Supplementary Information for 2005-03-03447A:

Cis-trans isomerisation at a proline opens the pore of a neurotransmitter–gated ion channel

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Methods

Mutagenesis and preparation of cRNA and Oocytes – Mutant 5-HT_{3A} receptor subunits were developed using pcDNA3.1 (Invitrogen, Abingdon, U.K.) containing the complete coding sequence for the 5-$HT_{3A(b)}$ subunit from mouse neuroblastoma N1E-115 cells as previously described [1]. For nonsense suppression the proline codon at 308 was replaced by TAG as previously described [2]. Wild type and mutant receptor subunit coding sequences were then subcloned into pGEMHE. This was linearized with *Nhe1* (New England Biolabs) and cRNA synthesised using the T7 mMESSAGE mMACHINE kit (Ambion). Oocytes from *Xenopus laevis* were prepared and maintained as described previously [2].

Synthesis of tRNA and dCA-amino acids- Unnatural amino acids were chemically synthesised as nitroveratryloxycarbonyl (NVOC) protected cyanomethyl esters and coupled to the dinucleotide dCA, which was then enzymatically ligated to 74-mer THG73 $tRNA_{CUA}$ as detailed previously [3]. Immediately prior to co-injection with mRNA, tRNA-aa was deprotected by photolysis. Typically 5 ng mRNA and 25 ng tRNA-aa were injected into Stage V-VI oocytes in a total volume of 50 nl. For control experiments, mRNA was injected 1) in the absence of tRNA and 2) with the THG73 74-mer tRNA. Experiments were preformed 18-36 h post injection.

Characterisation of mutant receptors- 5-HT-induced currents were recorded from individual oocytes using two-voltage electrode clamp with either a GeneClamp 500 amplifier or an OpusXpress system (Axon Instruments, Inc., Union City, CA). All experiments were performed at 22-25 ºC. Serotonin (creatinine sulphate complex,

Sigma) was stored as 25 mM aliquots at –80 °C, diluted in calcium-free ND96, and delivered to cells via computer-controlled perfusion systems. Glass microelectrodes were backfilled with 3 M KCl and had a resistance of approximately 1 MΩ. The holding potential was –60 mV unless otherwise specified. Data were analysed using PRISM software. EC_{50} data were obtained from at least 6 independent experiments using oocytes from at least 3 different batches. Expression of some unnatural amino acids was poor compared to insertion of Pro using the same technique: Pip 70-90% of Pro levels, Aze: 20-40%, Tbp 5-10%; Dmp 2-5%. For Dmp traces, baselines were normalized pre- and post-agonist application to determine 5-HT-induced depolarization at each [agonist]. We saw no significant distortion by desensitization in our response waveforms within the time resolution of the measurements (~0.5 s). There may be minor distortion for Pip, which appears to desensitize more rapidly than the other residues (Figure 2b).

Immunofluorescent Localization. This was modified from [4]. Briefly, oocytes were fixed using ice cold 4 % paraformaldehyde in phosphate buffer (PB: 66 mM Na_2HPO_4, 38 mM NaH_2PO_4 pH 7.2) and labelled using pAb120 antisera at 1:1000 dilution in Tris-buffered saline (TBS: 0.1 M Tris/HCl pH7.4, 0.9 % NaCl) overnight at 4ºC. Biotinylated anti-rabbit IgG (Vector) and Cy-5 avidin D (Vector) were used to detect bound antibody as per the manufacturer's instructions. Immunofluorescence was observed using a confocal microscope.

NMR. The NMR sample was prepared containing 1 mM peptide, 200 mM sodium dodecyl-d25 sulphate, 150 mM sodium chloride, 20 mM sodium phosphate, 1 mM EDTA, 20 μM 3,3,3-trimethylsilylpropionate, and 10% D_2O at pH 6.0 to a final volume of 550 μL in a 5 mm Ultra-Imperial grade NMR tube (Wilmad). All experiments were recorded at both 298 K and 303 K on a Bruker DRX500 spectrometer equipped with a *z*-shielded gradient triple resonance probe using standard procedures. [5] Two dimensional (2D) NOESY and TOCSY experiments, with mixing times of 200 and 72.5 ms, respectively, were collected with 256 and 1024 pairs of complex points and acquisition

times of 26 and 102 ms in the indirectly and directly acquired dimensions, respectively. Data processing and analysis were carried out on a Silicon Graphics O2 workstation using the programs AZARA and CCPNmr Analysis.

Table S1. [^{3}H]Granisetron affinity for 5-HT$_3$ receptors containing conventional mutations at Pro 308.

