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# Divergent Evolution in Metabotropic Glutamate Receptors

A NEW RECEPTOR ACTIVATED BY AN ENDOGENOUS LIGAND DIFFERENT FROM GLUTAMATE IN INSECTS\*

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The metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors involved in the regulation of glutamatergic synapses. Surprisingly, the evolutionarily distant *Drosophila* mGluR shares a very similar pharmacological profile with its mammalian orthologues (mGlu2R and mGlu3R). Such a conservation in ligand recognition indicates a strong selective pressure during evolution to maintain the ligand recognition selectivity of mGluRs and suggests that structural constraints within the ligand binding pocket (LBP) would hinder divergent evolution. Here we report the identification of a new receptor homologous to mGluRs found in *Anopheles gambiae*, *Apis mellifera*, and *Drosophila melanogaster* genomes and called AmXR, HBmXR, and DmXR, respectively (the mXRs group). Sequence comparison associated with three-dimensional modeling of the LBP revealed that the residues contacting the amino acid moiety of glutamate (the  $\alpha$ -COO<sup>-</sup> and NH<sub>3</sub><sup>+</sup> groups) were conserved in mXRs, whereas the residues interacting with the  $\gamma$ -carboxylic group were not. This suggested that the mXRs evolved to recognize an amino acid different from glutamate. The *Drosophila* cDNA encoding DmXR was isolated and found to be insensitive to glutamate or any other standard amino acid. However, a chimeric receptor with the heptahelical and intracellular domains of DmXR coupled to G-protein. We found that the DmX receptor was activated by a ligand containing an amino group, which was extracted from *Drosophila* head and from other insects (*Anopheles* and *Schistocerca*). No orthologue of mXR could be detected in *Caenorhabditis elegans* or human genomes. These data indicate that the LBP of the mGluRs has diverged in insects to recognize a new ligand.

activated by a wide variety of ligands (light, ions, neurotransmitters, odors, and hormones) and have evolved as one of the largest gene superfamilies (1). Pharmacological characterization of GPCRs phylogenetically related shows that the ligand recognition site has diverged during evolution. Generally, related receptors from different species recognize the same endogenous ligands but have different pharmacological profiles when one is considering synthetic ligands. In some cases, the divergence is so important that related receptors recognize different endogenous ligands (2).

However, such pharmacological divergences, as far as currently known, did not occur in the metabotropic glutamate receptor (mGluRs) subclass of GPCRs. The eight mammalian mGluRs (mGlu1R to mGlu8R) are involved in the regulation of many glutamatergic excitatory synapses (3, 4). They are classified into three groups based on their sequence homology, ligand recognition selectivity, and transduction pathway. Sequence analysis of the *Caenorhabditis elegans* genome revealed the presence of one homologue for each group (5), indicating that the three groups of mGluRs were already present in the common ancestor of nematodes and vertebrates. Functional data were obtained with the *Drosophila melanogaster* metabotropic glutamate receptor (DmGluAR) (6). Surprisingly its pharmacological profile was conserved, DmGluAR being activated or inhibited by the same natural and synthetic ligands as its mammalian mGluR orthologues (the group II mGluRs, mGlu2R and mGlu3R) (7). Such a conservation in the ligand recognition between invertebrates and mammals mGluRs, for the endogenous ligand and for different synthetic ligands, suggests the existence of structural constraints within the ligand binding pocket (LBP) or even in the whole ligand binding domain, called the Venus Flytrap module (VFTM) in mGluRs (8). These constraints would hinder further divergent evolution of the LBP.

Here we show that a strong divergence of the LBP and of the endogenous ligand has occurred during evolution. Indeed, we describe the identification of a new receptor belonging to the mGluR subclass. This receptor was found in the *Anopheles*, *Apis*, and *Drosophila* genomes and was called mXR (AmXR, HBmXR for honeybee, and DmXR, respectively). We isolated the *Drosophila* cDNA encoding DmXR. Comparison between the LBP sequence of mGluRs and mXRs associated with three-dimensional modeling of the LBP revealed that only part of the residues involved in the binding of glutamate was conserved,

Sensory and intercellular communications in the animal kingdom are often mediated by seven transmembrane G-protein-coupled receptors (GPCRs)<sup>1</sup> and their ligands. GPCRs are

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ581534.

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<sup>1</sup> The abbreviations used are: GPCRs, G-protein-coupled receptors; mGluR, metabotropic glutamate receptor; DmGluAR, *Drosophila* metabotropic glutamate receptor A; LBP, ligand binding pocket; VFTM,

Venus Flytrap module; mXR, metabotropic X receptor; AmXR, *Anopheles* mXR; HBmXR, *Apis* mXR; DmXR, *Drosophila* mXR; HEK, human embryonic kidney; IP, inositol trisphosphate; V1ahR, Vasopressine1a human receptor; GABA<sub>B</sub> receptor,  $\gamma$ -aminobutyric acid type B receptor; AVP, arginine vasopressin; RT, reverse transcriptase; HA, hemagglutinin.

suggesting that these receptors evolved to recognize an amino acid different from glutamate. We demonstrate that the DmXR could not be activated by glutamate but was activated by a compound with a primary amino group, found in extracts from *Drosophila* heads and from other insects (*Anopheles gambiae* and *Schistocerca gregaria*). No orthologue of this new receptor could be found in *C. elegans* and mammalian genomes. Our data show that the existence of receptor ligand-specific constraints cannot be generalized to the entire subclass of mGluRs. Moreover, in at least some insects, one mGluR has diverged to be activated by a new endogenous ligand.

#### EXPERIMENTAL PROCEDURES

**Materials**—All L-amino acids and D-aspartate, D-glutamate, D-serine, D-alanine, taurine, carnosine, and trichloroacetic acid were purchased from Sigma. L-Quisqualate,  $\gamma$ -aminobutyric acid (GABA), N-acetylaspartylglutamate, L-cysteinesulfonic acid,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, kainate, N-methyl-D-aspartate, and L-2-amino-4-phosphonobutyric acid were purchased from Tocris Neuramin (Bristol, UK). Glutamate pyruvate transaminase was from Roche Applied Science. Fetal bovine serum, culture medium, and other solutions used for cell culture were from Invitrogen. [ $^3$ H]Myoinositol was purchased from PerkinElmer Life Sciences.

