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12 August 1991; accepted 23 October 1991

A Single Amino Acid That Determines the Sensitivity of Progesterone Receptors to RU486

BRIGITTE BENHAMOU,* TERESA GARCIA,* THIERRY LEROUGE, AGNES VERGEZAC, DOMINIQUE GOFFLO, CLAIRE BIGOGNE, PIERRE CHAMBON, HINRICH GRONEMEYER†

The progesterone analog RU486, an abortifacient, inhibits the action of progestins in humans but not in chickens or hamsters. Substitution of cysteine at position 575 by glycine in the hormone binding domain (HBD) of the chicken progesterone receptor (cPR) generated a cPR that binds RU486 and whose activity is antagonized by that compound. In fact, all receptors that bind RU486 have a glycine at the corresponding position. The hamster PR, like cPR, has a cysteine. Only glycine—not methionine or leucine—at position 575 allowed binding of RU486 to cPR. Substitution of this glycine by cysteine in the human PR (hPR) abrogated binding of RU486 but not that of an agonist. The corresponding mutation in the human glucocorticoid receptor resulted in a loss of binding of both dexamethasone and RU486. Examination of a series of 11β-substituted steroids showed that antagonism is not an intrinsic property of an antihormone, because one hPR antagonist acted as an agonist for a mutated hPR. The positioning of an aromatic 11β-substitution in the PR HBD appears to be critical for generating agonistic or antagonistic activity.

UNDERSTANDING HOW AGONISTS and antagonists interact with their receptors is important for the design of pharmaceuticals. RU486 competitively binds to the progesterone receptor (PR) of certain species and inhibits progestin action (*1*). The PR is a member of the nuclear receptor family (*2*) and its DNA binding domain (DBD) and hormone-binding domain (HBD) have been identified (*3, 4*), as well as two transcription activation functions (TAFs), in the NH_2 terminal region A/B (TAF-1) and in the HBD (TAF-2) (*5*) (Fig. 1B). Binding of RU486 induces PR dimerization and DNA binding in vivo and in vitro (*5, 6*), but no activity of TAF-2 was observed in the presence of RU486 (*5*). In contrast to the human PR (hPR), both chicken (cPR) and hamster (haPR) PRs do not bind RU486. But several agonists bind similarly to all of these PRs. Moreover, RU486 inhibits the activity of the glucocorticoid receptor in both humans and chickens (*1*).

Regions other than the HBD do not appear to contribute to RU486 binding by hPR, because a fusion protein composed of β-galactosidase and the hPR HBD (amino acids 687 to 933) bound the antagonist with an affinity similar to that of the complete human PR. Binding of RU486 by a fusion protein containing the corresponding portion of cPR was barely detectable (7) (Fig. 2D). This result indicates that the 31 amino acids that differ in the HBDs of cPR and hPR are responsible for the differential RU486 binding and that no post-translational modification specific to eukaryotes is required for RU486 binding.

Whether a specific region (or regions) within the cPR HBD blocked RU486 binding was tested as follows. Portions of the cPR HBD were replaced with the corresponding sequences of hPR (Fig. 1B), and the effect of RU486 on the ability of the chimeras to induce transcription in the presence of agonist (RU27987) was investigated (Fig. 1A) by transiently transfecting HeLa cells with a reporter gene (MMTV-CAT) carrying the progestin responsive element (PRE) of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (*8*). RU486 is a complete hPR antagonist in this system (*5*). All chimeras (m1 to m4; Fig. 1B) induced transcription in the presence of the agonist, but only the transcription induced by RU27987 and m2, which has the hPR sequence 716 to 766 in place of the corresponding sequence of cPR (amino acids 569 to 619), was inhibited by RU486 (Fig. 1C). Immunoblots demonstrated that all chimeras were expressed at similar concentrations; thus, they activated transcription with similar efficiencies, indicating that the sequence differences within the HBDs of cPR and hPR do not significantly affect the structure or activity of TAF-2 or that TAF-2 is contained within one of the transposed sequences.

