTOR

SUMMARY

Previous work in our laboratory has demonstrated the blockade of the cation-selective 5-HT_{3A} receptor by the $GABA_A$ receptor antagonist picrotoxin (Das et al., 2003). Also, we have recently reported that the effect of PTX in the heteromers formed by 5-HT_{3A} and 5-HT_{3B} subunits is dramatically diminished as compared to the 5-HT_{3A} receptors alone (Das and Dillon, 2003). The goal of the present study was to identify key amino acid residues, which are involved in PTX interaction with the 5-HT_3 receptor. We investigated the role of several residues in the cytoplasmic aspect of the transmembrane domain 2 (TM2) of the 5-HT_{3A} receptor. While there was no significant effect on PTX sensitivity when the 2'S residue was mutated to A, mutation of the 6' T residue to F, in the 5-HT_{3A} subunit caused a 42-fold reduction in PTX sensitivity. Moreover, a converse mutation in the 5-HT_{3B} subunit at the 6' position (i.e. 6' N to T and S), allowed gain of partial sensitivity to PTX in the heteromeric receptors suggesting that this may one of the residues responsible for the decreased sensitivity of PTX in heteromeric receptors. We also examined the possible role of the adjacent 7' residue in PTX interaction with the 5-

HT_{3A} receptor. The $GABA_A$ and glycine receptors, to which PTX inhibits with higher affinity, have a conserved threonine at the 7' position. Interestingly, mutation of the 7' L to T in the 5-HT_{3A} receptor caused PTX to be a more potent inhibitor of the mutant receptor (IC_{50} for PTX =4.3μM). The affinity for PTX in the 5-HT_{3A} (L 7' T) receptor is comparable to that in the anionic $GABA_A$ receptors. Moreover, the mutation conferred fast activation and deactivation kinetics to the 5-HT_{3A} receptor. The role of 7' residue in the 5-HT_{3B} subunit was ascertained by mutation of 7'V to T and a "double mutation" at both 6' and 7' positions i.e. N6'T, V7'T in the 5-HT_{3B} subunit. The mutation V7'T 5-HT_{3B} subunit had a modest impact on PTX sensitivity, whereas the mutation V7'T along with N6'T (i.e. N6'T, V7'T), did not produce any additional sensitivity to PTX than obtained with N6'T alone. This suggested a nominal role of 7' residue in the 5-HT_{3B} receptor in PTX recognition. We have identified two residues in the TM2 domain of 5-HT_3 receptor, which are involved in PTX recognition. Our data corroborates previous evidence for the critical role of 6' residue and has identified a novel residue, which impacts PTX sensitivity in the TM2 domain of the 5-HT_3, receptor. Additionally, the radioligand [^{3}H]EBOB, which binds with high affinity to the $GABA_A$ receptors did not exhibit specific binding in the 5-HT_{3A} receptor suggesting that the domain of interaction of this and possibly related ligands is different than that in the anion-selective receptors.

ABBREVIATIONS:

5-HT, 5-hydroxytryptamine; 5-HT_{3A}, serotonin type 3A; 5-HT_{3B}, serotonin type 3B, DMSO, dimethysulfoxide; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetra acetic acid; 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; LGIC, ligand gated ion channels; nAChR, nicotinic acetylcholine receptor; PTX, picrotoxin.

KEYWORDS:

anion-selective, cation-selective, convulsant, ion channel, picrotoxin, whole-cell patch clamp

INTRODUCTION

Rapid neurotransmission in synapses of the central and peripheral nervous systems is mediated by neurotransmitter binding to membrane proteins belonging to the ligand-gated ion channel superfamily. 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 and Lummis, 2002; Karlin, 2002). From the therapeutic point of view, 5-HT_3 receptor antagonists are useful as antiemetics in chemotherapy induced emesis (Costall and Naylor, 1992 (b); Karim et al., 1997) and in irritable bowel syndrome (Berrada et al., 2003; Camilleri et al., 1999) but are also are thought to be potentially useful in neuropsychiatric diseases like anxiety, depression, drug dependence (Costall and Naylor, 1992 (a); Greenshaw and Silverstone, 1997) and in management of pain (Spath, 2002).

In the LGIC superfamily, multiple subtypes of each member exist, which contribute to functional diversity. The 5-HT_3 receptor is also composed of multiple subtypes, which include the 5-HT_{3A} (Maricq et al., 1991), 5-HT_{3B} (Davies et al., 1999) and the recently cloned 5-HT_3 like subunits, 5-HT_{3C}, $_{3D}$ and $_{3E}$ subunits (Niesler et al., 2003). Of all these subtypes, the 5-HT_{3A} and to some extent 5-HT_{3B}, have been characterized pharmacologically and functionally. While the 5-HT_{3A} receptors can efficiently form functional homomers in heterologous systems, the 5-HT_{3B} subunit by

itself is incapable of forming functional cell surface receptors (Dubin et al., 1999) and is retained in the endoplasmic reticulum (Boyd et al., 2002). Co-expression of the 5-HT_{3B} with the 5-HT_{3A} subunit alters agonist and antagonist pharmacology, although the effects are relatively modest (Brady et al., 2001; Dubin et al., 1999; Hanna et al., 2000). The most striking effect of co-expression 5-HT_{3B} with the 5-HT_{3A} is the change in biophysical properties (kinetics of activation and deactivation) of the heteromers (Davies et al., 1999). The pharmacological and functional properties of the other 5-HT_3 subtypes are currently unknown.

Like other members of this superfamily, the 5-HT_3 receptor is composed of five subunits, which co-assemble pseudosymmetrically to surround a central ion channel (Boess et al., 1995). The receptor shares structural features common to LGICs, which include a large extracellular N-terminal region, four putative 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 the TM2 forms the pore (Akabas et al., 1994; Brejc et al., 2001; Corringer et al., 1995; Ortells and Lunt, 1995).

The mechanism and site of action of the plant derived alkaloid picrotoxin, which inhibits all anionic LGICs has been the subject of research for decades. While the precise location of PTX binding site is unknown, evidence indicates that in all anion-selective LGICs, PTX acts at the highly conserved TM2 domain (Fig.1) of the receptor (ffrench-Constant et al., 1993; Gurley at al., 1995; Etter et al., 1999; Buhr et al., 2001; Shan et al., 2001; Dibas et al., 2002). Recently, we have for the first time, provided evidence that PTX also inhibits the cation-selective 5-HT_{3A} receptors (Das et al., 2003). Subsequently,

we also demonstrated that the interaction of PTX with the 5-HT_3 receptor is dependent on subunit composition (Das and Dillon, 2003). Upon co-expression of the 5-HT_{3B} subunit with the 5-HT_{3A} subunit, we observed a hundred-fold reduction in PTX sensitivity in the heteromeric receptors. The similarity in mechanism of action of PTX between the 5-HT_{3A} and $GABA_A$ receptor suggested a conserved site of action for this ligand.

Thus, following this observation, we sought to investigate the interaction of PTX at the molecular level and identify amino acid residue(s), which affect inhibitory effects of this ligand in the 5-HT_3 receptor. Since 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. A number of amino acid residues (2', 3' and 6') in the cytoplasmic aspect of the TM2 of the $GABA_A$ receptor have been implicated in the action of PTX (ffrench-Constant et al., 1993; Gurley at al., 1995; Buhr et al., 2001; Shan et al., 2001). In the present study, using site-directed mutagenesis, we demonstrate that 6'T residue is important in conferring PTX sensitivity to the 5-HT_{3A} receptor. Whereas, the 2' residue plays a critical role in PTX sensitivity in $GABA_A$ receptors, our results indicate it is not involved in PTX recognition with the 5-HT_{3A} receptor. Furthermore, we provide evidence that mutation of the 6' residue in the 5-HT_{3B} subunit restored PTX sensitivity partially, suggesting that the 6' residue may be one of the residues, which is responsible for the reduced sensitivity to PTX in the heteromers. We have also identified a novel residue i.e.7' in the 5-HT_{3A} receptor, which appears to play an important role in PTX interaction

EXPERIMENTAL PROCEDURES

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. 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, IA.

Cell culture, site-directed mutagenesis and expression of 5-HT$_3$-receptor cDNA. Mouse 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits (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,) 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 (200mM), penicillin and streptomycin (10,000U/ml). Site-directed mutagenesis in the 5-HT$_{3A}$ (S2'A, T6'F and L7'T) and 5-HT$_{3B}$ (N6'S, N6'T, and V7'T) subunits was performed using the QuickChange^{TM} mutagenesis kit (Stratagene, La Jolla, CA). For the double mutation in 5-HT$_{3B}$ subunit (N6'T, V7'T), mutagenic primers were designed using 5-HT$_{3B}$ (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 phospate transfection method (Chen and Okayama, 1989). For each transfection approximately 4-10 μg of DNA was used. For co-transfection of

receptor subunits (for e.g. 5-HT_{3A} + 5-HT_{3B} or 5-HT_{3A}+ 5-HT_{3A}(T6'F)) subunits, a 1:1 ratio of respective cDNA was used (unless otherwise stated). Cells were washed twice and were used for recordings 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 (200mM NaCl, 50mM $NaH_2PO_4.2H_2O$, pH 7.4) such that the final concentration was approximately 2mg/ml and stored at -80°C. Initial saturation experiments using tissue harvested from the stable cell line suggested high B_{max} values. Thus, relatively less 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 also carried out using the 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 triplicates and a minimum of 3 experiments was carried out for each saturation assay. Radioactivity

was measured using liquid scintillation (Packard). Data analysis was done 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): 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) was dissolved in the external solution (above) and applied to the target cell through a Y tube positioned within 100 μm of the cell. 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. 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, PTX is the test drug, 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 three individual experiments (typically 3-8) was conducted for each paradigm. All data were presented as mean ± S.E.M.

RESULTS

In order to compare the effects of PTX in wild-type and mutant receptors, it was necessary to activate the receptors with equipotent concentrations of 5-HT. Concentration-response curves (using 0.1-100 μM 5-HT) were initially constructed for the homomeric wild-type 5-HT_{3A}, heteromeric wild-type 5-HT_{3A}+ 5-HT_{3B} and all combinations of mutant receptors. The EC_{50} for 5-HT and the Hill-coefficient values are summarized in Table 1. Fig.2 shows the concentration-response curves for 5-HT in wild-type 5-HT_{3A}, and all mutant 5-HT_{3A} receptors. With exception of the 5-HT_{3A}(T6'F) mutation, changes in EC_{50} values of mutant receptors as compared to wild-type (Table 1), indicated that the mutations were well-tolerated and did not cause any gross alterations in channel structure. The 5-HT_{3A}(T6'F) mutation produced non-functional receptors but these receptors were "rescued" when co-expressed with the wild-type 5-HT_{3A} subunit exhibiting EC_{50} and Hill co-efficient values similar to the wild-type receptor.

The 2'S residue does not influence PTX sensitivity in the 5-HT_{3A} receptor. In the field isolated *Drosophila* GABA receptor, mutation of the 2' alanine in the β subunit to serine conferred PTX resistance (ffrench-Constant et al., 1993). In the 5-HT_{3A} receptor, a serine is present in the 2' position. We thus considered the possibility that mutation of the 2'S to A may enhance sensitivity to PTX. The effect of PTX in the 5-HT_{3A} (S2'A) is shown in Figs. 3B and 3D. Mutation at this position did not produce any significant effect on PTX sensitivity as the IC_{50} for PTX in the mutant receptor was similar to the wild-type

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receptor (IC_{50}=49 and 42μM respectively, Figs.3A, D). Thus, our data suggest that the 2' residue in the 5-HT_{3A} receptor may not be critical for determining PTX sensitivity.

The role of 6'T residue in determining PTX sensitivity in the 5-HT_{3A} receptor. In $GABA_A$ and glycine receptors, mutation of the conserved T to F, at the 6' position 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. The mutation T 6' F resulted in a loss of function as > 100 μM 5-HT failed to activate the receptor. Hence this mutant was initially studied by co-transfection of 5-HT_{3A}(T6'F) cDNA and wild-type 5-HT_{3A} cDNA in equal ratios (1:1) respectively. In later experiments, co-transfection of these subunit cDNAs was done using different ratios of cDNAs. A ≥ 3:1 ratio of 5-HT_{3A}(T6'F) and wild-type 5-HT_{3A} cDNA respectively did not produce functional receptors. Co-transfection of these subunit cDNAs in a ratio of 2:1 respectively produced functional receptors, the properties (i.e. EC_{50} values and PTX sensitivity) of which, were not significantly different from those in receptors formed by 1:1 cDNA ratios. The data presented in this study are obtained from receptors formed using T6'F:W.T cDNA ratio of 2:1. The receptor thus formed could be activated with 5-HT and the EC_{50} value was determined to be 0.9 μM (Fig.2, Table 1). Traces showing the effect of PTX on the 5-HT_{3A} + 5-HT_{3A} (T6'F) receptor are shown in Fig. 3C. The blockade by PTX in cells expressing the 5-HT_{3A} + 5-HT_{3A}(T6'F) receptors was reduced ~ 42 fold relative to the wild-type receptors alone. While the IC_{50} for PTX in the wild-type receptor was 42 μM (Table 1), the IC_{50} for PTX in the 5-HT_{3A} + 5-HT_{3A} (T6'F) was ~ 1.8 mM (Fig. 3D). Thus, while PTX sensitivity was not completely abolished by the

mutation, the ~42 fold change in sensitivity to PTX strongly suggests that the 6' residue is a critical determinant of PTX sensitivity in the 5-HT_{3A} receptor.

