Evolution-guided discovery and recoding of allosteric pathway specificity determinants in psychoactive bioamine receptors

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Edited by Robert J. Lefkowitz, Duke University Medical Center/Howard Hughes Medical Institute, Durham, NC, and approved March 16, 2010 (received for review December 22, 2009)

G protein-coupled receptors for dopamine and serotonin control signaling pathways targeted by many psychoactive drugs. A puzzle is how receptors with similar functions and nearly identical binding site structures, such as D2 dopamine receptors and 5-HT2A serotonin receptors, could evolve a mechanism that discriminates stringently in their cellular responses between endogenous neurotransmitters. We used the Difference Evolutionary Trace (DIT) and residue-swapping to uncover two distinct sets of specificity-determining sequence positions. One at the ligand-binding pocket determines the relative affinities for these two ligands, and a distinct, surprising set of positions outside the binding site determines whether a bound ligand can trigger the conformational rearrangement leading to G protein activation. Thus one site specifies affinity while the other encodes a filter for efficacy. These findings demonstrate that allosteric pathways linking distant interactions via alternate conformational states enforce specificity independently of the ligand-binding site, such that either one may be rationally rekeyed to different ligands. The conversion of a dopamine receptor effectively into a serotonin receptor illustrates the plasticity of GPCR signaling during evolution, or in pathological states, and suggests new approaches to drug discovery, targeting both classes of sites.

A core of residues of high evolutionary importance, including a subset unique to the dopamine receptor, was implicated in the allosteric pathway. The authors used ET to rank sequence positions in the transmembrane domain according to their evolutionary importance and identified a core of residues of high evolutionary importance, including those specific for the dopamine receptor, that are in structural contact with the ligand-binding site and thus accessible to small external modifications. Remarkably, the results identified a set of positions important for specificity of ligand recognition by G protein-coupled receptors (GPCR), critical both for their biological functions and for the effects of therapeutic drugs that target them. The authors provide an example of how ET can be used to discover new strategies to produce novel allosteric agents and to explore the allosteric mechanisms of these receptors.

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S pecificity of ligand recognition by G protein-coupled receptors (GPCR) is critical both for their biological functions and for the effects of therapeutic drugs that target them (1, 2). The receptors for the neurotransmitters dopamine and serotonin mediate very different physiological responses to their respective ligands in regions of the brain where both neurotransmitters are present. Numerous psychoactive drugs target either the transporters for these ligands (3) or the receptors themselves (4), but all display limited efficacy and multiple side effects. Remarkably, the receptors for these functionally distinct neurotransmitters are predicted, based on 44% sequence identity, to have very similar structures in their transmembrane (TM) domain, where ligand binding causes conformational changes that trigger G protein activation. Biochemical studies of D2 dopamine receptors (5–8), D2R, and comparisons to crystal structures for the β-adrenergic receptor (9, 10), adenosine receptor (11), and rhodopsin (12) delineate a set of residues forming the dopamine binding site, all but three of which are identical in the closely related 5-HT2A serotonin receptor, 5-HT2AR. Thus it seems unlikely that ligand-contacting residues alone are sufficient to confer specificity. A full understanding of the mechanism for ligand discrimination is of intense interest given that 7TMRs make up about 30% of all therapeutic drugs (13). Currently, most therapeutic drugs are thought to bind at the orthosteric site, although many compounds have been shown to bind elsewhere, at allosteric sites, and to impact ligand binding or receptor efficacy (14).

Here, on the assumption that discrimination between serotonin and dopamine has been important for survival throughout evolution of metazoan organisms, we used the Evolutionary Trace (ET) (15) to rank sequence positions in the transmembrane domain according to their evolutionary importance and identified a core of residues of high evolutionary importance, including those specific for the dopamine receptor, that are in structural contact with the ligand-binding site through no more than two intervening residues. Those whose identities differ between D2R and 5-HT2AR were swapped by site-directed mutagenesis so that each ET residue in the 5-HT2AR sequence replaced the corresponding residue in the D2R. The residue-swapped D2R constructs, mostly single point mutants, were then tested for their abilities to activate G proteins in response to dopamine or serotonin by using a cell-based high-throughput fluorescence assay. Ligand-binding affinities were also assessed using radioligand competition assays, and cell surface expression levels were quantified as well, to ensure that each construct was not grossly misfolded and that similar numbers were available for binding externally supplied ligands.

