the connections between areas of the cerebral cortex. The importance of recurrent connections in the pathways that mediate the VOR[9] allows us to model how recurrent connections could contribute to learning and memory. The model raises the possibility that subtle changes in the function of individual cellular mechanisms may have profound effects on the output from specific behavioural systems and emphasizes the importance of understanding the architecture of the neural networks that convert cellular changes into changes in behavioural output. □

Received 5 May; accepted 18 September 1992.

1. Churchland, P. S. & Sejnowski, T. J. *The Computational Brain* (MIT Press, Cambridge, MA, 1992).
2. Storm, J. F. *Nature* **336,** 379–381 (1988).
3. Bradley, K. & Somjen, G. G. *J. Physiol., Lond.* **156,** 75–92 (1961).
4. Strong, J. A. & Kaczmarek, L. K. in *Neuromodulation: The Biochemical Control of Neuronal Excitability* (eds Kaczmarek, L. K. & Levitan, I. B.) (Oxford University Press, 1987).
5. Lisberger, S. G. *Science* **242,** 728–735 (1988).
6. Miles, F. A., Braitman, D. J. & Dow, B. M. *J. Neurophysiol.* **43,** 1477–1493 (1980).
7. Lisberger, S. G. & Pavelko, T. A. *Science* **242,** 771–773 (1988).
8. Miles, F. A., Fuller, J. H., Braitman, D. J. & Dow, B. M. *J. Neurophysiol.* **43,** 1437–1476 (1980).
9. Lisberger, S. G., Morris, E. J. & Tychsen, L. *A. Rev. Neurosci.* **10,** 97–129 (1987).
10. Fuchs, A. F. & Kimm, J. *J. Neurophysiol.* **38,** 1140–1161 (1975).
11. Gonshor, A. & Melvill Jones, G. *J. Physiol., Lond.* **256,** 381–414 (1976).
12. Miles, F. A. & Lisberger, S. G. *A. Rev. Neurosci.* **4,** 273–299 (1981).
13. Ito, M. *Brain Res.* **40,** 81–84 (1972).
14. Watanabe, E. *Brain Res.* **297,** 169–174 (1984).
15. Lisberger, S. G. & Sejnowski, T. J. *Univ. California San Diego Inst. Neural Comput. Tech. Rep.* 9201 (1992).
16. Lisberger, S. G. & Pavelko, T. A. *J. Neurosci.* **6,** 346–354 (1986).

ACKNOWLEDGEMENTS. We thank numerous colleagues for their helpful comments. Research was supported by a grant from the Defense Advanced Research Project Agency, awarded through the Office of Naval Research.

A single amino-acid difference confers major pharmacological variation between human and rodent 5-HT$_{1B}$ receptors

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NEUROPSYCHIATRIC disorders such as anxiety, depression, migraine, vasospasm and epilepsy may involve different subtypes of the 5-hydroxytryptamine (5-HT) receptor[1,2]. The 1B subtype, which has a unique pharmacology, was first identified in rodent brain[3–7]. But a similar receptor could not be detected in human brain[6], suggesting the absence in man of a receptor with equivalent function. Recently a human receptor gene was isolated (designated 5-HT$_{1B}$ receptor[8,9], 5-HT$_{1D\beta}$ receptor[10,11], or S12 receptor[12]) which shares 93% identity of the deduced protein sequence with rodent 5-HT$_{1B}$ receptors[13–15]. Although this receptor is identical to rodent 5-HT$_{1B}$ receptors in binding to 5-HT, it differs profoundly in binding to many drugs. Here we show that replacement of a single amino acid in the human receptor (threonine at residue 355) with a corresponding asparagine found in rodent 5-HT$_{1B}$ receptors renders the pharmacology of the receptors essentially identical. This demonstrates that the human gene does indeed encode a 1B receptor, which is likely to have the same biological functions as the rodent 5-HT$_{1B}$ receptor. In addition, these findings show that minute sequence differences between homologues of the same receptor from different species can cause large pharmacological variation. Thus, drug–receptor interactions should not be extrapolated from animal to human species without verification.

