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# Expression of Functional G Protein-Coupled Receptors in Photoreceptors of Transgenic *Xenopus laevis*<sup>†</sup>

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**ABSTRACT:** G protein-coupled receptors (GPCRs) constitute the largest superfamily of transmembrane signaling proteins; however, the only known GPCR crystal structure is that of rhodopsin. This disparity reflects the difficulty in generating purified GPCR samples of sufficient quantity and quality. Rhodopsin, the light receptor of retinal rod neurons, is produced in large amounts of homogeneous quality in the vertebrate retina. We used transgenic *Xenopus laevis* to convert these retina rod cells into bioreactors to successfully produce 20 model GPCRs. The receptors accumulated in rod outer segments and were homogeneously glycosylated. Ligand and [<sup>35</sup>S]GTPγS binding assays of the 5HT<sub>1A</sub> and EDG<sub>1</sub> GPCRs confirmed that they were properly folded and functional. 5HT<sub>1A</sub>R was highly purified by taking advantage of the rhodopsin C-terminal immunoaffinity tag common to all GPCR constructs. We have also developed an automated system that can generate hundreds of transgenic tadpoles per day. This expression approach could be extended to other animal model systems and become a general method for the production of large numbers of GPCRs and other membrane proteins for pharmacological and structural studies.

Structural and biophysical studies require a large amount of protein in a homogeneous form. However, GPCRs<sup>1</sup> are typically expressed at very low levels in natural sources, with the exception of rhodopsin (1). The heterologous expression systems commonly used to purify GPCRs such as bacteria (2), yeast (3), insect cells (4), and transformed mammalian cells often lead to heterogeneous receptors, due to heterogeneous glycosylation, misfolding, etc., which hampers crystallographic approaches.

The expression of rhodopsin in retina is driven by a strong rod photoreceptor-specific promoter (5) to compensate for the daily shedding of the rod outer segments (ROS) (Figure 1A). In human retina, nearly 10<sup>8</sup> opsin molecules are produced every day (1). Rhodopsin molecules in ROS are

uniquely homogeneous (98% chemically homogeneous) compared to GPCRs expressed elsewhere, having the exact glycan always covalently bound to two residues (1). The same biochemical machinery is also capable of folding several other GPCRs expressed in rod neurons, including 5HT<sub>2A</sub>R (6), cannabinoid CB<sub>1</sub> receptor (7), dopamine D<sub>2</sub> receptor (8), and metabotropic glutamate receptor 8 (mGluR<sub>8</sub>) (9). In fact, a mGluR was heterologously expressed in high yield in the *Drosophila* photoreceptors (10–12). Recombinant rhodopsin and other GPCRs purified from either mammalian or baculovirus/insect cells exhibit heterogeneity of N-glycosylation compared with rhodopsin isolated from bovine rods (13, 14). However, removing the glycosylation sites affects the stabilization of membrane proteins such as GPCRs (15, 16).

The eight C-terminal amino acids of rhodopsin are necessary for vectorial transport of rhodopsin to ROS (17). When green fluorescent protein (GFP) was attached to the rhodopsin C-terminus, it was transported to ROS of *Xenopus* (18) and zebrafish (19). Moreover, rhodopsin–GFP fusions with the rhodopsin C-terminus attached were transported to ROS in *Xenopus* (20) and mouse (21). Here, we developed an expression system for human serotonin receptors (5HTRs) and endothelial differentiation gene receptors (EDGRs) that utilizes the specific biosynthetic machinery of *Xenopus laevis* rods, and employs rhodopsin's C-terminal sequence for dual functions: ROS targeting and purification by affinity chromatography. Recombinant 5HT<sub>1A</sub> and EDG<sub>1</sub> receptors retained their abilities to bind to their specific ligands and/or properly couple to G proteins. We also developed an automated injection system for producing large numbers of transgenic *Xenopus* tadpoles expressing these recombinant GPCRs in their ROS, which could generate receptor samples

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<sup>2</sup> Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDGR, endothelial differentiation gene receptor; EGFP, enhanced green fluorescent protein; GPCR, G protein-coupled receptor; 5HTR, serotonin receptor; MPPF, 4-(2'-methoxy)phenyl-1-[2'-(n-2''-pyridinyl)-10-p-fluorobenzamido]ethyl]piperazine; ROS, rod outer segments; WAY 100635, N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide.

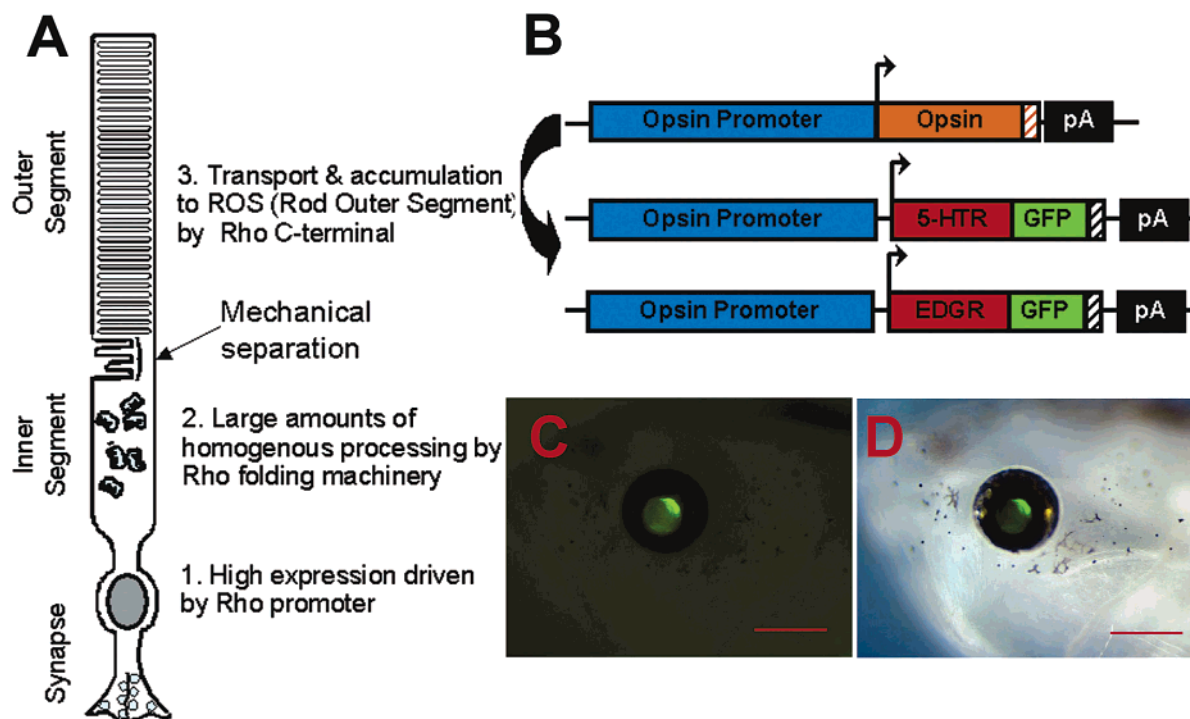


FIGURE 1: Expression of EGFP-tagged human 5HTRs or EDGRs in transgenic tadpoles. (A) Morphology and characteristics of a highly differentiated vertebrate rod photoreceptor cell. (B) GPCR-EGFP fusion constructs expressed in the rods of *X. laevis*. The opsin promoter is derived from *X. laevis* (38, 39), and the hatched box represents the mouse RHO<sub>15</sub> epitope. (C) Fluorescence field image of the eye of a 5HT<sub>1A</sub>R-GFP-RHO<sub>15</sub> transgenic tadpole. (D) Combination of a bright field image and a fluorescence field image of the same eye shown in panel C. Scale bars represent 0.5 mm.