Results

Initial ET analysis identified positions important for specificity of ligand recognition by G protein-coupled receptors. The authors compared their ET analysis to other existing computational approaches and found that their method provided unique insights into the allosteric mechanism of GPCR.

Materials and Methods

The authors describe their methods for using ET to rank sequence positions in the transmembrane domain of GPCRs, and for using site-directed mutagenesis and fluorescence assays to test the ability of mutant receptors to activate G proteins in response to dopamine or serotonin.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.0914877107/-/DCSupplemental.
Responsiveness above that displayed by I48T alone. Thus all 15 double mutation yielded dramatic enhancement of serotonin affinity by at least 40%. One swap, V91S had its most substantial (greater than twofold) increases in affinity for serotonin (Fig. S3). Receptor internalization in cells overexpressing a selective for G protein activation or applies to other downstream (Fig. S3). To test whether the enhanced serotonin responsiveness is selective for G protein activation or applies to other downstream

**Fig. 1.** Identification of candidates for specificity-determining residues. (A) The Ca\(^{2+}\) transients elicited in response to 10 \textmu M dopamine, as measured by fluorescence intensity (relative fluorescence units, RFU) changes in a microplate reader. HEK 293-derived cells stably expressing G\(_{\text{a16}}\) of top 20th percentile positions after ET analysis of all 2512 class A GPCR sequences. (B) Bioamine-specific determinants suggested by subtracting set B (the Class A trace) from set A (the bioamine trace). The residues that cluster in C define a bioamine-specific core that was further extended to include more bioamine-specific neighbors (D) or more residues important in all class A receptors (E) at ranks up to the 40th percentile (see Fig. S1 and Table S1 for details). This set represents 48 candidates for functionally important positions in bioamine receptors (f), of which 15 have different residues in D2R and 5-HT2AR (G).

Activation of G\(_{\text{a16}}\) in response to dopamine was diminished for six of the residue swaps: C118S, S193G, T205M, L379F, V381L, and N418S. Dose-response curves revealed that both the potency and maximal response to dopamine were diminished for C118S and S193G, while L379F showed almost no response to dopamine (Fig. 2C). Binding affinity for dopamine, as monitored by competition for binding of radiolabeled spiperone (19), an antagonist that binds with high affinity to all the D2 variants studied except for V91S, was significantly diminished for four of the residue swaps with diminished activation, C118S, S193G, L379F, N418S, and for an additional four residues, V83L, M117F, Y199F, and L414I (Table S2). Thus 10 of the 15 residue swaps lead to impaired interactions of D2R with dopamine, indicating that the corresponding residues in D2R contribute to dopamine recognition. Because all positions mutated are located within the transmembrane domains and at least two turns from the cytoplasmic face, they are not expected to act through direct alterations in G protein contacts.

The striking observation that all of the residues with diminished dopamine binding still bind with high affinity to spiperone (Table S2) provides further support for the specificity of our evolution-based approach. Both D2R and 5-HT2AR bind spiperone with high affinity, so swapping residues selected for discrimination between dopamine and serotonin would not be expected to exclude a common ligand.

**Fig. 2.** Mutation of ET positions in D2R alters dopamine response. (A) Typical C\(^{a}\) transients elicited in response to 10 \textmu M dopamine, as measured by fluorosence intensity (relative fluorescence units, RFU) changes in a microplate reader. HEK 293-derived cells stably expressing G\(_{\text{a16}}\) were transiently transfected with plasmids expressing D2R WT or indicated mutants, loaded with dye, and treated with dopamine at t = 10 s. (B) Dopamine maximal responses normalized by receptor surface expression. D2R WT response was defined as 100% (Dashed Line). Eight mutations (Red) reduced receptor responsiveness to dopamine. Data represent mean ± S.E.M. (*, P < 0.05; **, P < 0.001). (C) Dopamine-dose-response curves for D2RWT (\(\bullet\)), I48T (\(\Delta\)), N124H (\(\mathcal{D}\)) and S193G (\(\checkmark\)). Curves are four parameter fits as described in SI Text. (D) Positions that reduced dopamine response (\(\bullet\), C) mapped onto the \(\beta\)-adrenergic receptor structure.

