

Probing the Role of a Conserved M1 Proline Residue in 5-Hydroxytryptamine₃ Receptor Gating

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ABSTRACT

A conserved proline residue is found in the first transmembrane domain (M1) of every subunit in the ligand-gated ion channel superfamily. The position of this proline between the N-terminal extracellular agonist binding and the second transmembrane (M2) channel lining domains in the primary sequence suggests its possible involvement in the gating of the receptor. Replacing this proline with alanine, glycine, or leucine in the 5-hydroxytryptamine (5-HT)_{3A} homomeric receptors expressed in *Xenopus laevis* oocytes resulted in the absence of 5-HT-induced whole-cell currents, although there were normal levels of specific surface [³H]granisetron ([³H]BRL-43694) binding sites. To determine what properties of the conserved proline are critical for the function of the channel, two imino acids and an α -hy-

droxy acid were incorporated at the proline position using the nonsense suppression method. *trans*-3-Methyl-proline, pipecolic acid, and leucic acid were able to replace the conserved proline to produce active channels with EC₅₀ values similar to that for the wild-type receptor. These trends are preserved in the heteromeric receptors consisting of 5-HT_{3A} and 5-HT_{3B} subunits in oocytes. The prominent common feature among these residues and proline is the lack of hydrogen bond donor activity, potentially resulting in a flexible secondary structure in the M1 region. Thus, lack of hydrogen bond donor activity may be a key element in channel gating and may explain the high degree of conservation of this M1 proline.

A conserved proline (Pro) residue is found in the first transmembrane domain (M1) of all known subunits of the ligand-gated ion channel (LGIC, www.pasteur.fr/LGIC/LGIC.html for latest information) superfamily typified by the nicotinic acetylcholine receptors (nAChRs; Ortells and Lunt, 1995). The superfamily also includes 5-HT₃, γ -aminobutyric acid type A, and glycine receptors, which are all believed to be pseudosymmetric pentamers of various subunit compositions (Karlin and Akabas, 1995). All subunits have a similar structure of four putative transmembrane domains with a large extracellular N-terminal region (~200 amino acids) and a variable cytoplasmic loop between the transmembrane domains M3 and M4. The agonist binding sites are formed by the N-terminal region (~200 amino acids) and lie about 50 Å from the channel pore, which consists largely of the M2 domains (Unwin, 1993; Karlin and Akabas, 1995). The binding of agonist to the extracellular domain is communicated to the pore domain (M2) and results in conformational changes corresponding to the opening/closing of the channel. This

process is often termed "gating." The M1 domain, where the conserved Pro is located, provides the only covalent link and therefore may be a physical link between the binding sites and the pore.

Membrane-buried Pro residues are far more common in ion channels or transporter proteins than structural membrane proteins, and it has been suggested that this bias reflects an important functional role for Pro in proteins that control regulated transmembrane fluxes (Brandl and Deber, 1986). Mutagenesis studies show that replacing membrane-buried Pro with other amino acids strongly modifies the functional properties of the protein (e.g., reversing the polarity of the voltage-gated gap junction channel; Suchyna et al., 1993).

Structural aspects of the ligand binding and the channel lining regions of the nAChRs have been extensively studied with conventional mutagenesis (Karlin and Akabas, 1995). However, our knowledge of the gating mechanism of these and other members of the LGIC is incomplete due to both the dynamic nature of the gating process and the lack of direct measurements of conformational changes. Furthermore, conventional mutagenesis cannot probe many aspects of the role

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ABBREVIATIONS: M1, transmembrane domain 1; 5-HT, 5-hydroxytryptamine (serotonin); 5-HT_{3A}, 5-HT_{3B}, serotonin receptor type 3 A, B subunits; m5-HT_{3A}-UAG, mouse serotonin receptor type 3 A mRNA containing the UAG stop codon rather than the M1 Pro256 codon; nAChR, nicotinic acetylcholine receptor; tRNA-THG73-Xaa, tRNA THG73 acylated with amino or hydroxy acid; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate; P3m, *trans*-3-methyl-proline; Pip, pipecolic acid; Lah, leucic acid.

of Pro because Pro is unique among the natural amino acids in that its α -nitrogen is part of a pyrrolidine ring. Such a structure imparts unique constraints on the peptide backbone and prevents the nitrogen from serving as a hydrogen bond donor. Also, the Xaa-Pro bond has both a slightly lower activation energy for *cis-trans* isomerization and a significantly lower equilibrium energy between the *cis* and *trans* conformations than the corresponding energies for peptide bonds between other amino acids, raising the possibility of a functional role for a *cis*-prolyl bond. To determine the chemical properties important for the function of the conserved Pro, we replaced the Pro residue with unnatural amino acid analogs, thus introducing subtle changes.

The nonsense codon suppression method (Noren et al., 1989) has been successfully applied to study the muscle nicotinic receptor expressed in *Xenopus laevis* oocytes (Nowak et al., 1995; Kearney et al., 1996; Saks et al., 1996; Zhong et al., 1998). The nonsense codon suppression method provides the opportunity to replace the conserved M1 Pro residue with unnatural analogs, so subtle chemical changes can be investigated. One concern in applying the nonsense codon suppression method to homomeric membrane proteins is that the often low efficiency of suppression may be exacerbated by the number of identical subunits in the complex, producing even fewer completely assembled receptors. Another potential problem in general is reacylation of the nonsense suppressor tRNA after it has delivered its synthetic residue, which may result in incorporation of other natural amino acids at the site of interest

(Saks et al., 1996; Nowak et al., 1998). However, previous positive results with the homomeric Shaker K⁺ channel (England et al., 1997) and Kir2.1 (P.M. England, D.A. Dougherty, and H.A. Lester, unpublished observations) K⁺ channels encouraged us to attempt unnatural amino acid mutagenesis with the homomeric 5-HT_{3A} receptor.

The 5-HT_{3A} subunit forms a functional homomeric receptor when expressed in oocytes (Maricq et al., 1991) and was used for most of our experiments. However, while our studies were under way, cloning of the 5-HT_{3B} subunit (Davies et al., 1999) suggested that the native 5-HT₃ receptors are likely to be hetero-oligomers containing both the A and B subunits. We therefore conducted experiments on both the homomeric and the heteromeric receptors containing the mouse m5-HT_{3A} with and without the human h5-HT_{3B}.