7' L to T mutation in the 5-HT_{3A} receptors confers increased sensitivity to PTX and alters gating kinetics. From a previous study, we found that the affinity for picrotoxin and related ligands for the 5-HT_{3A} receptor is ~5-10 fold less compared to the anionic $GABA_A$ receptor (Das et al., 2003). In an attempt to understand the molecular basis of this difference, we examined the effect of mutation on the adjacent residues in the 2'-6' vicinity. 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 (Xu et al., 1995;Reeves et al., 2001). Hence it was tempting to speculate that this residue may also be part of the PTX-recognition-site. We, thus mutated the 7'L (in 5-HT_{3A} receptor) to T (the corresponding residue in $GABA_A$ and glycine receptors). Concentration-response curves for 5-HT in L7'T mutants revealed a very modest shift in sensitivity as compared to wild-type receptors (Fig.2, Table 1). A 10-fold increase in PTX sensitivity in the mutant L7'T was observed as compared to the wild-type receptors (Figs.4A, B, C). The sensitivity to PTX (i.e. 4.3 μM) conferred by this mutation was comparable to that in $GABA_A$ and glycine receptors.

A notable result of this mutation was a dramatic change in kinetics of activation, desensitization and deactivation as compared to wild-type 5-HT_{3A} receptor. Figs. 5A and B show the traces of activation and deactivation respectively in both receptor configurations. Fig. 5C and D show quantification of activation and deactivation times. While the time to achieve peak current and deactivation time in 5-HT_{3A} receptor was 11 ±

0.82 and 29.87 ± 2.34 seconds respectively, those in the mutant L7'T receptors were 3.3 ± 0.22 and 5.13 ± 0.721 respectively. 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, the L7'T receptors exhibited pronounced desensitization. Thus, mutation of 7'L to T in the 5-HT_{3A} receptor not only modified the action of PTX but also altered gating kinetics.

PTX sensitivity is restored partially in heteromeric receptors by a "converse mutation" in the 6' position of the 5-HT_{3B} subunit.

Recently, we have reported that heteromeric receptors formed by 5-HT_{3A} and 5-HT_{3B} subunits are ~100-fold less sensitive to PTX than homomeric 5-HT_{3A} receptors (Das and Dillon, 2003). Following our observation that the 6'T residue in 5-HT_{3A} was critical in PTX-recognition, we sought to examine if the 6' residue in 5-HT_{3B} subunit was responsible for the reduced sensitivity to PTX in the heteromeric receptors. Unlike the 5-HT_{3A} receptor, the 5-HT_{3B} subunit has an atypical asparagine at the 6' position (Fig.1). Thus, we mutated the 6'N residue in the 5-HT_{3B} subunit with the residue corresponding to the 6' position in the 5-HT_{3A} subunit. Also, unlike the mouse 5-HT_{3B} subunit, the human 5-HT_{3B} subunit has a serine in the 6' position. Thus, to test if there is a species difference in PTX interaction, we generated another mutation i.e. 5-HT_{3B}(N6'S). The concentration-response curves for 5-HT generated for the wild-type 5-HT_{3A}+5-HT_{3B} heteromers and these mutants are shown in Fig. 6. As compared to the wild-type heteromeric receptors, roughly, a two-fold increase in sensitivity to 5-HT was observed in each of these mutants. Figs. 7A and 7B show the traces obtained in presence of PTX in

the 5-HT_{3A} + 5-HT_{3B} (N6'T) and 5-HT_{3A} + 5-HT_{3B} (N6'S) mutants respectively. The 6'N to T/S mutation in the 3B subunit partly restored PTX sensitivity of the heteromeric receptor as evidenced by ~ 17 fold shift to the left of the PTX concentration-response curve. While the IC_{50} for PTX in the wild-type heteromeric receptors was ≥ 3mM that obtained in N6'T and N6'S mutants were 176 μM and 213 μM respectively. The mean results of the above experiments are summarized in Fig.7C. Our results demonstrate that the 6'N in 5-HT_{3B} subunit is at least partially responsible for the reduced sensitivity of heteromeric receptors to PTX. The lack of complete restoration of PTX sensitivity in the heteromeric receptors suggests that other TM2 residues may be involved in producing reduced sensitivity to PTX in the heteromeric receptors. Furthermore, our data also suggests that the striking loss of sensitivity to PTX observed in mouse heteromeric receptors might not be evident in human heteromeric receptors.

Unlike 5-HT_{3A} receptor, the 7' residue in 5-HT_{3B} subunit does not appreciably affect PTX sensitivity.

As shown earlier, the 7' residue is an important determinant of PTX action in the 5-HT_{3A} receptor. In an attempt to identify additional residues that contribute to differential effects of PTX in the heteromeric 5-HT_{3A}+5-HT_{3B} receptors, we focused on the 7' residue in the 5-HT_{3B} subunit. While evidence suggests that the amino acid residue in 7' position of the 5-HT_{3A} receptor is exposed to the channel lumen, there is no evidence for the same in the 5-HT_{3B} subunit. However, because of its proximity to the 6' residue, and its importance in PTX recognition in the 5-HT_{3A} receptors (present study), we mutated the 7'V to T in 5-HT_{3B} subunit. The effect of this mutation on channel properties (Fig.6)

(i.e. EC_{50} and Hill co-efficient) was modest when compared to wild-type heteromeric receptors. Fig. 8A shows traces obtained in 5-HT_{3A}+ 5-HT_{3B}(V7'T) heteromeric mutant receptors in presence of increasing concentrations of PTX. These receptors exhibited a modest 4-fold increase in sensitivity to PTX (Fig. 8C). If 7'V residue in 5-HT_{3B} subunit indeed contributed to loss of PTX-sensitivity in the heteromeric receptors, then mutation of both 6' and 7' residues in this subunit would be expected to restore full sensitivity to the heteromeric receptors. To test this possibility, we generated a 5-HT_{3B} (N6'T, V7'T) "double mutant". As shown in Figs. 8B and C, mutation in the 7' position of the 5-HT_{3B} subunit did not display any additional sensitivity to PTX than what was obtained with a single mutation (i.e. N6'T/S). Thus, in contrast to the 5-HT_{3A} receptor, the amino acid residue in 7' position of 5-HT_{3B} receptor appears to play only a marginal role in modulating the effects of PTX.

The high-affinity radioligand [^{3}H]EBOB, which competes for the PTX-site in $GABA_A$ receptor, does not exhibit specific binding in the 5-HT_{3A} receptor.

[^{3}H]EBOB is a high affinity radioligand that binds to the convulsive site in $GABA_A$ receptors. The binding site for [^{3}H]EBOB is believed to overlap with convulsant drugs like PTX. Picrotoxin and other convulsant drugs like PTZ, TBOB, TBPS and insecticides like lindane, dieldrin interact at this site and displace specific [^{3}H]EBOB binding (Cole et al., 1995). Our site-directed mutagenesis studies suggested that the site of action of convulsants in 5-HT_3 receptors was comparable to that in the anion-selective receptors. To determine if the binding site of [^{3}H]EBOB in the 5-HT_{3A} receptor was similar to the $GABA_A$ receptor, we performed saturation binding assays (see methods) using

membranes harvested from a stable cell line expressing the 5-HT_{3A} receptor. While saturation studies using recombinant $GABA_A$ receptors revealed a K_d value of 9nM (Yagle et al., 2003), saturation studies with this ligand exhibited no specific binding in the 5-HT_{3A} receptor. Saturation studies with [^{3}H]GR65630, a ligand which binds specifically to the agonist binding site in the 5-HT_3 receptor revealed a K_d of 8 ± 1.7 nM and B_{max} value of 23.8 ± 1.6 pMol/mg protein (Table 2).

The lack of specific [^{3}H]EBOB binding suggested that the binding site of this ligand and possibly related ligands in the 5-HT_{3A} receptors is different from the $GABA_A$ receptors. In addition, TBPS, which is believed to bind in the cytoplasmic aspect of the $GABA_A$ receptor (Jursky et al., 2000), did not appreciably inhibit 5-HT currents in whole-cell patch clamp recordings (data not shown) further supporting this notion.

DISCUSSION

Effects of mutations on PTX action in 5-HT_3 receptor: comparison with anion-selective receptors. Evidence from recent studies in anionic LGICs indicate that PTX acts at the highly conserved TM2 domain of the receptor (ffrench-Constant et al., 1993; Gurley at al., 1995; Etter et al., 1999; Buhr et al., 2001; Shan et al., 2001; Dibas et al., 2002). In particular, the 2' and 6' residues and more recently 3' near the cytoplasmic aspect of TM2 domain have been implicated as determinants of PTX sensitivity (Buhr et al., ffrench-Constant et al., 1993; Gurley at al., 1995; Shan et al., 2001). Our recent finding that PTX also inhibits the cation-selective 5-HT_{3A} receptor in a manner similar to the anionic $GABA_A$ receptor suggested a conserved site of action for this ligand (Das et al., 2003). We also discovered that while homomeric receptors formed from the 5-HT_{3A} subunits were sensitive to PTX, heteromers consisting of the 5-HT_{3A}+5-HT_{3B} subunits showed a hundred-fold reduction in sensitivity to PTX (Das and Dillon, 2003). Thus, the focus of the present investigation was to determine the molecular basis for action of PTX in the 5-HT_3 receptor.

The role of 2' residue in conferring PTX sensitivity has been documented in anionic receptors. In the *Drosophila* GABA receptor, a natural mutation, which occurs at the 2' position (A2'S) which confers resistance to cyclodiene insecticides also conferred resistance to PTX (ffrench-Constant et al., 1993). Using substituted cysteine accessibility method, Xu et al, (1995) demonstrated that PTX prevented modification of the

engineered cysteine residue in $GABA_A\alpha 1$ V2'C receptor. Replacement of the 2'A with T in the invertebrate GluRβ receptor resulted in receptors resistant to PTX (Etter et al., 1999). In the $GABA_C$ $\rho 1$ receptors, mutation of the 2'P to S, V, G or A (corresponding residues in $GABA_C$ $\rho 2$, $GABA_A\alpha 1$, glycine α and *Drosophila* GABA receptors respectively) resulted in increased sensitivity of the $GABA_C$ $\rho 1$ receptors (Wang et al., 1995). While the $5\text{-}HT_{3A}$ receptor is sensitive to PTX, the affinity of this and related ligands is approximately 5-10 fold less that the anionic $GABA_A$ receptors (Das et al., 2003). The $5\text{-}HT_{3A}$ receptor has serine in the 2' position and thus based on previous studies we mutated the 2' S to A, anticipating an increase in sensitivity to PTX. The picrotoxin sensitivity of the S2'A mutant however, was not significantly different from the wild-type $5\text{-}HT_{3A}$ receptor. Mutation of the 2'P to G and A in the β subunit of glycine receptor did not change the PTX sensitivity of the heteromeric glycine$\alpha\beta$ receptors (Shan et al., 2001). The authors concluded that the 2' residue was not critical in determining PTX resistance in these receptors. Our results thus appear consistent with the conclusions of Shan et al. (2001).

The crucial role of 6' residue in determining PTX sensitivity is evident from a number of studies in the anionic receptors. PTX resistance is conferred if the wild-type 6' threonine residue is mutated to phenylalanine in the β subunit of the $\alpha 1\beta 2\gamma 2$ $GABA_A$ receptors (Gurley et al., 1995). In the $GABA_C$ receptors, co-expression of $\rho 1$ and $\rho 2$ subunits produce receptors insensitive to PTX and substituting the 6' methionine residue in $\rho 2$ subunit with threonine (the corresponding residue in $\rho 1$) produces heteromeric receptors which gain sensitivity to PTX (Zhang et al., 1995). Shan et al, (2001)

determined that in glycine$\alpha\beta$ heteromeric receptors, the 6' phenylalanine to threonine mutation in β subunit conferred increased PTX sensitivity to the PTX-resistant heteromeric receptors. Based on their results and comparison with data obtained from other groups, they proposed that a ring of threonine residues at the 6' position was essential and a common requirement for PTX sensitivity amongst members of the LGICs. Moreover, they suggested that if the site of action of PTX is identical in all members of the LGICs, then it is likely to be formed by the threonine residues in the 6' position. Based on these studies we mutated the conserved 6' T to F in the 5-HT_{3A} receptor. Since mutant T6'F receptors failed to respond to > 100μM of 5-HT, we co-expressed these with the wild-type receptor in order to study the role of this residue in PTX interaction. The co-assembled receptors thus formed (5-HT_{3A}+ 5-HT_{3A} (T6'F)), exhibited ~ 42-fold decrease in sensitivity to PTX, strongly suggesting the importance of 6'T residue in PTX inhibition of the 5-HT_{3A} receptor. Since the stoichiometry of the 5-HT_{3A}+ 5-HT_{3A} (T6'F) receptor is unknown, the number of phenylalanines needed to confer this decrease in sensitivity to PTX is not clear. Also, it is probable that we might be underestimating the resistance conferred by PTX due to the fact that some amount of currents may have come from the PTX-sensitive wild-type 5-HT_{3A} receptors. As for the reason why the mutant T6'F receptor failed to respond to agonist is speculative. The 6'T residue in the $GABA_A$ and glycine receptors lines an important region of the pore, in that, it is close to the activation gate (Unwin, 1995; Wilson and Karlin, 1998) and selectivity filter (Cohen et al., 1992). Hence, structural perturbation in this region of the protein may be expected to have significant effects on function. Insertion of five bulky phenylalanines in the

homomeric 5-HT_{3A} receptor at this position may have produced sufficient alteration in the protein structure to affect function. However, neither insertion of five phenylalanines in homomeric glycineα1 receptors (Shan et al., 2001) nor insertion of five cysteines in homomeric 5-HT_{3A} receptor (Reeves et al., 2001 Panicker et al., 2002) produce non-functional receptors. Contrary to this, in our studies, T6'C mutation in 5-HT_{3A} receptor (see Table 1) also did not produce receptors which respond to agonist and had to be co-expressed with wild-type receptor to restore function. Recent findings in the $GABA_A$ and glycine receptor suggest that structure of pore at the 6' level in these two receptors may be different (Horenstein et al., 2001; Shan et al., 2001). Moreover, the pore structure at the 6' level may be distinct in different expression systems (Shan et al., 2002). Thus, our findings might in part be explained by differences in pore structure in the 6' region of 5-HT_3 receptor and/or the expression system used.