**Cloning of the DmX Receptor and In Vitro Mutagenesis**—DmXR cDNAs with incomplete 5' end were obtained after screening a *Drosophila* wild-type Oregon R head library as in Ref. 6. These cDNAs were first called DmGluBR (corresponding to CG30361, Flybase) (9). All the cDNAs had the same sequence corresponding to nucleotides coding for residue 336 to the last residue in Fig. 1A followed by a 265-bp 3'-untranslated sequence. The missing N-terminal part of the DmXR was cloned by RT-PCR from *Drosophila* Oregon R heads using the sense primer BC522 (5'-ACAACATGAACCTAATGCTGCC-3') and the antisense primer 6V (5'-CACTGAAAGTGATCCTCC-3'). Several independent clones were sequenced in their entire length in order to verify the correctness of the amplification by comparison to the DmXR genomic sequence. The full-length coding sequence was assembled in pBS plasmid (Stratagene), and the sequence was verified by sequencing. The entire coding sequence was subcloned in the mammalian expression vector pRK5 and tagged N-terminally with the hemagglutinin epitope (HA-DmXR/pRK5) as in Ref. 10.

The chimeric receptor that contains the extracellular domain of DmGluAR and the 7TM and C-terminal regions of DmXR was constructed with the PCR overlap extension method using DmGluRA and DmXR as template. The choice of the limits of the different domains was done as in Ref. 7. For all constructs, the sequences were verified using the appropriate primers and the "dNA sequencer, Long Readir 4200 Li-COR" from Sciencetec.

For the construction of the mutant receptors, amino acid changes in the DmXR LBP were introduced using the PCR overlap extension method as described previously (11) for the tagged HA-DmXR/pRK5 plasmid. The presence of each mutation of interest and the absence of undesired ones were confirmed by sequencing. The resulting expression constructs were used for transient expression in human embryonic kidney (HEK 293) cells.

**Cell Culture, Transfection, and Inositol Phosphate (IP) Assay**—HEK 293 cells were cultured as described in Ref. 12 and transiently transfected by electroporation with either 14  $\mu$ g of carrier DNA (pRK), plasmid DNA containing HA-DmXR wild-type or HA mutants DmXR (4  $\mu$ g), plasmid DNA containing  $G\alpha_{q19}$  (2  $\mu$ g) (into pcDNA3.1, Invitrogen) (to enable the DmXR and the DmGluAR coupling to phospholipase C) (13), and for positive controls, plasmid DNA containing DmGluAR (6) (2  $\mu$ g) or plasmid DNA containing V1ahr (14) (1  $\mu$ g) for  $10^7$  cells.

Determination of IP accumulation in transfected cells was performed after labeling the cells overnight with [ $^3$ H]myoinositol (23.4Ci/mol) as described previously (15).

**Immunocytochemistry**—HEK 293 cells were grown on 8-well glass slides coated with poly-D-lysine and transfected (2  $\mu$ g of HA-DmX/pRK5 or 2  $\mu$ g of HA mutant DmXR/pRK5). The immunocytochemistry was performed as described previously (13). Cell-surface receptor expression was assayed by labeling with anti-HA monoclonal mouse 12CA5 antibody for 2 h at 1.3  $\mu$ g/ml in phosphate-buffered saline/gelatin (0.2%), as described previously (15).

**RT-PCR Experiments**—*Drosophila* poly(A)<sup>+</sup> mRNA was used with the One-step RT-PCR PLATINIUM Taq kit (Invitrogen). DmXR sense primer was XRY2 (5'-TGT ATT GCC ATC AAG GAG AAG-3') and antisense primer was 6V. PCR products of the expected size (380 bp)

were sequenced. Positive control reactions were set up in parallel using phosphoglycerate kinase sense primer 5'-GGC CAA GAA GAA TAA CGT GCA GTT GC-3' and phosphoglycerate kinase antisense primer 5'-CGC TGG TCA ATG CAC GCA CGC-3' that amplified a fragment of 430 bp (16).

**Protocol of Amino Acid Extraction**—We harvested *Drosophila* heads or other tissues frozen in liquid nitrogen. The tissues were weighed, ground, and sonicated. To separate DNA and membranes from small molecules, a first centrifugation was performed 15 min at  $10,000 \times g$ . Proteins precipitation was then performed by treating the supernatant with 10% trichloroacetic acid, to a final concentration of 5% for at least 2 h at 4 °C (17). The extracts were then submitted to a 30-min centrifugation at  $38,000 \times g$ . The supernatant was recovered, evaporated, and dissolved in HEPES saline buffer. The pH was adjusted to 7.4 by adding NaOH. The final concentration was about 40  $\mu$ g of tissues/ $\mu$ l. In the pharmacological assays, 1 $\times$  corresponds to the amount obtained with 2 mg of tissues.

**Sequences Analysis and Molecular Modeling**—The sequence alignment between the rat mGlu1R, the *Drosophila* DmGluAR, and the three mXRs was produced using ClustalW and the default parameters (18). The inferred amino acid sequences of class III GPCRs from rat, *Drosophila*, and *Anopheles* were obtained from Swiss Protein, Flybase, and NCBI data banks, respectively. The AmXR sequence (AAAB01008900) was deduced from *Anopheles gambiae* genome using TblastN (19) and the DmXR sequence. The HBmXR sequence was deduced from *Apis mellifera* genome sequence (Baylor College of Medicine) using TblastN (19) and the DmXR sequence.

The phylogenetic tree was constructed using an exhaustive number of class III GPCR sequences from various species retrieved from data banks using TblastN searches (see Fig. 2). Sequences were aligned using the default parameters of ClustalW (protein weight matrix, Blossum30; Gap open penalty, 10.0; Gap extension penalty, 0.1). The resulting multialignment was then used for construction of an evolutionary tree using the Neighbor Joining method (20), and the positions with gaps were excluded. Bootstrap values were calculated using 1000 trials and a seed number of 111. The unrooted tree was then drawn from the .phb file using TreeView (21).

A homology model of the closed form of the VFTM of DmXR was generated using the x-ray crystal structure of the VFTM of rat mGlu1R as a template (Protein Data Bank accession number 1EWKA). The three-dimensional model was built by using Modeler 5 (22) in the Insight-II environment (Accelrys, San Diego) as described previously (23), based on the alignment shown in Fig. 1. The Verify3D plots (24) were generated using Profile3D in the Insight-II environment.