We show that replacement of the M1 Pro (residue 256) using conventional mutagenesis with alanine (Ala), glycine (Gly), or leucine (Leu) resulted in inactive receptors. However, two imino acids, *trans*-3-methyl-proline (P3m) and pipecolic acid (Pip; Fig. 1A), were able to substitute for the Pro and produce active receptors that have EC₅₀ values similar to that of the wild-type receptor. Importantly, functional receptors were also produced by leucic acid (Lah; Fig. 1A), the α -hydroxy analog of Leu, which forms an ester instead of an amide bond when incorporated into proteins (Ellman et al., 1992; Chapman et al., 1997). These results support the model that normal gating requires a residue without a backbone hydrogen bond at this position.

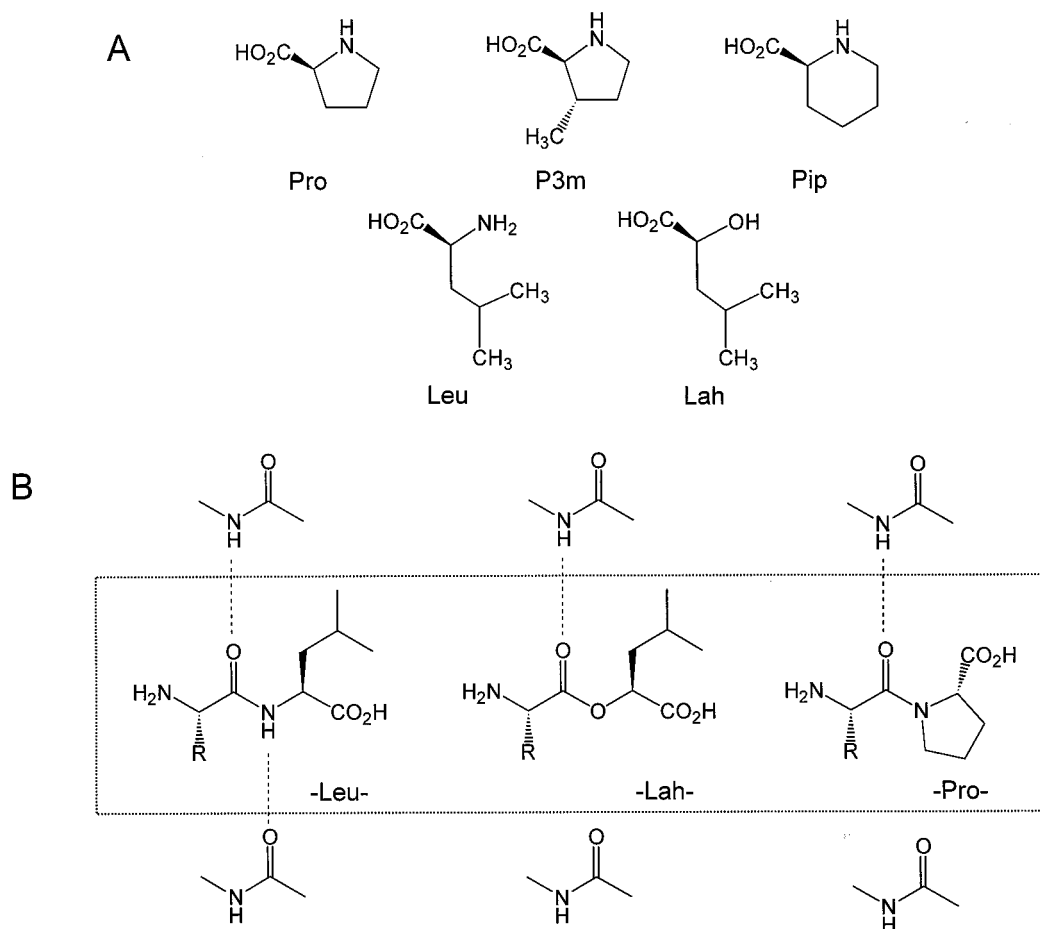


Fig. 1. Amino acid analogs used in the nonsense codon suppressions. A, structures of the unnatural amino acid analogs P3m, Pip, and Lah that form functional 5-HT₃ receptors when incorporated at the M1 Pro site. B, possible hydrogen bonds for Leu, Lah, and Pro residues in a polypeptide.

Materials and Methods

Chemicals. Metoclopramide, tropisetron, and [8-(diethylamino)octyl-3,4,5]-trimethoxybenzoate (TMB-8) were purchased from Research Biochemicals Inc. (Natick, MA). [9-methyl-³H]BRL-43694 (granisetron; 84.5 Ci/mmol, 1 μ Ci/ μ l) was purchased from New England Nuclear (Boston, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

The nitrobenzyl-protected, cyanomethyl ester of Lah (Nb-Lah-CN) was prepared as previously described (Chapman et al., 1997; England et al., 1999a; P.M. England, H.A. Lester, and D.A. Dougherty, unpublished observations). The NVOC-protected, cyanomethyl ester of P3m (NVOC-P3m-CN) was a generous gift from P. G. Schultz (University of California at Berkeley). The NVOC-protected, cyanomethyl ester of Pip (NVOC-Pip-CN) was prepared using standard synthetic transformations (Nowak et al., 1998).

Molecular Biology. A cDNA clone of the mouse 5-HT_{3A} (m5-HT_{3A}) subunit was provided by Dr. D. Julius (University of California at San Francisco; Maricq et al., 1991). The human 5-HT_{3B} (h5-HT_{3B}) cDNA was provided by Dr. E. Kirkness (The Institute for Genetic Research; Davies et al., 1999). These cDNAs were subcloned into the oocyte expression vector plasmid pAMV (Nowak et al., 1995). Mutations in the cDNA were made using the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA). Plasmids were linearized with *NotI* and used as template to produce mRNAs using the T7 mMESSAGE mMACHINE kit from Ambion (Austin, TX). Acylated tRNAs were prepared by ligating THG73 with amino- or hydroxyacylated dinucleotides (Xaa-dCA) as described previously (Nowak et al., 1998; England et al., 1999b) to form tRNA-THG73-Xaa. Immediately before injection, the α -amino or α -hydroxy protecting group (Nb or NVOC) on the acylated tRNA was removed by a 5-min irradiation at room temperature with a 1-kW xenon arc lamp fitted with WG-335 and UG-11 filters.