The segment of the PR HBD present in cPR m2 differs at six residues from the corresponding sequence of cPR (Fig. 3A). Two of these residues are conserved in hPR and human glucocorticoid receptor (hGR), both of which bind RU486 (hPRGly^{722}, hGRGly^{567}; hPRLeu^{763}, hGRLeu^{608}). The rabbit PR (rbPR), which also binds RU486, has a sequence identical to the hPR HBD sequence present in m2 (Fig. 3, A and B). We therefore analyzed the effect of replacements of these two residues with the corresponding amino acids from the hPR (*9*). Whereas cPR(Met^{616}-Leu) still behaved like cPR (Fig. 1D), RU27987-induced transcription by the double mutant cPR(His^{573}-Gln, Cys^{575}-Gly) was inhibited by RU486 (Fig. 1D). This sensitivity to RU486 was solely due to the mutation of cPRCys^{575} to Gly, because cPR(His^{573}-Gln) behaved like wild-type cPR (Fig. 1, C and D), whereas RU486 efficiently inhibited transcription induced by cPR(Cys^{575}-Gly) in the presence of RU27987 (Fig. 1D). Analysis of RU486 binding to bacterially expressed β-galactosidase fusion proteins confirmed that the HBDs of m2 and cPR(Cys^{575}-Gly) bound RU486 (Fig. 2D). That these two chimeras bound RU486 with an affinity lower than that of hPR indicates that additional divergent amino acids contribute to the binding of RU486 by hPR. However, the single amino acid

B. Benhamou, T. Garcia, A. Vergezac, D. Gofflo, C. Bigogne, Roussel-Uclaf, Recherche Santé, Departement Endocrinologie, Centre de Recherche Roussel-Uclaf, 93230 Romainville, France.
T. Lerouge, P. Chambon, H. Gronemeyer, Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 67085 Strasbourg-Cédex, France.

*These authors contributed equally to this study.
†To whom correspondence should be addressed.

substitution in cPR(Cys^{575}-Gly) increased its affinity for RU486 by more than 1000-fold, up to 20% of progesterone's affinity (Fig. 2D). The cPR m2 has a five to six times higher binding affinity for RU486 than cPR(Cys^{575}-Gly) has but both cPRs have similar binding affinities for RU39628, which has no 11β-substitution (Fig. 2A). This indicates that the additional five divergent amino acids, which are present in the hPR sequence of chimera m2, are involved in accommodating bulky 11β substitutions. This observation suggests that this hPR HBD portion may, at least in part, correspond to the so-called "11β-pocket" of the receptor (*10*). We conclude that Cys^{575} is the sole residue critically responsible for the inability of cPR to bind RU486.

RU486 might not bind to cPR if Cys^{575} formed a disulfide bridge with Cys^{581} (another divergent amino acid of the hPR cassette present in m2) and restricted the putative 11β-pocket of cPR. However, cPR(Cys^{581}-Ser) was insensitive to RU486, thus excluding this possibility (Fig. 1D).

To investigate the function of the Gly^{722} of hPR in RU486 binding and antagonism, we replaced it by a cysteine residue in hPR; we also replaced cPRCys^{575} by either a Met or a Leu residue (*9*). In the presence of the agonist RU27987, all of these mutants activated transcription as efficiently as the corresponding wild-type receptors, indicating that TAF-2 was not affected by these mutations; however, this activation was not inhibited in any of the cases by excess RU486 (Fig. 1E). Binding analyses revealed that the binding affinity of hPR for RU486 was 40,000 times lower when Gly^{722} was replaced by Cys indicating that, even in the presence of the remaining sequence of the hPR, a Cys does not permit RU486 binding. Replacement of Cys^{575} in the cPR with two other residues also did not permit RU486 binding (Fig. 1E). Because Gly is the only amino acid without a side chain, these results suggest that hPR Gly^{722} (or cPR Cys^{575}) is at a critical position in the 11β-pocket, and that the presence of amino acid side chains may sterically impede RU486 binding.

If Gly were the only amino acid that allowed RU486 binding, all PRs that do not bind RU486 should have a different residue at the corresponding position (Fig. 3A). It is known that RU486 is not an antagonist for the hPR of at least three species: chicken, hamster, and tammar wallaby (*1*). Cloning and sequencing of the corresponding portion of the haPR cDNA (*11*) confirmed that it also contains a Cys at the position corresponding to hPR Gly^{722} (Fig. 3B). The hGR and human androgen (hAR) receptors also bind RU486 and RU486 acts as an antagonist of their cognate ligands (*1*). A sequence alignment of all receptors that bind RU486 as an antagonist revealed the presence of a Gly residue at the position corresponding to hPR Gly^{722} (Fig. 2).