Mutation of the 6'T to F in the 5-HT_{3A} receptor resulted in loss of sensitivity to PTX. A more reliable method of addressing the importance of a residue in PTX interaction would be to create a "gain of function" mutation. Previously, we found that heteromeric receptors composed of 5-HT_{3A}+5-HT_{3B} were hundred-fold less sensitive to PTX (Das and Dillon, 2003). The 5-HT_{3B} subunit has a valine residue in the 2' position which is identical to the residue present in PTX-sensitive $GABA_A\alpha$1 receptors. In contrast to this, the 6'N residue in the 5-HT_{3B} is not conserved and thus we hypothesized that this residue may have contributed to the loss of PTX-sensitivity in the heteromeric 5-HT_3 receptors. Indeed, mutation of the 6'N to S and a conserved T partially restored sensitivity to the relatively PTX-resistant heteromeric receptors. The "gain of function"

conferred by mutation of 6'N to T/S in the 5-HT_{3B} subunit, reinforced the thought that this residue is a critical determinant of PTX sensitivity. On the other hand, the lack of full restoration of sensitivity to PTX in the heteromers to match that in the wild-type 5-HT_{3A} receptor suggested that other residue(s) were involved in conferring decreased resistance to PTX in heteromeric receptors. From molecular modeling studies, Zhorov et al, (2000) proposed that threonine at the 6' position contributed to hydrogen bonding with the hydrophillic domain of PTX. Thus, based on this, our data suggests PTX sensitivity in the N 6' T/S mutant receptor increased due to the ability to form –H bond interaction with PTX.

Comparison of the amino acid sequence of TM2 domain between the 5-HT_{3A} and 3B subunits and with $GABA_A$ and glycine receptors, suggested that the adjacent 7' residue could potentially be involved in PTX interaction with the 5-HT_3 receptor. Studies in both the $GABA_A$ and 5-HT_3 receptors indicate that 7' residue is exposed to the lumen of the channel (Xu et al., 1995; Reeves et al., 2001). Due to the evidence that 7'L is exposed to the lumen of the ion channel and its proximity to the 6' residue, which is an important recognition-site for PTX, we hypothesized that it may also be a part of the PTX-recognition-site. Mutation of 7'L in the 5-HT_{3A} receptor to T (corresponding residue in the $GABA_A$ and glycine receptors), resulted in a 10-fold increase in sensitivity to PTX in the 5-HT_{3A} receptor. The affinity of the mutant L7'T receptor for PTX matched that of the anionic $GABA_A$ receptor (i.e. IC_{50}~5μM). Furthermore, the L7'T mutation resulted in receptors exhibiting distinct kinetics of activation, desensitization

and deactivation as compared to the wild-type receptors. To our knowledge, this is the first report of the role of 7' residue in PTX interaction, with a member of the LGICs.

To determine if the 7' residue was an additional residue responsible for the decreased sensitivity of heteromeric 5-HT_{3A}+5-HT_{3B} receptors, we mutated 7'V to T in the 5-HT_{3B} subunit. This mutation resulted in modest increase in sensitivity to PTX. If indeed 7' residue was involved in PTX-resistance of the heteromeric receptor, then mutation of both 6' and 7' residues in the 5-HT_{3B} subunit (i.e. N6'T, V7'T) would be expected to a fully restore PTX sensitivity of heteromeric receptors to match that of the wild-type 5-HT_{3A} receptor. However, mutation of 7'V to T concomitant with 6'N to T did not produce additional increase in sensitivity to PTX suggesting that 7'V in the 5-HT_{3B} does not play a prominent role in conferring PTX resistance in the heteromeric receptors. Thus, while the novel role of 7'L residue in PTX interaction in 5-HT_{3A} subunit is evident, there appears to be a minimal role of this residue in conferring resistance in the heteromeric receptors. An alternative explanation of our data could be that in the heteromers, the 7' residue in the 5-HT_{3B} subunit might be oriented away from the channel lumen and hence may not contribute to PTX recognition. However, due to lack of experimental evidence this reasoning is merely speculative. Additional mutations in the 5-HT_{3B} subunit will be required to determine the role of other TM2 residues in PTX sensitivity.

Is the binding site for PTX in 5-HT_3 receptor the same as anionic receptors? The fact that PTX-mediated block in the 5-HT_{3A} receptor is (a) non-competitive (b) enhanced with increasing concentrations of agonist (use-dependent) (c) negligible in

absence of agonist (Das et al., 2003) suggested PTX binds in the pore of the channel. Mutational data obtained from the present study also lends credence to the hypothesis that PTX binds in the ion-channel. However, some interesting differences in the residues involved in PTX-recognition are evident between 5-HT_3 and $GABA_A$ receptors. In our study, the 2' residue did not appear to play an important role in PTX sensitivity but the 6' did.

The picrotoxin-site or convulsive site in the $GABA_A$ receptor has been characterized by variety of radioligands which include [^{3}H]DHP, [^{35}S]TBPS, [^{3}H]TBOB and [^{3}H]EBOB (Lawrence and Casida, 1985; Ticku et al., 1978; Squires et al., 1983; Huang and Casida, 1996). Amongst all the available radioligands, [^{3}H]EBOB is most suitable for characterizing the PTX-site due to its high affinity for both insect (particularly *Drosophila*) and mammalian $GABA_A$ receptors Convulsant drugs like PTX, PTZ, TBOB, TBPS and insecticides like lindane, dieldrin interact at this site and displace specific [^{3}H]EBOB binding (Cole et al., 1995). In the field isolated *Drosophila* GABA receptor which has A2'S mutation, [^{3}H]EBOB exhibited reduced affinity and apparent number of binding sites (B_{max}) (Cole et al., 1995). A recent study by Jursky et al, (2000) using radioligand binding assays suggested that the 3'L in $GABA_A$ *β3* subunit may contribute to a high affinity binding-site for another convulsant, TBPS. 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. i.e. the 2'-3' vicinity. [^{3}H]EBOB, which exhibits specific binding in the $GABA_A$ receptors and is displaced by PTX, did not specifically bind to the 5-HT_{3A} receptors. In addition,

TBPS, which presumably binds at the 3' region in $GABA_A$ receptors, did not inhibit whole-cell currents induced by 5-HT in the $5\text{-}HT_{3A}$ receptor (data not shown). Also, mutation of the 3'F to L in the $5\text{-}HT_{3A}$ receptor did not alter PTX sensitivity (unpublished observation). Thus, taken together, our data suggest that the binding site for PTX may not be in the 2'-3' region but possibly in the 6'-7' region. However, an allosteric effect cannot be ruled out. Also, PTX, in anion-selective receptors is believed to interact at multiple binding sites i.e. at the cytoplasmic aspect (2'-6' vicinity) and towards the extracellular aspect (15'-17') vicinity. Such additional binding sites for PTX in $5\text{-}HT_3$ receptors may also exist. Mutation of residues 15' and 17' would be required to address this possibility.

FIGURE LEGENDS

Figure IV-1. Map of the Second Transmembrane Domain, which Forms the Ion Channel, for Several Members of the LGICs Superfamily. The 0' position represents the most cytoplasmic aspect of the channel, while the 19' position represents the extracellular aspect of the channel. Residues in the TM2 domain of the LGICs are highly conserved. Residues shown to influence sensitivity to picrotoxin include the 2', 3', 6', 15' and 19' positions.

Receptor	0'	1'	2'	3'	4'	5'	6'	7'	8'	9'	10'	11'	12'	13'	14'	15'	16'	17'	18'	19'
nACh α1	K	M	T	L	S	I	S	V	L	L	S	L	T	V	F	L	L	V	I	V
$GABA_A$ 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 IV- 2. 5-HT Sensitivity in Wild-type and Mutant 5-HT_{3A} Receptors. Mutations in 2', 7' positions in the 5-HT_{3A} did not produce any significant changes to the functional properties indicating that the gross structure of the mutant receptors was unaltered. 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 (discussion). See Table IV-1 for the EC_{50} values and Hill co-efficients. All data points are from a minimum of 4-6 cells.

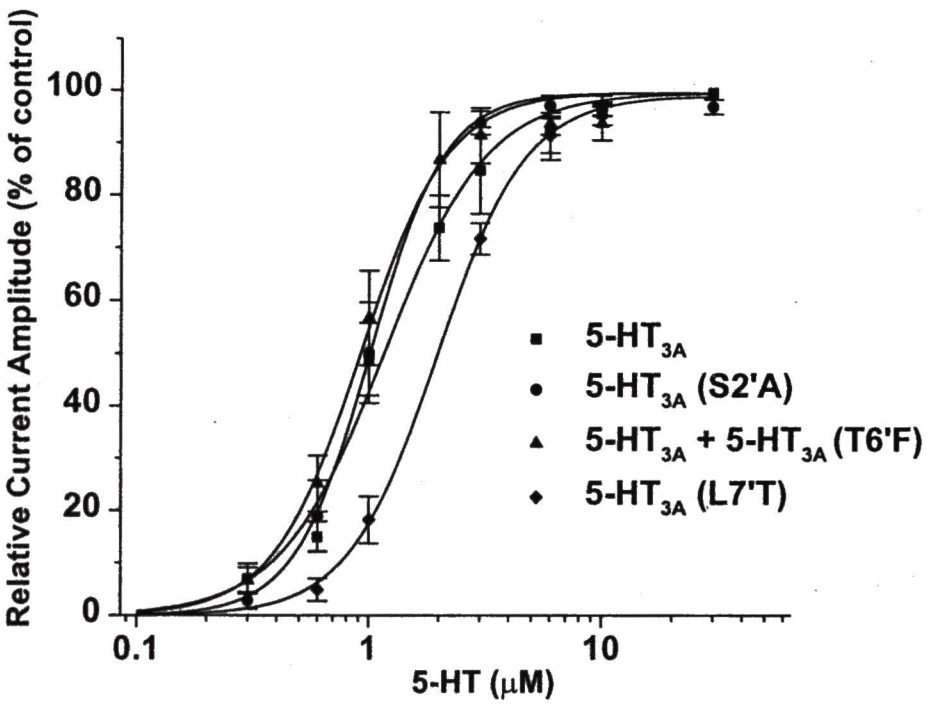

Relative Current Amplitude (% of control)
100
80
60
40
20
0
0.1
1
10
5-HT (μM)
5-HT3A
5-HT3A (S2'A)
5-HT3A + 5-HT3A (T6'F)
5-HT3A (L7'T)

FIGURE IV-3 Effect of S2'A and T6'F Mutation in the 5-HT_{3A} Receptor. ***A,*** Typical traces obtained when cells expressing wild-type 5-HT_{3A} receptors were gated with an EC_{50} concentration (1.2 μM) of 5-HT. ***B,*** recordings obtained in cells expressing the 5-HT_{3A} (S2'A) receptors when gated with 1 μM 5-HT. The IC_{50} for PTX in the mutant receptor was similar to the wild-type receptor (49 and 42 μM respectively). ***C,*** Recordings in cells co-expressing 5-HT_{3A} and 5-HT_{3A}(T6'F) obtained when cells were gated with 0.9 μM 5-HT. PTX sensitivity was substantially reduced in these receptors compared to the wild-type. The IC_{50} for PTX in the T6'F mutant receptor was ~1.8mM. ***D,*** Mean PTX inhibitory curves for wild-type and mutant receptor configurations. n=4-6 for each data point. All recordings were obtained in cells gated at respective EC_{50} concentration of 5-HT.