#### RESULTS

***Anopheles*, *Apis*, and *Drosophila* mX Receptors Are New Homologues of mGluRs with a Divergent LBP**—We identified a new receptor homologous to mGluRs, called mXR, in genomic sequences from *A. gambiae*, *A. mellifera*, and *D. melanogaster* using TblastN searches against all genomic sequences available at NCBI, with the complete sequence of mammalian mGluRs as a probe. In order to make sure that these sequences were actually transcribed, we cloned the cDNA encoding the *Drosophila* receptor (DmXR), as described under "Experimental Procedures." The DmXR sequence is shown in Fig. 1 as well as the sequence of the *Anopheles* (AmXR) and *Apis* (HBmXR) receptors deduced from the genome sequence. The mXRs (AmXR, HBmXR, and DmXR) displayed about 75% sequence identity between themselves, indicating that they encode orthologous insect receptors. A direct comparison between amino acid sequences of the mXRs and members of the mGluRs subclass (DmGluAR and mGlu1bR in Fig. 1) revealed that all the structural features characteristic of mGluRs were conserved. The mGluRs share sequence similarity with the GABA<sub>B</sub> receptors, the calcium-sensing receptor, some taste receptors, and a class of mammalian putative pheromone receptors and constitute the class III within the large GPCR family (1). The sequence of the seven-transmembrane domain of mXRs displayed 32–40% overall amino acid identity with the mGluRs and only 17–25% with the other members of the class III receptors, suggesting that the mXRs are part of the mGluR subclass. To further analyze this, a multialignment of mXRs and many

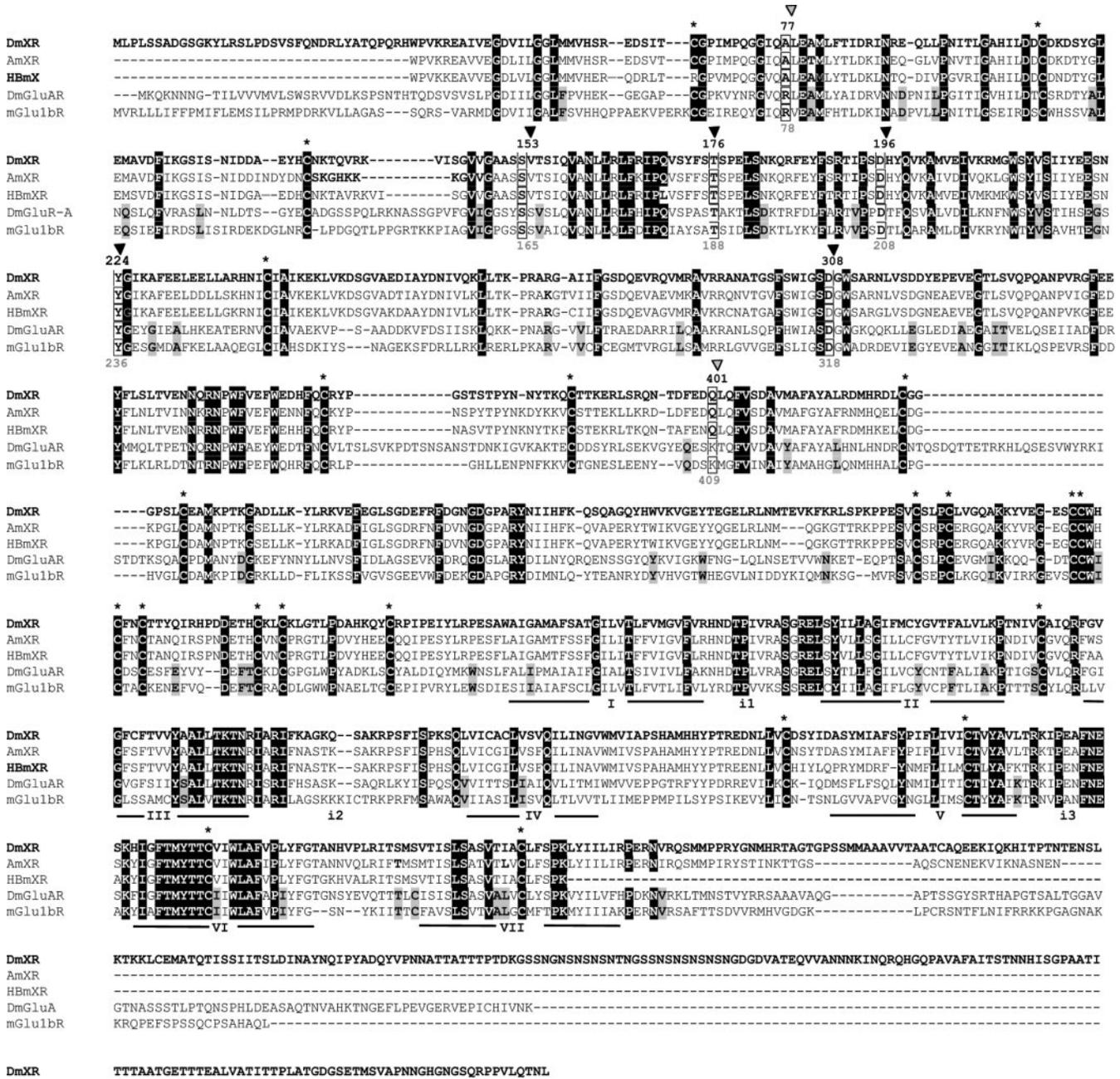


FIG. 1. Multiple alignment of DmXR, AmXR, and HBmXR with the *Drosophila* DmGluAR and the mammalian mGlu1bR. Amino acid sequences were aligned using ClustalW. Residues shaded in black are conserved in all mGluRs (including those not represented in the figure) and in the mXRs. Substitutions in mXRs of residues conserved in all mGluRs are highlighted in gray. Predicted transmembrane segments are indicated by numbered bars below the sequence. Conserved cysteines are indicated with an asterisk. LBP residues involved in glutamate binding conserved in all known mGluRs compared with the residues at equivalent positions in mXRs are indicated by an arrowhead (black, conserved in mXRs; gray, non-conserved in mXRs). Numbers below the alignment show the position in mGlu1bR of the LBP conserved residues involved in the glutamate binding. Numbers above the alignment show the position in DmXR of the LBP homologue residues. The non-conserved (in mGluRs) N-terminal and C-terminal parts of the AmXR and HBmXR sequences are not shown in this alignment.

other members of the class III GPCRs from various species was generated and used to generate a phylogenetic tree (Fig. 2). This analysis was restricted to members of the class III GPCRs containing both the ligand binding domain and the seven-transmembrane domain. This analysis clearly revealed three main subclasses of ligand binding domain-containing class III GPCRs, as indicated with bootstrap values for the branches defining these groups of 1000, 988, and 1000 (Fig. 2). These subclasses correspond to the mGlu receptors, the sensory receptors, and the GABA<sub>B</sub> receptor subunits, respectively. The DmXR, AmXR, and HBmXR sequences are clearly part of the mGlu receptors subclass but define a group different from the group I, II, and III mGluRs, and each of these groups was

defined by bootstrap values of 1000 (887 for the group II if the DmGluAR sequence is included). The same conclusion was obtained when the phylogenetic tree was calculated without excluding positions with gaps or when only the sequences of the 7TM domain of all class III GPCRs (even those not containing a known ligand binding domain) were used for the analysis. Taken together these observations demonstrate the mXRs derive from an ancestral mGlu receptor. However, all three identified mXR sequences differed from all other mGluRs at the level of some key residues involved in glutamate binding, suggesting that they are not activated by this acidic amino acid. Previous mutagenesis and modeling studies as well as data obtained from the crystallization of the