Electrophysiology. Stage V to VI *X. laevis* oocytes were harvested and injected with 50 nl/oocyte of a mixture containing 10 to 25 ng of mRNA plus 20 to 50 ng of tRNA. For wild-type and some conventional mutants, much reduced amounts of mRNA (~0.5 ng/oocyte) were used. The ratio between the m5-HT_{3A} and h5-HT_{3B} mRNA in the conventional mutagenesis experiments was 1:1, whereas in suppression experiments, a 50:1 excess of the stop codon-containing m5-HT_{3A}-UAG mRNA was used.

Two-electrode voltage-clamp recordings were performed 24 to 36 h after injection using a GeneClamp500 circuit and a Digidata 1200 digitizer from Axon Instruments, Inc. (Foster City, CA) interfaced with an IBM-compatible PC running pCLAMP6 or CLAMPEX7 software from Axon. The recording solutions contained 96 mM NaCl, 2 mM KCl, 2 mM MgCl₂, and 5 mM HEPES, pH 7.4 (ND96). Whole-cell current responses to various drug concentrations at indicated holding potentials (typically -60 mV) were fitted to the Hill equation, $I/I_{\max} = 1/(1 + (EC_{50}/[A])^n)$, where I is agonist-induced current at concentration $[A]$, I_{\max} is the maximum current, EC_{50} is the concentration inducing half-maximum response, and n is the Hill coefficient.

Surface Binding Assay. Two days after injection with 50 ng of mRNA, intact oocytes were used in ligand binding assays (Chang and Weiss, 1999). Briefly, individual oocytes were incubated in ND96 and 5 nM [³H]BRL-43694 for 60 s, washed three times in ND96 within a period of 15 s, and placed in scintillation vials for counting with a Beckman LS5000TA counter. Nonspecific binding was determined with oocytes from the same batch by including 10 μ M tropisetron in the binding solution.

Results

Conserved M1 Pro Is Essential for Receptor Function: Conventional Mutagenesis. To demonstrate the importance of the conserved Pro in the function of the homomeric m5-HT_{3A} receptor, the Pro256 codon in the cDNA was

replaced by that of either Ala, or Gly, or Leu using conventional mutagenesis. Mutant mRNAs transcribed from the cDNA templates, when injected into *X. laevis* oocytes (at 50 ng/oocyte), failed to produce any detectable serotonin responses at -60 mV holding potential with ≤ 1 mM serotonin.

To determine whether the mutant receptors reach the cell surface, we examined binding of the 5-HT₃ specific antagonist [³H]BRL-43694 to intact oocytes (Fig. 2). Oocytes injected with either the wild-type or the Pro256Gly mutant mRNA showed [³H]BRL-43694 binding that could be blocked by another 5-HT₃ specific antagonist, tropisetron (10 μ M). The level of nonspecific binding, defined by tropisetron, was similar to that of uninjected oocytes. Oocytes expressing the Pro256Gly receptor display 2-fold higher specific surface binding than the wild type. However, the mRNAs used in these experiments were from different batches, which could cause this variability.

To test whether the mutant receptors that reach the cell surface could be activated under any circumstances, we introduced a second mutation, Val13'Ser (or Val290Ser), into the M2 channel-lining domain. The notation refers to a convention that numbers the M2 region from its putative cytoplasmic N terminus. The Val13'Ser mutation has a profound effect on the channel gating similar to a class of M2 mutations at the 9' site described previously at the $\alpha 7$ nicotinic (Revah et al., 1991), muscle-type nicotinic (Filatov and White, 1995; Labarca et al., 1995; Ohno et al., 1995), and m5-HT_{3A} (Yakel et al., 1993) receptors. In the $\alpha 7$ nAChR, the Leu9'Thr (or Leu247Thr) mutation in the channel domain produced a mutant receptor that is much more sensitive to agonists with much slower desensitization kinetics than the wild-type receptor (Revah et al., 1991; Bertrand et al., 1992). Similarly, the corresponding Leu9'Ser mutation in the mouse muscle nAChR expressed in oocytes produced a lower EC₅₀ value, and this reduction was roughly multiplicative with the number of Leu9'Ser or Leu9'Thr subunits in the pentameric receptor (Filatov and White, 1995; Labarca et al., 1995). Although the aligning mutation, Leu9'Thr (or Leu286Thr), in the m5-HT_{3A} receptor showed a decreased EC₅₀ value, the ~3-fold decrease was much less than that for the nAChRs (Yakel et al., 1993). We therefore tested other positions in the M2 domain of the m5-HT_{3A} homomeric receptor and found that the Val13'Ser (Val290Ser) mutant receptor has an EC₅₀ value ~70-fold less than the wild type

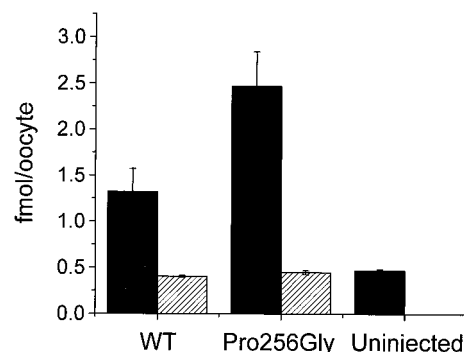


Fig. 2. The nonfunctional Pro256Gly mutant forms specific 5-HT₃ binding sites on the cell surface. Total binding of [³H]BRL-43694 to intact oocytes 2 days after injection with the indicated mRNA is shown as filled columns (mean \pm S.E., $n = 5-7$). Nonspecific binding of [³H]BRL-43694, determined in the presence of 10 μ M tropisetron, is shown as hatched columns.

