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Ancestral reconstruction of the ligand-binding pocket of Family C G protein-coupled receptors

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The metabotropic glutamate receptors (mGluRs) within the Family C subclass of G protein-coupled receptors are crucial modulators of synaptic transmission. However, their closest relatives include a diverse group of sensory receptors whose biological functions are not associated with neurotransmission, raising the question of the evolutionary origin of amino acid-binding Family C receptors. A common feature of most, if not all, functional Family C receptors is the presence of an amino acid-binding site localized within the large extracellular Venus flytrap domain. Here, we used maximum likelihood methods to infer the ancestral state of key residues in the amino acid-binding pocket of a primordial Family C receptor. These residues were reconstructed in the background of the fish 5.24 chemosensory receptor, a broad-spectrum amino acid-activated receptor. Unlike the WT 5.24 receptor, which was not activated by mGluR agonists and displayed low sensitivity toward L-glutamate, the reconstructed ancestral receptor possessed a pharmacological profile characterized by high affinity for both L-glutamate and selective Group I mGluR agonists. This pharmacological phenotype could be largely recapitulated by mutating only two residues in the 5.24 receptor-binding pocket. Our results suggest that this primordial Family C receptor may have arisen early in metazoan evolution and that it already was preadapted as a glutamate receptor for its later use at excitatory synapses in glutamate-mediated neurotransmission.

metabotropic glutamate receptor | primordial receptor | olfactory receptor | calcium-sensing receptor | taste receptor

Glutamate and GABA-mediated neurotransmission likely evolved early in the metazoan lineage concurrent with the evolution of the nervous system. Although basal metazoan lineages such as those leading to present-day sponges do not have fully developed nervous systems, they do possess primitive neural nets and hints of glutamate and GABA-mediated transmission (1, 2). These two neurotransmitters act at ligand-gated ion channels and the metabotropic glutamate receptors (mGluRs) and $GABA_B$ receptors in the Family C class of proteins within the G protein-coupled receptor (GPCR) superfamily (3). Family C receptors mediate a wide spectrum of physiological processes ranging from the modulation of synaptic transmission to the perception of sensory stimuli. In addition to the mGluRs and the $GABA_B$ receptor, other Family C members include the calcium-sensing receptor (CaSR), which participates in the regulation of calcium homeostasis in the body (4, 5), and a diverse group of sensory receptors. The latter group encompasses the fish 5.24 chemosensory receptor expressed in olfactory epithelium and other sensory tissues of the fish, the mammalian T1R taste receptors present on taste cells, and the V2R class of putative pheromone receptors expressed in the vomeronasal organ (6–9).

The ligand-binding domain of Family C receptors, also known as the "Venus flytrap domain," is distantly related to the prokaryotic periplasmic-binding proteins involved in amino acid and nutrient transport in bacteria (10, 11). Free amino acids act at Family C receptors as either direct-acting orthosteric agonists or as allosteric modulators of receptor activity. The mGluRs, the 5.24 receptor, the closely related GPRC6A receptor (8, 12, 13), and the T1R1/T1R3 heteromeric taste receptor are all directly activated by amino acids. Amino acids also allosterically regulate the activity of the CaSR and $GABA_B$ receptors (14, 15). For example, although calcium is considered the primary endogenous ligand at the CaSR, the calcium-induced responses are enhanced in the presence of amino acids, which are thought to bind to a site analogous to the glutamate site in the mGluRs (16).

A key parameter in the study of the evolution and interrelatedness of Family C receptors is the amino acid selectivity for one or several amino acids. The mGluRs are activated by glutamate, whereas other Family C receptors, such as the T1R taste receptors and the fish 5.24 chemosensory receptor (8, 9, 17, 18), are broadly tuned to recognize most amino acids. Other Family C receptors appear to be activated by smaller subsets of amino acids, for example, GPRC6A, which is activated most potently by arginine and lysine, and the CaSR, which is modulated most potently by hydrophobic aromatic amino acids such as phenylalanine and tryptophan.