for crystallization trials in low-volume systems. This expression method could also be applied to other animal model systems with similar rhodopsin production machinery. Thus, it is potentially applicable to all members of the GPCR superfamily, and may constitute a solution to the current bottleneck for structural studies of GPCRs.

## EXPERIMENTAL PROCEDURES

**cDNA Clones of 5HTRs and EDGRs.** The coding sequences corresponded with their GenBank accession numbers: 5HT receptors, 1A (M83181), 1B (M81590), 1D (M89955), 1E (M91467), 1F (AF498981), 2A (X57830), 2B (X77307), 2C (M81778), 4B (Y10437), 5A (AF498985), 6 (L41147), and 7A (L21195); and EDG receptors, 1 (M31210), 2 (U78192), 3 (X83864), 4 (AF233092), 5 (AF034780), 6 (AJ000479), 7 (AF127138), and 8 (AF317676). The most predominant isoform was chosen for 5HT<sub>4</sub>R and 5HT<sub>7</sub>R.

***X. laevis* Expression Constructs.** The pXOP-5HTR (or EDGR)-EGFP-RHO<sub>15</sub> transgene plasmids were constructed as follows. The pXOP-C1-EGFP vector (a gift from B. Knox, State University of New York Upstate Medical University, Syracuse, NY) was cut by AgeI and AccIII to remove the EGFP sequence, and religated to generate the pXOP-C1(-) plasmid. A DNA fragment encoding the last 15 amino acids of mouse rhodopsin (RHO<sub>15</sub>) and EGFP were inserted into pXOP-C1(-) to produce pXOP-N1-EGFP-RHO<sub>15</sub>. The cDNAs encoding 5HTRs and EDGRs were amplified from pCRII-5HTRs and pCRII-EDGRs, respectively, and the Kozak sequence was added before the start codon. The amplified products were inserted into the *srf*I site of pXOP-N1-EGFP-RHO<sub>15</sub> to generate pXOP-5HTR (or EDGR)-EGFP-RHO<sub>15</sub>.

**Transgenesis.** Transgenic *X. laevis* embryos were generated by intracytoplasmic sperm injection (ICSI) as described previously (22), with minor modifications (23). The transgene fragments were released by restriction digestion, and purified from an agarose gel. Sperm nuclei were permeabilized using digitonin. Properly gastrulating embryos were raised in 0.1× MMR (Marc's Modified Ringer's, 100 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES) until they were collected. Tadpoles were anesthetized in 0.01% 3-aminobenzoic acid ethyl ester (Sigma-Aldrich, St. Louis, MO) and monitored for EGFP expression using a Leica MZFL III fluorescence stereoscope. Developmental stages of embryos were determined according to the description of Nieuwkoop and Faber (24).

**Immunostaining and Microscopy of Transgenic *X. laevis* Eyes.** Transgenic tadpoles were fixed in freshly prepared fixative [2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3)] for 3 h at 4 °C, washed in 5, 10, 15, and 20% SPB (sucrose phosphate buffer), equilibrated in 20% SPB/OCT (2:1) at 4 °C overnight, and embedded in 20% SPB/OCT (2:1). Embedded tadpoles were cryosectioned through the eye. Immunostaining of rhodopsin was performed as previously described (25), using B6-30N (1:400) (26) as the primary antibody and Cy5-conjugated goat anti-mouse IgG (1:200) (Jackson ImmunoResearch Laboratories, West Grove, PA) as the secondary antibody. The sections were counterstained with 1 μg/mL Hoechst 33342 (Sigma). Fluorescence was visualized under a Nikon fluorescence microscope.

**Crude ROS Purification.** Transgenic tadpole eyes (200–400) were homogenized in 38% sucrose in 1× MMR and centrifuged at 3000g and 4 °C for 15 min. The supernatant

was collected and diluted with 7 volumes of  $1 \times$  MMR, and centrifuged at 20000g for 30 min to recover the pellets. The protein concentration was measured with the Lowry method.

**Solubilization and Purification of the 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> Fusion Protein.** For receptor purification, 600 eyes from 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein-expressing tadpoles were homogenized in 5 mM BTP (pH 7.0), containing protease inhibitors and DNase. The membranes were recovered by centrifugation, washed with  $10 \times$  TBS (Tris-buffered saline), and solubilized by adding 1.75 mL of 20 mM *n*-dodecyl  $\beta$ -maltoside in TBS with rocking at 4 °C for 30 min. Insoluble material was removed by centrifugation, and two 50  $\mu$ L aliquots of the extract were taken. One of the aliquots was incubated with glycopeptidase F (PNGase F, Sigma) overnight at 16 °C. Immunoblotting with different antibodies was performed to confirm the identity of the receptor. Immobilized 1D4 antibody on Sepharose gel (100  $\mu$ L) was added to the detergent-solubilized 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein and rocked for 30 min at 4 °C, and then loaded onto a column and washed with 10 mL of washing buffer [10 mM MES (pH 6.0), 10 mM *n*-dodecyl  $\beta$ -maltoside, and 1 M NaCl]. The elution was carried out by adding a competing nonapeptide to the washing buffer and collecting  $5 \times 100 \mu$ L fractions. Fractions were immediately neutralized with 1 M Tris (pH 7.5) and analyzed by electrophoresis and silver staining.

**Purification of the 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> Fusion Protein for the Radioligand Binding Assay.** To purify receptors for binding assays, ~2600 eyes from 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein-expressing tadpoles and 18 eyes from 1-year-old 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein-expressing frogs were homogenized in 5 mM BTP (pH 7.5), containing protease inhibitors and DNase. The membranes were recovered by centrifugation, washed with 100 mM BTP (pH 7.5), and solubilized by adding 7.2 mL of solubilization buffer [1 mM *n*-dodecyl  $\beta$ -maltoside, 100 mM BTP (pH 7.5), protease inhibitors, and DNase] and rocking at 4 °C for 30 min. Insoluble material was removed by centrifugation. Sepharose gel with immobilized 1D4 antibody (300  $\mu$ L) was added to the detergent-solubilized 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein; the mixture was rocked for 60 min at 4 °C, and then the Sepharose gel was washed six times with 1 mL of washing buffer [1 mM CHAPS and 100 mM BTP (pH 7.5)]. The Sepharose gel with the purified 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein was then directly used for binding assays. The 5HT<sub>1A</sub>R-RHO<sub>15</sub> protein expressed in HEK293T cells was purified with the same methods as the control.