(Fig. 3). The Val13/Ser m5-HT_{3A} homomeric receptor has a similar current-voltage profile and voltage jump relaxation kinetics (Fig. 7C) but much slower desensitization kinetics than the wild type (Fig. 2A). Furthermore, overexpression of the mutant receptor with the injection of large amounts (~25 ng/oocyte) of mRNA produced a standing voltage-clamp current (1–2 μ A at –60 mV) in the absence of agonist (data not shown), indicating constitutive activation of a subpopulation of receptors. Interestingly, this mutant receptor is at least 10-fold less sensitive to blockade by the open channel blocker TMB-8 than the wild type (Fig. 4B).

The double-mutant receptors, containing M1 Pro256→Ala, Gly, or Leu coupled with the Val13/Ser mutation, produce substantial standing currents in the oocytes; and these currents are changed little by 5-HT₃ agonists or antagonists (Fig. 4, C and D). In addition, the standing currents are blocked by the channel blocker TMB-8 at concentrations near those that block the Val13/Ser single-mutant receptor (Fig. 4, B and C). These results indicate that the M1 Pro256Xaa (e.g., Ala, Gly, Leu) mutation does not prevent assembly, surface expression, or (if an appropriate additional mutation is present) activation of the receptor and instead suggest a special functional role for the M1 Pro in channel gating.

Unique Hydrogen Bonding Properties of Pro May Account for Its Importance in Gating: Unnatural Amino Acid Mutagenesis. To establish the feasibility of the nonsense codon suppression method for homomeric LGICs, m5-HT_{3A} mRNA containing the UAG stop codon rather than the M1 Pro256 codon (abbreviated here as m5-HT_{3A}-UAG) was injected into oocytes along with either the full-length nonsense suppressor tRNA charged with Pro or some other amino acid (termed tRNA-THG73-Xaa) or full-length uncharged tRNA (termed tRNA-THG73). These oocytes were then assayed for serotonin-induced current over a concentration range of 0.3 to 1000 μ M under two-electrode voltage-clamp at –60 mV holding potential. For tRNA charged with Pro, the oocytes showed a serotonin response, similar to the wild type (Fig. 5B), that peaked at ~30 h after RNA injection and diminished by 48 h (Fig. 5A). No serotonin responses (at concentrations up to 1 mM) were detected after the injection of uncharged tRNA-THG73 or with tRNA-THG73-Ala, tRNA-THG73-Leu, tRNA-THG73-Phe, or tRNA-

THG73-Thr. These results are consistent with the above conventional mutagenesis experiments, which showed that replacing the conserved Pro256 with other amino acids produces inactive receptors.

The time-sensitive expression pattern (Fig. 5A) was not seen when wild-type m5-HT_{3A} receptor mRNA alone was injected. Presumably, receptor synthesis stops when the pool of tRNA-THG73-Pro has been exhausted and the receptors are removed from the membrane or otherwise inactivated. The data allow the conclusion that such turnover processes proceed with a time constant of 1 to 2 days.

Several synthetic analogs (structures given in Fig. 1A) were able to replace the conserved M1 Pro256 and produce active receptors in oocytes when incorporated through the nonsense suppression method (Fig. 6A); these are P3m, Pip, and Lah. The P3m receptor showed an EC₅₀ value of 1.3 μ M, similar to that of the wild type (1.4 μ M), whereas the Pip receptor had a slightly higher EC₅₀ value of 3.6 μ M, and the Lah receptor had a slightly lower EC₅₀ value of 0.8 μ M (Fig. 6A). In addition, the P3m and Lah receptors also showed slight differences in their kinetic properties compared with the wild-type receptor. The P3m receptor inactivates more slowly than the wild type after agonist washout (Fig. 6B), whereas the Lah receptor recovers more slowly from desensitization (Fig. 6D). Because of the slow recovery of the Lah receptor from desensitization, the dose-response relation (Fig. 6A) was determined by normalizing responses to various concentrations with those to 1 μ M 5-HT applied between each test dose.

Similar results were obtained when the wild-type h5-HT_{3B} mRNA was included in the nonsense codon suppression experiments. A previous report shows that the effects of incorporating the B subunit in the 5-HT₃ receptor in vitro include a larger single-channel conductance, a slight right shift of dose-response, reduced rectification in the current-voltage relation, lowered Ca²⁺ permeability, and changes in pharmacology (such as *d*-tubocurarine sensitivity) compared with the h5-HT_{3A} homomer (Davies et al., 1999). In this experiment, functional heteromeric receptors were also obtained when the h5-HT_{3B} mRNA was coinjected with m5-HT_{3A}-UAG along with suppressor tRNA-THG73-P3m, tRNA-THG73-Pip, tRNA-THG73-Lah, or tRNA-THG73-Pro. Dose-

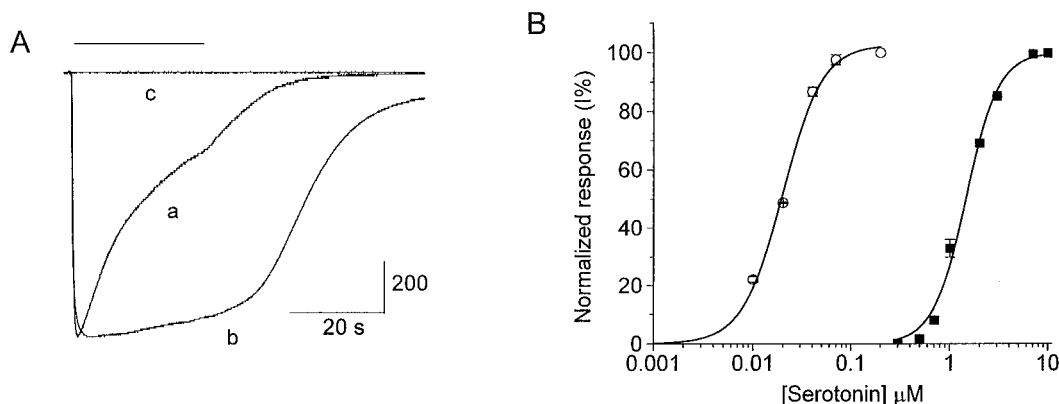


Fig. 3. The Val13/Ser mutant receptor is more sensitive to agonist than the wild type. Whole-cell currents induced by perfusion of agonist (serotonin) were recorded using two-electrode voltage-clamp as described in *Materials and Methods*. A, representative traces (normalized to peak amplitudes of 1000) from oocytes injected with either the wild-type m5-HT_{3A} mRNA (a) or Val13/Ser mutant mRNA (b) at 3 μ M serotonin. The horizontal line above the current traces indicates the duration of ligand applications. Trace c shows the complete block of the whole-cell current responses when serotonin was coapplied with 100 nM tropisetron, 100 nM *d*-tubocurarine, or 10 μ M metoclopramide for both the wild type and the mutant. B, normalized dose-response relations (mean \pm S.E.) of the wild-type (■) and the Val13/Ser receptors (○), with EC₅₀ values of 1.4 and 0.02 μ M, respectively.