**Positions Outside the Orthosteric Binding Site Control Specificity For D2R Activation Without Altering Ligand Affinities.** ET-identified residues also contribute to discrimination against serotonin responses. The swaps I48T, M117F, N124H, and T205M all resulted in significantly enhanced responses to serotonin (Fig. 3). Three different swaps, L41T, F110W, and S193G, all led to substantial (greater than twofold) increases in affinity for serotonin, and three others, M117F, C118S, and T205M, increased serotonin affinity by at least 40%. One swap, V91S had its most dramatic effect when paired with I48T; as described below, this double mutation yielded dramatic enhancement of serotonin responsiveness above that displayed by I48T alone. Thus all 15 residue swaps enhanced interactions with serotonin, or diminished interactions with dopamine, or both (SI Text and Table S3).

In sharp contrast to the results obtained with ET residues ranked as highly important is the lack of effect of swapping less important residues. From among 111 transmembrane residues that are different between D2R and 5-HT2AR, we selected five residues that met our distance criteria but ranked in the lowest 20th percentile of evolutionary importance and made the corresponding sequence swaps: I105K, A188N, V191L, I195K, and S409N. Despite their nonconservative character, all of them yielded dopamine responses similar to those of wild-type D2R, and none of them yielded enhanced serotonin responsiveness (Fig. S2). Thus ET rank is a strong predictor of the likelihood that a residue contributes substantially to ligand specificity.

Because the I48T substitution results in enhanced serotonin responsiveness without diminished dopamine responsiveness, we tested the possibility that this substitution simply renders D2R more promiscuous with respect to ligand discrimination using natural and synthetic ligands. No effect of this mutation was observed for responses to histamine, isoproterenol (\(\beta\)-adrenergic agonist), quinpirole (D2R agonist), or DOI (2,5-dimethoxy-4-iodoamphetamine, a partial agonist for 5-HT2AR), supporting the idea that the evolutionary selection of this residue was associated with discrimination between dopamine and serotonin (Fig. S3).

To test whether the enhanced serotonin responsiveness is selective for G protein activation or applies to other downstream signaling pathways, we measured \(\beta\)-arrestin-dependent agonist-induced receptor internalization in cells overexpressing a \(\beta\)-arrestin-EGFP fusion protein. The results revealed that the I48T mutation, which yielded dramatic enhancement of G protein activation by serotonin, gave rise to almost no effect on this alternate pathway.

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**Table S2.** Positions that are different between D2R and 5-HT2AR, we selected five residues that met our distance criteria but ranked in the lowest 20th percentile of evolutionary importance and made the corresponding sequence swaps: I105K, A188N, V191L, I195K, and S409N. Despite their nonconservative character, all of them yielded dopamine responses similar to those of wild-type D2R, and none of them yielded enhanced serotonin responsiveness (Fig. S2). Thus ET rank is a strong predictor of the likelihood that a residue contributes substantially to ligand specificity.
and responsiveness. Moreover, combining N124H and I48T receptor and less like a dopamine receptor in both its affinities N124H or I48T yields a receptor that is more like a serotonin out much effect on affinities. Combining S193G with either dopamine responsiveness and enhance serotonin responses with-

The S193G mutation confers diminished dopamine affinity and swaps can lead to additive or possibly synergistic effects (Fig. 4). All 15 residue swaps. However, we did find that combining ligand residues, perhaps further still from the ligand-binding site, may need to be swapped to allow proper folding in the context of

Our working hypothesis is that the 15 ET residues targeted in this study work together to confer responsiveness to dopamine while minimizing responsiveness to serotonin. Unfortunately, a version of D2R with residue swaps at all positions appears to be improperly folded, as judged by a complete lack of response to either dopamine or serotonin. Although this mutation reduces binding affinity for both dopamine and serotonin, the concentrations of these used in the internalization assays were high enough to be saturating. Thus this residue allosterically controls conformational switches required for both G protein activation and internalization.