**Radioligand Binding Assay.** Crude tadpole ROS expressing the 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein, CHO cells (Amersham Biosciences, Piscataway, NJ) expressing 5HT<sub>1A</sub>R, or immobilized 1D4 antibody on Sepharose gel with purified 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein was resuspended in binding buffer [50 mM Tris-HCl (pH 7.4), 2 mM MgCl<sub>2</sub>, 1 mM EDTA, and protease inhibitor cocktail]. [methyl-<sup>3</sup>H]MPPF (PerkinElmer Life Science, Boston, MA) (27) or [methoxy-<sup>3</sup>H]WAY 100635 (Amersham Biosciences) (28) was used as a specific radioligand. The membranes (20  $\mu$ g/well) were incubated with 0.05% saponin in a final volume of 50  $\mu$ L at room temperature for 10 min, and then incubated with 50  $\mu$ L of [methyl-<sup>3</sup>H]MPPF (0–50 nM) for 90 min. Nonspecific binding was assessed in the presence of 10  $\mu$ M serotonin. Sepharose gel with bound receptors (10  $\mu$ L/well) was

incubated with 50  $\mu$ L of [methoxy-<sup>3</sup>H]WAY 100635 (0–10 nM) for 90 min. Nonspecific binding was assessed in the presence of 10  $\mu$ M spiroxatrine. Assays were terminated by rapid filtration through a GF/C filter plate and four rinses with washing buffer [20 mM Tris-HCl (pH 7.4)]. The radioactivity was measured by a TopCounter (PerkinElmer). Assays were performed in duplicate. Saturation experiments were analyzed by nonlinear regression curves using Prism (GraphPad). Values of the apparent equilibrium dissociation constant ( $K_d$ ) and the maximal number of binding sites ( $B_{max}$ ) for radioligands were derived from the calculated curves.

**[<sup>35</sup>S]GTP $\gamma$ S Binding Assay.** The 5HT<sub>1A</sub>R or EDG<sub>1</sub>R cDNAs were cloned into expression vector pcDNA4TO (Invitrogen), with a T7 tag (corresponding to the N-terminus of the major capsid protein from the T7 bacteriophage) and the RHO<sub>15</sub> tag attached. CHO cells were transfected with the 5HT<sub>1A</sub>R (or EDG<sub>1</sub>R)–T7–RHO<sub>15</sub> expression construct by using Lipofectamine 2000 reagents (Invitrogen). Transgenic tadpoles or CHO cells expressing 5HT<sub>1A</sub>R or EDG<sub>1</sub>R were homogenized in binding buffer [20 mM HEPES (pH 7.4), 10 mM MgCl<sub>2</sub>, 1 mM EDTA, and 100 mM NaCl]. To reduce the level of binding of [<sup>35</sup>S]GTP $\gamma$ S to the rhodopsin-activated G protein (transducin) in the retina, the tadpole membrane was preincubated with 10 mM hydroxylamine in binding buffer at room temperature for 60 min. The membrane (25  $\mu$ g/well) was incubated with serotonin, spiperone, or sphingosine 1-phosphate (S1P) for 30 min in a total volume of 150  $\mu$ L of binding buffer (10  $\mu$ M GDP, 0.5% BSA, and 1 mM DTT) before 50  $\mu$ L of 800 pM [<sup>35</sup>S]GTP $\gamma$ S was added to each well to make a final concentration of 200 pM. The binding assays were carried out at room temperature for 60 min under gentle shaking. The final concentration of hydroxylamine was lower than 1 mM. Nonspecific binding was assessed in the presence of 10  $\mu$ M unlabeled GTP $\gamma$ S. Assays were terminated by centrifugation at 4000g for 15 min and the removal of supernatant. The radioactivity was measured by a TopCounter (PerkinElmer). Assays were performed in triplicate.

## RESULTS

In our studies, we have chosen two subfamilies of GPCRs: 5HTRs and EDGRs. With at least 14 distinct members grouped into seven subfamilies, 5HTRs represent one of the most complex families of neurotransmitter receptors (29). The eight EDGRs are grouped into two subclasses based on their endogenous ligands, sphingosine 1-phosphate (S1P) receptors and lysophosphatidic acid (LPA) receptors, each of which is composed of closely related receptor subtypes (30).

**Expression of 5HTRs and EDGRs in ROS of Transgenic *Xenopus* Tadpoles.** To assess the feasibility of our approach to expression of GPCRs in ROS, we expressed the 12 human 5HTRs (5HT<sub>1A</sub>, 5HT<sub>1B</sub>, 5HT<sub>1D</sub>, 5HT<sub>1E</sub>, 5HT<sub>1F</sub>, 5HT<sub>2A</sub>, 5HT<sub>2B</sub>, 5HT<sub>2C</sub>, 5HT<sub>4B</sub>, 5HT<sub>5A</sub>, 5HT<sub>6</sub>, and 5HT<sub>7A</sub>) and the eight human EDGRs (EDG<sub>1–8</sub>) in transgenic *X. laevis*. The transgene construct for each tested GPCR was composed of five fragments: a previously characterized *Xenopus* opsin promoter containing a 1.3 kb sequence upstream of the start codon (20), a 5HTR or EDGR coding sequence, enhanced green fluorescent protein (EGFP) fused for easy visualization of transgenic tadpoles, the 15-residue C-terminus from mouse



Table 1: Summary of Transgenic Efficiency and Transgene Expression Level in the *X. laevis* Tadpoles Injected with XOP-GPCRs-EGFP-RHO<sub>15</sub> Transgenes