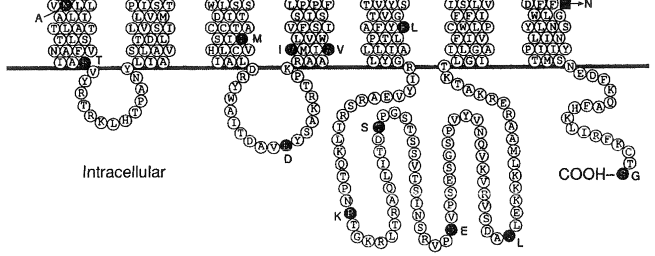
The human and rodent 5-HT$_{1B}$ receptors bind to 5-HT with comparably high affinity. But the human receptor binds with much lower affinity to the serotonergic agonist RU 24969 and to the β-adrenergic receptor antagonists propranolol, pindolol and alprenolol, and with higher affinity to several serotonergic drugs, including sumatriptan and 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT)[8–14] (Table 1).

The 32 amino-acid differences between the human and rat receptors are scattered throughout the molecule, but only eight are found in the transmembrane domains, which are thought to contain the ligand-binding pocket (Fig. 1). An asparagine residue in the seventh transmembrane segment has been implicated in β-antagonist binding to the β-adrenergic receptor[16] and to human 5-HT$_{1A}$ receptors[17]. Notably, an asparagine

DHE	6 ± 1	2 ± 1	4 ± 2	NA
RU24969	44 ± 4	2 ± 1	2 ± 1	10
Metergoline	25 ± 3	200 ± 40	129 ± 33	NA
Sumatriptan	38 ± 3	560 ± 100	465 ± 85	NA
Methysergide	130 ± 7	970 ± 130	1,823 ± 297	NA
8-OH-DPAT	1,600 ± 100	25,000 ± 1,000	>10,000	30,000
Methiothepin	12 ± 1	38 ± 8	13 ± 4	NA
β-adrenergic				
(−)Propranolol	8,100 ± 400	17 ± 1	57 ± 4	NA
(−)Pindolol	11,000 ± 1,000	20 ± 3	153 ± 62	69
(−)Alprenolol	11,000 ± 800	13 ± 1	NA	NA

The values are depicted as mean ± s.e.m. from 4 (wild type) and 3 (T355N) independent experiments done in triplicates. NA, data not available. Complementary DNAs encoding the wild-type and T355N mutant human receptors were inserted into the mammalian expression vector pRK5 and introduced by transient transfection into the human embryonic kidney 293 cell line by a modified calcium phosphate precipitation method[23]. The cells were collected by centrifugation 48 h after transfection, lysed in ice-cold buffer (50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA), homogenized, and sonicated for 10 s. Nuclei and intact cells were removed by centrifugation at 1,000*g* for 10 min. The supernatant was spun at 35,000*g* for 30 min and the resulting pellet, containing the microsomal membrane fraction, was resuspended in binding buffer containing 50 mM Tris-HCl (pH 7.4), 4 mM $CaCl_2$, 0.1% ascorbic acid, 10 mM pargyline and 1 μM leupeptine. Microsomal membranes (50 μg protein) were incubated with the ligands in binding buffer (30 min, 25 °C). Binding was terminated by the addition of 5 ml ice-cold 50 mM Tris-HCl (pH 7.8), rapid vacuum filtration through glass fibre filters, and two subsequent 5-ml washes. Specific binding was defined as the excess over blanks taken in the presence of 10^{-6} M cold 5-HT. Scatchard analyses of saturation binding of [^{3}H]5-HT showed two populations of binding sites; equilibrium dissociation constants (K_D) for the wild-type and mutant receptor, respectively, were 4.6 ± 1.4 and 3.9 ± 1.2 nM (high-affinity sites); 72 ± 24 and 75 ± 14 nM (low-affinity sites). The respective receptor densities in pmol per gram of protein were 1,000 ± 320 and 1,440 ± 610 (high-affinity sites), 13,670 ± 1,860 and 25,330 ± 4,480 (low-affinity sites). Specific binding of [^{3}H]5-HT was not detectable in untransfected cells (not shown), indicating that these cells do not express significant levels of endogenous 5-HT receptors. Equilibrium inhibition constants (K_i) were determined according to the following equation: $K_i = IC_{50}/(1 + [T]/K_D)$, where IC_{50} is the concentration of competing ligand required for 50% inhibition of [^{3}H]5-HT binding, [T] is the concentration of the [^{3}H]5-HT tracer (3 nM), and K_D is the high-affinity constant of [^{3}H]5-HT, as determined by saturation binding. The data were analysed by nonlinear least-square fitting using the EBDA[24] and LIGAND[25] programs.