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and T205M, none is in a ligand-contacting position, and except for M117F, all are at positions at least 10 Å from the ligands in the β-adrenergic receptor structures. T205M paradoxically displays enhanced dopamine binding but reduced dopamine responsiveness. Thus a set of proximal residues largely determine binding affinities, whereas more distant residues constitute a specificity-encoding conformational filter needed to transduce ligand binding into G protein-activating conformational changes at the cytoplasmic face. Of great interest is the observation that the filtering function may or may not be specific for G protein activation. Agonist binding also induces activation of an alternate signaling pathway via GPCR kinases (GRKs), β-arrestin binding, and internalization (23). The observation that one residue, L379, is essential for the conformational changes directing both pathways, whereas another, I48, is selective for G protein activation suggests the intriguing possibility that manipulating specific ET residues by mutagenesis (24) or targeting them with allosteric ligands could be used to bias ligand activation toward specific effectors and downstream pathways, i.e., to enhance functional selectivity or “biased agonism” (25, 26) for experimental or therapeutic purposes.

These conclusions have important implications for our understanding of how allosteric coupling of agonist binding to distant G protein activation has evolved to discriminate between related ligands and for improving rational approaches to drug design. Considerable prior evidence has pointed to the importance of structural features distinct from the binding site that are important for allosteric coupling of agonist binding and G protein activation. These include the DRY motif near the cytoplasmic end of TM3 (27–29) and a glutamate residue (E368 in D2R) in TM6 that forms a salt bridge (“ionic lock”) to the arginine in DRY, as well as tryptophan and proline residues involved in a “rotamer toggle” in TM6 (30, 31), and the NPXXY motif in TM7. Unlike the residues identified in this study, however, these conformational switches are largely invariant, suggesting they mediate universal mechanisms within Class A GPCR that are unlikely to contribute to ligand specificity. By contrast, the present results highlight a previously undescribed set of more variable residues that communicate ligand binding to these generic switch motifs in a ligand-specific way. TM3 residues C118 and M117 are in direct contact with the tryptophan of the rotamer toggle switch, and TM2 residue V63L may communicate with this switch through intervening water molecules that occupy a cavity in the structure (Fig. S4). Likewise TM6 residues L379 and V381, TM7 residue N418, and TM1 residue I48 are positioned so as to influence the NPXXY motif residues through intervening waters. Several of the 15 residues are located on TM3 or TM6, helices that undergo relative motion upon activation (32, 33). These results not only reveal the existence of residues with the unique function of conferring ligand specificity through conformational filtering but also demonstrate that they can be identified computationally and mutated rationally to reprogram efficacy in an allosteric pathway to respond to alternate bound ligands, much as the tumblers of a lock may be adjusted to different keys. The spatial separation of the residues identified here emphasizes the cooperative nature of the allosteric switching induced by ligand-binding in GPCR, with a requirement for concerted movements by multiple structural elements within the transmembrane domain.

From a therapeutic perspective, our results also suggest a paradigm shift in rational, structure-based drug design. Currently such efforts are largely focused on optimizing interactions between ligand functional groups and contact residues in the binding pocket. The results presented here imply that only limited improvement in drug selectivity is likely to be obtained by focusing on such interactions, but that drugs directed to sites outside the orthosteric binding pocket and formed by residues selected during evolution to maximize response specificity might yield dramatic improvements in specificity and/or efficacy either alone or in combination with conventional binding-pocket-directed drugs. Allosteric modulators of this type have already been identified for metabotropic glutamate receptors (34) and in a cannabinoid receptor (35). The sites defined by the residues identified in this study represent potential targets for rational drug design.

In addition to the ligand-specificity determinants identified here in D2R, ET has previously successfully identified residues...
critical for function in rhodopsin and related Class A GPCRs (16) as well as residues that discriminate between G protein signaling and arrestin signaling in the β2-adrenergic receptor (24). Combining the evolutionary trace with crystal structures or homology models of target receptors will be an important part of this approach to drug discovery.

The essential features of the approach are as follows. ET ranks the importance of every residue based on whether its sequence variations correlate mostly with major or with minor evolutionary divergences (15). This is the computational equivalent of laboratory experiments that measure residues importance by how much their mutations perturb the functional response of an assay. In practice, starting with a protein family, its aligned sequences, and the associated divergence tree, ET identifies and ranks best alignment positions that vary between major branches but that have low variation entropy within each one of these branches. Conversely, it will rank poorly positions that vary even between closely related species (36). Important properties of top-ranked ET residues are that they cluster spatially within the structure of a representative family member (37), that these clusters identify functional sites (38), and that they efficiently guide experiments to functional determinants (24, 39-42). Although individual case studies cannot guarantee generality, structural motifs of just a few top-ranked ET residues also prove sufficient to predict protein function on a structural protomeric scale, which is consistent with accurate and large-scale identification of key functional determinants (43-45).