<i>Xenopus</i> transgene	transgenic efficiency (%) <sup>a</sup>	relative intensity (%) <sup>b</sup>
<i>XOP-5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub></i>	42.3 (885/2092)	81
<i>XOP-5HT<sub>1B</sub>R-EGFP-RHO<sub>15</sub></i>	13.1 (14/107)	69
<i>XOP-5HT<sub>1D</sub>R-EGFP-RHO<sub>15</sub></i>	17.0 (33/194)	62
<i>XOP-5HT<sub>1E</sub>R-EGFP-RHO<sub>15</sub></i>	29.5 (69/234)	73
<i>XOP-5HT<sub>1F</sub>R-EGFP-RHO<sub>15</sub></i>	20.1 (39/194)	74
<i>XOP-5HT<sub>2A</sub>R-EGFP-RHO<sub>15</sub></i>	11.5 (25/217)	55
<i>XOP-5HT<sub>2B</sub>R-EGFP-RHO<sub>15</sub></i>	5.6 (22/396)	44
<i>XOP-5HT<sub>2C</sub>R-EGFP-RHO<sub>15</sub></i>	7.9 (16/203)	46
<i>XOP-5HT<sub>4B</sub>R-EGFP-RHO<sub>15</sub></i>	53.6 (105/196)	80
<i>XOP-5HT<sub>5A</sub>R-EGFP-RHO<sub>15</sub></i>	36.0 (58/161)	87
<i>XOP-5HT<sub>6R</sub>-EGFP-RHO<sub>15</sub></i>	31.5 (51/162)	59
<i>XOP-5HT<sub>7A</sub>R-EGFP-RHO<sub>15</sub></i>	56.8 (46/81)	83
<i>XOP-EDG<sub>1R</sub>-EGFP-RHO<sub>15</sub></i>	47.6 (39/82)	82
<i>XOP-EDG<sub>2R</sub>-EGFP-RHO<sub>15</sub></i>	39.0 (39/100)	84
<i>XOP-EDG<sub>3R</sub>-EGFP-RHO<sub>15</sub></i>	34.6 (27/78)	64
<i>XOP-EDG<sub>4R</sub>-EGFP-RHO<sub>15</sub></i>	26.0 (13/50)	65
<i>XOP-EDG<sub>5R</sub>-EGFP-RHO<sub>15</sub></i>	26.3 (25/95)	64
<i>XOP-EDG<sub>6R</sub>-EGFP-RHO<sub>15</sub></i>	25.9 (110/425)	100
<i>XOP-EDG<sub>7R</sub>-EGFP-RHO<sub>15</sub></i>	43.5 (64/147)	68
<i>XOP-EDG<sub>8R</sub>-EGFP-RHO<sub>15</sub></i>	12.4 (31/251)	41

<sup>a</sup> Average percentage of GPCR-EGFP-RHO<sub>15</sub> protein-expressing tadpoles (the first number in parentheses) among the surviving tadpoles (the second number) after stage 42. <sup>b</sup> The intensity of EGFP in the retina of transgenic tadpoles was arbitrarily classified into four levels, with the strongest expression observed as 4. A weighed average was calculated. The relative intensity was determined by comparing to the highest average intensity of *EDG<sub>6R</sub>-EGFP-RHO<sub>15</sub>*.

rhodopsin (RHO<sub>15</sub>) containing the ROS targeting signal and the epitope for the 1D4 antibody (31), and the polyadenylation site (Figure 1B).

We observed green fluorescence in the *Xenopus* tadpole eyes for all 12 5HTRs and eight EDGRs, suggesting successful expression of these GPCR-EGFP-RHO<sub>15</sub> fusion proteins (Figure 1C,D). Different levels of fluorescence intensity and transgenic efficiencies were observed among these receptors (Table 1). The 5HT<sub>1A</sub>R, which had one of the highest transgenic efficiencies and fluorescence intensities, was selected for further characterization (below). The 5HT<sub>2A</sub>, 5HT<sub>2B</sub>, 5HT<sub>2C</sub>, and EDG<sub>8</sub> subtypes had the lowest levels of expression and low transgenic efficiency (Table 1).

**Fluorescence and Immunocytochemical Evidence of GPCR Expression in Rods.** To confirm the restricted expression in rods and the extent of ROS targeting for these GPCR-EGFP-RHO<sub>15</sub> proteins, transgenic tadpoles were collected at developmental stage 48 or later, when their retinas had already differentiated (24), cryosectioned, and counterstained with Hoechst dye, which labels cell nuclei to identify the nuclear layers in the retina. A consistent expression of the EGFP signal was detected across the entire retina (Figure 2A). In most cases, we found that the expression of these GPCR-EGFP-RHO<sub>15</sub> fusion proteins was clearly restricted to ROS, without any discernible expression in the rest of the rods (Figure 2B). This means that the opsin promoter selectively drives expression of these GPCR-EGFP-RHO<sub>15</sub> constructs in rods, and that the RHO<sub>15</sub> targeting signal from mouse rhodopsin effectively and selectively targets receptors to the ROS. Some of the tested GPCRs displayed relatively weak expression, and 5HT<sub>2B</sub>R is shown as an example (Figure 2C). While the expression level of the fusion protein