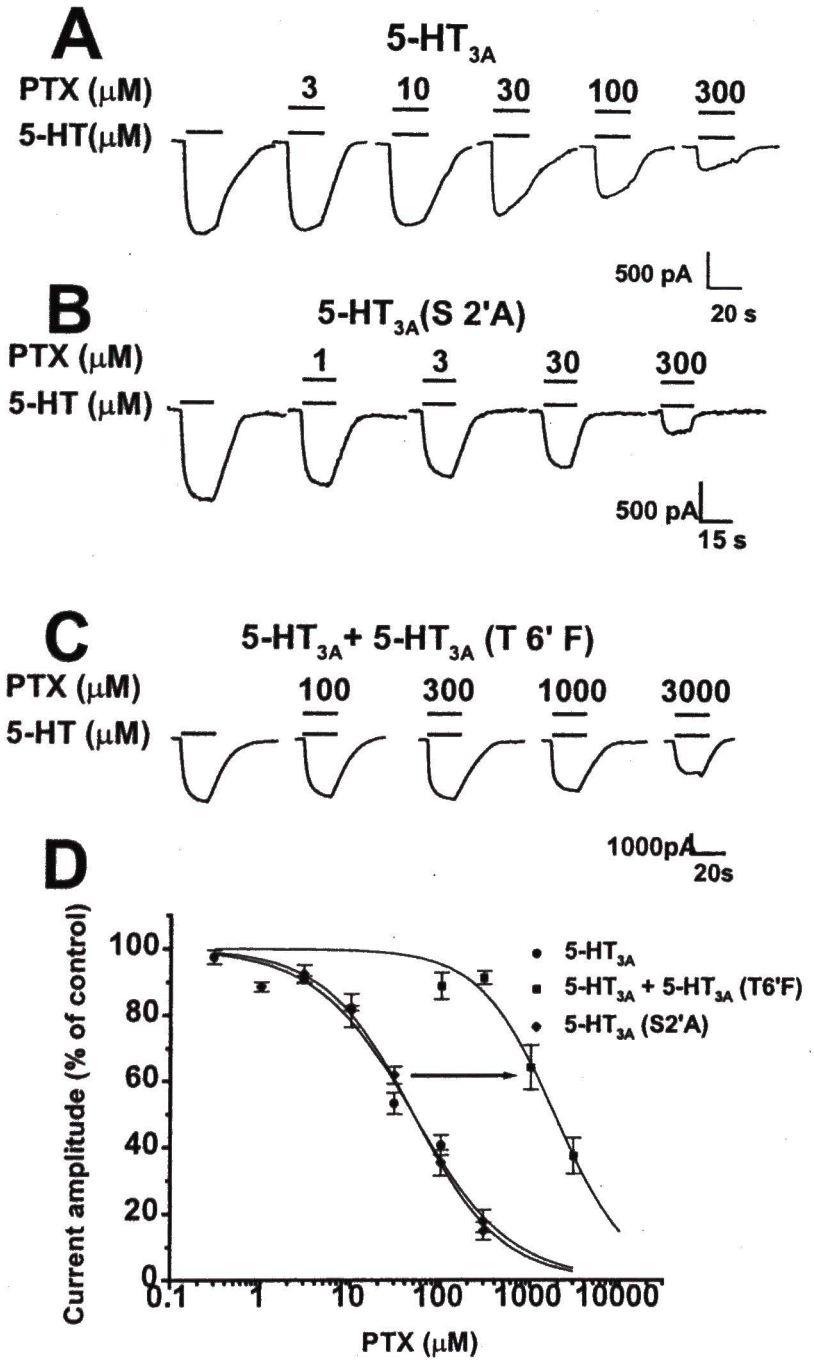

A
5-HT3A
PTX (μM)
3
10
30
100
300
5-HT(μM)
500 pA
20 s
B
5-HT3A(S 2'A)
PTX (μM)
1
3
30
300
5-HT (μM)
500 pA
15 s
C
5-HT3A+ 5-HT3A (T 6' F)
PTX (μM)
100
300
1000
3000
5-HT (μM)
1000pA
20s
D
Current amplitude (% of control)
100
80
60
40
20
0
0.1
1
10
100
1000
10000
PTX (μM)
5-HT3A
5-HT3A + 5-HT3A (T6'F)
5-HT3A (S2'A)

FIGURE IV-4. Effect of L7'T Mutation in the 5-HT_{3A} Receptor. ***A,*** Example of responses to varying concentrations of PTX in the wild-type 5-HT_{3A} receptor. ***B,*** Traces obtained in presence of PTX in L7'T mutant receptors. PTX sensitivity was increased 10-fold, and matched that typically seen in anionic receptors. The IC_{50} for PTX was 4.3 μM. The mutation also conferred distinct kinetics to the receptor as compared to wild-type. ***C,*** Mean PTX inhibitory concentration obtained in both receptor configurations. n=4-6 for each data point. Both receptor types were gated with an EC_{50} concentration of serotonin (i.e 1.2 and 2 μM respectively).

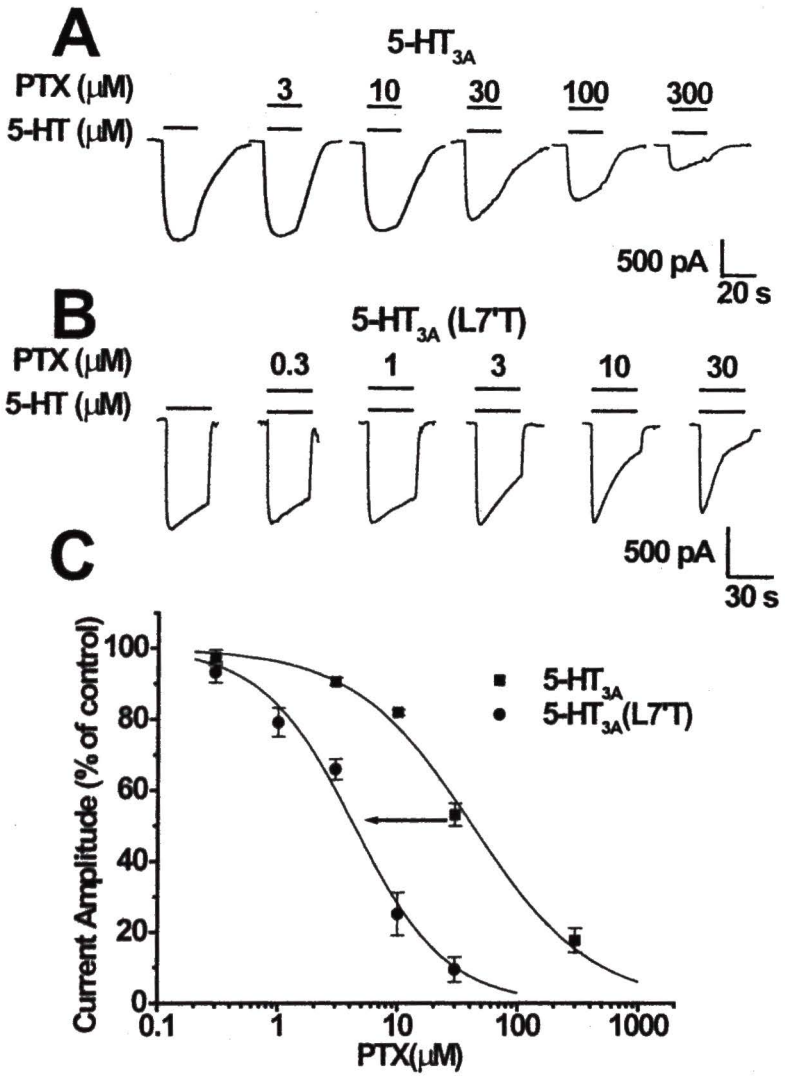

A
5-HT3A
PTX (μM)
5-HT (μM)
3
10
30
100
300
500 pA
20 s
B
5-HT3A (L7'T)
PTX (μM)
5-HT (μM)
0.3
1
3
10
30
500 pA
30 s
C
Current Amplitude (% of control)
100
80
60
40
20
0
0.1
1
10
100
1000
PTX(μM)
5-HT3A
5-HT3A(L7'T)

FIGURE IV-5 Effect of 5-HT_{3A}(L7'T) mutation on gating kinetics. ***A,*** Mean activation kinetics in 5-HT_{3A} and 5-HT_{3A}(L7'T) receptors (measured as time to peak current in seconds). While the time to achieve peak current amplitude in 5-HT_{3A} receptors was 11.179 ± 0.817 seconds, that in 5-HT_{3A}(L7'T) was 3.34 ± 0.218 seconds. ***B,*** Mean deactivation kinetics in 5-HT_{3A} and 5-HT_{3A}(L7'T) receptors. Time to deactivate (in seconds) was 5.13 ± 0.721 seconds in 5-HT_{3A}(L7'T) as compared to 29.87 ± 2.34 seconds in 5-HT_{3A} receptor. ** indicates significantly different from 5-HT_{3A}, $p<0.01$, Student's t-test in ***A*** and ***B***.

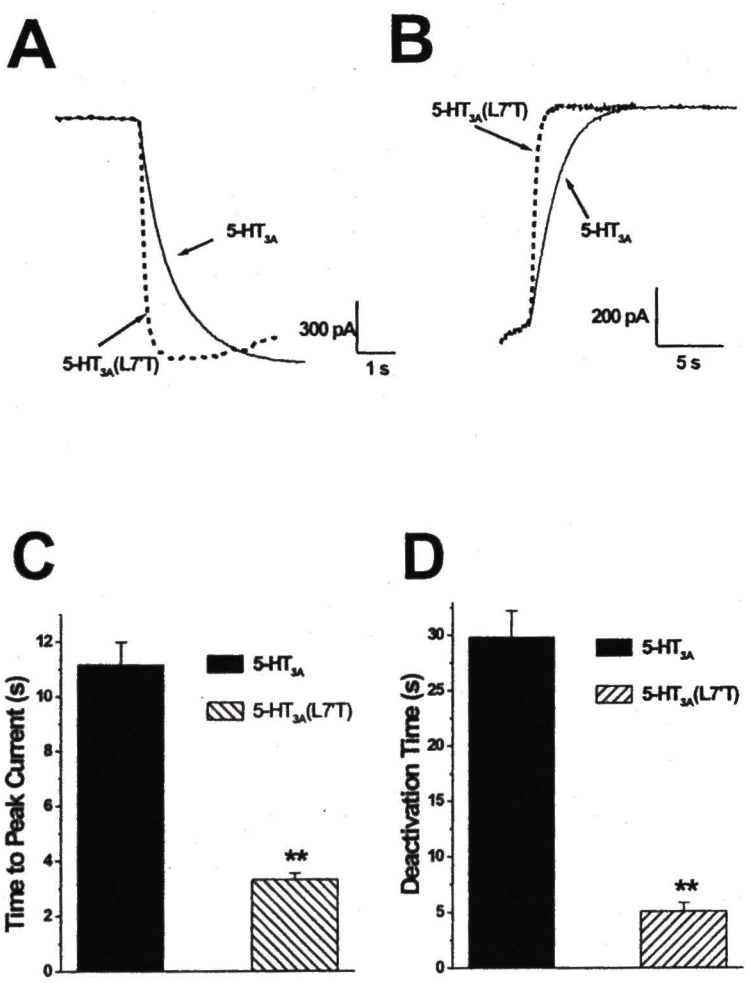

A
B
5-HT3A
5-HT3A(L7'T)
300 pA
1 s
5-HT3A(L7'T)
5-HT3A
200 pA
5 s
C
D
Time to Peak Current (s)
Deactivation Time (s)
5-HT3A
5-HT3A(L7'T)
**
**

FIGURE IV-6. Concentration Response Curves for 5-HT in Wild-type Heteromeric and Mutant Heteromeric Receptors. Agonist sensitivity was approximately two-fold increased in the heteromeric receptors with mutations in the 5-HT_{3B} subunit. The EC_{50} and Hill co-efficient values are given in Table 1.

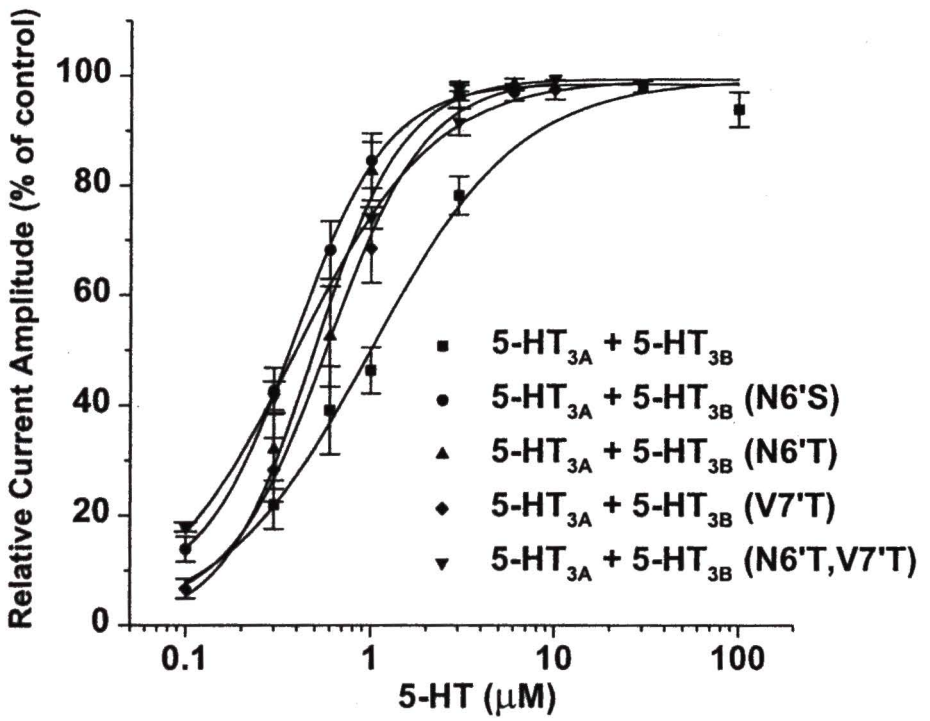

Relative Current Amplitude (% of control)
100
80
60
40
20
0
0.1
1
10
100
5-HT (μM)
5-HT3A + 5-HT3B
5-HT3A + 5-HT3B (N6'S)
5-HT3A + 5-HT3B (N6'T)
5-HT3A + 5-HT3B (V7'T)
5-HT3A + 5-HT3B (N6'T,V7'T)

FIGURE IV-7. PTX Sensitivity is Restored Partially in Heteromeric Receptors with a converse mutation in the 6' position. ***A,*** Traces obtained from cells expressing 5-HT_{3A} + 5-HT_{3B}(N6'T) in presence of PTX. An increase in sensitivity to PTX was observed in these receptors. The IC_{50} for PTX was 213 μM. (compared to wild-type heteromers with an IC_{50} of ~3100 μM. ***B,*** Traces obtained from cells expressing 5-HT_{3A} + 5-HT_{3B}(N6'S). The IC_{50} for PTX was 176 μM ***C,*** Mean PTX inhibitory effect in cells expressing wild-type 5-HT_{3A}, 5-HT_{3A} + 5-HT_{3B} and mutant heteromeric receptors. Cells were gated with an EC_{35} concentration of 5-HT.