The variable ligand activation profiles of Family C receptors raise the question as to the pharmacological phenotype of Family C receptors and how the amino acid-binding pockets may have evolved over time to fulfill specific roles in the context of neurotransmission versus sensory perception. In this study, molecular modeling of selected Family C receptors, together with the results of previous pharmacological studies, were used to identify key residues in the binding pocket responsible for conferring amino acid ligand selectivity. Maximum likelihood/Bayesian phylogenetic methods then were used to infer the residues in the binding pocket of the fish 5.24 chemosensory receptor, and the predicted ancestral-binding pocket was reconstructed by using site-directed mutagenesis. Our results show that the predicted ancestral receptor possessed high affinity for L-glutamate and, surprisingly, it also was potently activated by highly selective mGluRs agonists. These findings suggest that the primordial Family C receptor may have been preadapted for use in glutamate-mediated transmission.

Results

Phylogenetic Analysis and Ancestral Reconstruction. A phylogenetic analysis of the aligned sequences showed that members of Family C receptors fall into three major classes: the mGluR class; a second class consisting of the CaSR, the T1R taste receptors, and a group of olfactory and putative pheromone sensory receptors; and a third more distant class consisting of the two $GABA_B$ receptor subunits, which were used to root the tree (Fig. 1). This phylogeny resulted

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Abbreviations: CaSR, calcium-sensing receptor; (*S*)-DHPG, 3,5-dihydroxyphenylglycine; GPCRs, G protein-coupled receptors; mGluR, metabotropic glutamate receptor.

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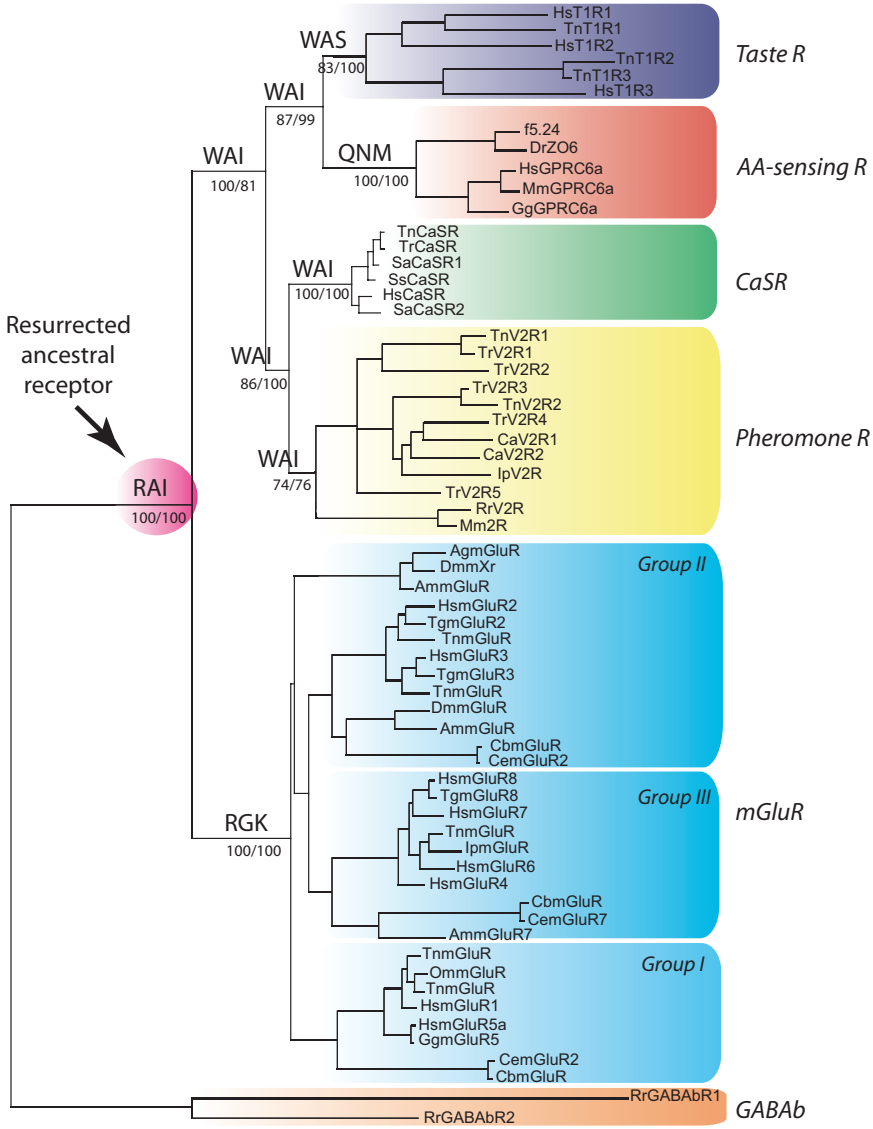