	K_d (nM)	SEM	n
wild type	0.63	0.02	5
P308A	0.70	0.12	3
P308C	0.52	0.09	3
P308G	0.37	0.19	3
P308K	0.57	0.09	3
P308V	0.53	0.15	3
P308Q	0.35	0.14	3

Supplementary Figures Captions

Figure S1. Immunofluorescence demonstrates 5-HT_3 receptors with unnatural amino acids at Pro8* reach the membrane of oocytes.[6]

Figure S2. Schild plots and IC_{50}s show the affinity of MDL72222 is similar to WT in receptors containing representative unnatural amino acids. A: Concentration response curves of wild type receptors in the presence of increasing concentrations of MDL72222. EC_{50} values are: 1.29 ± 0.08 (control), 2.99 ± 0.33 (30nM), 6.70 ± 1.21 (60nM), 8.69 ± 0.86 (100nM) and 19.29 ± 2.21nM (300nM) B: Schild plots from data in A and from similar data for receptors containing Pip. EC_{50} values for Pip: 0.64 ± 0.20(control), 1.07 ± 0.19 (30nM); 2.59 ± 0.08 (60nM); 3.18 ± 0.31 (100nM); 5.77 ± 1.3 (300nM). Both Schild plots show slopes not significantly different to 1, and similar K_ds for MDL72222 (18.1 nM and 28.9 nM for wild type and Pip respectively; derived from the x-intercepts of the plot). These values are similar to MDL72222 IC_{50}values determined at EC_{50} concentrations shown in C:WT pIC_{50}= 7.585 ± .175, IC_{50}= 25.9nM; Pip pIC_{50}= 7.689 ± 0.104, IC_{50}= 20.5nM. The IC_{50} for DMP containing receptors at its EC_{50} is also similar to WT: pIC_{50}= 7.35 ± 0.175, IC_{50}=44.7nM. The slope of the fit line on this plot is not significantly different to zero.

Figure S3. Deviations of H^a chemical shifts from those expected in a random coil, $Dd(H^a)$, plotted against residue number for (a) the major conformer and (b) resolved resonances of the minor conformer. (c) the pattern of nuclear Overhauser effects between sequential backbone amide sites, dNN(i, i+1)

Supplementary figures:

Figure S1.

Wild type **blank** **2-Me-Pro** **2,4-CH_2-Pro** **3-Me-Pro**

Figure S2A

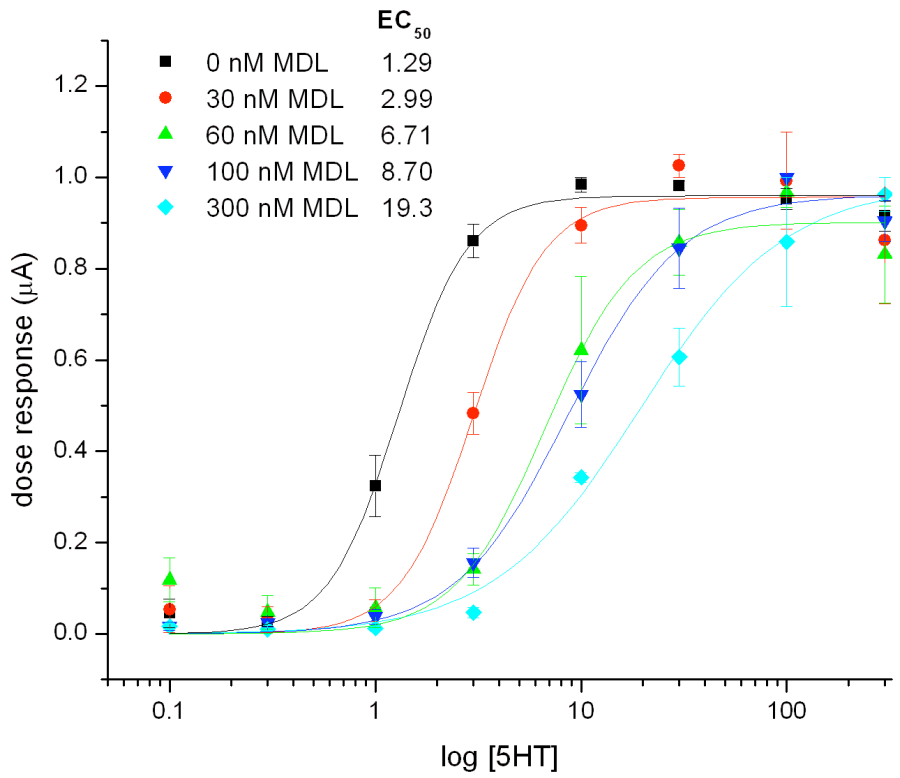

EC50
0 nM MDL 1.29
30 nM MDL 2.99
60 nM MDL 6.71
100 nM MDL 8.70
300 nM MDL 19.3
dose response (μA)
log [5HT]
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.1
1
10
100