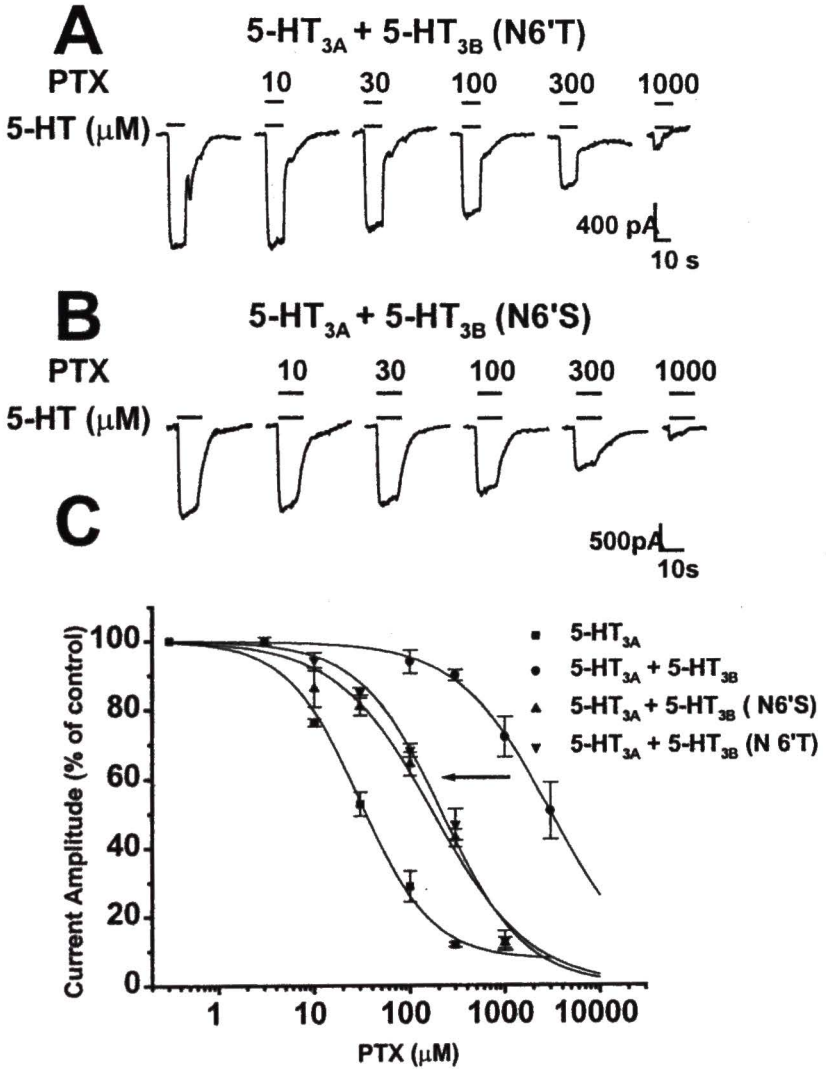

A
5-HT3A + 5-HT3B (N6'T)
PTX
10
30
100
300
1000
5-HT (μM)
400 pA
10 s
B
5-HT3A + 5-HT3B (N6'S)
PTX
10
30
100
300
1000
5-HT (μM)
C
500pA
10s
Current Amplitude (% of control)
100
80
60
40
20
0
1
10
100
1000
10000
PTX (μM)
5-HT3A
5-HT3A + 5-HT3B
5-HT3A + 5-HT3B (N6'S)
5-HT3A + 5-HT3B (N 6'T)

FIGURE IV-8. Effect of V7'T and N6'T, V7'T mutations in the 5-HT_{3B} subunit on PTX sensitivity. ***A,*** Traces obtained from cells expressing 5-HT_{3A} + 5-HT_{3B}(V7'T) in presence of PTX. A modest 4-fold increase in PTX sensitivity was observed in these receptors suggesting that the 7' residue may have minimal role in PTX recognition in the 5-HT_{3B} subunit. The IC_{50} for PTX was 763 μM compared to wild-type heteromers with an IC_{50} of ~3100 μM. ***B,*** Recordings from cells expressing the 5-HT_{3B}(N6'T, V7'T) double mutation. No additional gain in sensitivity in these receptors were observed when compared to receptors expressing N6'T mutation alone. The IC_{50} for PTX was 343 μM. ***C,*** Mean PTX inhibitory effect in cells expressing wild-type 5-HT_{3A}, 5-HT_{3A} + 5-HT_{3B} and mutant heteromeric receptors. Cells were gated with an EC_{35} concentration for 5-HT.

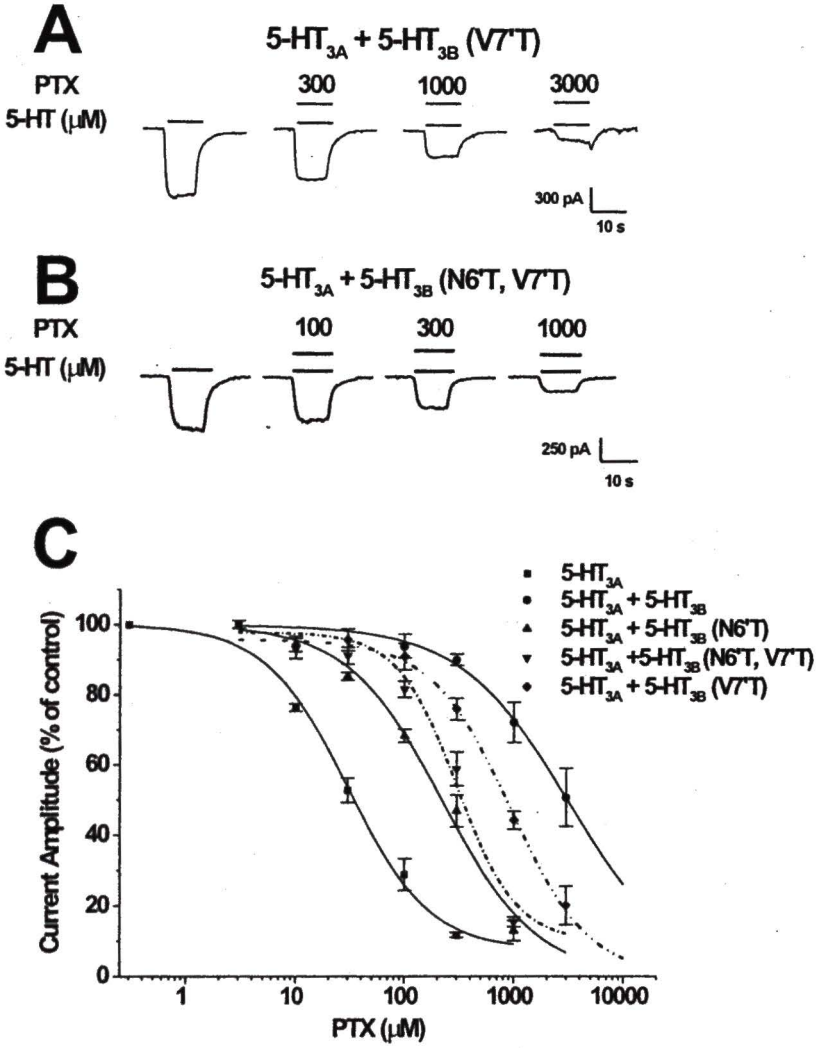

A
5-HT3A + 5-HT3B (V7'T)
PTX
5-HT (μM)
300
1000
3000
300 pA
10 s
B
5-HT3A + 5-HT3B (N6'T, V7'T)
PTX
5-HT (μM)
100
300
1000
250 pA
10 s
C
5-HT3A
5-HT3A + 5-HT3B
5-HT3A + 5-HT3B (N6'T)
5-HT3A +5-HT3B (N6'T, V7'T)
5-HT3A + 5-HT3B (V7'T)
Current Amplitude (% of control)
PTX (μM)

TABLE IV-1. Summary of Functional Properties of Wild-type 5-HT_3 Receptor and Mutant Receptors. EC_{50} values for 5-HT and IC_{50} values for PTX are reported in μM. IC_{50} values for PTX were obtained by gating the receptors with either an EC_{35} (^{A}) or an EC_{50} (^{B}) concentration of 5HT. ND= not determined. n= number of cells, nH= Hill co-efficient, ± = standard error of mean.

	5-HT			PICROTOXIN		
RECEPTOR	**EC_{50}**	**nH**	**n**	**IC_{50}**	**nH**	**n**
3A	1.2±0.06	1.9±0.19	4	30±3.1[A]	1.1±0.12	4
				42±5.8[B]	0.87±0.09	4
3A + 3B	0.99±0.07	1.06±0.1	4	3100±300[A]	0.88±0.05	6
3A+3A(T6'F)	0.9±0.09	2.3±0.25	5	1839±302[B]	1.04±0.19	5
3A (S2'A)	1.0±0.03	2.5±0.22	5	49±6[B]	0.88±0.09	5
3A+3B(N6'T)	0.49±0.03	1.8±0.27	5	213±24[A]	0.99±0.1	5
3A+3B(N6'S)	0.39±0.0	1.7±0.04	5	173±28[A]	0.85±0.12	5
3A (L7'T)	1.96±0.05	2.3±0.09	6	4±0.5[B]	1.1±0.1	4
3A+3B(V7'T)	0.59±0.09	1.65±0.29	4	763±9[A]	1.27±0.01	3
3A+3B(N6'T, V7'T)	0.39±0.01	1.16±0.05	4	343±45[A]	1.3±0.2	3
3A+3A(T6'C)	1.3±0.04	2.8±0.2	4	ND	ND	

TABLE IV-2. Summary of Saturation Binding Data from 5-HT_{3A}/HEK-t cells. Radioligand binding studies were performed using either [^{3}H]GR65630 or [^{3}H]EBOB using membranes harvested from a stable cell line expressing the mouse 5-HT_{3A} receptor. Non specific binding was defined by excess of m-CPBG or PTX respectively. At least 3 experiments were performed per saturation assay for each radioligand. s.e.m = standard error of mean.

Parameters	**[^{3}H[GR65630**	**[^{3}H]EBOB**
Kd ± s.e.m (nM)	8 ± 1.7	No specific binding
Bmax ± s.e.m (pmol/mg protein)	23 ± 1.6	No specific binding

REFERENCES

Akabas, M.H., Kaufmann, C., Archdeacon, P., and Karlin, A., 1994. Identification of acetylcholine receptor channel lining residues in the entire M2 segment of alpha subunit. Neuron 13, 919-927.

Bell-Horner, C.L., Dibas, M.I., Huang, R.Q., Drewe, J.A., and Dillon, G.H., 2000. Influence of subunit configuration on the interaction of picrotoxin site ligands with recombinant $GABA_A$ receptors. Molecular Brain Research 76, 47-55.

Berrada, D., Canenguez, K., Lembo, T., 2003. New approaches to the medical treatment of irritable bowel syndrome. Current Gastroenterology Report 5(4), 337-342.

Boess, F.G., Beroukhim, R., and Martin, I.L., 1995. Ultrastructure of the 5-hydroxytryptamine$_3$ receptor. Journal of Neurochemistry 64, 1401-1405.

Boyd, G.W, Low, P., Dunlop, J.I, Robertson, L.A, Vardy, A., Lambert, J.J, Peters, J.A, Connolly, C.N., 2002. Assembly and cell surface expression of homomeric and heteromeric 5-HT_3 receptors: the role of oligomerization and chaperone proteins. Molecular and Cellular Neuroscience 21(1), 38-50.

Brejc, K., van Dijk, W.J, Klaassen, R.V., Schuurmans, M., van Der Oost, J., Smit, A.B., and Sixma, T.K., 2001. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature 411(6835), 269-276.

Buhr, A., Wagner, C., Fuchs, K., Sieghart, W., and Siegel, E., 2001. Two novel residues in M2 of the γ-aminobutyric acid type A receptor affecting gating by GABA and picrotoxin affinity. Journal of Biological Chemistry 276, 7775-7781.

Camilleri, M., Mayer, E.A., Drossman, D.A., Heath, A., Dukes, G.E., McSorley, D., Kong, S., Mangel, A.W., Northcutt, A.R., 1999. Improvement in pain and bowel function in female irritable bowel patients with alosetron, a 5-HT_3 receptor antagonist. Aliment Pharmacology Therapy 13(9),1149-1159.