Fig. 1. Phylogeny of Family C receptors used for statistical inference of binding pocket residues in ancestral proteins, with inferred ancestral residues at sites 78, 310, and 389 (amino acid numbering based on the 5.24 receptor sequence) indicated above the nodes, and bootstrap percentage value for parsimony/distance analyses indicated below the nodes (see Fig. 4 for tree sequences, accession numbers, and bootstrap values within each subgroup). Branch lengths of the phylogeny were estimated by using likelihood methods under the *wag*+*G* model.

from analyses of aligned protein sequences by using a variety of methods, including maximum parsimony as well as neighbor-joining and minimum evolution distance analyses. Only one node was found to differ across these different types of analyses (within the V2R pheromone/olfactory receptor group); this node was collapsed to form a polytomy in further ancestral reconstruction studies. Average bootstrap values across all nodes shown in the phylogeny were 85% and 91% in distance and parsimony analyses, respectively, an indication of the robustness of the phylogeny (see Fig. 4, which is published as supporting information on the PNAS web site).

For conducting the ancestral reconstruction, key residues in the amino acid-binding pockets of Family C receptors were selected based on the crystal structure of mGluR1 (19), molecular modeling of the Venus flytrap domains of the mGluRs, 5.24 receptor, GPRC6A, and the CaSR (20–22), and results from additional previous studies examining the ligand-binding pockets of Family C receptors. Extensive pharmacophore, molecular modeling, and mutagenesis studies have established that the molecular basis for agonist binding to the mGluRs and the amino acid selectivity of other Family C receptors is mediated via contacts between residues in the distal portion of the binding pocket and the side chains of the amino acid ligands (12, 13, 16–18, 20–26). Collectively, these studies have demonstrated that only a small subset of binding-pocket residues act as critical determinants of ligand selectivity.

We focused on the amino acids occupying three key positions in the binding pocket that are known to confer amino acid ligand selectivity of Family C receptors: in the 5.24 receptor, these residues are glutamine 78, asparagine 310, and methionine 389; these positions are equivalent to arginine 78, glycine 319, and lysine 409 in mGluR1. Ancestral residues were reconstructed by using maximum likelihood methods (27, 28) under the amino acid model found to have the best fit to the data, as determined by using nested likelihood ratio tests. In comparing models with different fixed substitution rates among the different amino acid changes, the *wag* model (29) was found to have the highest likelihood score (Table 2, which is published as supporting information on the PNAS web site); moreover, the addition of parameters to allow for among-site

rate heterogeneity significantly increased the statistical fit to the data ($+\gamma$: $2\delta L = 2857.79$, $\chi^2_{[1]} = 6.63$ at the significance level $P < 0.01$), whereas the addition of a parameter to account for unequal amino acid frequencies did not ($+F$:$2\delta L = 32.81$, $\chi^2_{[19]} = 36.19$ for $P < 0.01$), making $wag+\gamma$ the best fitting model of those tested for this data set. Reconstructions with the highest posterior probabilities under the $wag+\gamma$ model were found to be arginine 78, alanine 310, and isoleucine 389 (Fig. 1), with average posterior probabilities ranging from 0.76 to 0.96.