In contrast, the human mineralocorticoid receptor (hMR), a close relative of hGR, has an Ala at position 773 (Fig. 3) and does not bind to RU486. We constructed the mutant hGR(Gly^{567}-Cys) to test whether a Cys would prevent binding of RU486 by the hGR (*12*). This mutant did not activate transcription in the presence of dexamethasone when assayed in COS cells (Fig. 1F). In fact, hGR Gly^{567}-Cys was unable to bind either dexamethasone or RU486 (*13*). Thus, a Cys at position 567 may alter the structure of the hGR HBD more dramatically than in the case of hPR (for example by generating another disulfide bridge). Alternatively, the presence of a Cys residue at position 567 may be incompatible with the binding of the 11β-hydroxylated dexamethasone.

The antagonistic activity of RU486 is due

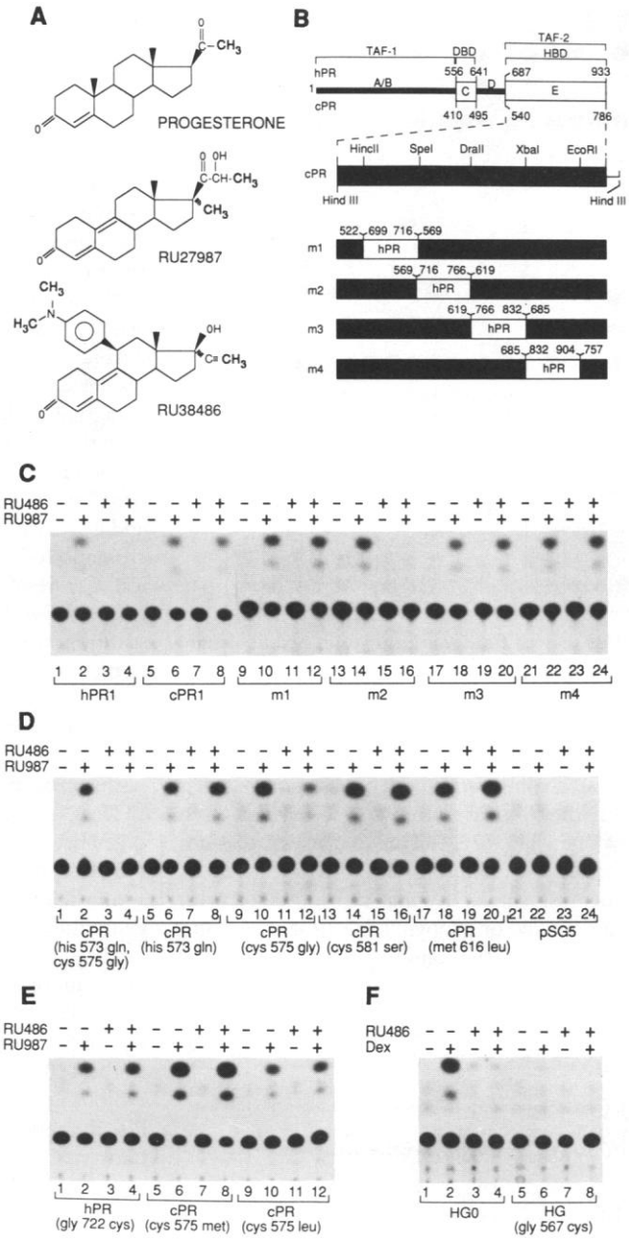
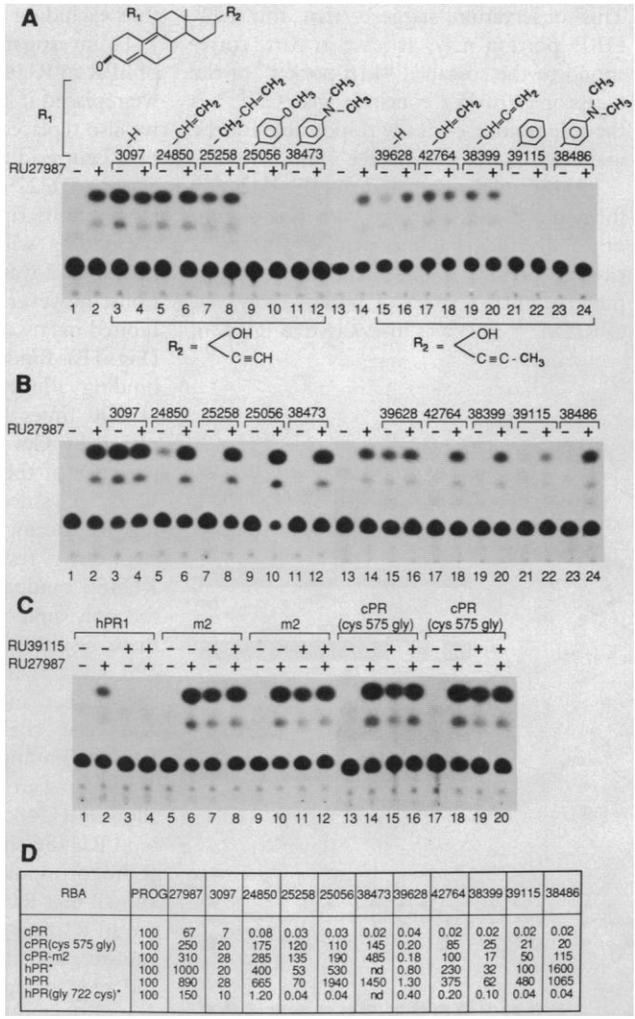