Although there are several useful approaches for identifying functionally important sites in GPCR, the efficiency of the approach described here for finding specificity determinants rests on the likelihood that virtually every amino acid substitution at every position has been tested in the course of evolution so that the evolutionary record of variation and of divergence provides orders of magnitude more information than any strictly experimental approach such as random or targeted mutagenesis or accessibility to chemical modification (46). In particular, these approaches are unlikely to predict residues critical for ligand-selective allosteric conformational coupling and have tended to yield information primarily about the orthosteric binding site. Beyond its practical implications, the recoding of efficacy by individual mutations casts a unique light on GPCR evolutionary mechanisms. Our observations that single residue variations trigger diverse, sometimes opposite, efficacy changes in distinct ligands implies that, during evolution, single point variations can perturb simultaneously ligand affinities, efficiencies, and potentially bias toward downstream signaling pathways, as suggested by the successful use of ET-guided mutations to reprogram effector bias (24). Over evolutionary time, variations would probe a large array of functional changes that reprogram the type, distribution, and strengths of couplings between extracellular upstream ligands and intracellular downstream responses, thus providing, from a network perspective, an evolutionary switchboard mechanism to constantly reconfigure the inputs and outputs at GPCR nodes.

Material and Methods

Evolutionary Trace Analysis. Evolutionary trace analyses were performed with the real-value ET (38) in bioamine and class A GPCRs using 402 and 2512 sequences, respectively. These sequences were gathered from GPCRDB and aligned separately using ClustalW. As before, alignments were performed on 195 gapless seven transmembrane helix residues, because the loops are highly divergent (16). The residues ranked in the top 20th percentile of importance in the bioamine specific and Class A traces were mapped onto the β2-adrenergic receptor (PDB 2RH1, Fig. 5I) and clustered significantly: with z scores of 12.7 and 12.5, respectively (47). The difference between them revealed a significant cluster (z score = 5.4) of 10 amino acids traced in bioamine receptors and not in Class A overall (Fig. 5I). Eight of these bioamine-specific positions clustered within 4.5 Å of each other and thus formed a structurally determined core of residues (z score 7.1, Fig. 5I) that should be especially important to bioamine receptor specific functions. An additional nine residues that are important at the 20th percentile for class A GPCRs as a whole, are within 4.5 Å of this initial set (Fig. 5I). Sequence comparison between D2R and 5-HT2AR reveals that all but one of these 17 positions are identical. Extending the criteria to include both Class A and bioamine-specific ET residues to the 40th percentile and within 4.5 Å of any of the above 17 positions expanded the target residues to 48 (Fig. 5J and M). Of these, 15 vary between D2 and 5-HT2A receptors and were used for functional-swap experiments (Fig. 5I). These positions, and alignments inputs, as well as the trace rank file outputs used for these analyses, may be found and reproduced on our GPCR difference-ET server at http://mammoth.bcm.tmc.edu/gPCR/diff/GPCRpaper.pdf. (The visual tool available in beta version.)

Mutagenesis and Cell Transfection. The cDNA clone for human dopamine D2Rlong receptor was obtained from the Missouri, (www.cnca.org). D2R mutants were generated using the QuikChange mutagenesis kit (Strategene, La Jolla, CA) and confirmed by DNA sequencing (Lone Star Labs, Houston). All mutants were generated in the human D2Rlong tagged in the N-terminal with the HA epitope.

HEK cells (American Type Culture Collection, Manassas, VA) were stably transfected with the Gα16 cDNA (a gift from M. Simon, Caltech) using lipofec-tamine 2000 (Invitrogen, Carlsbad, CA) as described by the manufacturer. Cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum supplemented with penicillin, streptomycin, L-glutamine, and G418 (25 μg/ ml). Cells were grown in a 37 °C humidified environment with 5% CO2. For receptor activation assays, HEK-Gα16 cells were reverse transfected in poly-D-lysine-coated 96-well plates and assays performed 48 hrs post transfection.