Phenotypic Analysis of Wild-Type and Ancestral Receptors. To establish baseline pharmacological parameters for the wild-type receptors, the amino acid selectivity profiles of the WT 5.24 receptor were compared with the mGluRs. For the mGluRs, the mGluR1, mGluR2, and mGluR6 receptor subtypes representing Groups I, II, and III mGluRs, respectively, were studied in live transiently transfected HEK-293 cells by using a fluorescence-based functional assay measuring intracellular calcium release. In agreement with previous studies (8, 17, 18), the 5.24 receptor was most potently activated by arginine followed by glutamine, alanine, and lysine; with the exception of aspartic acid, tryptophan, and proline, which showed no activation at 500 μM, all other amino acids induced responses at concentrations of 100 μM or lower (Table 3, which is published as supporting information on the PNAS web site). In contrast, all three mGluRs responded to glutamate but not to any other amino acid tested (21, 30). Dose–response analyses yielded EC_{50} values for glutamate equal to 1.4, 1.1, 55, and 406 μM for mGluR1, mGluR2, mGluR6, and the 5.24 receptor, respectively (Table 3). These results demonstrate that (*i*) compared with the mGluRs, the 5.24 receptor displays low affinity for L-glutamate and (*ii*) that the mGluRs and the 5.24 receptor are fundamentally different in their activation profiles. In the context of ligand recognition, the mGluRs and the 5.24 receptor represent two ends of the amino acid selectivity spectrum; the 5.24 receptor is broadly tuned to most amino acids, whereas the mGluRs are activated exclusively by glutamate.

To examine the phenotype of the partially reconstructed receptor, we introduced the inferred amino acids into the binding pocket of the 5.24 chemosensory receptor via site-directed mutagenesis. The 5.24 receptor was chosen as the template for mutagenesis because of its high divergence from the mGluRs in terms of both its amino acid selectivity (broad vs. narrow amino acid selectivity, respectively) and because of its sequence dissimilarity to the mGluRs. Thus, glutamine 78, asparagine 310, and methionine 389 in the 5.24 receptor were mutated to arginine, alanine, and isoleucine, respectively; this triple mutant is herein referred to as the "ancestral receptor." Immunoblotting indicated that the ancestral receptor was expressed at levels similar to the parent 5.24 receptor (Fig. 2*a*). Pharmacological analysis of the predicted ancestral receptor demonstrated that it retained broad sensitivity to most amino acids, whereas its affinity for glutamate was increased >130-fold over that of the 5.24 receptor (Fig. 2*c* and Table 3). This large increase in affinity for glutamate was accompanied by a decrease in the affinities for the basic amino acids arginine and lysine (Fig. 2*b* and Table 3).

Further experiments were conducted by using subgroup selective mGluR agonists. The Group II selective mGluR agonist dicarboxycyclopropylglycine, and the Group III selective agonist L-serine-*O*-phosphate, did not activate the 5.24 receptor or the ancestral receptor. The nonselective mGluR agonist (2S,1′S,2′S)-2-(carboxycyclopropyl)glycine (L-CCG-I) and the Group I selective compounds, quisqualic acid and ibotenic acid, displayed very low potency at the WT 5.24 receptor, whereas no activation was observed with the highly specific Group I agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG) (Fig. 2*d* and Table 1). However, L-CCG-I and all three Group I agonists potently activated the ancestral receptor. Remarkably, the potency of DHPG at the ancestral receptor (EC_{50} = 2.4 μM) was slightly higher than that of the Group I receptor, mGluR1a (EC_{50} = 3.3 μM; Table 1). Together, these results indicate that the predicted ancestral Family C receptor was potently stimulated by L-glutamate and by Group I selective but not Group II or III selective mGluR agonists.