Chen C., and Okayama H. 1987. High efficiency transformation of mammalian cells by plasmid DNA. Molecular Cell Biology 7, 2745-2750.

Corringer, P.J., Galzi, J.L., Eisele, J.L., Bertrand, S., Changeux, J.P., Bertrand, D., 1995. Identification of a new component of the agonist binding site of the nicotinic alpha 7 homooligomeric receptor. Journal of Biological Chemistry 270(20), 11749-11752.

Cole, L.M., Roush, R.T., Casida, J.E., 1995. Drosophila GABA-gated chloride channel: modified [^{3}H]EBOB binding site associated with Ala-->Ser or Gly mutants of Rdl subunit. Life Sciences 56(10),757-765.

Costall, B., and Naylor., R.J., 1992 (a). Anxiolytic potential of 5-HT_3 receptor antagonists. Pharmacology and Toxicology 70(3),157-162.

Costall, B., and Naylor, R.J., 1992 (b). Neuropharmacology of emesis in relation to clinical response. British Journal of Cancer Supplements 19,S2-7; discussion S7-8.

Das, P., Bell-Horner, C.L., Machu, T.K., and Dillon, G.H., 2003. The $GABA_A$ receptor antagonist picrotoxin inhibits the 5-hydroxytryptamine type 3 receptors. Neuropharmacology 44(4),431-438.

Das, P., and Dillon, G.H., 2003. The $5\text{-}HT_{3B}$ subunit decreases picrotoxin sensitivity when co-expressed with the $5\text{-}HT_{3A}$ receptor. Molecular Brain Research, In press.

Davies, P.A., Pistis, M., Hanna, M.C., Peters, J.A., Lambert, J.J., Hales, T.G., and Kirkness, E.F., 1999. The $5\text{-}HT_{3B}$ subunit is a major determinant of serotonin receptor function. Nature 397, 359-363.

Dibas, M.I., Gonzales, E.B., Das, P., Bell-Horner, C.L., and Dillon, G.H., 2002. Identification of a key residue within the second transmembrane domain that confers use-facilitated block by picrotoxin in glycine $\alpha 1$ receptors Journal of Biological Chemistry 277, 9112-9117.

Dubin, A.E., Huvar, R., Andrea, M.R., Pyati, J., Zhu, J.Y., Joy, K.C., Wilson S.J., Galindo, J.E., Glass, C.A., Luo, L., Jackson, M.R., Lovenberg, T.W., and Erlander, M.G., 1999. The pharmacological and functional characteristics of the serotonin $5\text{-}HT_{3A}$ receptor are specifically modified by the $5\text{-}HT_{3B}$ receptor subunit. Journal of Biological Chemistry 274(43), 30799-30810.

Etter, A., Cully, D.F., Liu, K.K., Reiss, B., Vassilatis, D.B., Schaeffer, J.M., and Arena J.P., 1999.Picrotoxin blockade of invertebrate glutamate gated chloride channels: subunit dependence and evidence for binding within the pore. Journal of Neurochemistry 72, 318-326.

ffrench-Constant, R.H., Rocheleau, T.A., Steichen, J.C., and Chalmers, A.E., 1993. A point mutation in a Drosophila GABA receptor confers insecticide resistance. Nature 363, 449-451.

Greenshaw, A.J., and Silverstone, P.H., 1997. The non-antiemetic uses of serotonin 5-HT_3 receptor antagonists. Clinical pharmacology and therapeutic applications Drugs 53(1), 20-39.

Gurley, D.J., Amin, P.C., Ross, D.S., Weiss, D.S., and White, W., 1995. Point mutations in the M2 region of the α, β, or γ subunit of the $GABA_A$ channel that abolish block by picrotoxin. Receptors Channels 3, 12-30.

Hanna, M.C., Davies, P.A., Hales, T.G., and Kirkness, E.F., 2000. Evidence for expression of heteromeric serotonin 5-HT_3 receptors in rodents. Journal of Neurochemistry 75(1), 240-247.

Huang, J., and Casida, J.E., 1996. Characterization of [^{3}H]ethynylbicycloorthobenzoate ([^{3}H]EBOB) binding and the action of insecticides on the gamma-aminobutyric acid-gated chloride channel in cultured cerebellar granule neurons. Journal of Pharmacology and Experimental Therapeutics 279(3), 1191-1196.

Jursky, F., Fuchs, K., Buhr, A., Tretter, V., Sigel, E., and Sieghart, W., 2000. Identification of amino acid residues of $GABA_A$ receptor subunits contributing to the formation and affinity of the tert-Butylbicyclophophorothinate binding site. The Journal of Biological Chemistry 74, 1310-1316.

Karim, F., Roerig, S.C., and Saphier, D. 1996. Role of 5-hydroxytryptamine $_3$ antagonists in the prevention of emesis caused by anticancer therapy. Biochemical Pharmacology 52(2), 685-692.

Kume, A., and Albin, R.L., 1994. Quantitative autoradiography of 4'-ethynyl-4-n-[2,3-3H2]propylbicycloorthobenzoate binding to the $GABA_A$ receptor complex. European Journal of Pharmacology 263(1-2),163-173.

Lawrence, L.J., and Casida, J.E., 1983. Stereospecific action of pyrethroid insecticides on the gamma-aminobutyric acid receptor-ionophore complex. Science 221(4618), 1399-1401.

Maricq, A.V., Peterson, A.S., Brake, A.J., Myers, R.M., and Julius, D., 1991. Primary structure and functional expression of the 5-HT_3 receptor, a serotonin-gated ion channel. Science 254, 432-437.

Niesler, B., Frank, B., Kapeller, J., and Rappold, G.A., 2003. Cloning, physical mapping and expression analysis of the human 5-HT_3 serotonin receptor like genes HTR3C, HTR3D and HTR3E. Gene 310, 101-111.

Ortells, M.O., and Lunt, C.G., 1995. Evolutionary history of the ligand-gated ion channel superfamily of receptors. Trends in Neuroscience 18(3), 121-127.

Panicker, S., Cruz, H., Arrabit, C., Slesinger P.A., 2002. Evidence for a centrally located gate in the pore of a serotonin-gated ion channel. Journal of Neuroscience 22(5),1629-1639.

Perret, P., Sarda, X., Wolff, M., Wu, T.-T., Bushey, D., and Goeldner, M., 1999. Interaction of non-competitive blockers within the γ-aminobutyric acid type A

chloride channel using chemically reactive probes as chemical sensors for cysteine mutants. Journal of Biological Chemistry 274, 25350-25354.

Ramanjaneyulu, R., and Ticku, M.K., 1984. Binding characteristics and interactions of depressant drugs with [^{35}S]t-butylbicyclophosphorothionate, a ligand that binds to the picrotoxinin site. Journal of Neurochemistry 42, 221-229.

Reeves, D.C., Goren, E.N., Akabas, M.H., and Lummis, S.C.R., 2001. Structural and electrostatic properties of the 5-HT_3 receptor pore revealed by substituted cysteine accessibility mutagenesis. Journal of Biological Chemistry 276, 42035-42042.

Reeves, D.C., and Lummis, S.C.R., 2002. The molecular basis of the structure and function of the 5-HT_3 receptor:a model ligand-gated ion channel. Molecular Membrane Biology 19, 11-26.

Shan, Q., Haddrill, J.L., and Lynch, J.W., 2001. A single β subunit M2 domain controls the picrotoxin sensitivity of αβ heteromeric glycine receptor chloride channels. Journal of Neurochemistry 76, 1109-1120.

Shan, Q., Hadrill, J.L., and Lynch, J.W., 2002. Comparitive surface accessibility of a pore-lining threonine residue (T6') in the glycine and $GABA_A$ receptors. The Journal of Biological Chemistry 277(47), 44845-44853.

Spath, M., 2002. Current experience with 5-HT_3 receptor antagonists in fibromyalgia Rheumatology Disorder Clinic North America 28(2), 319-328.

Squires, R.F., Casida, J.E., Richardson, M., Saederup, E., 1983. ^{35}S]t-butylbicyclophosphorothionate binds with high affinity to brain-specific sites

coupled to gamma-aminobutyric acid-A and ion recognition sites. Molecular Pharmacology 23(2),326-336.

Ticku, M.K., Ban, M., Olsen, RW., 1978. Binding of [^{3}H]alpha-dihydropicrotoxinin, a gamma-aminobutyric acid synaptic antagonist, to rat brain membranes. Molecular Pharmacology 14(3), 391-402.

Unwin, N., 1993. Neurotransmitter action: opening of ligand gated ion channels. Cell 72, 31-41.

Wang, T.L., Hackam, A.S., Guggino, W.B., and Cutting, G.R., 1995. A single amino acid in gamma-aminobutyric acid rho 1 receptors affects competitive and noncompetitive components of picrotoxin inhibition. Proceedings of the National Academy of Sciences U.S.A. 92(25), 11751-11755.

Wilson, G., and Karlin, A., 2001. Acetylcholine receptor channel structure in the resting, open and desensitized states probed with substituted-cysteine-accessibility method. Proceedings of the National Academy of Sciences U.S.A. 98(3), 1241-1248.

Xu, M., Covey, D.F., and Akabas, M.H., 1995. Interaction of picrotoxin with $GABA_A$ receptor channel-lining residues probed in cysteine mutants. Biophysics Journal 69, 1858-1867.

Yagle, M.A., Martin, M.W., de Fiebre, C.M., de Fiebre, N.C., Covey, D.F., and Dillon, G.H., 2003. [^{3}H] ethylnylbicycloorthobenzoate ([^{3}H]EBOB) binding in rat cerebellum and recombinant GABA receptors. Neurotoxicology, In press.

Zhang, H.G., ffrench-Constant, R.H., Brideau, A.D., and Lipton, S.A., 1995. Cloning of a γ-aminobutyric acid type $_C$ receptor subunit in rat retina with a methionine residue critical for picrotoxinin channel block. Proceedings of National Academy Sciences 92, 11756-11760.

Zhorov, B., and Bregestovski, P., 2000. Chloride channels of glycine and GABA receptors with blockers: Monte Carlo minimization and structure-activity relationships. Biophysics Journal 78, 1786-1803.

CHAPTER V

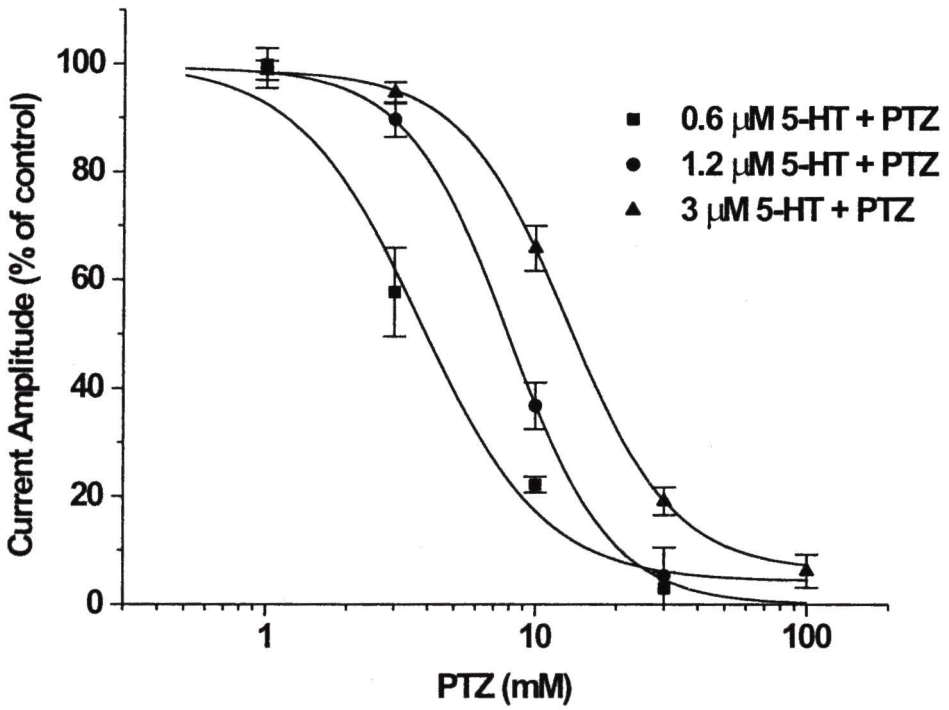
ADDITIONAL STUDIES

In addition to experiments described in earlier chapters, we conducted other studies geared towards understanding the mechanism of convulsant drug action and also possibly defining a binding site for these ligands in the 5-HT_3 receptor. These studies were not included in manuscripts because they did not fit the general theme of the manuscripts and hence have been reported separately in this chapter.

The CNS convulsant pentylenetetrazole inhibits the 5-HT_{3A} receptor in a competitive manner. While the mechanism of block produced by PTX in $GABA_A$ receptors is predominantly non-competitive, the pharmacologic mechanism of PTZ inhibition is competitive in nature (Huang et al., 2001). PTZ-induced inhibition of the $GABA_A$ receptor is not due to actual competition with GABA at the agonist recognition site, because PTZ does not displace the GABA agonist [^{3}H]muscimol in binding assays (Ticku and Maksay, 1983). Instead, the "competitive" inhibition of GABA currents is likely due to PTZ preferentially binding to and stabilizing a receptor state whose probability is influenced by GABA concentration. Other examples of allosteric "competitive" antagonism have been reported (Bertrand et al., 1992; Lynch et al., 1995). To determine if the mechanism of block produced by PTZ in the 5-HT_{3A} receptors is similar to the $GABA_A$ receptors, we evaluated the effect of PTZ using different gating

concentrations of 5-HT. As illustrated in Fig. 1., the PTZ IC_{50} was dependent on the 5-HT gating concentration. Thus, the PTZ IC_{50} increased four-fold when the channel was gated with 0.6 μM compared to 3 μM 5-HT (Fig. 1). The similarity in mechanism of action of PTZ in the $5\text{-}HT_{3A}$ receptor and $GABA_A$ receptors suggests a conserved site of action for this ligand.