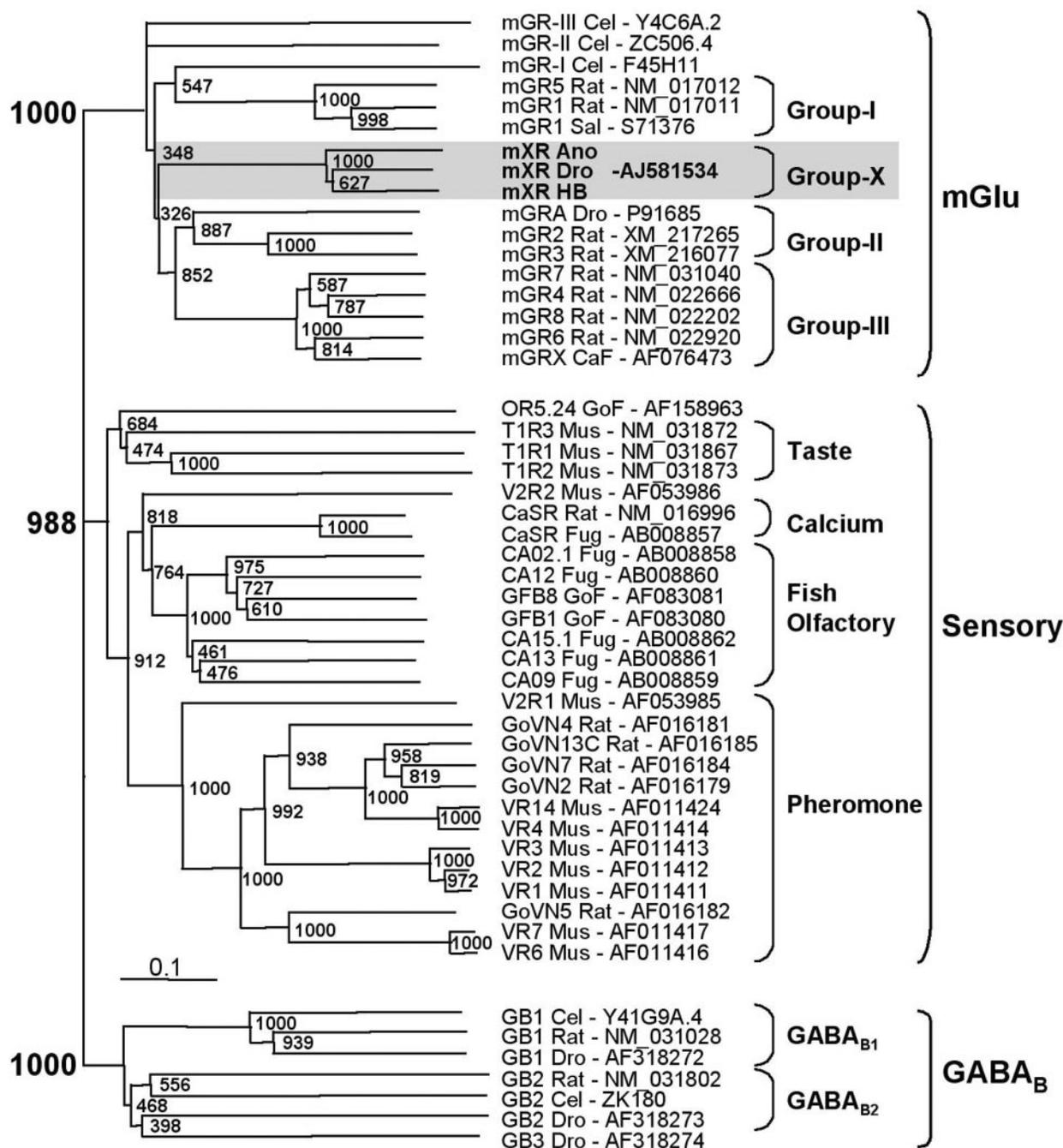


FIG. 2. **Phylogenetic relationship between the mXRs and other members of class III GPCRs.** The sequences of the indicated class III GPCRs (GenBank™ accession numbers indicated in the figure, except for the *C. elegans* sequences for which the name of the cosmid where the sequence was found is shown) from rat or mouse (*Mus*) (chosen as mammalian representatives), goldfish (*GoF*, *Carassius auratus*), catfish (*CaF*, *Ictalurus punctatus*), Fugu fish (*Fug*, *Takifugu rubripes*), salmon (*Sal*, *Oncorhynchus masou*), *C. elegans* (*Cel*), and *D. melanogaster* (*Dro*) were aligned and the tree calculated with positions with gaps excluded. Bootstrap values for each branch are indicated. The mXR group is highlighted in gray.

mGlu1R VFTM identified several key residues involved in the binding of glutamate (25, 26). All these are conserved among all mGluRs, including the *Drosophila* receptor DmGluAR (Fig. 1) and the *C. elegans* mGluR homologues (5), except mXRs. In mGlu1R, Ser-165 and Thr-188 on one hand and Asp-208, Tyr-236, and Asp-318 on the other hand are involved in the binding of the  $\alpha$ -carboxylic and  $\alpha$ -amino groups of glutamate, respectively. In addition Arg-78 and Lys-409 (in mGlu1R) are involved in the binding of the  $\gamma$ -carboxylic group of glutamate (Fig. 1 and Fig. 3). In mXRs, the residues that directly contact the  $\alpha$ -carboxylic (Ser-153 and Thr-176 in DmXR) and the  $\alpha$ -amino groups (Asp-196, Tyr-224, and Asp-308 in DmXR) of glutamate were all conserved (Fig. 1 and Fig. 3). However, the

residues interacting with the  $\gamma$ -carboxylic group of glutamate (Arg-78 and Lys-409 in mGlu1R) were not conserved in the mXRs. The homologous residues in mXRs were Ala (77 in DmXR) and Gln (401 in DmXR), respectively (Figs. 1 and 3). Previous mutagenesis experiments have shown that Arg-78 is required for a high affinity binding of glutamate to mGlu1R (27). Therefore, the replacement of an Arg residue by Ala in mXRs suggested that these receptors were not glutamate receptors.