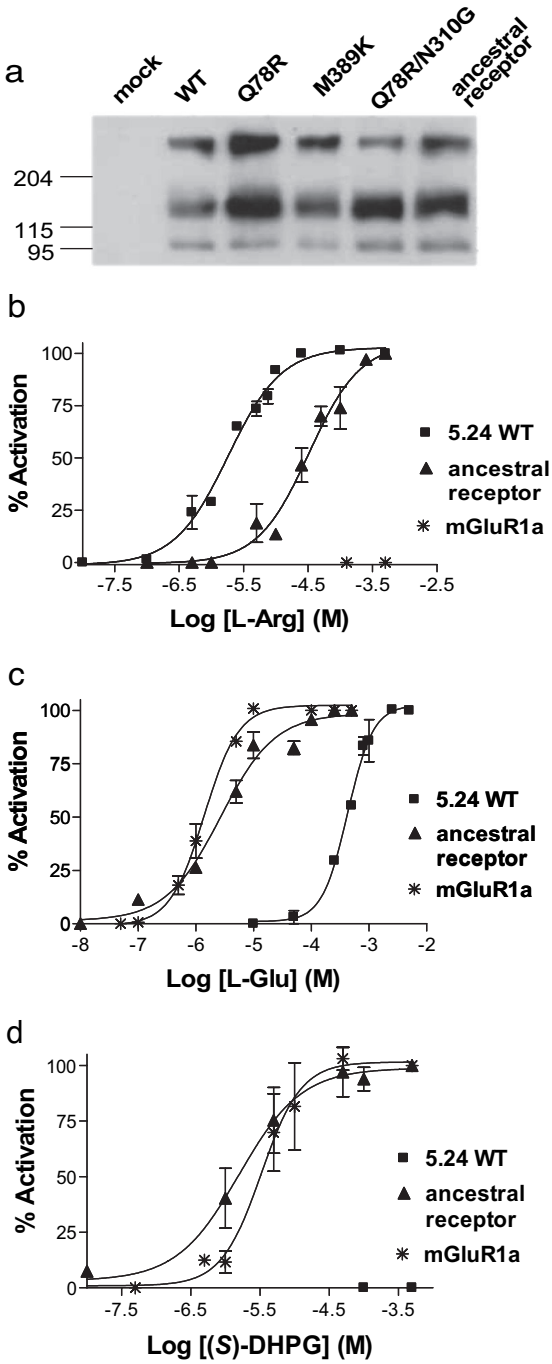

Fig. 2. Pharmacological characterization of the 5.24 and mutant receptors. (*a*) Protein expression in transfected HEK 293T cells. Samples were separated on SDS/polyacrylamide gels, transferred to nitrocellulose, and probed with an anti-*c-myc* monoclonal antibody. (*b*) Comparison of L-arginine activation of the 5.24 receptor, the ancestral receptor, and rat mGluR1a; the EC_{50} values are listed in Table 3. (*c*) L-glutamate activation curves for the 5.24 receptor, the ancestral receptor, and rat mGluR1a. (*d*) Activation curves for the Group I mGluR-specific agonist (S)-3,5-DHPG; the EC_{50} values are shown in Table 1.

To further probe the molecular basis of agonist recognition, a double mutant (Q78R/N310G) where only two of the three target residues were mutated, and a single mutant (Q78R) where only one

Table 1. Comparison of EC_{50} values for mGluR agonists

Agonists	5.24 receptor	Ancestral receptor	Q78R/ N310G	Q78R	mGluR1a
L-CCG-1	+	4.9 ± 1.2	3.5 ± 0.8	1.3 ± 0.1	24.7 ± 1.9
ibotenate	183.7 ± 22.5	0.8 ± 0.3	3.2 ± 0.4	6.4 ± 0.3	4.4 ± 0.9
quisqualate	230.0 ± 58.0	0.8 ± 0.2	3.5 ± 0.8	8.3 ± 0.4	0.4 ± 0.2
(*S*)-DHPG	–	2.4 ± 1.3	3.8 ± 1.1	+	3.3 ± 0.7
DCG-IV	–	–	–	–	–
L-SOP	–	–	–	–	–

The values (in micromolar) are means ± SEM of three experiments. –, no response at 500 μM; +, response at 500 μM but not 100 μM. Additional compounds tested that did not activate the 5.24 receptor or the ancestral receptor at 500 μM include kainate, *S*-AMPA, NMDA, and GABA. L-CCG-1, (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl)glycine; DCG-IV, dicarboxycyclopropylglycine.

of the three was mutated, were generated and characterized. Similar to the ancestral receptor, both the double and single mutants retained high affinity for glutamate (Table 3). Thus, the switch from very low glutamate potency in the WT 5.24 receptor to high glutamate potency in the ancestral receptor was largely recapitulated by mutating a single residue (at position 78) located in the distal region of the binding pocket. Although a similar shift in ligand selectivity was seen upon mutation of methionine 389 to lysine in the 5.24 receptor (18), the ancestral reconstruction indicates that the amino acid at this position in the predicted precursor was isoleucine (Fig. 1), which would not be expected to confer the arginine-preferring to glutamate-preferring ligand selectivity switch.