FIGURE V-1. Mechanism of Inhibition of 5-HT_{3A} Receptors by the CNS convulsant Pentylenetetrazole. PTZ inhibition curves obtained at three different gating concentrations of 5-HT. At successively higher 5-HT gating concentrations the PTZ inhibition curves were rightward shifted, suggesting a competitive mode of inhibition. The IC_{50} values for PTZ (in mM) obtained against 0.6, 1.2 and 3 μM 5-HT were 3.7 ± 0.6, 7.9 ± 0.15 and 13.3 ± 0.16, respectively. Hill coefficients were 1.8 ± 0.5, 2.3 ± 0.09 and 2.1 ± 0.05, respectively (n= 4-6).


Current Amplitude (% of control)
100
80
60
40
20
0
1
10
100
PTZ (mM)
0.6 μM 5-HT + PTZ
1.2 μM 5-HT + PTZ
3 μM 5-HT + PTZ

SCAM studies in the 5-HT_{3A} receptor. The substituted cysteine accessibility method (SCAM) devised by Akabas and Karlin (1992) is a combination of biochemical and genetic approach to study ion-channel function. In this method, the native amino acid in membrane embedded channel is mutated to cysteine one at a time, and the cys-substituted mutants are tested for their ability to react with a small, charged sulfhydryl reagent. This strategy can be used to identify amino acid residues, which line the ion-channel pore, to locate selectivity filters and gates and to assess channel structure in different functional states. SCAM is based on certain assumptions a) only residues which line the ion channel are in the water accessible region of the protein b) residues exposed to the water accessible region will react rapidly with sulfhydryl reagents than residues exposed to the hydrophobic lipid layer.

Initially, the wild-type receptor is tested with sulfhydryl reagents to determine if any native cysteines are reactive to sulfhydryl reagents. In case they are, they have to be suitably mutated to retain ion-channel function. Next, each of the residues in the membrane embedded region of the channel are mutated to cysteine one at a time, expressed in a heterologous system and tested with sulfhydryl reagents. If channel function is irreversibly altered, then it is assumed to be due to reaction of engineered cysteine with sulfhydryl reagent. The cysteine residue is thus inferred to line the ion-channel. In case, ion channel function is not altered irreversibly, then it could indicate a lack of reaction or reaction that has occurred but with no detectable effect on function.

This strategy has also been used to locate binding sites for channel blockers (Gross and MacKinnon, 1996; Pascual and Karlin, 1998). If cysteine substituted mutants are protected from sulfhydryl modification by a putative channel blocker, then one can infer that the binding site of the blocker is at that site or at a site distally located from the blocker binding site. Using this technique, Xu and Akabas (1995) showed that the 2' valine and 6' threonine in the rat $GABA_A$ $\alpha 1$ subunit are water accessible and exposed to the lumen of the channel. PTX protected the sulfhydryl of the 2'$\alpha 1$V257C from modification by sulfhydryl reagent suggesting that the 2' residue may form part of the binding pocket of PTX.

Mutation of the 6'T to F in the 5-HT_{3A} resulted in a loss of sensitivity to PTX. In addition mutation of the 6'N to T or S in the 5-HT_{3B} subunit resulted in a gain of sensitivity to PTX (Chapter IV). Thus, similar to $GABA_A$ and glycine receptors, the 6' residue is an important determinant of PTX sensitivity in the 5-HT_3 receptors. The 2' and 6' residues are exposed to the channel in the 5-HT_{3A} receptors (Reeves et al., 2002). To determine if the binding site of PTX in the 5-HT_{3A} receptor is in the 2'-6' region, we mutated the 2'S to C and 6'T to C and tested the effect of the small positively charged sulfhydryl reagent MTSET in the engineered cysteine mutants. The premise for this experiment was that if the 2' or 6' residue formed the putative binding site of PTX, then they would be protected from modification by sulfhydryl reagent. While mutation of 2'S to C revealed near normal function, mutation of the 6'T to C resulted in non-functional receptors. This mutant could however be "rescued" by co-expression with wild-type 5-HT_{3A} (see Table 1 for EC_{50} values and Hill co-efficients). The reagents were applied to

cells expressing the cysteine-engineered mutants using the following protocol: 10X EC_{50} of 5-HT (10s), saline (3min), 10X EC_{50} of 5-HT (10s), 1mM MTSET + 10X EC_{50} of 5-HT (1 min), saline (3 min) and 10X EC_{50} of 5-HT (10s).

Treatment of the wild-type 5-HT_{3A} receptor with MTSET did not exhibit modification of currents (Fig. 2). We assumed that the 8 native cysteine residues did not react with MTSET or the reaction did not result in any change in function. Subsequent SCAM studies using the cysteine-engineered mutants at the 2' and 6' position also did not display any irreversible modification. The result were the same using both saturating and an EC_{50} concentration of the agonist. Thus, we were unable to test if PTX could protect these residues from irreversible modification. To determine if the experimental protocol was adequate to perform these studies, we used the same protocol to study sulfhydryl modification of an engineered cysteine residue in the N-terminal region of $GABA_A$ $\beta 2$ receptor. As shown in Fig.3, the positively charged sulfhydryl reagent MTSEA altered GABA activated currents irreversibly in $GABA_A$ $\alpha 1\beta 2$(R207C) receptors.

The lack of sulfhydryl modification of cysteine residues at both 2' and 6' positions was unanticipated. Previous SCAM studies in the 5-HT_{3A} receptor that have reported reaction at both 2' and 6' residues were done in oocyte expression system (Reeves et al., 2002; Panicker et al., 2002). There is evidence suggesting that the pore structure at this region of the channel in $GABA_A$ receptor may be different in different expression systems (Shan et al., 2002). Thus, our results may be explained by the different expression system used (HEK-293 cells).

FIGURE V-2. Effect of Sulfhydryl Reagent MTSET in the Wild-type 5-HT_{3A} Receptor. ***A,*** HEK-293 cells expressing wild-type 5-HT_{3A} receptor treated with 1mM MTSET in saline for 1 min. No modification of current was observed after sulfhydryl treatment. ***B,*** Wild-type 5-HT_{3A} receptor was treated with 1mM MTSET in saturating concentration of 5-HT. No modification of current was observed in the open state of the channel. Agonist application time was 10s and sulfhydryl reagent application time was 1 minute.

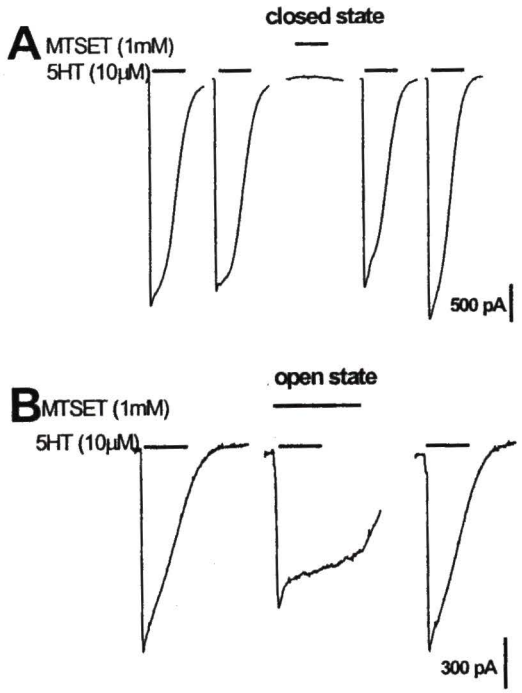

closed state
A
MTSET (1mM)
5HT (10μM)
500 pA
open state
B
MTSET (1mM)
5HT (10μM)
300 pA

FIGURE V-3. Effect of Sulfhydryl Reagent MTSEA on $GABA_A$ $\alpha 1\beta 2$(R207C) Receptors. Treatment of $GABA_A$ $\alpha 1\beta 2$(R207C) with MTSEA resulted in irreversible blockade of the receptor suggesting reaction of engineered cysteine with MTSEA. A saturating GABA concentration was used to gate the channel. Application time of agonist is 10s.

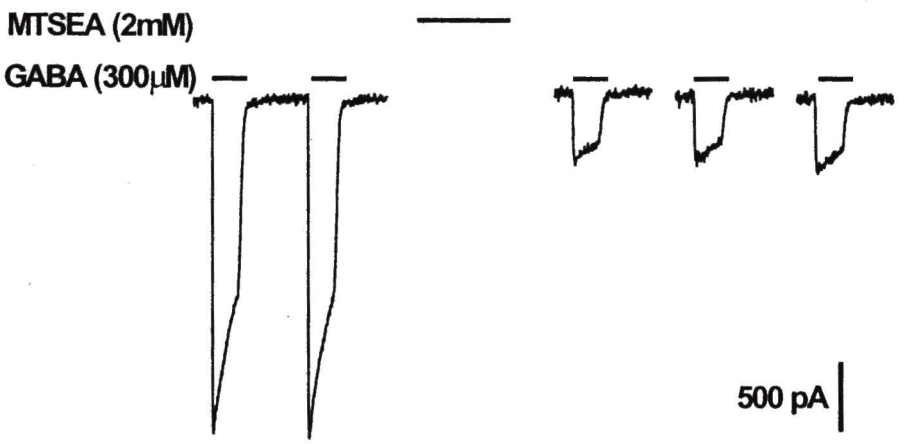

MTSEA (2mM)
GABA (300μM)
500 pA

TABLE V-1 EC_{50} **and Hill co-efficient values (nH) for wild-type 5-**HT_{3A} **and cysteine engineered mutants.**

RECEPTOR	EC_{50} (μM)	nH
5-HT_{3A}	1.2 ± 0.06	1.9 ± 0.19
5-HT_{3A} (S2'C)	1.17 ± 0.05	2.2 ± 0.18
5-HT_{3A}+5-HT_{3A} (T 6'C)	1.3 ± 0.04	2.8 ±0.2

REFERENCES

Akabas, M.H., Stauffer, D.A., Xu, M., and Karlin, A., 1992. Acetylcholine receptor channel structure probed in cysteine substitution mutants. Science 258, 307-310.

Bertrand, D., Devillers-Thiery, A., Revah, F., Galzi, J.L., Hussy, N., Mulle, C., Bertrand, S., Ballivet, M., and Changeux, J.P., 1992. Unconventional pharmacology of a neuronal nicotinic receptor mutated in the channel domain. Proceedings of the National Academy of Sciences 89, 1261-1265.

Gross, A., MacKinnon, R., 1996. Agitoxin footprinting the shaker potassium channel pore. Neuron 16(2), 399-406.

Huang, R.Q., Bell-Horner, C.L., Dibas, M.I., Covey, D.F., Drewe, J.A., and Dillon, G.H., 2001.Pentylenetetrazole induced inhibition of recombinant gamma-aminobutyric acid type A receptors: mechanism and site of action. Journal of Pharmacology and Experimental Therapeutics 298, 986-995.

Lynch, J.W., Rajendra, S., Bany, P.H., and Schofield, P.R., 1995. Mutations affecting the glycine receptor agonist transduction mechanism convert the competitive antagonist, picrotoxin, into an allosteric potentiator. Journal of Biological Chemistry 270, 13799-13806.

Pascual, J.M., Karlin, A., 1998. Delimiting the binding site for quaternary ammonium lidocaine derivatives in the acetylcholine receptor channel. Journal of General Physiology 112(5),611-621.

Panicker, S., Cruz, H., Arrabit, C., Slesinger P.A., 2002. Evidence for a centrally located gate in the pore of a serotonin-gated ion channel. Journal of Neuroscience 22(5),1629-1639.

Reeves, D.C., Goren, E.N., Akabas, M.H., and Lummis, S.C.R., 2001. Structural and electrostatic properties of the 5-HT_3 receptor pore revealed by substituted cysteine accessibility mutagenesis. Journal of Biological Chemistry 276, 42035-42042.

Shan, Q., Hadrill, J.l., and Lynch, J.W., 2002. Comparative surface accessibility of a pore-lining threonine residue (T6') in the glycine and $GABA_A$ receptors.

Ticku, M.K., and Maksay, G., 1983. Convulsant/depressant site of action at the allosteric benzodiazepine-GABA receptor-ionophore complex. Life Science 33, 2363-2375.

Xu, M., Covey, D.F., and Akabas, M.H., 1995. Interaction of picrotoxin with $GABA_A$ receptor channel-lining residues probed in cysteine mutants. Biophysics Journal 69, 1858-1867.

CHAPTER VI

SUMMARY AND CONCLUSIONS

While it was known for decades that PTX inhibits all members of anion-selective ion channels, its effects, if any, on cation-selective 5-HT_3 channels was previously unknown. Our study, for the first time, provided evidence of PTX's inhibitory effects in these receptors. The primary goal of this dissertation was to attain an understanding of the mechanism of action of PTX and identify amino acid residues, which are involved in PTX-recognition.