*The Venus Flytrap Module of DmXR Can Adopt the Same Structure as the Venus Flytrap Module of mGlu1R*—In order to verify the hypothesis that these new receptors could not bind glutamate, we tested whether the VFTM of DmXR could lead to

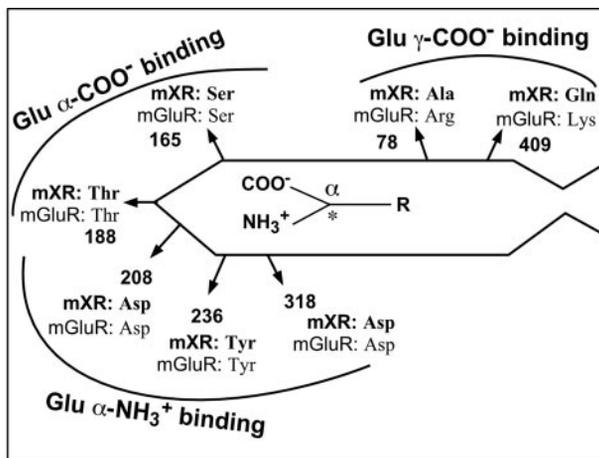


FIG. 3. Schematic drawing of an  $\alpha$ -amino acid surrounded by the LBP residues. Residues conserved in all known mGluRs are compared with the residues at equivalent positions in mXRs (in boldface).

a three-dimensional structure similar to the mGlu1R VFTM, with the residues involved in the binding of the  $\alpha$ -amino and  $\alpha$ -carboxylic groups of amino acids in a correct position. A three-dimensional model of the VFTM of DmXR was generated using the coordinates of the mGlu1R VFTM structure as template (1ewk:A) (26). Our model (Fig. 4A) presented Verify3D scores similar to those determined with the mGlu1R VFTM structure (Fig. 4B), indicating that the extracellular domain of DmXR very likely folds like that of mGlu1R. In this model, Ser-153 and Thr-176 (and Asp-196, Tyr-224, and Asp-308, not shown) are in such a position that they can bind the  $\alpha$ -amino acid function (Fig. 4D compared with mGlu1R in Fig. 4C). However, changes in the residues that lined the other side of the binding pocket (Arg to Ala and Lys to Gln) in DmXR prevented the binding of the  $\gamma$ -carboxylic group of glutamate (Fig. 4D compared with mGlu1R in Fig. 4C). Moreover, there was no other obvious residue in the LBP that could replace the role of Arg and Lys in the binding of the  $\gamma$ -carboxylic group of glutamate. This further suggested that glutamate could not bind in this binding site and that DmXR might be activated by another amino acid. We also noticed the presence of a phenylalanine (Phe-174) side chain inside the binding pocket of DmXR (Fig. 4D) replacing a Ser or an Ala for the mGluRs. This Phe was also found in the AmXR and in HBmXR LBPs.

**DmXR Is Not a Glutamate Receptor**—In order to test the hypothesis that DmXR might not be activated by glutamate, we expressed an N-terminal tagged version of this receptor (see “Experimental Procedures”) in HEK cells cotransfected with different G-protein  $\alpha$ -subunits. The DmXR transduction pathway was analyzed by testing its coupling to wild-type  $G_{\alpha_i}$  and to chimeric G-protein  $\alpha_{i/o}$ -subunits ( $G_{\alpha_{q19}}$  and  $G_{\alpha_{q05}}$ ). These chimeric G-proteins allow many  $G_{i/o}$ -coupled receptors negatively coupled to adenylate cyclase to activate phospholipase C (13). We did not get any stimulation of DmXR with 1–10 mM glutamate (Fig. 5A), although this receptor was properly addressed at the plasma membrane (see Fig. 7A). In contrast, glutamate elicited a 3-fold stimulation of the IP production in cells expressing DmGluAR and  $G_{\alpha_{q19}}$  (Fig. 5A). We then verified whether DmXR could couple to G-proteins. In mGluRs, the heptahelical and intracellular domain are involved in the coupling to G-proteins (28). A chimeric receptor composed of the extracellular domain of DmGluAR, which contains the glutamate VFTM, and the heptahelical and intracellular domain of DmXR was constructed (see “Experimental Procedures”). After application of 1 mM glutamate, this chimeric receptor induced a 150% IP stimulation above control when cotransfected with

$G_{\alpha_{q19}}$  (chimera in Fig. 5A) and  $G_{\alpha_{q05}}$  (not shown) but not with  $G_{\alpha_i}$  (not shown). These results indicated that the DmXR was coupled in HEK cells to  $G_{\alpha_i}$  and  $G_{\alpha_o}$  like group II and III mGluRs (7). This also indicated that the lack of glutamate stimulation obtained with DmXR was not due to the inability of this receptor to activate these mammalian G-proteins. We then examined whether other ionotropic and metabotropic glutamate receptors agonists ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, kainate, *N*-methyl-D-aspartate, quisqualate, and L-2-amino-4-phosphonobutyric acid) could activate DmXR cotransfected with  $G_{\alpha_{q19}}$ , but none of these compounds displayed any significant activity (not shown). Finally, because the  $\alpha$ -amino acid-binding motif was conserved in mXRs, the agonist activity of all other standard and some unusual amino acids (see legend of Fig. 5A) were also examined. None of these molecules induced any detectable activation of the receptor. We thus decided to probe the activity of the DmXR with tissue extracts that should contain the natural ligand of this receptor.