found in the rat and the mouse 5-HT_{1B} receptors. METHODS. Threonine codon 355 of the human receptor cDNA[8] was replaced with an asparagine codon by oligonucleotide-directed mutagenesis. A 39-base synthetic oligonucleotide carrying the asparagine codon (containing two mismatches with the threonine codon) (GTTGAGATAGCCCAGCCAGTTGAAGAAGTCAAAGATGGC) was used as the mutagenesis primer. This primer was hybridized to a single-stranded DNA containing the wild-type sequence and extended by T4 DNA polymerase, and the double-stranded DNA plasmid was introduced into *Escherichia coli*. Bacterial colonies containing the T355N mutation were identified by two consecutive hybridization analyses and a single clone carrying the mutation was selected and confirmed by DNA sequencing.



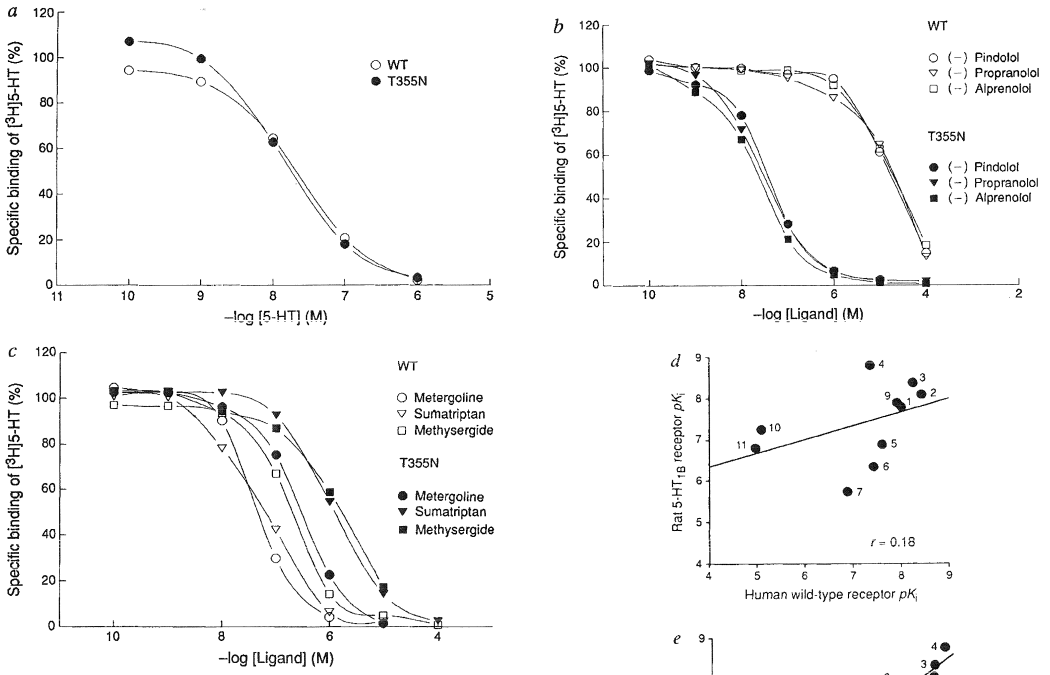


FIG. 2 Comparison of the ligand-binding properties of the wild type (WT; open symbols) and T355N mutant (filled symbols) human receptors expressed in human embryonic kidney 293 cells. *a*, Competition of 5-HT (○, ●) for [^{3}H]5-HT binding. *b*, Competition of the β-adrenergic receptor antagonists (−)pindolol (○, ●), (−)propranolol (▽, ▼) and (−)alprenolol (□, ■) for [^{3}H]5-HT binding. *c*, Competition of the serotonergic ligands metergoline (○, ●), sumatriptan (▽, ▼) and methysergide (□, ■) for [^{3}H]5-HT-binding. *d*, Correlation between pK_i values for the human wild-type receptor and the rat 5-HT_{1B} receptor. (*e*) Correlation between pK_i values for the human T355N mutant receptor versus the rat 5-HT_{1B} receptor. Ligand numbers and pK_is are based on their order of appearance in Table 1.