Although PTX blocks $GABA_A$ and glycine receptors, the pharmacologic mechanism of block produced by PTX is distinct in these receptors. While PTX-mediated inhibition in $GABA_A$ receptors is largely non-competitive (Yoon et al., 1993), inhibition in glycine receptors is competitive (Lynch et al., 1995). Moreover, use-facilitated blockade by PTX, a characteristic typically observed in $GABA_A$ receptors (Dillon et al., 1995) is not observed in glycine receptors (Dibas et al., 2002). As shown earlier, the mechanism of block produced by PTX in the 5-HT_{3A} receptor was demonstrated to be non-competitive and use-facilitated. Furthermore, the ligands PTZ and U-93631, which are believed to interact at the PTX-site or in overlapping domains in the $GABA_A$ receptor, also inhibited the 5-HT_{3A} receptor. PTZ mediated inhibition was found to be competitive (allosteric competition) in the 5-HT_{3A} receptors similar to the $GABA_A$

receptors. Thus, taken together, it was reasonable to speculate that the site of action of PTX in the 5-HT_3 receptors would be similar to the $GABA_A$ receptor.

The block mediated by PTX in anion-selective glycine, $GABA_C$ and GluR receptors is dependent on subunit configuration. We provided evidence that heteromeric 5-$HT_{3A/3B}$ receptors were a hundred-fold less sensitive to PTX as compared to homomeric 5-HT_{3A} receptors. At low and high gating concentrations of 5-HT, the degree of block produced by PTX in heteromeric receptors was marginal as compared to homomeric 5-HT_{3A} receptors. As yet, there are no ligands, which exhibit appreciable selectivity between the homomeric and heteromeric receptors. The remarkable selectivity of PTX for the homomeric receptors can be exploited to detect expression of these receptors not only in heterologous expression systems but also in native tissue preparations.

Evidence from studies in anion-selective LGICs strongly suggests that PTX interacts in the TM2 domain, which forms the pore of the receptor. The fact that PTX-mediated blockade in the 5-HT_3 receptor is non-competitive, is increased in presence of increasing concentrations of agonist (use-dependent), is nominal in absence of agonist suggested that PTX interacts in the ion channel. The focus of subsequent studies presented in this thesis was to identity the molecular determinants involved in PTX recognition. The residues at 2', 3' and 6' in TM2 domain of anion-selective receptors have been shown to modulate the sensitivity of PTX and related ligands. In addition, residues in the 15' and 17' positions are also involved in PTX recognition (Dibas et al., 2002; Perret et al., 1999). We focused on the residues located at the cytoplasmic aspect of the 5-HT_3 receptor as probable determinants of PTX sensitivity.

A naturally occurring mutation at the 2' position (i.e. A2'S), in a field isolated *Drosophila* GABA receptor, conferred resistance to PTX (ffrench-Constant et al., 1993). We, thus mutated the 2'S to A, in the 5-HT_{3A} receptor anticipating an increase in sensitivity to PTX. Mutation of the 2'S to A residue however, did not alter sensitivity to PTX. This observation was consistent with that of Shan et al, (2001), who demonstrated that the 2' residue is not involved in PTX in glycine receptors. Thus, in this respect, the effect of PTX in 5-HT_3 receptors resembles that in glycine receptors.

We tested if the 6' position contributed to PTX recognition in the 5-HT_{3A} receptors by mutating the 6'T to F. This mutation resulted in a 42-fold loss of sensitivity to PTX, demonstrating that this residue is a key determinant of PTX sensitivity, not unlike the $GABA_A$ and glycine receptors. Additionally, a converse mutation in the 6' position in the 5-HT_{3B} subunit partially restored PTX sensitivity in PTX-resistant heteromeric receptors. The IC_{50} for PTX in mutant N6'T receptors was 220 μM compared to ~3100 μM in the wild-type heteromeric receptors. This convincingly demonstrated the critical role of 6' residue in conferring PTX sensitivity. Another mutation at the 6' position in the 5-HT_{3B} subunit, i.e. N6'S also increased the sensitivity to PTX. While the mouse and rat receptors have N at the 6' position, the human 3B subunit has a serine residue. Our results also suggest that the 100-fold shift in PTX sensitivity observed in mouse heteromeric receptors may not be seen in human heteromeric receptors due to the presence of serine residue at the 6' position. The mechanism by which sensitivity to PTX is lost or gained is not known. Based on molecular modeling studies of Zhorov and Bregestovski, (2000), we can surmise that,

hydrogen bond formation by threonine residue with the hydrophilic domain of PTX is disrupted upon mutation of 6'T to F. Conversely, mutation of 6'N to T or S in the 5-HT_{3B} subunit, may allow formation of hydrogen bond thus explaining the increase in sensitivity to PTX in the N6'T/S mutants.

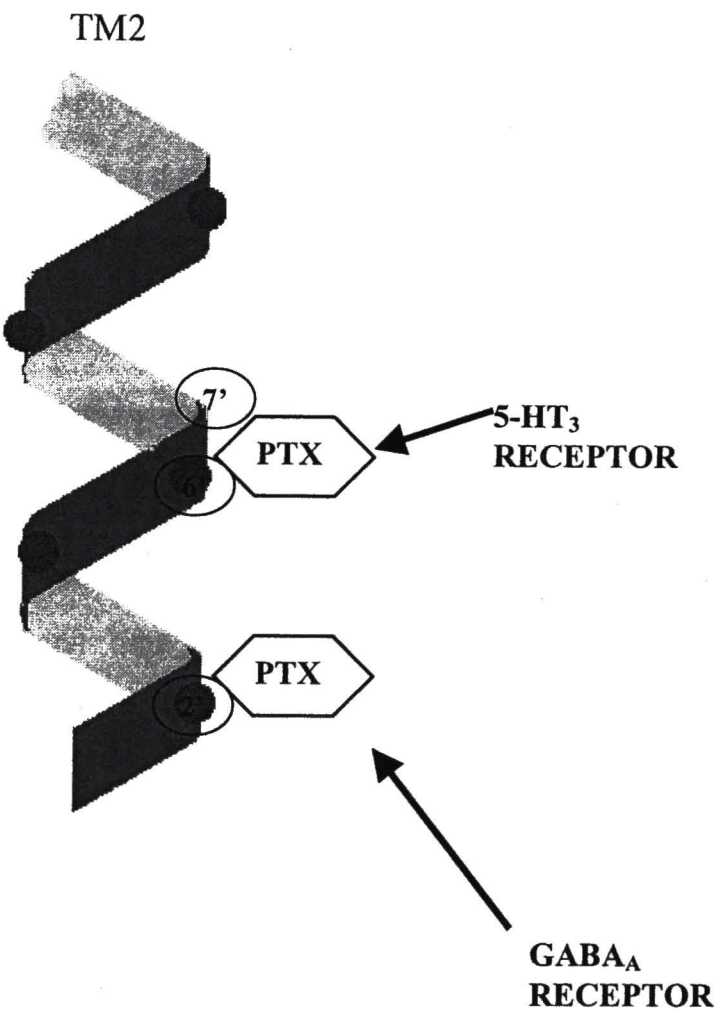
We also reported the novel finding that the 7' residue in 5-HT_{3A} subunit is also a determinant of PTX sensitivity. This is the first report of the involvement of this residue in PTX recognition in LGICs. Interestingly, the involvement of this residue in PTX recognition in the 5-HT_{3B} appeared minimal. While the single mutation V 7'T conferred a marginal increase in sensitivity to PTX, the double mutation N6'T, V7'T, did not produce any additional sensitivity to PTX than what was seen with the single N6'T mutation. A striking effect of this mutation was altered gating kinetics. The mutation conferred rapid activation and deactivation kinetics as compared to the wild-type 5-HT_{3A} receptors. Additionally, the mutant receptors exhibited prominent desensitization, which was minimal in wild-type receptors. The role of 7' residue in channel gating needs to be explored further.

The high affinity radioligand [^{3}H]EBOB, which interacts at the PTX site in the $GABA_A$ receptor (Cole et al.,1995), did not bind specifically with the 5-HT_{3A} receptor. Moreover, TBPS, which displaces [^{3}H]EBOB binding produced very little inhibition of the 5-HT_{3A} receptor (data not shown). In addition, 3'F to L mutation in the 5-HT_{3A} receptor did not alter PTX sensitivity (mutation of 3'L to F decreases sensitivity to PTX in $GABA_A$ receptor) (Buhr et al., 2001). Overall, the data from our studies suggested that the recognition site for PTX in the 5-HT_3 receptors is different from that in the $GABA_A$

receptor. While evidence suggests that in $GABA_A$ receptor, PTX and related ligands may bind at the 2'-3' region in the cytoplasmic aspect of the ion channel, our data suggest that they may interact in the 6'-7' region in the 5-HT_3 receptors (Fig.1, page 187). However, whether 6'-7' region is the actual binding site for PTX, or a domain allosterically coupled to the binding site is not clear. One way of more clearly answering this question would be to use SCAM technique to determine if PTX could protect sulfhydryl modification of engineered cysteine residues in the 2' and 6' position. The lack of sulfhydryl modification of cysteines at both 2' and 6' position did not allow us to investigate PTX protection of these residues.

The possibility for multiple binding sites for PTX in 5-HT_3 receptors exists. Evidence obtained from anion-selective receptors is suggestive of PTX recognition sites being in the cytoplasmic and extracellular aspect of the channel. Mutation of residues in the extracellular region of 5-HT_3 TM2, particularly at the 15' and 17' position would be required to address this possibility.

FIGURE VI–1 Proposed Model Showing the Site of Action of PTX in the 5-HT_3 Receptor. The domain of action of PTX in the cation-selective 5-HT_3 receptor is comparable but not identical to that in the anion-selective $GABA_A$ receptor


TM2
7'
6'
2'
PTX
PTX
5-HT3
RECEPTOR
GABAA
RECEPTOR

Future directions

In the present study we showed for the first time that PTX and related ligands like PTZ and U-93631 inhibit the 5-hydroxytryptamine type 3 receptors. While the pharmacologic mechanism of block produced by these ligands in this receptor appeared similar to the $GABA_A$ receptors, the molecular determinants governing PTX sensitivity appeared different. We identified the 6'-7' region as determinants of PTX sensitivity. The possibility of multiple PTX-recognition sites in the $5\text{-}HT_3$ receptors exists. In future studies, we would like to investigate the effect of mutation of 15' and 17' residue in PTX interaction. As described earlier, mutation of the 7'L to T residue in $5\text{-}HT_{3A}$ receptors conferred not only increased sensitivity to PTX but also fast activation and deactivation kinetics. Conversely, mutation of 7'T to L in glycine α1 receptors decreased the activation and deactivation time significantly (unpublished observation). Thus, a systematic replacement of the 7' residue with amino acids of different sizes and hydrophobicity will allow elucidation of the role of 7' residue in channel gating.

REFERENCES

Buhr, A., Wagner, C., Fuchs, K., Sieghart, W., and Siegel, E., 2001. Two novel residues in M2 of the γ-aminobutyric acid type A receptor affecting gating by GABA and picrotoxin affinity. Journal of Biological Chemistry 276, 7775-7781.

Cole, L.M., Roush, R.T., Casida, J.E., 1995. Drosophila GABA-gated chloride channel: modified [^{3}H]EBOB binding site associated with Ala-->Ser or Gly mutants of Rdl subunit. Life Sciences 56(10),757-765.

Dibas, M.I., Gonzales, E.B., Das, P., Bell-Horner, C.L., and Dillon, G.H., 2002. Identification of a key residue within the second transmembrane domain that confers use-facilitated block by picrotoxin in glycine α1 receptors Journal of Biological Chemistry 277, 9112-9117.

Dillon, G.H., Im, W.B., Carter, D.B., and McKinley, D.D., 1995. Enhancement by GABA of the association rate of picrotoxin and *tert*-butylbicyclophosphorothionate to the rat cloned α1ß2γ2 $GABA_A$ receptor subtype. British Journal of Pharmacology 115, 539-545.

ffrench-Constant, R.H., Rocheleau, T.A., Steichen, J.C., and Chalmers, A.E., 1993. A point mutation in a Drosophila GABA receptor confers insecticide resistance. Nature 363, 449-451.

Lynch, J.W., Rajendra, S., Bany, P.H., and Schofield, P.R., 1995. Mutations affecting the glycine receptor agonist transduction mechanism convert the competitive

antagonist, picrotoxin, into an allosteric potentiator. Journal of Biological Chemistry 270,13799-13806.

Perret, P., Sarda, X., Wolff, M., Wu, T.-T., Bushey, D., and Goeldner, M., 1999. Interaction of non-competitive blockers within the γ-aminobutyric acid type A chloride channel using chemically reactive probes as chemical sensors for cysteine mutants. Journal of Biological Chemistry 274, 25350-25354.

Shan, Q., Haddrill, J.L., and Lynch, J.W., 2001. A single β subunit M2 domain controls the picrotoxin sensitivity of αβ heteromeric glycine receptor chloride channels. Journal of Neurochemistry 76, 1109-1120.

Yoon, K.W., Covey, D.F., and Rothman, S.M., 1993. Multiple mechanisms of picrotoxin block of GABA induced currents in rat hippocampal neurons. Journal of Physiology 464, 423-439.

Zhorov, B., and Bregestovski, P., 2000. Chloride channels of glycine and GABA receptors with blockers: Monte Carlo minimization and structure-activity relationships. Biophysics Journal 78, 1786-1803.