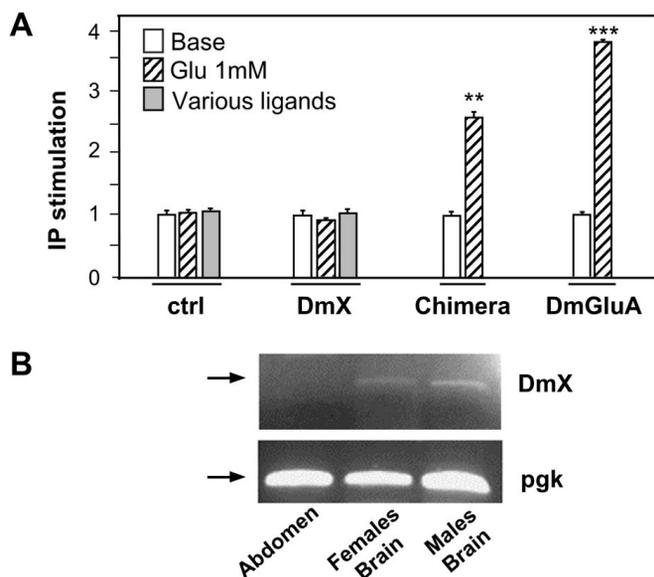
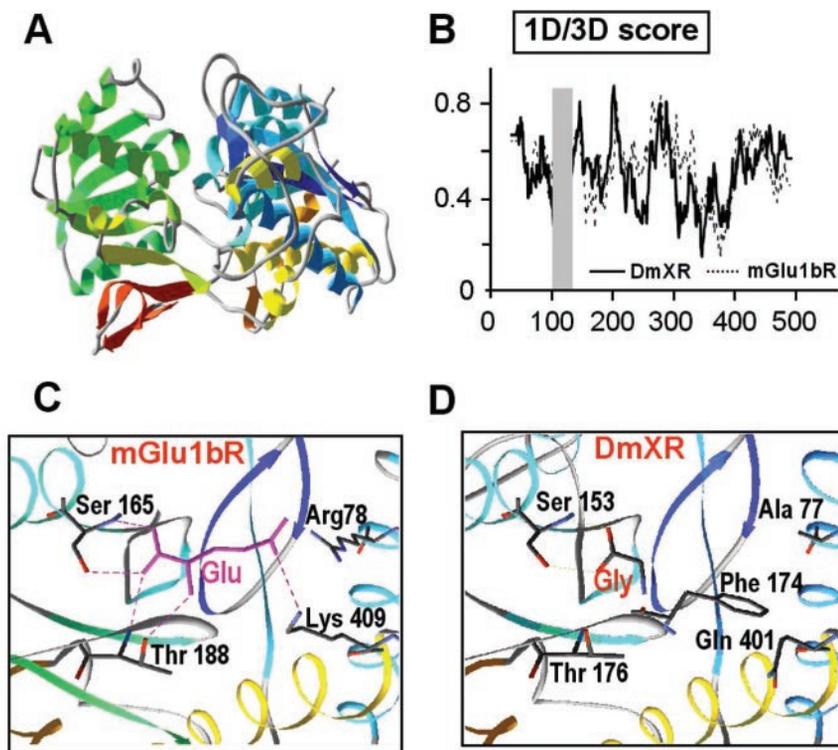
**DmXR Is Expressed in the Brain**—To establish which tissues would contain the endogenous ligand of DmXR, we studied the expression of the receptor, assuming that the ligand would be present in the tissue where the receptor is present. We performed RT-PCR experiments on brain or abdomen RNA extracts of adult flies and could amplify DmXR messenger RNA in female and male brain but not in the abdomen (Fig. 5B). These results suggested that the ligand of DmXR should also be found in the brain.

**A Drosophila Endogenous Compound with a Primary Amino Group Activates DmXR**—*Drosophila* head extracts enriched in small hydrophilic molecules were prepared after removal of proteins with 10% trichloroacetic acid and assayed on HEK cells coexpressing DmXR and  $G_{\alpha_{q19}}$ . As shown in Fig. 6A, 2 mg ( $1\times$ ) of fresh head extracts activated DmXR. Increasing the concentration of head extract leads to increased DmXR-triggered response (Fig. 6A). The same head extract also stimulated DmGluAR (Fig. 6A). This last result indicated that glutamate (and likely other amino acids) was indeed present in the extract.

To determine whether the active molecule present in the *Drosophila* head extract possessed a primary amino group, we treated the extract with formaldehyde which should mask this amino group (29) (Fig. 6B). As shown in Fig. 6C, the treated *Drosophila* head extract was unable to activate DmXR and DmGluAR. As expected, 1 mM glutamate treated with formaldehyde was also unable to activate DmGluAR. We verified that the effect of formaldehyde at the used concentration was not due to a toxic action on the HEK cells because the IP production of the control HEK cells in the presence of formaldehyde was not modified (Fig. 6C). However, our amino acid extraction protocol did not allow the removal of small peptides. We therefore wanted to determine whether the active molecule in the extract had a peptide bond. To answer this question, we hydrolyzed the extract with hydrochloric acid 6 N at 120 °C for 24 h, a procedure that should disrupt all peptide bonds. As control for this reaction, the nonapeptide arginine vasopressin (AVP), an agonist of the human vasopressin V1a receptor (14), was hydrolyzed in the same way. As shown in Fig. 6D, the hydrolyzed *Drosophila* head extract still activated both the DmXR and the DmGluAR. In contrast, the hydrolyzed AVP was unable to activate the V1a receptor, as opposed to the untreated AVP (Fig. 6D). This showed that the active molecule in the extract did not require any peptide bond to activate the receptor. Taken together, these results were in accordance with our hypothesis that the DmXR endogenous agonist might be an  $\alpha$ -amino acid-like molecule.

**The Endogenous Ligand Acts into the LBP**—Because the

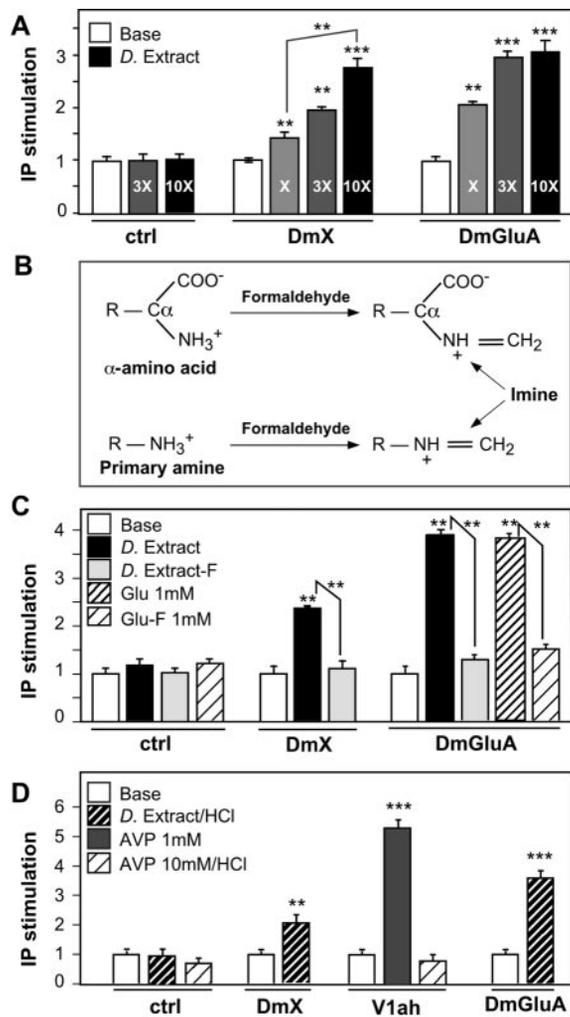
**FIG. 4. Three-dimensional model of DmXR VFTM.** *A*, ribbon view of the DmXR VFTM. *B*, verify three-dimensional plot generated using the DmXR and mGlu1R VFTM models. *Gray band* represents a region of mXR, which could not be modeled because the structure of the equivalent region in mGlu1R VFTM has not been solved yet. mGlu1R, *dotted line*; DmXR, *full line*. *C*, view of some of the mGlu1R LBP residues interacting with the  $\alpha$ -amino acid moiety of glutamate and the mGlu1R LBP residues interacting with the  $\gamma$ -amino acid moiety of glutamate. *D*, equivalent region as that shown in *C* for the DmXR LBP. Note the Phe-174 lateral side chain is lying inside the DmXR LBP.