Figure S2B

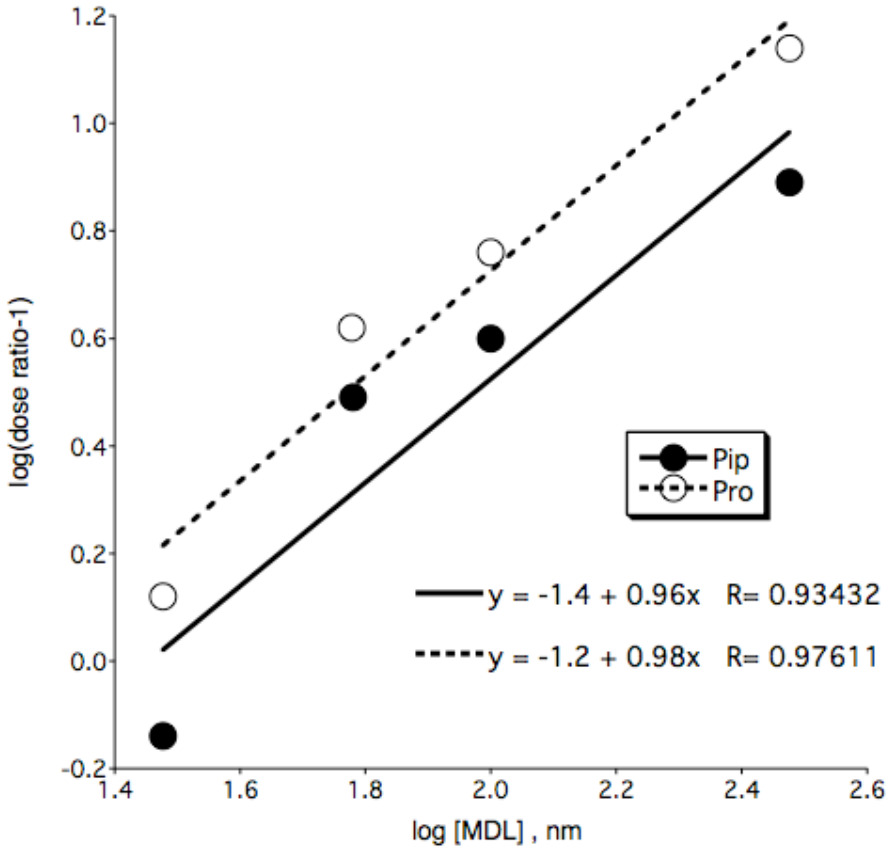

1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
log(dose ratio-1)
1.4
1.6
1.8
2.0
2.2
2.4
2.6
log [MDL] , nm
Pip
Pro
y = -1.4 + 0.96x R= 0.93432
y = -1.2 + 0.98x R= 0.97611