Fig. 1. Influence of the corresponding amino acids cPR Cys^{575} and hPR Gly^{722} on binding of the RU486 and RU27987. (**A**) Structures of the agonists progesterone and RU27987 (RU987), and of the antagonist RU38486 (RU486). (**B**) Illustration of the domain structure (A/B to E) of the PR (*16*). The TAFs, DBD, and HBD are indicated. Below, an expanded diagram of the cPR HBD (black) and of chimeric HBDs of the mutant cPRs (m1 to m4) carrying the specified segments (white) of the hPR HBD. Restriction sites, generated by silent mutations of the wild-type cDNA, are indicated; the flanking Hind III sites are natural (*3*). (**C**) A segment of the hPR HBD (amino acids 716 to 766; Fig. 3) confers RU486 sensitivity onto the cPR. The cPR (lanes 5 to 8), hPR (lanes 1 to 4), and chimeric PR expression vectors m1 to m4 (lanes 9 to 25) were cotransfected into HeLa cells with MMTV-CAT as reporter gene and transcriptional activation was monitored in the presence of RU27987 (10 nM), RU-486 (1 μM), or both as indicated. (**D**) A single amino acid confers RU486 binding and antagonism onto cPR. Analyses were as follows: cPR-(His^{573}-Gln, Cys^{575}-Gly) (lanes 1 to 4), cPR(His^{573}-Gln) (lanes 5 to 8), cPR-(Cys^{575}-Gly) (lanes 9 to 12), cPR(Cys^{581}-Ser) (lanes 13 to 16), and cPR-(Met^{616}-Leu) (lanes 17 to 20). (**E**) Only Gly, but not Cys, Met, or Leu permit RU486 binding. Analysis as in (C) of hPR(Gly^{722}-Cys) (lanes 1 to 4), cPR(Cys^{575}-Met) (lanes 5 to 8), and cPR(Cys^{575}-Leu) (lanes 9 to 12). (**F**) Analysis, in transiently transfected COS cells, of transcriptional activation by the wild-type hGR (HG0) (*17*) (lanes 1 to 4) or mutant hGr Gly^{567}-Cys HG (lanes 5 to 8) in the presence of 100 nM dexamethasone (Dex), 10 μM RU486, or both, as indicated at the top. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.



RBA	PROG	27987	3097	24850	25258	25056	38473	39628	42764	38399	39115	38486
cPR	100	67	7	0.08	0.03	0.03	0.02	0.04	0.02	0.02	0.02	0.02
cPR(cys 575 gly)	100	250	20	175	120	110	145	0.20	85	25	21	20
cPR-m2	100	310	28	285	135	190	485	0.18	100	17	50	115
hPR*	100	1000	20	400	53	530	nd	0.80	230	32	100	1600
hPR	100	890	28	665	70	1940	1450	1.30	375	62	480	1065
hPR(gly 722 cys)*	100	150	10	1.20	0.04	0.04	nd	0.40	0.20	0.10	0.04	0.04