response relations for the heteromeric P3m, Pip, and the wild-type (Pro) receptors are also slightly right-shifted compared with their homomeric counterparts (Fig. 6A). The heteromeric Lah receptor showed slower recovery from desensitization, similar to the homomeric receptor (data not shown). In addition, it is expressed at a lower level (100–200 nA peak amplitude at -60 mV holding potential), vitiating systematic dose-response studies. In general, we found that the heteromeric expression levels in the nonsense codon suppression experiments are lower than those for homomeric receptors, possibly because the A and B subunits were expressed at nonoptimal ratios. Our results are summarized in Table 1.

Discussion

The highly conserved Pro in the M1 domain of the LGIC superfamily was replaced in the 5-HT₃ receptors by both conventional and unnatural amino acid mutagenesis. None of the selected naturally occurring amino acids were able to substitute for the M1 Pro256 in generating functional channels, although these receptors were expressed on the cell surface. In contrast, replacing this conserved Pro with the unnatural residues, P3m, Pip (structural analogs of Pro), and Lah (which forms an ester instead of an amide bond) produced functional receptors that are similar to the wild type. These findings extend results from the muscle-type nAChR (England et al., 1999b) and point to an important role of the

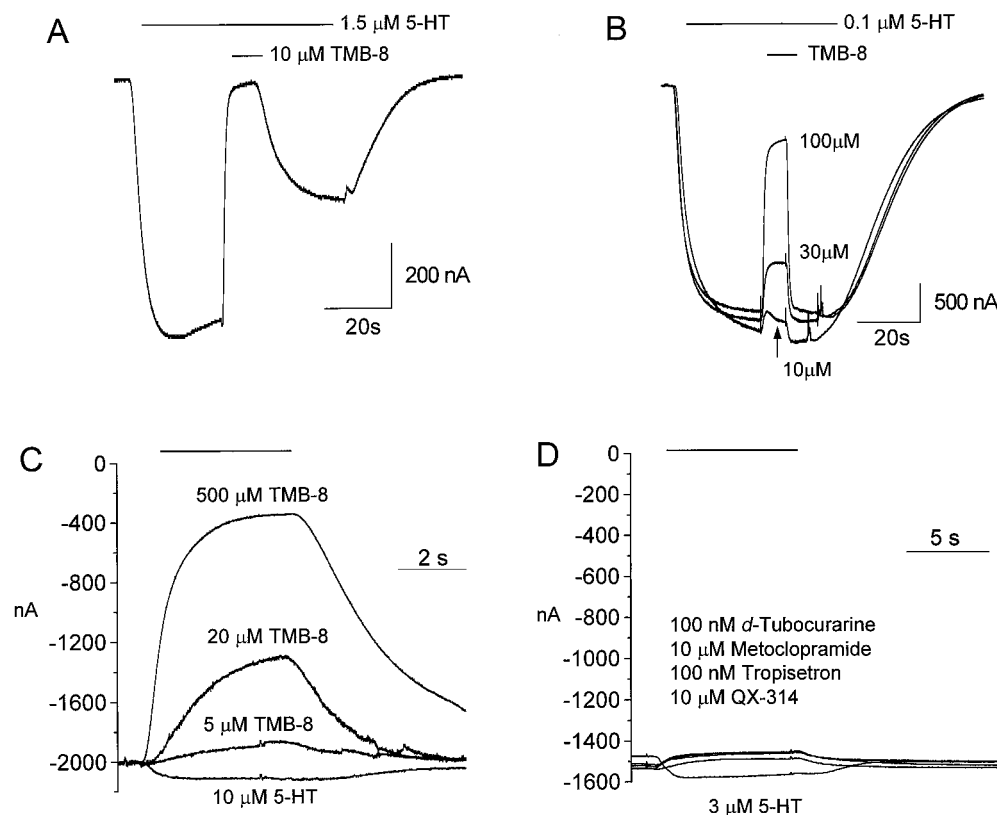


Fig. 4. Double-mutant receptors produce constitutively open channels blocked by TMB-8. Representative recordings from oocytes injected with wild type (WT; A), Val13/Ser (B), and Pro256Gly-Val13/Ser (C and D) mRNA at holding potential -60 mV. In each case (A–C), TMB-8 blocked the current when applied. A, the WT receptor was almost completely blocked by 10 μ M TMB-8. B, the Val13/Ser receptor was only slightly blocked by 10 μ M and was further blocked by increasing concentrations of TMB-8. C, in oocytes injected with Pro256Gly-Val13/Ser double-mutant mRNA, the standing current showed sensitivity to TMB-8 block resembling that for the Val13/Ser receptor. Mean while, the double-mutant receptor showed only small additional responses to saturating agonist (10 μ M 5-HT, C), and small inhibition by antagonists (D).