**FIG. 5. Pharmacological and expression studies of DmXR.** *A*, agonist properties of glutamate or various small molecules on DmXR, DmGluAR/DmXR chimera, and control (*ctrl*) DmGluAR. IP production in cells expressing the indicated receptor under basal (*open bars*), glutamate (*hatched bars*), and various small molecules; each of the 19 remaining L-amino acids, D-glutamate, D-aspartate, D-serine, D-alanine, Gd3+ (1 mM), Ca<sup>2+</sup>, GABA, taurine, L-cysteinesulfonic acid, dipeptide *N*-acetylaspartylglutamate, carnosine, each at a 1 mM concentration, are indicated by *shaded bars*. IP stimulation is calculated relatively to IP production in basal conditions. The effect of drugs was compared with basal activity using a two-tailed Student's *t* test. The statistically significant effects were always observed in three independent experiments at least. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Data are means  $\pm$  S.E. from triplicate determinations from typical experiments. *B*, RT-PCR analysis of DmXR expression in adult abdomen, female brain, and male brain. DmXR RT-PCR were performed with primers that allow us to distinguish authentic cDNA (380 bp) from genomic DNA (1100 bp) by product size. RT-PCR products were sequenced to verify the amplification of DmXR cDNA. In all cases the integrity of the RNA preparation was ascertained by a control RT-PCR with primers specific for the *Drosophila* phosphoglycerate kinase (*pgk*) that amplify a coding region of 430 bp.

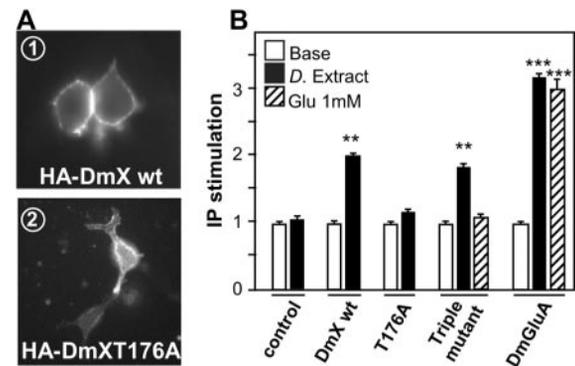
majority of the residues involved in the binding of glutamate in the mGluRs were conserved in DmXR and were in a correct position to interact with the ligand according to our three-dimensional model, we asked whether this conserved part of the LBP was also involved in the binding of the DmXR ligand. To this aim we constructed a mutant receptor containing an alanine substitution of Thr-176, the DmXR homologue of the crucial residue Thr-188 (in mGlu1R). The mutation of this residue is known to completely inactivate the glutamate-induced response of mGlu1R (30) and other mGluRs (31, 32). The DmXR mutant was well expressed in HEK cells and was addressed at the plasma membrane of these cells (Fig. 7A). As shown in Fig. 7B, the mutated receptor was no longer stimulated by the extract, indicating that the Thr residue was also essential for the activation of DmXR by the endogenous compound in the extract. We then tested the role of the new residues Ala (77 in DmXR) and Gln (401 in DmXR) found in the mXRs LBP as well as the role of the phenylalanine side chain that was found inside the ligand pocket according to the three-dimensional model of the DmXR LBP (Phe-174). These three residues were mutated to Arg, Lys, and Ala, respectively. This triple mutant could still be activated by the *Drosophila* head extracts (Fig. 7B), indicating that these residues played no major role in the binding of the endogenous ligand. This is also consistent with these three residues not being important for the correct folding of the DmXR, in agreement with our three-dimensional model. Although the LBP of the triple DmXR mutant contained all key residues directly contacting glutamate in all other mGluRs, glutamate was still unable to stimulate this receptor (Fig. 7B). This suggests that more general changes than the substitution of two residues had occurred in the structure of the DmXR LBP.

**DmXR Is Activated by Other Insect Extracts**—Because mXR-like sequences were not found in *C. elegans* nor in the mouse and human genome sequences, we examined the effect of extracts from *C. elegans* or mouse brain on DmXR. Transfected HEK cells did not respond to the addition of these extracts (Fig. 8), whereas the positive control DmGluAR was already fully activated with 5 times less concentrated ex-