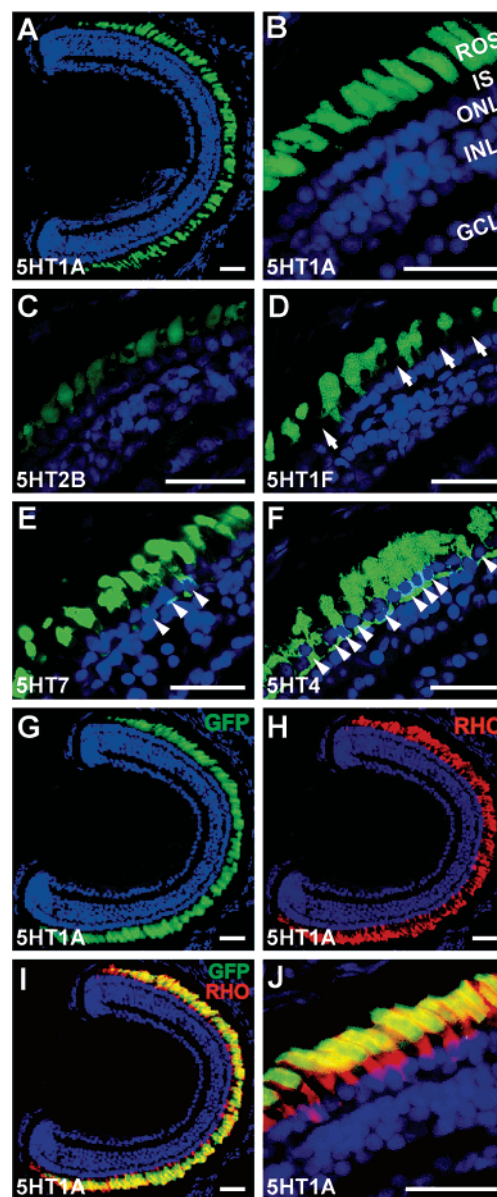


FIGURE 2: Expression of EGFP-RHO<sub>15</sub>-tagged human 5HTRs in the rods of transgenic *X. laevis*. The expression of EGFP-RHO<sub>15</sub>-tagged human 5HTRs (green) was imaged directly by fluorescence microscopy on the cryosections of transgenic tadpole eyes. Each section was counterstained with Hoechst to reveal nuclei (blue). Panels A, B, and G–J show expression of 5HT<sub>1A</sub>R, and panel C shows expression of 5HT<sub>2B</sub>R, panel D 5HT<sub>1F</sub>R, panel E 5HT<sub>7A</sub>R, and panel F 5HT<sub>4B</sub>R. (A and B) Typical expression of EGFP-RHO<sub>15</sub>-tagged human 5HT<sub>1A</sub>R in transgenic tadpole ROS. (C) Occasionally, lower expression levels were found with some of the 5HTRs, as shown here with 5HT<sub>2B</sub>R. (D) Mosaic expression was detected with some of the 5HTRs, represented here by 5HT<sub>1F</sub>R. Arrows indicate photoreceptor cells with very low levels of transgene expression. (E and F) Transgenic tadpoles expressing some of the 5HTRs in inner segments and cell body at different levels. Arrowheads indicate the photoreceptors with the ectopic expression. (G–J) Localization of the 5HTR-EGFP-RHO<sub>15</sub> protein in the ROS of transgenic tadpoles was confirmed by immunocytochemistry. (G) Eye section of a transgenic tadpole showing the expression of 5HT<sub>1A</sub>R (green). (H) The same section shown in panel G was immunostained with anti-rhodopsin antibody B6-30N (red). (I) Overlapped images of panels G and H, demonstrating the expression of the 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein in ROS. (J) Higher magnification of the image shown in panel I. Abbreviations: ROS, rod outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars represent 30  $\mu$ m.

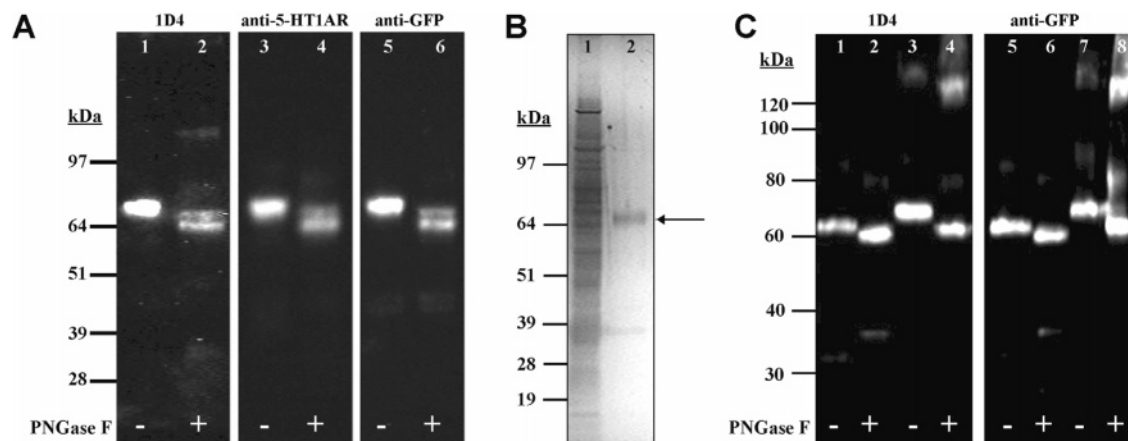


FIGURE 3: EGFP-RHO<sub>15</sub>-tagged human 5HT<sub>1A</sub>R, EDG<sub>1</sub>R, and EDG<sub>6</sub>R are homogeneously glycosylated in the rods of transgenic *X. laevis*. (A) Expression of 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> fusion protein in *Xenopus* eyes detected by immunoblotting. Membrane extracts containing 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein were probed with 1D4, anti-5HT<sub>1A</sub>R, or anti-GFP antibodies, with (+) or without (–) PNGase-F treatment. (B) Purification of 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> fusion protein. Silver-stained gel showing a membrane extract of transgenic tadpole eyes expressing 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> protein (lane 1) and the fusion protein purified with immobilized 1D4 antibody (lane 2, arrow). (C) Expression of EDG<sub>1</sub>R-EGFP-RHO<sub>15</sub> and EDG<sub>6</sub>R-EGFP-RHO<sub>15</sub> fusion protein in *Xenopus* eyes detected by immunoblotting. Membrane extracts containing EDG<sub>1</sub>R-EGFP-RHO<sub>15</sub> (lanes 1, 2, 5, and 6) and EDG<sub>6</sub>R-EGFP-RHO<sub>15</sub> proteins (lanes 3, 4, 7, and 8), probed with 1D4 or anti-GFP antibodies, with (+) or without (–) PNGase-F treatment.

varied between individual tadpoles, it occasionally also varied from one rod to another within the same retina (Figure 2D). This finding is consistent with the similar mosaic expression pattern of the RHO<sub>15</sub>-tagged rhodopsin-EGFP fusion protein found in *Xenopus* retina using the same promoter (20). While most fusion proteins were transported to ROS, some receptors such as 5HT<sub>4B</sub>R and 5HT<sub>7A</sub>R were mislocalized to the inner segment membranes and synapses (Figure 2E,F). A possible explanation is that these receptors form complexes with synaptic proteins, overriding the vectorial RHO<sub>15</sub> signal. To further demonstrate the restricted expression of these GPCR-EGFP-RHO<sub>15</sub> fusion proteins in the ROS of transgenic tadpoles, we used antibodies against rhodopsin to identify the ROS in the transgenic retinas (Figure 2H). As shown in Figure 2G–J, the EGFP fluorescence colocalized with rhodopsin expression, confirming that the expression of GPCR-EGFP-RHO<sub>15</sub> fusion proteins was limited to ROS.

**Homogeneity in the Molecular Forms and Purification of 5HT<sub>1A</sub>R.** The identity of the 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> fusion protein was confirmed by three parallel immunoblots of eye extract from transgenic tadpoles. The blots in Figure 3A (lanes 1, 3, and 5) show a band present in the crude extract recognized by 1D4, anti-5HT<sub>1A</sub>R, and anti-GFP antibodies, respectively. In addition, the partially deglycosylated fusion proteins (lanes 2, 4, and 6) demonstrate that the transgenic receptor was expressed in a uniformly glycosylated form. 1D4 antibody did not recognize *Xenopus* rhodopsin because of sequence differences from mammalian rhodopsin. This experiment confirmed that the band at ~70 kDa corresponds to the 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> fusion protein. The receptor from solubilized eye membranes was subjected to chromatographic purification using immobilized 1D4 antibody as a stationary phase. The electrophoresis developed by silver staining (Figure 3B) shows the crude extract (lane 1) compared with the purified receptor (lane 2). Note that only a minor contaminant (most probably frog rhodopsin, which is very abundant in the extract) was observed after a single chromatographic step. The expression level of the fusion protein was quantified by comparing it with bovine serum

albumin standards (data not shown). We calculated that each transgenic tadpole expressed 1–5 ng of fusion protein. All 11 other 5HTRs also exhibited homogeneous glycosylation, as opposed to the heterogeneous glycosylation we observed in these same 12 5HTRs expressed in HEK293 cell lines (data not shown).