mutagenesis (Fig. 1). Scatchard analysis showed that the wild-type and mutant (T355N) receptors bind [^{3}H]5-HT with similar affinity, each with two binding-site populations (K_D = 4.6 and 72 nM, wild type; 3.9 and 75 nM, mutant; data not shown). Competition analysis also showed similar 5-HT affinities for the wild-type and mutant receptors (Fig. 2*a*; Table 1). Thus, the T355N substitution does not affect binding to the natural ligand 5-HT.

To compare the pharmacology of the wild-type and mutant human receptors with that of rodent 5-HT$_{1B}$ receptors, we carried out competition assays with serotonergic and β-adrenergic drugs from various chemical classes. Like 5-HT, 5-carboxyamidotryptamine (5-CT) and dihydroergotamine (DHE), which have similar inhibition constants (K_i) with the wild-type human and the rodent receptors, showed comparable K_is for T355N (Table 1). In contrast, the β-antagonists propranolol, pindolol and alprenolol had markedly reduced K_is with T355N, which were comparable to the K_is seen with the rodent receptors (Fig. 2*b*; Table 1). The K_i of the serotonergic ligand RU 24969 was ~20-fold lower with T355N than with the wild type, and similar to the K_is for the rodent receptors (Table 1). Metergoline, sumatriptan, methysergide and 8-OH-DPAT bound to T355N with K_is that were 7–15 times higher than those seen with the wild type, and more similar to K_is observed for the rodent receptors (Fig. 2*c*; Table 1). Methiothepin, which has similar K_is with the wild-type human and the rodent receptors, showed a fourfold higher K_i with T355N (Table 1). The K_is of 11 ligands showed no correlation between the human wild type and T355N (r = 0.03), or the human wild-type and rat receptors (r = 0.18), but showed a significant correlation between T355N and the rat receptor (r = 0.86; P < 0.0025) (Fig. 2*d*, *e*).

Thus, substitution of threonine 355 of the human receptor with the corresponding asparagine of rodent 5-HT$_{1B}$ receptors confers the pharmacology of a 1B subtype on the human receptor. Taken together with the high sequence homology between these receptors, this demonstrates that the 5-HT receptor gene investigated here does indeed encode the human species variant of the 1B 5-HT receptor subtype. Therefore, given that the human and rodent 5-HT$_{1B}$ receptors bind the natural ligand, 5-HT, with the same affinity, it is likely that despite their dramatically different pharmacology, these receptors are equivalent in function.

The identification of a human 5-HT$_{1B}$ receptor is of biological and clinical significance. Whereas this receptor and the human 5-HT$_{1D}$ receptor[22] share only 68% amino-acid sequence identity and are encoded on different chromosomes, their pharmacological properties are virtually indistinguishable[8–12]. Therefore, biological functions such as appetite control, migraine reduction and regulation of 5-HT release, attributed previously to the 5-HT$_{1D}$ receptor on the basis of drugs presumed to be selective for this subtype, indeed may be mediated by either the 5-HT$_{1D}$ or the 5-HT$_{1B}$ receptor, or by both. Understanding the individual functions of these receptors thus may help improve the clinical application of compounds such as the antimigraine drug sumatriptan, which binds to both the 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors.