Additional experiments demonstrated that DHPG also displayed high potency at the Q78R/N310G double mutant but not the single Q78R mutant (Table 1). Inspection of the molecular models of the 5.24 receptor (Fig. 3*a*) and the ancestral receptor (Fig. 3*b*) indicated that the inability of DHPG and other Group I agonists to activate the 5.24 receptor is likely due to steric block of the bulky side chains of the Group I mGluR agonists by the large side chain of asparagine 310 (Fig. 3*c*). In the ancestral receptor and the double mutant, the asparagine at this position was replaced with the small side-chain amino acids alanine and glycine, respectively; the model indicates that this change eliminates the steric hindrance and provides sufficient space for the docking of DHPG (Fig. 3*d*).

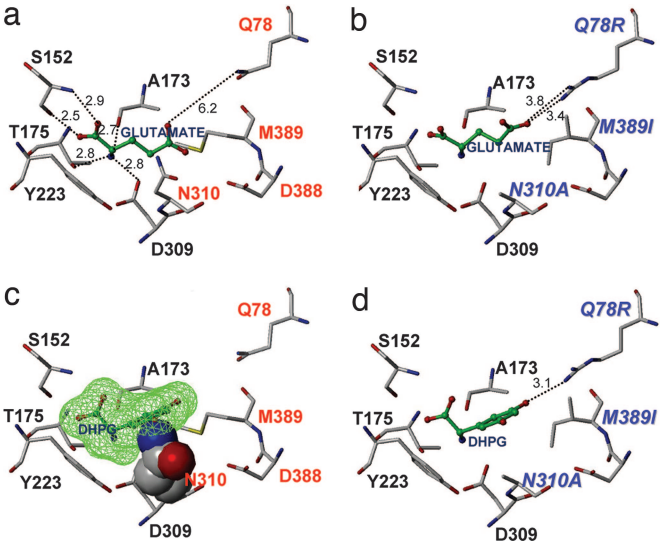

Fig. 3. *In silico* docking of ligands in the 5.24 and ancestral receptors. The ligands are displayed as ''ball and stick'' representations with the carbon atoms in green; the carbon atoms in the receptor-binding pocket are shown in gray. Color coding for other atoms: nitrogen, blue; oxygen, red; sulfur, yellow. Dashed lines depict atom pairs with the distances between atoms listed in angstroms. (*a* and *c*) The conserved residues in the binding pocket establishing bonds with the α amino and α carboxyl groups of the bound ligand are numbered in black, whereas residues in the distal region of the pocket that mediate amino acid selectivity are numbered in orange. Ligand selectivity depends on the ability of the distal functional group of the ligand to reach and establish an interaction with residues in the distal region of the pocket. (*a*) Glutamate docked into the 5.24 receptor-binding pocket. (*b*) Glutamate in the ancestral receptor pocket. (*c*) The inability of DHPG to dock into the 5.24 receptor is illustrated. The Connolly surface of DHPG is shown as a green meshwork. The side-chain atoms of asparagine 310, which are depicted in a space-filling mode, cause steric blockade of DHPG binding. (*d*) DHPG in the ancestral reconstructed receptor with the residues mutated shown in bold blue type.