**Homogeneity in the Molecular Forms of EDG<sub>1</sub>R and EDG<sub>6</sub>R.** The identity of these two EDGR-EGFP-RHO<sub>15</sub> fusion proteins was confirmed by two parallel immunoblots incubated with different antibodies. The blots in Figure 3C show a band present in the crude extract that binds to anti-GFP and 1D4 antibodies. This experiment confirmed that the bands at ~62 kDa (lanes 1 and 5) and ~68 kDa (lanes 3 and 7) correspond to the EDG<sub>1</sub>R-EGFP-RHO<sub>15</sub> and EDG<sub>6</sub>R-EGFP-RHO<sub>15</sub> fusion proteins, respectively. In addition, deglycosylation with PNGase F (lanes 2, 4, 6, and 8) demonstrated that these receptors are uniformly glycosylated. All six other EDGRs showed a similar level of homogeneous glycosylation, as opposed to the heterogeneous glycosylation we observed in these same EDGRs expressed in transformed mammalian cell lines (data not shown). The oligomerization observed in the deglycosidase-treated EDG<sub>6</sub>R-EGFP-RHO<sub>15</sub> protein (lane 8 in Figure 3C) is probably due to the prolonged incubation at 16 °C.

**Antagonist Binding Assay with 5HT<sub>1A</sub>R.** To test whether the 5HT<sub>1A</sub>R expressed as the 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> fusion protein in the ROS of *Xenopus* tadpoles was properly folded, we measured the binding affinity of [methyl-<sup>3</sup>H]MPPF {4-(2'-methoxy)phenyl-1-[2'-(n-2"-pyridinyl-10-p-fluorobenz-amido)ethyl]piperazine}, a radiolabeled selective ligand, for 5HT<sub>1A</sub>R. A crude ROS fraction was purified from tadpole eyes. Since the 5HT<sub>1A</sub>R expressed in ROS disk membranes is oriented with the extracellular side facing the interior of these disks and is thus shielded from the solution, we added the detergent saponin to disrupt the sealed disk membranes to allow the ligands access to the receptors. We found that saponin at a concentration of ≥0.05% could effectively increase the level of specific binding of [methyl-<sup>3</sup>H]MPPF to 5HT<sub>1A</sub>R transgenic ROS membranes, but not to control

ROS membranes (data not shown). On the basis of these observations, we included 0.05% saponin in all ligand binding assays with membranes. The level of specific binding of MPPF to the membranes from 5HT<sub>1A</sub>R transgenic tadpoles increased in a concentration-dependent manner, whereas control tadpole membranes did not show significant specific binding to up to 50 nM [*methoxy*-<sup>3</sup>H]MPPF (data not shown). This suggests that the receptor was expressed and folded correctly, since it could recognize its selective ligand. We also performed saturation binding assays to measure the binding affinity and compared it to that of 5HT<sub>1A</sub>R expressed in standard mammalian CHO cells. The binding affinity of [*methoxy*-<sup>3</sup>H]MPPF for the tadpole samples ( $K_d$ ) was  $29 \pm 0.7$  nM, and  $B_{max} = 2540$  fmol/mg. The equivalent measurement performed on membranes from CHO cells expressing the 5HT<sub>1A</sub>R yielded the following:  $K_d = 0.35 \pm 0.1$  nM and  $B_{max} = 1770$  fmol/mg (data not shown).

The difference observed in the dissociation constant  $K_d$  of transgenic tadpoles and CHO cells could be due to the different lipid composition of their respective membranes (10–12). To test this possibility, we measured the binding affinity of [*methoxy*-<sup>3</sup>H]WAY 100635, a radiolabeled selective ligand, for the purified 5HT<sub>1A</sub>R from both HEK 293T cells and transgenic *Xenopus* retinas, where the influence of the different lipid composition of membranes is eliminated. This approach would examine whether purified detergent-solubilized 5HT<sub>1A</sub>R from transgenic *Xenopus* retinas is in a native configuration, allowing ligand binding. We took advantage of the RHO<sub>15</sub> tag in the expressed 5HT<sub>1A</sub>R proteins, which can be recognized by the 1D4 antibody, and measured the binding affinity of [*methoxy*-<sup>3</sup>H]WAY 100635 for 5HT<sub>1A</sub>R bound to Sepharose gel with the immobilized 1D4 antibody. The level of specific binding of [*methoxy*-<sup>3</sup>H]WAY 100635 to the purified 5HT<sub>1A</sub>R from both transfected cells and transgenic *Xenopus* increased in a concentration-dependent manner (Figure 4A,B), whereas samples from control cells and tadpoles did not exhibit significant specific binding to up to 10 nM [*methoxy*-<sup>3</sup>H]WAY 100635 (not shown). The proteins purified from transfected cells and transgenic retinas showed similar binding affinity. The dissociation constant of [*methoxy*-<sup>3</sup>H]WAY 100635 for 5HT<sub>1A</sub>R purified from transgenic *Xenopus* retinas ( $K_d$ ) equaled  $3.34 \pm 1.13$  nM with an estimated  $B_{max}$  of 159.1 pmol/mg. The equivalent measurement performed on proteins from HEK 293T cells expressing the 5HT<sub>1A</sub>R yielded the following:  $K_d = 3.69 \pm 0.51$  nM with an estimated  $B_{max}$  of 88.0 pmol/mg. These results suggest that membrane composition may be the major factor accounting for the difference in the binding affinity observed with membranes from 5HT<sub>1A</sub>R-expressing cells and tadpoles. In addition, they also indicate that these 5HT<sub>1A</sub>R proteins expressed in retina can be purified in a properly folded form.

**[<sup>35</sup>S]GTPγS Binding Assay with 5HT<sub>1A</sub>R.** To examine whether 5HT<sub>1A</sub>R expressed in *Xenopus* rods can be functionally coupled with endogenous G proteins in retina rods, binding of [<sup>35</sup>S]GTPγS to membranes from tadpole eyes was assessed. To reduce the extent of rhodopsin-mediated [<sup>35</sup>S]GTPγS binding to photoreceptor G protein transducin, rhodopsin was inactivated by treatment of tadpole membranes with 10 mM hydroxylamine at room temperature (32). This treatment led to an ~50% reduction in the level of basal binding of [<sup>35</sup>S]GTPγS to tadpole eye membranes without a