Finally, our results show that a single amino-acid difference can cause dramatic pharmacological variation between species homologues of the same receptor. Thus, ligand-binding properties of a given receptor cannot be extrapolated across species without independent validation, even in the context of very high interspecies sequence identity. This has important implications

5. Hoyer, D., Engel, G. & Kalkman, H. O. *Eur. J. Pharmac.* **118,** 1–12 (1985).
6. Hoyer, D., Engel, G. & Kalkman, H. O. *Eur. J. Pharmac.* **118,** 13–23 (1985).
7. Hoyer, D. & Middlemiss, D. N. *Trends pharmac. Sci.* **10,** 130–132 (1989).
8. Jin, H. *et al. J. biol. Chem.* **267,** 5735–5738 (1992).
9. Hamblin, M. W., Metcalf, M. A., McGuffin, R. W. & Karpells, S. *Biochem. biophys. Res. Commun.* **184,** 752–759 (1992).
10. Weinshank, R. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **89,** 3630–3634 (1992).
11. Demchyshyn, L. *et al. Proc. natn. Acad. Sci. U.S.A.* **89,** 5522–5526 (1992).
12. Olav-Levy, F. *et al. J. biol. Chem.* **267,** 7553–7562 (1992).
13. Voigt, M. M., Laurie, D. J., Seeburg, P. H. & Bach, A. *EMBO J.* **10,** 4017–4023 (1991).
14. Adham, N. *et al. Molec. Pharmac.* **41,** 1–7 (1992).
15. Maroteaux, L. *et al. Proc. natn. Acad. Sci. U.S.A.* **89,** 3020–3024 (1992).
16. Suryanarayana, A., Daunt, D. A., VonZastrow, M. & Kobilka, B. K. *J. biol. Chem.* **266,** 15488–15492 (1991).
17. Guan, X. G., Peroutka, S. J. & Kobilka, B. K. *Molec. Pharmac.* **41,** 695–698 (1992).
18. Albert, P. R. *et al. J. biol. Chem.* **265,** 5825–5832 (1990).
19. Kobilka, B. K. *et al. Nature* **329,** 75–79 (1987).
20. Fargin, A. *et al. J. biol. Chem.* **264,** 4848–4852 (1989).
21. Findlay, J. & Eliopoulos, E. *Trends pharmac. Sci.* **11,** 492–499 (1990).
22. Hamblin, M. W. & Metcalf, M. A. *Molec. Pharmac.* **40,** 143–148 (1991).
23. Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J. & Capon, D. J. *Cell* **56,** 487–493.
24. McPherson, G. A. *Comp. Prog. Biomed.* **17,** 107–114 (1983).
25. Munson, P. J. & Rodbard, D. *Analyt. Biochem.* **107,** 220–239 (1989).

ACKNOWLEDGEMENTS. We thank C. Clark and R. Ward for comments on the manuscript. This work was supported by the NIH, the Kleiner Family Foundation and Genentech, Inc.

Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation

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GLUTAMATE is important in several forms of synaptic plasticity such as long-term potentiation, and in neuronal cell degeneration[1,2]. Glutamate activates several types of receptors, including a metabotropic receptor that is sensitive to *trans*-1-amino-cyclopenthyl-1,3-dicarboxylate, coupled to G protein(s) and linked to inositol phospholipid metabolism[3–6]. The activation of the metabotropic receptor in neurons generates inositol 1,4,5-trisphosphate, which causes the release of Ca^{2+} from intracellular stores and diacylglycerol, which activates protein kinase C[7–9]. In nerve terminals, the activation of presynaptic protein kinase C with phorbol esters enhances glutamate release[10]. But the presynaptic receptor involved in this protein kinase C-mediated increase in the release of glutamate has not yet been identified. Here we demonstrate the presence of a presynaptic glutamate receptor of the metabotropic type that mediates an enhancement of glutamate exocytosis in cerebrocortical nerve terminals. Interestingly, this potentiation of glutamate release is observed only in the presence of arachidonic acid, which may reflect that this positive feedback control of glutamate exocytosis operates in concert with other pre- or postsynaptic events of the glutamatergic neurotransmission that generate arachidonic acid. This presynaptic glutamate receptor may have a physiological role in the maintenance of long-term potentiation where there is an increase in glutamate release mediated by postsynaptically generated arachidonic acid[11].

The addition of the selective agonist for the metabotropic receptor, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid

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