Discussion

We investigated the pharmacological origins of Family C receptors by using phylogenetic methods to reconstruct key sites within the ancestral ligand-binding pocket of the predicted primordial receptor. The ancestral reconstruction was carried out in the background of the fish 5.24 chemosensory receptor. The WT 5.24 receptor was not activated by mGluR-selective agonists and displayed very low affinity for glutamate, whereas the predicted ancestral receptor was highly sensitive to glutamate and Group I mGluR-specific agonists. Further mutagenesis experiments demonstrated that this pharmacological switch could be accomplished by mutating only two residues in the binding pocket to those predicted based on the ancestral reconstruction. Together, these findings demonstrate (*i*) that minor perturbations in the binding pocket can have profound consequences on the pharmacological profile of Family C receptors and (*ii*) that the primordial precursor to Family C receptors had high affinity for glutamate.

The high affinity of the predicted ancestral receptor for glutamate, similar to present-day mGluRs, and its ability to be activated by highly specific Group I agonists, suggests that the key characteristics of the ligand pocket mediating high sensitivity to glutamate already were present in the ancestral receptor. Our data also imply that the Group I mGluRs may have retained a more primitive conformation of the binding pocket compared with the Group II and Group III mGluRs. The binding pocket residue occupied by glutamine 78 in the 5.24 receptor is predicted to be an arginine in the ancestral receptor. This arginine, which is strictly conserved in all extant functional mGluRs, establishes a bonding interaction with the γ carboxyl group of the bound glutamate ligand (Fig. 3*b*) and is buried within the core of the binding domain in the mGluRs (31). This arginine residue likely played a critical role in mediating an evolutionary progression to a glutamate-sensitive receptor that would eventually be used in neurotransmission.

The reconstructed ancestral receptor characterized in this study subsequently gave rise to two major groups of receptors. One lineage evolved into glutamate-sensitive receptors, and the other lineage radiated to become the diverse group of present-day sensory receptors, which lost high affinity for glutamate and evolved broad sensitivity to other amino acids. Members of the glutamate lineage have been found in basal chordates such as the ascidian *Ciona intestinalis*, as well as protostomes including the nematode *Caenorhabditis elegans*, and the fruitfly *Drosophila melanogaster* (32). In the *Ca. elegans* genome, three mGluR receptor sequences corresponding to the three subgroups of mGluRs are present, although pharmacological studies have not been reported (11). In insects, a single functional mGluR (DmGluR) and a Family C orphan receptor (DmXR) that is not activated by amino acids including glutamate have been described in refs. 33 and 34. Surprisingly, members of the sensory class of receptors including T1R taste, V2R, and 5.24/GPRC6A orthologs are either underrepresented or missing from invertebrate species, although a single sequence annotated as a CaSRs is present in *Ca. elegans* and in *Ci. intestinalis* (32). However, The presence of at least one member of

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the sensory receptor lineage in protosomes indicates that the resurrected ancestral receptor already would have existed at the time of the bilaterian ancestor, predating the protostome-deuterostome divergence.

The most basal Family C receptors identified to date include sequences cloned from the sponge (2) and the amoeba *Dictyostelium discoideum* (35). In the case of the *Dictyostelium* receptor, no activation by glutamate or other amino acids could be demonstrated. In fact, Taniura *et al.* (35) described the *Dictyostelium* receptor as an "mGlu precursor receptor" and suggested that the primordial Family C receptor may have resulted from the fusion of two genes, one that served as a precursor to the glutamate receptors and a second that served as the prototype for the transmembrane domain of the $GABA_B$ receptor lineage. In contrast, the sponge receptor did show weak activation by high (millimolar) concentrations of glutamate. Together these findings suggest that glutamate activation of Family C receptors may have arisen early in metazoan evolution, with the high glutamate affinity seen in the resurrected ancestral receptor fully present by the time of the bilaterian ancestor. The emergence of high-affinity activation of an ancestral Family C receptor by glutamate coincides with the evolution of nervous systems in higher animals and may have been a necessary prerequisite for the development of glutamatergic neurotransmission characteristic of excitatory synapses.