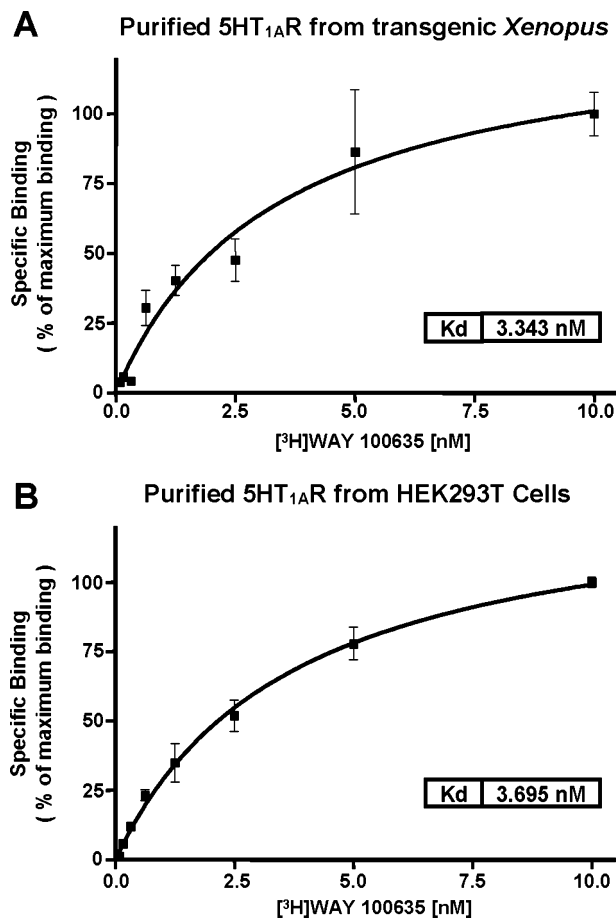


FIGURE 4: Ligand binding of purified 5HT<sub>1A</sub>R fusion proteins from *Xenopus* rods and HEK293T cells. (A) Specific binding of a 5HT<sub>1A</sub>R selective antagonist [*methoxy*-<sup>3</sup>H]WAY 100635 to 5HT<sub>1A</sub>R–EGFP–RHO<sub>15</sub> protein (from *Xenopus* retinas) bound to 1D4 antibody immobilized in Sepharose gel. Data show the results from two independent experiments. (B) Specific binding of a 5HT<sub>1A</sub>R selective antagonist [*methoxy*-<sup>3</sup>H]WAY 100635 to 5HT<sub>1A</sub>R–RHO<sub>15</sub> protein purified from HEK293T cells using 1D4 affinity gel. Data show the results from three independent experiments.

significant effect on the binding of [<sup>35</sup>S]GTPγS to CHO membranes expressing the 5HT<sub>1A</sub>R–RHO<sub>15</sub> protein (data not shown), corroborating specific inactivation of rhodopsin–transducin interaction in ROS membranes. As shown in Figure 5A, agonist serotonin stimulated binding of [<sup>35</sup>S]GTPγS to tadpole membranes by ~50%, similar to results obtained for CHO membranes transiently expressing the 5HT<sub>1A</sub>R–RHO<sub>15</sub> protein (Figure 5A). Interestingly, 5HT<sub>1A</sub>R-specific inverse agonist spiperone could significantly block binding of [<sup>35</sup>S]GTPγS to both CHO membranes (by 25%) and tadpole membranes (by 50%), indicating that 5HT<sub>1A</sub>R displayed constitutive G<sub>i</sub>-coupling activity in both CHO cells and *Xenopus* rods (Figure 5A). These results clearly indicate that the 5HT<sub>1A</sub>R–EGFP–RHO<sub>15</sub> fusion protein expressed in *Xenopus* rods could properly couple to G proteins in a manner very similar to that of receptors expressed in mammalian cells. These data also suggest that the GFP-fused version of 5HT<sub>1A</sub>R does not experience significant changes in its properties, as compared with those of the 5HT<sub>1A</sub>R expressed in CHO membranes.

**[<sup>35</sup>S]GTPγS Binding Assay with EDG<sub>1</sub>R.** To test whether the EDG<sub>1</sub>R expressed as the EDG<sub>1</sub>R–EGFP–RHO<sub>15</sub> fusion protein in the ROS of *Xenopus* tadpoles was properly folded,



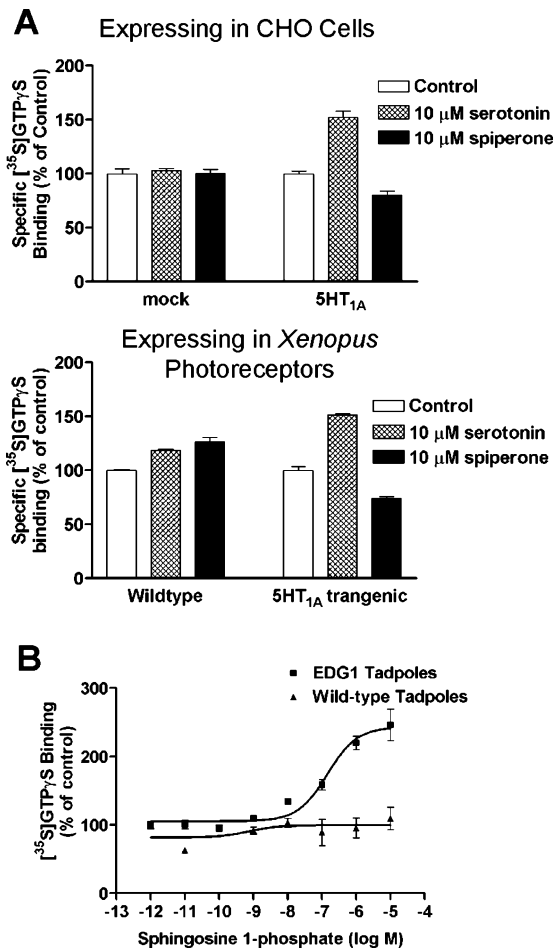


FIGURE 5: Functional characterization of 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub> and EDG<sub>1</sub>R-EGFP-RHO<sub>15</sub> fusion proteins from *Xenopus* rods. (A) Binding of [<sup>35</sup>S]GTP<sub>γ</sub>S to the CHO membrane expressing 5HT<sub>1A</sub>R (top) and to the membrane from 5HT<sub>1A</sub>R transgenic tadpoles (bottom). Agonist serotonin specifically stimulated binding of [<sup>35</sup>S]GTP<sub>γ</sub>S to 5HT<sub>1A</sub>R expressed in both CHO cells and transgenic tadpoles; the inverse agonist spiperone specifically inhibited binding of [<sup>35</sup>S]GTP<sub>γ</sub>S to 5HT<sub>1A</sub>R. Similar results were obtained in two independent experiments. (B) Binding of [<sup>35</sup>S]GTP<sub>γ</sub>S to the membranes from EDG<sub>1</sub>R transgenic tadpoles. Agonist S1P specifically stimulated binding of [<sup>35</sup>S]GTP<sub>γ</sub>S to EDG<sub>1</sub>R expressed in transgenic tadpoles in a dose-dependent manner. In panels A and B, 100% represents basal [<sup>35</sup>S]GTP<sub>γ</sub>S binding of CHO or tadpole rod membrane in the absence of S1P.

we measured the extent of EDG<sub>1</sub>R activation by the GTP<sub>γ</sub>S assay, given the difficulties associated with binding studies of lipid ligands. Agonist S1P stimulated binding of [<sup>35</sup>S]GTP<sub>γ</sub>S to tadpole membranes by ~150% (Figure 5B), results similar to those obtained from cell membranes expressing EDG<sub>1</sub>R (not shown), indicating that the expressed EDG<sub>1</sub>R in transgenic tadpoles is properly coupled to G proteins. These results suggest that the EDG<sub>1</sub>R expressed in *Xenopus* ROS is properly folded, and can activate downstream signaling upon binding to its agonist.