Calcium Release Assays. At the time of assay, cells were washed once with Krebs/Ringer/Hepes (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 10 mM HEPES, 1.2 mM KH2PO4, 1.2 mM MgSO4, pH 7.4) supplemented with 1.8 g/l glucose. Cells were incubated with 2.5 μM Fluo-4 AM (Invitro-gen) for 1 hr at room temperature with pluronid acid F-127 (final conc. 0.02% v/v), and maintained in 1 mM probenecid to inhibit dye efflux. Ca2+-en-hanced fluorosence was recorded using a Synergy HT plate reader (Winooski, VT).Fluorescence was measured for 10 s to establish baseline, and then at t = 10 seconds, drugs (diluted in KRH) were injected into the well and fluorescence measured every half second for 1 min. On the same plate, receptor surface expression was detected using immunofluorescence. Briefly, cells were fixed with 4% paraformaldehyde for 1 hr at RT. Cells were incubated with Phosphate Saline buffer (PBS) supplemented with 5% donkey serum (Jackson ImmunoResearch, West Grove, PA) followed by incubations with a mouse anti-HA epitope (Santa Cruz Biotechnologies, Santa Cruz, CA) and Alexa Fluor 488-conjugated donkey antitoxo antibody (Invitrogen). Receptor activation data were normalized by the receptor surface expression and traces plotted using GraphPad Prism. Baseline was subtracted to all traces. In some experiments immunolabeling was also carried out in the presence of detergent (0.1% Triton X in TBST with 5% Donkey serum) to check for any major differences in the ratios of surface receptor to total receptor.

Internalization Assays. HEK WT or HEK-Gα16 cells were transfected, fixed, and protein expression (total and surface) determined as described above. Cells were cotransfected either with receptor-expressing plasmids alone, or with equal amount of receptor and j-arrin-GEP-cDNAs. Forty eight h after transfection, either HEK WT or HEK-Gα16 were treated at 37 °C with 10 μM dopamine or 5-HT at different time points. Immunodetection was performed by incubating the cells with mouse anti-HA antibody followed by incubations with an Alexa Fluor 594-conjugated donkey antitoxo antibody. Endpoint fluorescence values were detected using a Synergy HT plate reader. Cells transfected with pcDNA 3.1(+) instead of receptor cDNA were used to subtract nonspecific fluorescence.

[^H] Spiperone Binding. Membranes were prepared from transiently transfected HEK-Gα16 cells. 48 h after transfection, cells were scraped and centrifuged at 1000 x g for 5 min at 4 °C, and the pellets were resuspended in 1 ml of hypertonic buffer (10 mM Tris, 3 mM MgCl2, 2 mM EDTA, pH 7.4) supplemented with protease inhibitors. The homogenates were centrifuged at 13, 500 x g for 10 min at 4 °C, and the membrane pellets (from a confluent 100-mm dish) were resuspended by homogenization in 1 ml of binding buffer (10 mM Tris, 150 mM NaCl, pH 7.4). Aliquots of membrane suspension were incubated in binding buffer either with different concentrations of the antagonist [3H] spiperone (in saturation binding experiments) or with increasing concentrations of agonists or antagonists in the presence of 0.6 mM [3H] spiperone (105 Ci/mmole) (in competition binding experiments). Samples were incubated for 90 min at RT prior to filtration through Whatman GF/C filters using a
96-well Inotech filtration apparatus and counted using a Beckman LS1701 scintillation counter. Specific [3H] spiperone binding was defined as total binding less nonspecific binding in the presence of 1 μM sulpiride (Sigma Aldrich, St. Louis). Saturation and competition binding data were analyzed by nonlinear regression analysis using GraphPad Prism 3.0 (GraphPad Software, San Diego), and the equation, \[ B = B_{max}L/(1 + K_D), \] where \( B \) is bound spiperone, and \( L \) is the spiperone concentration. \( IC_{50} \) values were obtained by fitting the data from the competition studies to a one-site competition model. \( K_i \) values were determined using the equation \[ K_i = IC_{50}/(1 + L/K_D), \] where \( L \) is the concentration of radioligand, and \( K_D \) is the spiperone concentration for half-maximal binding of [3H] spiperone in the absence of competitor.

ACKNOWLEDGMENTS. We thank Monica Galaz Montoya for performing some assays and Ronhall Lua for help with the difference-ET server. This work was supported by grants from the National Institutes of Health (GM066099 and GM079636 to G.L.) and by the Welch Foundation (Q0035 to T.G.W.) and NIH training fellowships (T90 DK070109 and T15 LM007093 to G.J.R.).