Experimental Procedures

Sequence Alignments and Phylogenetic Analyses. The protein sequences for Family C GPCRs were obtained from the National Center for Biotechnology Information database by using the human mGluRs and CaSR and the fish 5.24 sequences as probes. After elimination of redundant and truncated sequences from an initial collection of 95 sequences, the remaining 62 sequences from 19 genera, along with the rat $GABA_B$-R1 and R2 sequences used as the outgroup, were aligned by using ClustalW (see Fig. 4 for accession numbers). For the alignment, the short segment of the N termini (encompassing the signal peptide) and the C termini (after the end of the transmembrane domains) were highly variable and excluded from the multiple alignment. Phylogenetic analyses of protein sequences by using maximum parsimony and distance methods, including neighbor joining (36) and minimum evolution (37), were performed by using the PAUP* program (38). Bootstrap methods were used to assess the degree of confidence of nodes in the phylogeny (39).

Ancestral reconstructions of the residues in the binding pocket were performed by using maximum likelihood methods (27, 28) as implemented in the PAML program (40). Average marginal posterior probabilities at each site across the phylogeny were calculated by using an empirical Bayesian approach, incorporating the maximum likelihood estimates of parameter values including branch lengths. Likelihood scores of amino acid-based likelihood models differing only in fixed substitution rate matrices were compared directly; otherwise pairwise likelihood ratio tests were used to assess among nested models, which provided a better fit to the data (41). The amino acid-based model found to have the best fit to the data then was used for inference of ancestral states.

Expression Constructs, Site-Directed Mutagenesis, and Protein Expression. A *c-myc*-His-tagged expression construct of the goldfish 5.24 receptor cDNA (8) was generated as described in pcDNA3.1 (17). Mutations were made by using the QuikChange site-directed mutagenesis procedure (Stratagene, La Jolla, CA) with either a 665-bp EcoRV cassette or a 1.6-kb EcoRI-XbaI cassette of 5.24 in pBluescript KS^+ (Stratagene) as the template. After mutagenesis, the cassettes were subcloned back into the *myc*-His-tagged 5.24 receptor. The cDNAs were transiently transfected into HEK-293T cells by using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and SDS/PAGE and immunoblotting with an anti-*c-myc* mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) were conducted as described in ref. 17.

Functional Analysis of WT and Mutant Receptors. A fluorescence-based assay measuring the release of intracellular Ca^{2+} was performed as described by Kuang *et al.* (17). The responses were recorded on a FLEXstation fluorescence plate reader (Molecular Devices, Sunnyvale, CA). (*S*)-3,5-DHPG and other mGluR agonists were obtained from Tocris Bioscience (Ellisville, MO). In the experiments with the $G_{\alpha i}$-coupled receptors mGluR2 and mGluR6, HEK-293T cells were cotransfected with the promiscuous G protein subunit $G_{\alpha 15}$ as described in ref. 20.

Molecular Modeling and Ligand Docking. Homology models of the WT 5.24 and ancestral receptor were generated by using the x-ray crystal structure of the extracellular domain of rat mGluR1 as a template (ref. 19; PDB ID code 1EWK) by using version 6.0 of the MODELER program (42). Sybyl 6.9 (Tripos, St. Louis, MO) was used to view, analyze, and manipulate the structure. The ligand arginine was manually docked into the WT 5.24 model and the structure of the complex was subjected to molecular dynamics refinement by using AMBER 7.0 as described in ref. 21. The refined structure then was used for ligand docking. For ligand docking, triionized L-glutamate and diionized DHPG were constructed and minimized by using Sybyl. The ligand molecules were docked into the binding pocket, defined as all residues within 6.5 Å of the ligand, by using the docking program FlexX. The optimally docked conformer was selected based on the scores calculated from the scoring function in the CSCORE module of Sybyl.

This work is dedicated to the memory of Dr. Hubert H. van Tol. We thank Drs. E. Rosemond for assistance; J. Ngai (University of California, Berkeley, CA) for the 5.24 cDNA; and D. M. Broussard, D. Guttman, and H. H. van Tol for insightful comments on the manuscript. This work was supported by operating grants and a Strategic Training Grant on Membrane Proteins Linked to Disease from the Canadian Institutes for Health Research (to D.R.H.) and an operating grant from the Natural Sciences and Engineering Research Council of Canada (to B.C.). D.K. was supported by a Graduate Scholarship in Pharmacy from the Canadian Rx and D Foundation.

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