Fig. 2. Requirement of a phenyl group in the 11β position and critical positioning in the PR HBD for steroids to act as antagonists of PR-induced transcription. Aliphatic substitutions in the 11β position generate PR agonists. (**A**) Two series [differing in position 17 (R2), bottom] of steroids with the general structure depicted at the top and the indicated 11β substitutions (R1) were assayed for their ability to stimulate or antagonize MMTV-CAT transcription in the presence of hPR, transiently expressed in HeLa cells. (**B**) Similar analysis for hPR(Gly^{722}-Cys). The signal in lane 22 was stronger in three other experiments, which is consistent with the observation that RU39115 does not bind to hPR(Gly^{722}-Cys) as in (D). (**C**) RU-39115 is an antagonist for transcription induced by hPR in the presence of RU27987 (lanes 1 to 4), but it is an agonist with m2 (lanes 5 to 12) or cPR(Cys^{575}-Gly) (lanes 13 to 20). (**D**) Relative binding affinities of progestins and antagonists to wild-type and mutant HBDs from cPR and hPR. The HBDs were expressed in *Escherichia coli* as fusion proteins with β-galactosidase (*7*). Binding affinities are given relative to progesterone (PROG). Asterisks indicate data for the wild-type and mutant hPRs derived from transiently expressed full-length receptors.

to the 11β-dimethylaminophenyl substitution (*10*). To investigate the characteristic feature of this substitution that confers antagonistic properties onto the steroid, we analyzed a series of PR ligands (Fig. 2) for their ability to induce or antagonize transcription by wild-type and chimeric PRs. All those steroids that contained an aromatic 11β substitution were antagonists of transcription induced by hPR in the presence of RU27987 (Fig. 2A), whereas the other tested compounds behaved as agonists (that is, they activated transcription when given alone; Fig. 2A). This behavior was not affected by altering the 17α substitution, because similar results were obtained with an ethynyl or propynyl substituent. A comparison of the binding and agonistic or antagonistic properties of these compounds with hPR, hPR Gly^{722}-Cys, cPR, m2, and cPR Cys^{575}-Gly revealed the following. (i) In the hPR, Gly^{722} is a critical amino acid for binding of bulky aliphatic and aromatic 11β-substitutions, because the mutation Gly^{722}-Cys (*14*) results in a 40,000-fold lower affinity for RU486 (Fig. 2D). (ii) In the cPR, Cys^{575} is the amino acid which is most critically involved in impeding the binding of bulky 11β substitutions (Fig. 2D; the mutation Cys^{575}-Gly increased the affinity of that receptor for RU38486 1000-fold). (iii) Compared to ethynyl, a propynyl group in the 17α position reduced the affinity of binding to all receptors (Fig. 2D). Thus, as proposed previously (*10*), the antagonistic activity of 11β-substituted steroids has two mechanistically different aspects: the acceptance of the substituent by the 11β-pocket of the receptor and the antagonism itself, which appears to require the presence of an aromatic 11β group in the steroid.

The antagonistic effect of RU486 on hPR activity results from the inability of hPR TAF-2 to activate transcription (*5*) in its presence. The aromatic substitution in the antagonists shown in Fig. 2A could interfere with the formation of an active TAF-2 within the HBD. Alternatively, TAF-2 could be constitutively active in the absence of ligand and be structurally altered by the presence of aromatic residues in the vicinity of the critical Gly residue. The study of RU39115 (Fig. 2) revealed that an aromatic 11β substitution may be necessary, but not sufficient, for antagonistic activity. RU39115 antagonized transcription induced by hPR in the presence of RU27987 (Fig. 2C) but acted as an agonist for activation of transcription by m2 and cPR(Cys^{575}-Gly).

Thus, some of the 25 amino acids that differ between the HBDs of hPR and cPR m2 determine whether this steroid has an agonistic or antagonistic effect. These divergent amino acids must contribute to ligand binding, because RU39115 binds about ten times more strongly to hPR than to m2 (Fig. 2D). We assume that the steroid, and its phenyl group, are differently positioned in the HBDs of hPR and m2. All steroids exhibiting the general structure depicted in Fig. 2A appear to be agonists of PR-induced transcription (provided they bind to the receptor), unless the 11β substitution contains a phenyl group, which has to be in a critical position within the 11β-pocket. This hypothesis, if confirmed by the three-dimensional structures of agonist and antagonist-liganded PR HBDs, may account for the observation that some RU486 derivatives bearing a particular substitution at position 17 behave as agonists (*15*), because this