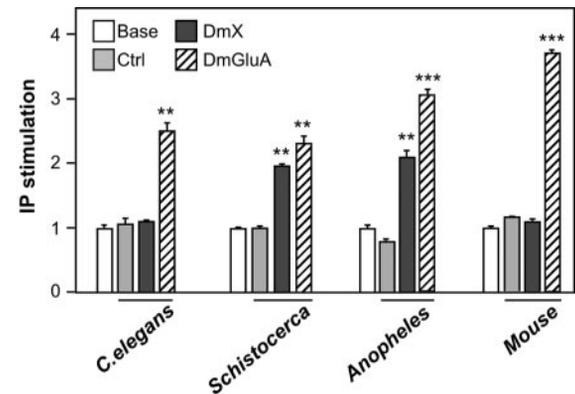


**FIG. 6. Activation responses of the DmX receptor.** *A*, effect of increasing concentration (basal, X, 3X, and 10X) of *Drosophila* head extracts on IP stimulation in HEK cells transfected with  $G\alpha_{q19}$  without receptor (*ctrl*),  $G\alpha_{q19}$  and DmXR (*DmX*), and  $G\alpha_{q19}$  and DmGluAR (*DmGluA*). Note that the extract contained a concentration of glutamate largely sufficient to activate DmGluAR and consequently that glutamate is not acting as an antagonist on the DmXR. *B*, schematic representation of formaldehyde action on an  $\alpha$ -amino acid and on a primary amino molecule. *C*, effect of *Drosophila* head extract or glutamate treated with formaldehyde (10 mM) on IP stimulation in HEK cells transfected with  $G\alpha_{q19}$  without receptor (*ctrl*),  $G\alpha_{q19}$  and DmXR (*DmX*), and  $G\alpha_{q19}$  and DmGluAR (*DmGluA*). Basal (open bars), 3 $\times$  head extract (solid bars), 3 $\times$  head extract treated with formaldehyde (shaded bars), 1 mM glutamate (hatched bars), 1 mM glutamate treated with formaldehyde (widely hatched bars). *D*, effect of *Drosophila* head extracts (at a 5 $\times$  concentration) and control nonapeptide AVP hydrolyzed by 6 N HCl at 120 °C for 24 h on IP accumulation in HEK cells transfected with  $G\alpha_{q19}$  without receptor (*ctrl*),  $G\alpha_{q19}$  and DmXR (*DmX*), human vasopressin V1a receptor (*V1ah*), and  $G\alpha_{q19}$  and DmGluAR (*DmGluA*). Basal (open bars), hydrolyzed head extracts (hatched bars), hydrolyzed AVP10 (widely hatched bars). 1, 3, 5, and 10 $\times$  = extract from 2, 6, 10, and 20 mg of fresh tissues, respectively. Effect of drugs was compared with basal activity using a two-tailed Student's *t* test, unless stated otherwise (*t* test between two drug concentrations and between treated and non-treated extracts). The statistically significant effects were always observed in three independent experiments at least. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Data are means  $\pm$  S.E. of triplicate determinations from typical experiments.

tracts (Fig. 8). These results suggested that the DmXR ligand was either not present or present at a very low concentrations in these extracts. However, extracts from two other insects, *S. gregaria* brain and *Anopheles*, were also able to induce a clear response (Fig. 8).



**FIG. 7. Targeting and site-directed mutagenesis of DmXR.** *A*, expression and surface targeting of the T176A DmX mutant (panel 1) and the wild-type (panel 2) receptor. Cells expressing the mutant and the wild-type receptors epitope-tagged at their N-terminal extracellular end were labeled with the HA antibody. The cells were not permeabilized in order to detect only the surface receptors. *B*, IP stimulation after incubation of *Drosophila* head extract (2 $\times$ ) in HEK cells expressing  $G\alpha_{q19}$  without receptor (*ctrl*),  $G\alpha_{q19}$  and wild-type DmXR (*DmX wt*),  $G\alpha_{q19}$  and T176A DmXR mutant (*T176A*),  $G\alpha_{q19}$  and A77R,Q401K, F174A DmX triple mutant receptor (*Triple mutant*),  $G\alpha_{q19}$  and DmGluAR (*DmGluA*). Basal (open bars), *Drosophila* head extract (solid bars), and glutamate (hatched bars). Effect of drugs was compared with basal activity using a two-tailed Student's *t* test. The statistically significant effects were always observed in three independent experiments at least. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Data are means  $\pm$  S.E. of triplicate determinations from typical experiments.



**FIG. 8. Effect of different species extracts on DmXR.** Detection of IP stimulation of HEK cells transfected with  $G\alpha_{q19}$  (shaded bars),  $G\alpha_{q19}$  and DmXR (solid bars), and  $G\alpha_{q19}$  and DmGluAR (hatched bars). Control (*Ctrl*) cells transfected with  $G\alpha_{q19}$  without extract (open bars). Results obtained with extracts from 10 mg of fresh tissue (5 $\times$ ) are shown. *C. elegans*, whole organism of *C. elegans*; *Schistocerca*, dissected brain of the locust *S. gregaria*; *Anopheles*, whole mosquitoes *A. gambiae*; *Mouse*, dissected brain of adult female mouse *M. musculus*. Effect of drugs was compared with basal activity using a two-tailed Student *t* test. The statistically significant effects were always observed in three independent experiments at least. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Data are means  $\pm$  S.E. of triplicate determinations from typical experiments.

## DISCUSSION

Our data show that a strong divergence in the LBP and in the endogenous ligand of the mGluRs can occur during evolution, leading to a new group of mGluR-like protein called mXR. The new LBP has evolved so divergently that the receptor lost its ability to be activated by glutamate. Furthermore, our results indicate that the mXRs are activated by a new natural ligand, not identified yet. Indeed a large range of amino acids, GABA, and calcium that would activate other class III receptors were inactive on DmXR. It appears that the structural changes in the new LBP have occurred mostly in the  $\gamma$ -carboxylic binding part of the pocket, whereas the  $\alpha$ -amino acid binding part was conserved. This suggests that the mXRs natural ligand might be an amino acid-like molecule. Our DmXR triple

mutagenesis shows that the mXR new residues found instead of the glutamate binding consensus are not critical for the activation on the one hand, whereas on the other hand the triple mutant with the rebuilt glutamate binding consensus is still not activated by glutamate. These results suggest that the chemical environment of the  $\gamma$ -carboxylic binding part of the pocket has been strongly modified during divergent evolution.

The availability of the whole genome sequences enables exhaustive comparisons of a protein family between different model organisms. There are three mGlu-like receptors in the *C. elegans* genome, and the comparisons of the LBP sequences of these evolutionary distant receptors show that each *C. elegans* receptor can be assigned to an mGluR group (5). This clearly indicates that a receptor for each group existed in the common ancestor of the nematodes and the vertebrates. We found only two mGluR homologues in insects like *Anopheles*<sup>2</sup> and *Drosophila*, mGluAR and mXR. Two evolutionary scenarios can be hypothesized to explain this situation. In the first scenario, the mXR has diverged from a group III receptor that is also coupled to  $G\alpha_{\nu}$  proteins. The group I receptor, which is coupled to  $G\alpha_q$  protein, would have disappeared either before or after this divergence. In the second scenario, the group II receptor gene would have been duplicated so that one of the two genes could have evolved divergently, and the group I and group III receptors would have disappeared either before or after these duplication and divergence events. Phylogenetic analysis indicated that the mXR belonged to the branch leading to the group II and III receptors. However, we could not assign the mXR to either group II or III receptors with a sufficiently good bootstrap score. Thus, to date we are still unable to choose between one of the two scenarios presented.

This new metabotropic non-glutamate receptor was not found in *C. elegans* nor in mammalian genomic sequences. Furthermore, only extracts from insects have been able to stimulate DmXR. Taken together, these observations suggest that the mXR would be specific to insects and the cognate mX ligand would also be specific to insects. We show that DmXR is expressed in the adult brain of *Drosophila*. Whether the function of mXR in the insect central nervous system is completely new or whether it takes the place of glutamatergic neurotransmission remains to be determined.

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