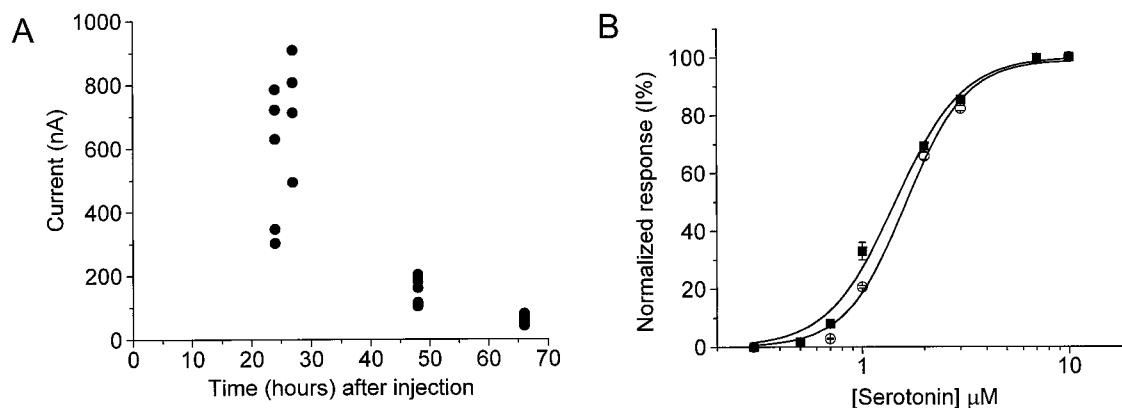


Fig. 5. Pro-suppressed and wild-type receptors have similar dose-response relations. A, peak currents induced by 10 μ M serotonin at -60 mV holding potential were recorded at the indicated times from oocytes injected with a mixture of m5-HT_{3A}-UAG mRNA and prolyl-suppressor tRNA, tRNA-THG73-Pro. B, the receptors expressed via nonsense codon suppression by prolyl-tRNA (○) have a dose-response relation (mean \pm S.E.) similar to that of the wild type (■).

conserved M1 Pro, particularly its hydrogen bonding characteristics, in channel gating.

Conventional Mutagenesis. The conservation of the M1 Pro in every subunit of this superfamily of LGICs (Ortells and Lunt, 1995) suggests that natural mutation at the site is not tolerated. This requirement appears to hold for the m5-HT_{3A} receptor subunit as well as the nAChR α -subunits (Dang et al., 1995; England et al., 1999b), although natural

amino acids can substitute for the M1 Pro in non- α -subunits. The unique properties of Pro among the naturally occurring amino acids exclude the introduction of subtle modifications using conventional mutagenesis. The null phenotype observed with conventional mutants could be a result of failed gating of the receptor channel or failure in receptor assembly/maturation onto the plasma membrane. Indeed, oocytes injected with the 5-HT₃ Pro256Gly mRNA showed no seroto-

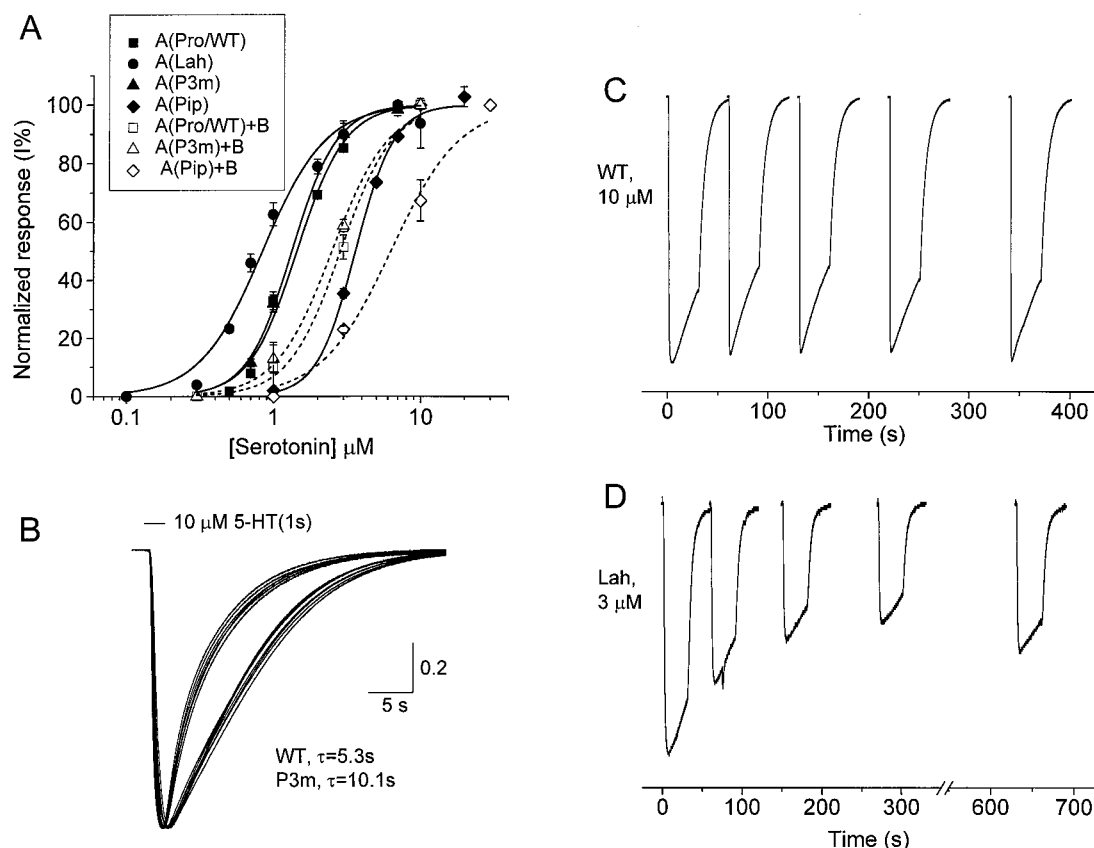


Fig. 6. Properties of unnatural residues at the M1 Pro position. A, currents were recorded at -60 mV holding potential from oocytes injected with the mixture of m5-HT_{3A}-UAG mRNA (homomeric, solid symbols and solid lines) or in combination with h5-HT_{3B} mRNA (heteromeric, open symbols and dashed lines) plus tRNA-THG73-P3m, tRNA-THG73-Pip, or tRNA-THG73-Lah. Data for wild-type subunits are also shown. Normalized dose-response relations are shown as mean \pm S.E. Because of the slow recovery from desensitization of the Lah receptor (D), the peak current responses were normalized against responses to 1 μ M serotonin measured in alternation with test concentrations. B, comparison of washout kinetics between the homomeric wild-type and the P3m receptors in response to a 1-s pulse of 10 μ M 5-HT. Traces recorded from four oocytes for each group were normalized to peak amplitudes of 1 and superimposed. The slower and faster decaying traces are from oocytes expressing P3m and wild-type receptors, respectively. C and D, responses for the homomeric wild-type and the Lah receptors during a series of five successive 30-s 5-HT applications (10 μ M for wild type, 3 μ M for Lah). Note that the Lah receptor desensitizes progressively during the first four applications and recovers only partially during a 330-s wash period and note the break on the time axis in D.