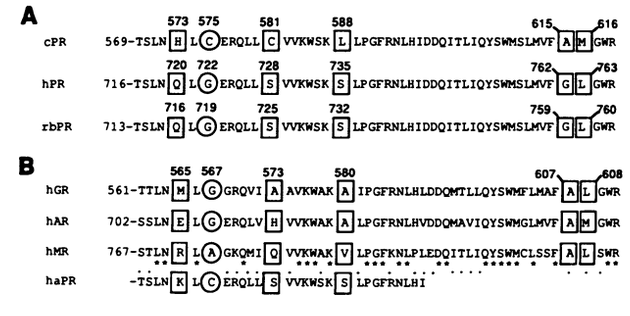


Fig. 3. Alignment of the hPR fragment generating the m2 chimera with the corresponding sequences of the PRs of other species (**A**) and other steroid receptors (**B**). Residues divergent between hPR and cPR are boxed, amino acids corresponding to hPR Gly^{722} and cPR Cys^{575} are circled. Sequences of the haPR HBD are available on request; sequences of the cPR (*3*), hPR (*16, 18*), rbPR (*19*), human glucocorticoid receptor (hGR) (*20*), human androgen receptor (hAR) (*21*), and human mineralocorticoid (hMR) (*22*) receptor have been described. Asterisks below the alignment indicate identity, dots indicate amino acid similarity.

substitution may affect the positioning of the dimethylaminophenyl group in the 11β-pocket.

Approximately 1% of women do not respond to the antagonistic action of RU486 (*1*), but respond normally to progestins. In these cases a mutation at position 722 might be responsible for the failure of RU486 to induce abortion.

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7. The construction and expression of β-galactosidase fusion proteins with the cPR HBD has been described (*4*). Fusion proteins with chimeric or mutant cPR HBDs were constructed accordingly. To generate fusion proteins with the wild-type hPR, we inserted the Bcl I to Bgl II fragment of hPR1 (*16*) encompassing the hPR HBD into the Bam HI site of PUR (*4*). Extracts were prepared as described in (*4*), and binding affinities were determined by the incubation of receptors with 5 nM [^{3}H]RU27987 with or without various concentrations of competitor steroids for 24 hours at 0°C. Free ligand was removed by means of the dextran-coated charcoal method.
8. The Hind III fragment of cPR1 (*3*), encoding the cPR HBD, was cloned into a modified Bluescript KS+ (Stratagene), lacking all polylinker sites except Hind III. Silent mutations were introduced by site-directed mutagenesis (Amersham) generating unique restriction sites (Fig. 1B). Cassettes corresponding to the hPR fragments were constructed from synthetic oligonucleotides (sequences are available on request) and introduced into the corresponding sites of the vector. All mutations and sequences generated by oligonucleotides were sequenced and the chimeric cPR-hPR Hind III fragments were used to replace the corresponding cPR fragment in the expression vector cPR21 (*3*). HeLa cells at about 40% confluence were cotransfected with 1 μg of receptor expression vector, 1 μg MMTV-CAT reporter gene, and 3 μg of pCH110 [a β-galactosidase expression vector (Pharmacia) used as reference gene to normalize for variations in transfection efficiency]. Transfection, preparation of cell extracts, and quantitative determination of chloramphenicol transferase activity have been described (*23*).
9. Point mutations (verified by sequencing) were introduced by site-directed mutagenesis. The corresponding oligonucleotides are available on request.
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11. Total RNA (7.5 μg) from the uterus of estrogen-stimulated female hamsters was denatured for 5 min at 70°C in the presence of 100 ng of primer NT47 and 0.5 mM deoxyribonucleotide triphosphates (dNTPs) in a final volume of 30 μl. The mixture was placed on ice and 200 units of M-MLV reverse transcriptase, 25 units of RNasin and M-MLV reverse transcriptase buffer (Bethesda Research Laboratories) were added. After incubation for 45 min at 37°C in a final volume of 40 μl the reaction was terminated by boiling for 5 min. The polymerase chain reaction was done with 100 pmol each of primer NY39 and NY40 in 10 mM tris-HCl (pH 8.3), at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM dNTPs in a final volume of 100 μl, with 5 μl of the reverse transcription reaction and 2.5 units of AmplitaqTM DNA polymerase (Perkin-Elmer Cetus Instruments). The thermal conditions were as follows: 5 cycles of 40 s at 94°C, 2 min at 45°C, 2 min at 72°C; 30 cycles of 40 s at 94°C, 1 min at 65°C, 2 min at 72°C, and 7 min at 72°C. The major 500-bp amplification product was purified on a polyacrylamide gel (8%), digested by Not I and Xba I, and inserted into pBluescript II SK+ (Stratagene). Three recombinants of two different amplifications were sequenced using the Sanger dideoxy method, all inserts gave the sequence in Fig. 3B. The primers used were as follows: NT47, 5′-CATCATYTCNGGRAA; NY39, 5′-ATTCTAGAGAGAGTTATCTGGTCATC; NY40, 5′-ATGCGGCCGCGTHTTCTTYAARAGGGCAATG; R = A or G; Y = C or T, and H = A, C, or T.
12. The hGR (Gly^{567}-Cys) was constructed by site-directed mutagenes of the human glucocorticoid receptor expression vector HG1 (*17*). To assure that no mutations occurred outside the sequenced area, the sequenced Xho I to Bsp MI fragment, containing the point mutation, was reinserted into the corresponding sites of HG1. Western blots of extracts prepared from transfected cells confirmed that a protein of the expected size was expressed as efficiently as the wild-type receptor from HG0 (*17*).
13. No specific binding of tritiated dexamethasone or RU486 could be detected in extracts of COS cells transiently transfected with hGR(Gly^{567}-Cys), whereas specific binding was observed when transfecting HG0 (*17*).
14. The pattern seen for the agonistic or antagonistic properties of the various steroids with hPR(Gly^{722}-Cys) (Fig. 2B) was identical to that obtained with cPR.
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24. We thank our colleagues at Roussel-Uclaf, in particular J. Nierat for oligonucleotide synthesis, G. Teutsch and D. Philibert for providing steroids, M. Moguilewski and M. T. Bocquel for advice and encouragement, and C. Benicourt for his contributions in the early phase of this work. C. Benicourt and C. Bigogne are now with Jouveinal. Supported by grants from the INSERM, CNRS, the Association pour la Recherche sur le Cancer, and the Fondation pour la Recherche Médicale.