Figure S2C

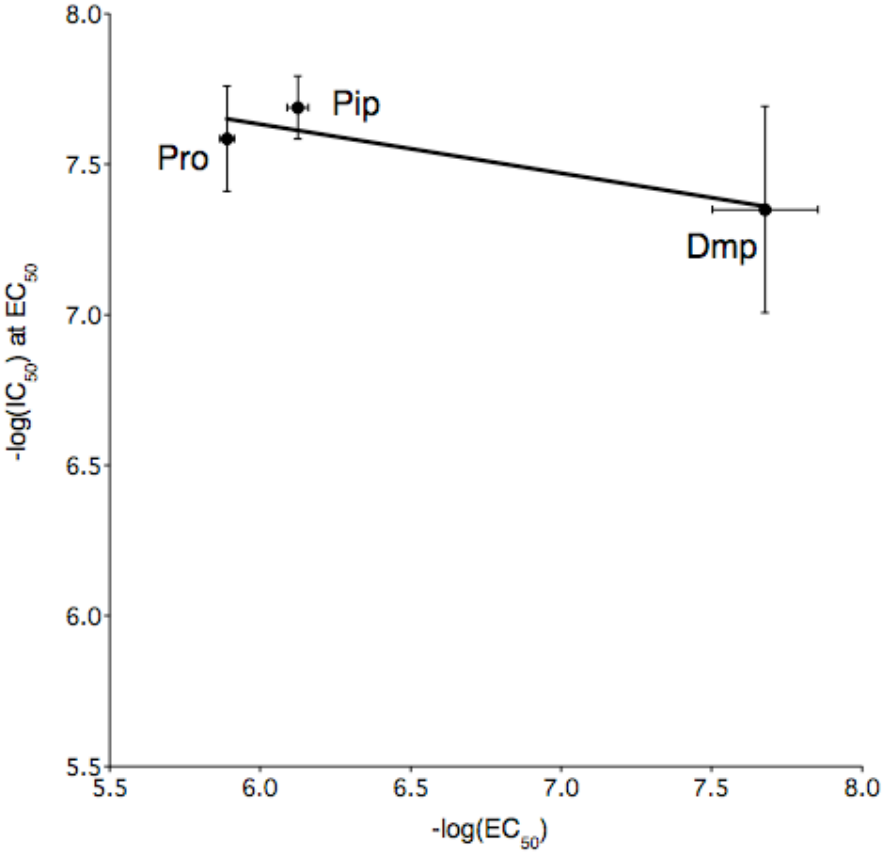
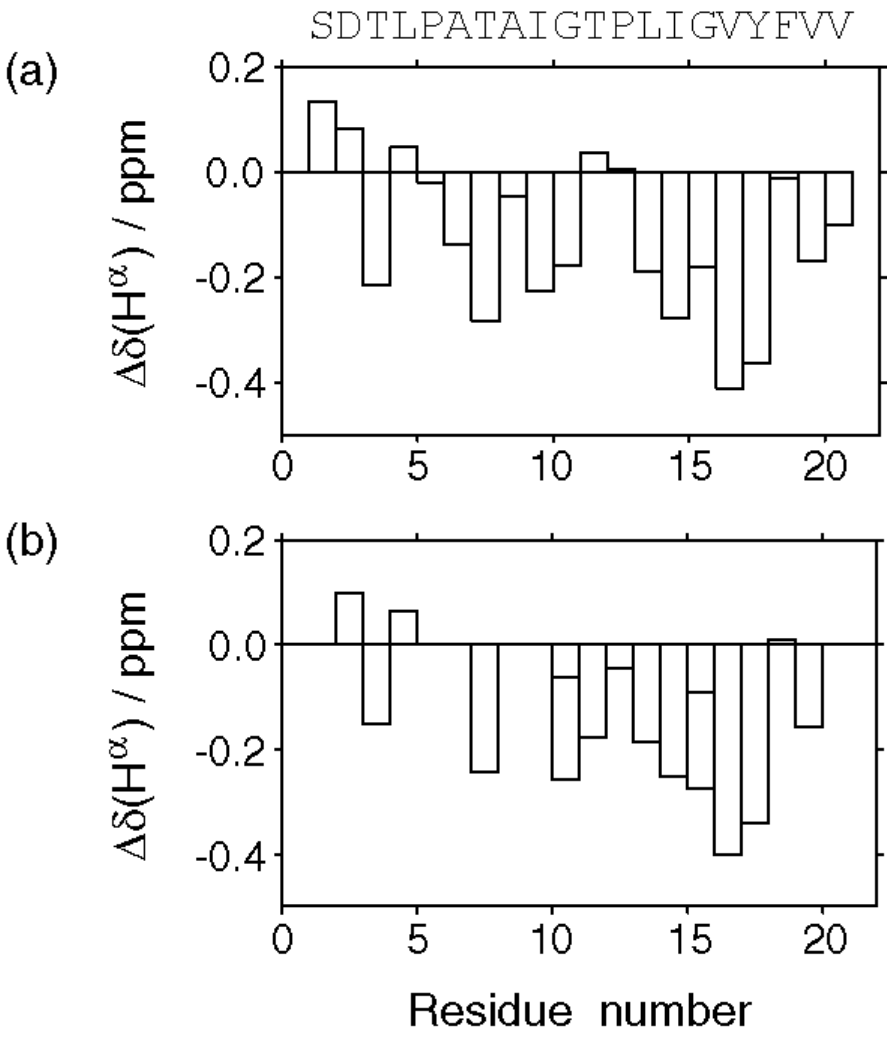
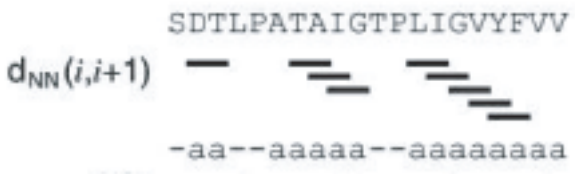

8.0
7.5
7.0
6.5
6.0
5.5
5.5
6.0
6.5
7.0
7.5
8.0
Pip
Pro
Dmp
-log(IC50) at EC50
-log(EC50)

Figure S3


SDTLPATAIGTPLIGVYFVV
(a)
(b)
$\Delta\delta(H^{\alpha})$ / ppm
0.2
0.0
-0.2
-0.4
0
5
10
15
20
Residue number

(c)


SDTLPATAIGTPLIGVYFVV
$d_{NN}(i,i+1)$
-aa--aaaaa--aaaaaaaa

References to Supplementary Information

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