TABLE 1
Values of EC₅₀ and Hill coefficients for several residues at the M1 Pro position

	5-HT Response	Homomer		Heteromer	
		EC ₅₀	n^H	EC ₅₀	n^H
		μ M		μ M	
WT (Pro)	+	1.4	2.7	2.9	2.0
Lah ^a	+	0.8	2.4	N.D. ^b	N.D.
P3m ^c	+	1.3	2.9	2.5	2.0
Pip	+	3.6	3.0	6.3	1.6
Ala	—				
Gly	—				
Leu	—				
Thr	—				

^a Slow recovery from desensitization.

^b Not determined.

^c Slow washout.

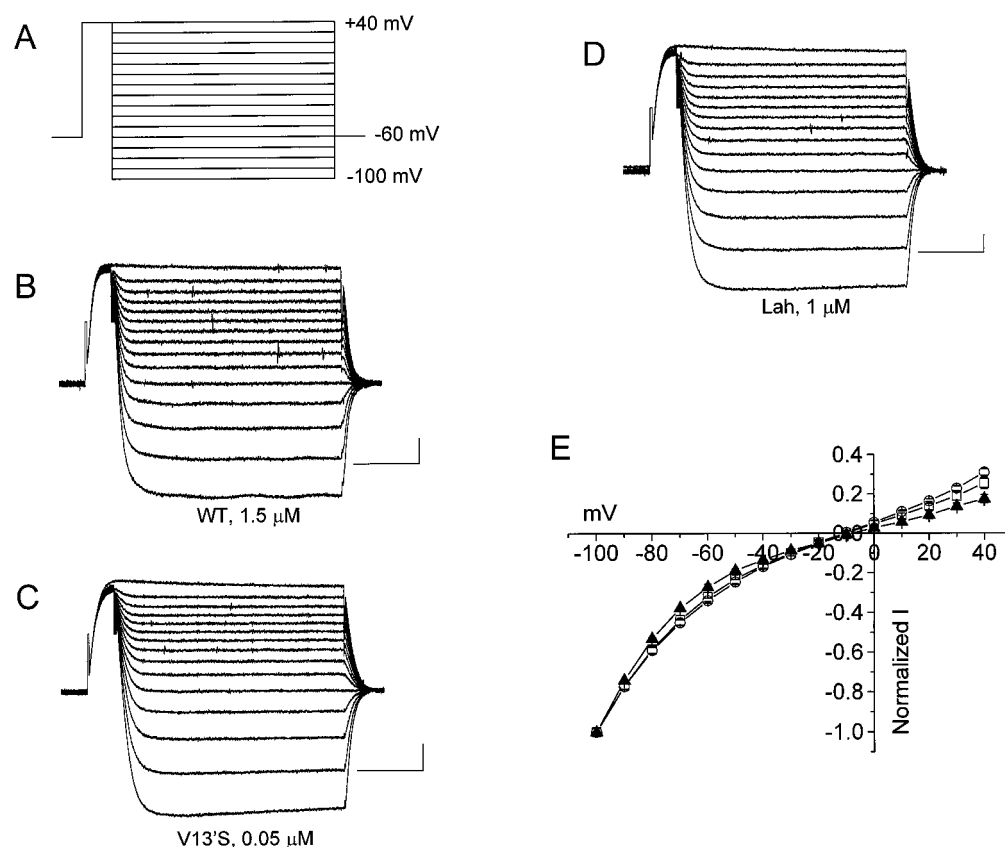


Fig. 7. Mutations do not affect voltage dependence of the serotonin responses. A, voltage-jump relaxation protocol used in these experiments. The membrane potential was held at -60 mV, stepped to $+40$ mV for 8 ms, and then stepped to test potentials between -100 and $+40$ mV in 10-mV increments. The current traces obtained for the same oocyte in the absence of agonist were subtracted from those in the presence of agonist to yield agonist-induced currents. B, wild type, 1.5 μ M 5-HT. C, Val13'Ser, 0.05 μ M 5-HT. D, Lah, 1 μ M. Scale bars, 200 nA and 20 ms. E, average peak current-versus-voltage relations from three or four oocytes in each group are plotted as mean \pm S.D. Data were normalized to the response at -100 mV.

nin-induced whole-cell current but ample numbers of specific binding sites on the cell surface (Fig. 2). Similarly, surface α -bungarotoxin binding sites corresponding to nAChRs were found when the M1 Pro was replaced by Leu (England et al., 1999b) or Gly (Dang et al., 1995), and the mutant nAChRs were also defective in channel gating.

One approach to evaluate the gating process of the M1 Pro mutant receptors is to use a class of mutations in the M2 region that dramatically alter gating kinetics (Revah et al., 1991; Yakel et al., 1993; Filatov and White, 1995; Labarca et al., 1995). The most extensively studied of such mutations involves the Leu at the 9' position in M2. Replacing the bulky hydrophobic Leu with smaller hydrophilic residues such as Thr and Ser increases sensitivity to ACh by >100 -fold (Revah et al., 1991; Labarca et al., 1995). However, the increase in sensitivity to agonist was much less dramatic in the m5-HT_{3A} homomeric receptor at the aligning Leu286 (Yakel et al., 1993). In the muscle-type nAChR, it was discovered that the Val13'Ser mutation produces a greater increase in agonist sensitivity than the Leu9'Ser mutation (C. Labarca, personal communication). In addition, the M2 13' position is believed to face the channel lumen (Akabas et al., 1992; White and Cohen, 1992). We also found that the Val \rightarrow Ser mutation at the 13' position produced a ~ 70 -fold increase in agonist sensitivity in the m5-HT_{3A} homomer, and the Val13'Ser mutant m5-HT_{3A} receptor also desensitized more slowly than the wild-type m5-HT_{3A} receptor (Fig. 3A). For another well-studied receptor, the $\alpha 7$ nAChR, 13' and 9' mutations produce roughly comparable shifts in EC_{50} values (Devillers-Thiery et al., 1992). Thus, at the level of detailed changes in physiology produced by aligning mutations, there are differences among receptors within the LGIC superfamily.