12 June 1991; accepted 8 October 1991

Selection of Intrinsic Horizontal Connections in the Visual Cortex by Correlated Neuronal Activity

SIEGRID LÖWEL* AND WOLF SINGER

In the visual cortex of the brain, long-ranging tangentially oriented axon collaterals interconnect regularly spaced clusters of cells. These connections develop after birth and attain their specificity by pruning. To test whether there is selective stabilization of connections between those cells that exhibit correlated activity, kittens were raised with artificially induced strabismus (eye deviation) to eliminate the correlation between signals from the two eyes. In area 17, cell clusters were driven almost exclusively from either the right or the left eye and tangential intracortical fibers preferentially connected cell groups activated by the same eye. Thus, circuit selection depends on visual experience, and the selection criterion is the correlation of activity.

LONG-RANGE TANGENTIAL CONNECTIONS are a constituent feature of the intrinsic circuitry of neocortex (*1*). They consist mainly of axon collaterals of pyramidal cells (*2*), and in the visual cortex they interconnect discrete, regularly spaced groups of cells (*3*), which appear to show similar functional properties (*4*). It has been proposed that these horizontal pathways (i) form large receptive fields (*5*), (ii) mediate inhibitory and subliminal excitatory effects from outside the classical receptive field (*6*), and (iii) generate functionally coherent cell assemblies by synchronizing the responses of spatially distributed neurons (*7*). These functions require highly specific interactions, and it has been proposed that the tangential connections attain their specificity through experience-dependent selection (*8–11*). In kitten visual cortex, tangential connections develop mainly after birth (*9–13*), and final selectivity is achieved by pruning: either by elimination of inappropriate collaterals (*10, 12*) or transitory axons (*13*) or by decreases in the tangential extent and number of clusters (*11*). This pruning is influenced by visual deprivation (*9–12*). Here we test whether the selective stabilization of tangential connections is influenced by experience such that the selection criterion is the correlation of activity in interconnected cells (*14*).

To restrict correlated activity to defined

Max-Planck-Institut für Hirnforschung, Deutschordenstrasse 46, D-6000 Frankfurt am Main 71, Federal Republic of Germany.

*To whom correspondence should be addressed.