Previous data with natural and unnatural residues in the M2 region suggest that some M2 side chains move into a more polar, presumably aqueous, environment in the open state; furthermore, subtle distinctions among the effects of various side chains argued for a somewhat structured environment (Kearney et al., 1996). Apparently, details of these motions and structures vary among the members of LGIC superfamily.

In addition, the Val13'Ser receptor demonstrated at least a 10-fold decrease in sensitivity to the cationic open channel blocker TMB-8 (Fig. 4B), presumably because a hydrophilic substitution in the channel reduces its affinity for the hydrophobic moiety of TMB-8 (Charnet et al., 1989). Although we are not aware of previous studies on 5-HT₃ receptors showing that M2 region mutations affect the sensitivity to open-channel blockers, there are many such reports for nAChR. Thus, the Val13'Ser mutation in the m5-HT_{3A} homomeric receptor causes changes in channel behavior resembling those for M2 mutations in the nAChRs: increased sensitivity to agonists, slower desensitization, and altered sensitivity to a class of channel blockers. However, it was recently reported that TMB-8 acts as a competitive antagonist at the wild-type 5-HT₃ receptor (Sun et al., 1999), possibly at the ligand binding sites. In contrast, our results suggest that TMB-8 interacts with the channel at the Val13'Ser site either directly or allosterically.

When the M1 Pro256Ala, or Pro256Gly, or Pro256Leu mutations were combined with the Val13'Ser mutation in the channel domain by conventional mutagenesis, mRNA injection into oocytes produced standing currents (1 – 2 μ A at -60 mV) that were blocked by TMB-8 and responded marginally to 5-HT₃ ligands (Fig. 4, C and D). Moreover, these standing

currents showed sensitivity to TMB-8 block similar to that for the Val13/Ser receptor, strongly suggesting that the standing current is produced by the doubly mutated m5-HT_{3A} receptor. These data reinforce the concept that the conserved M1 Pro is an important element in the conformational transitions that gate the channel, but in this case, the channels cannot close.

Unnatural Amino Acid Mutagenesis: Lack of H-Bond Donor. The conventional mutagenesis experiments suggest that the M1 Pro is important for channel gating. However, all of the natural amino acid replacements studied to date produced inactive receptors (Dang et al., 1995; England et al., 1999b), vitiating further experiments on such substitutions. The nonsense suppression method provides a tool to incorporate synthetic amino acid analogs into specific positions of a given protein. By using such an approach, we can begin to examine which properties of the M1 Pro are important for the gating of these channels.

This study reports the first use of the nonsense suppression methodology with the 5-HT₃ receptor. In important control experiments, tRNA acylated with the native Pro was injected into *X. laevis* oocytes along with the m5-HT_{3A}-UAG mRNA. This manipulation was intended to reconstruct the wild-type receptor using the suppression methodology; indeed, the expressed receptor has EC₅₀ and Hill coefficient values identical to those of the wild-type receptor (Fig. 5B), establishing the viability of the nonsense suppression approach in this system.

Among the synthetic amino acid analogs investigated, three (two Pro analogs, P3m and Pip, and Lah) suppressed the UAG codon in m5-HT_{3A}-UAG to produce functional receptors that were gated by serotonin. The two Pro analogs (Fig. 1A) produce subtle changes in the side chain structure while maintaining many characteristics of Pro, such as the hydrogen-bonding properties. The third unnatural residue, Lah, produces a backbone ester in place of the backbone amide and imparts a more severe change in the side chain structure (Fig. 1A). The fact that each of these analogs can substitute for Pro256 suggests that the size of the side chain of the conserved M1 Pro256 is not important in channel gating. All natural amino acids, except Pro, can participate as both hydrogen bond donors and acceptors, providing important stabilizing energy in both α -helix and β -sheet structures (Fig. 1B). The three unnatural residues (P3m, Pip, and Lah) share one major common feature with Pro in that none of them can function as a hydrogen bond donor (Fig. 1B). Because these structurally distinct unnatural residues could substitute for the M1 Pro256 and produce functional receptors, we suggest that normal receptor gating requires the absence of a backbone hydrogen bond with the M1 Pro as donor. If M1 is α -helical except for a kink at the proline, then there is by definition an uncompensated acceptor at i-4. Apparently, "compensating" this with a conventional amino acid locks the M1 helix into a form that prevents gating.

On the other hand, cysteine scanning accessibility studies of the extracellular half of the M1 domain of the muscle-type nAChRs suggest that this region indeed has an irregular secondary structure (Akabas and Karlin, 1995). Furthermore, the accessibility patterns at several positions appeared to change in the presence versus the absence of the agonist acetylcholine, indicating that this part of the receptor does undergo agonist-induced conformational changes. Without

the stabilizing hydrogen bond, the resulting structure would be more flexible to support the conformational changes induced by ligand binding.

Other mechanisms, including the fact that the Xaa-Pro bond is more likely to take up the *cis* conformation than is a peptide bond, cannot be ruled as the basis for the effects of mutating the M1 Pro. However, the ester linkage does not share this feature with the Pro residue, making the lack of a hydrogen bond donor the simplest and most straightforward interpretation of our results.

Substituting M1 Pro With Unnatural Residues Has Similar Effects on Heteromeric 5-HT₃ Receptor. Recent discovery of the 5-HT_{3B} subunit suggests that native 5-HT₃ receptors are likely to be heteromers of the A and B subunits (Davies et al., 1999). The M1 Pro is also conserved in the 5-HT_{3B} subunit. Like the β -subunits of neuronal nAChRs, the B subunit of the 5-HT₃ receptor does not form functional homomeric channels *in vitro* and appears to play a structural role, modifying the pharmacological and physiological properties of the A subunit when coexpressed (Davies et al., 1999). The suppressed 5-HT₃ heteromeric receptors showed slight right shifts in dose-responses compared with the suppressed homomeric receptors (Fig. 5A), similar to the wild-type homomeric and heteromeric receptors (Davies et al., 1999). Our findings suggest that the B subunit plays a similar role in the wild-type and the suppressed heteromeric receptors, where the M1 Pro256 of the A subunit was replaced by unnatural residues.

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