**Automated Injection System for Production of Transgenic *Xenopus* Tadpoles.** To obtain protein samples from transgenic tadpoles for crystallization trials, a robotic system was developed to scale up the production of transgenic tadpoles. The eggs to be injected are positioned in spherical indentations (wells) in an agarose plate, which is cast by a plastic mold. When allowed to sediment for a few minutes in the wells, more than 99% of the eggs will have their pigmented

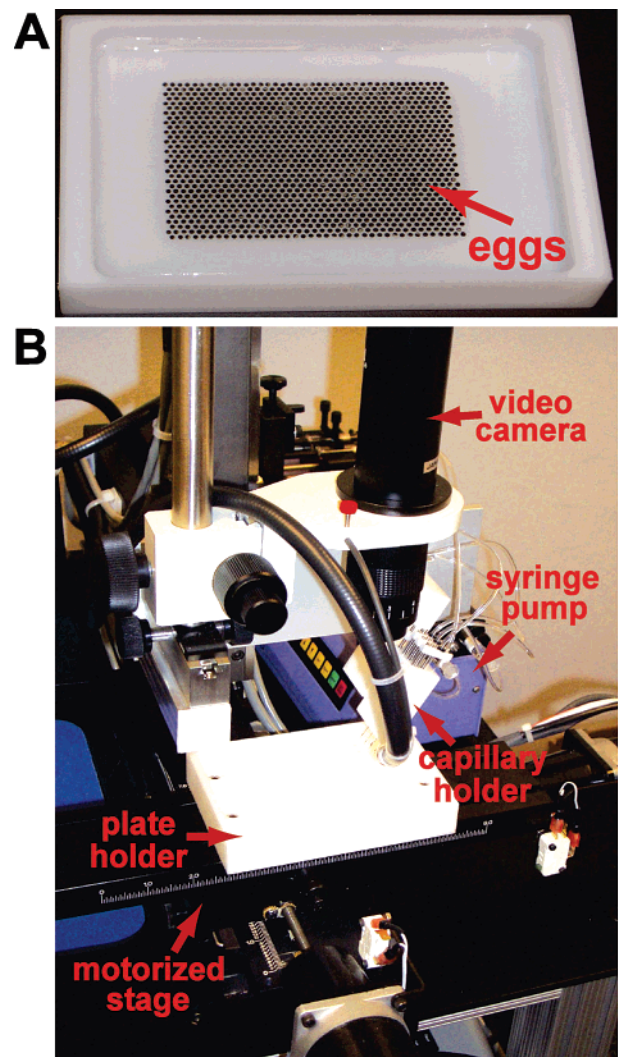


FIGURE 6: Automated injection system for producing large numbers of transgenic *Xenopus* tadpoles. (A) An agarose plate cast by a plastic mold to make spherical indentations in the gel. The red arrow points to the eggs held in the indentations. Note that the pigmented animal pole of more than 99% eggs is facing up after the eggs are allowed to sediment. (B) The automated system is composed of a motorized stage, an agarose plate holder, a video camera, a four-position adjustable capillary holder, two dual-channel syringe pumps, and a control system. The four-position adjustable capillary holder is comprised of four bored guide holes for capillary needles and four adjusters that allow precise alignment of capillary tips to each other. The current system allows automatic injections of four eggs at the same time. The current injection speed is 5000 egg injections per hour.

animal pole facing up toward the injection point, a result of the lower density of the animal pole portion (Figure 6A). The automated injection system is composed of a motorized stage with an agarose plate holder, a video camera for aligning the needles and monitoring the injection, a four-position adjustable capillary holder for holding needles, two dual-channel syringe pumps, and a computerized control system (Figure 6B). This system allows automatic injections of four eggs at the same time. The current injection speed is 5000 egg injections per hour. Under optimal conditions, we can inject 40 000 eggs per week by this automated system, resulting in ~2000 developed transgenic tadpoles. The quantification of 5HT<sub>1A</sub>R indicates that 3 ng of transgenic protein can be purified from each transgenic tadpole, which means that 6 μg of purified receptor may be produced per week.



## DISCUSSION

The GPCR system of signal transduction is ubiquitous and important for life in organisms from yeast to humans (33). GPCRs are also the most common targets for drug intervention, and are thus extremely important to the pharmaceutical industry (34). The structures of GPCRs other than rhodopsin are the most sought-after targets for the discovery of new, potent, and selective agonists and antagonists, and by researchers who are investigating G protein signaling at the molecular level. Despite such interest, and the fact that high-level expression systems have been developed for several GPCRs, the only high-resolution structure of a GPCR is that of rhodopsin. This suggests that, in addition to quantity, sample quality is also necessary for GPCR crystallization, and the answer may lie in the native system that has evolved to produce large amounts of the GPCR rhodopsin in a uniquely homogeneous form. We have chosen *X. laevis* as our model expression system, which allowed us to have an extensive screening for the expression of a large number of GPCRs. During the course of this study, a similar expression system was tested for human endothelin receptor subtype B [hET(B)R] fused with the 10 C-terminal residues of rhodopsin, under the control of the mouse opsin promoter/enhancer, by gene-targeted replacement (knock-in) (35).

**Expression of GPCRs in *X. laevis* Tadpoles.** Our results suggest that the constructs used in this work effectively convert retina of *X. laevis* tadpoles into a bioreactor for GPCR expression. We did not detect an accumulation of fusion proteins in the ER or Golgi apparatus, suggesting that all the fusion proteins were properly folded and transported to the ROS disk membranes. This was remarkable, since it is common for receptor overexpressed in mammalian cells to accumulate biosynthetic intermediates in the ER and Golgi apparatus. To achieve sufficient expression of a membrane protein for crystallization, it is not just higher-level expression that is necessary but the equivalent scaling up of the biochemical processing machinery. Rods are unique in their high level of production of membrane proteins, as large amounts of rhodopsin and other membrane proteins are produced and processed continuously in the retina. This property is missing in the standard mammalian cell expression systems utilized for membrane protein production. Interestingly, the ROS-targeting signal from mouse rhodopsin that the *Xenopus* receptor constructs carry is recognized by the transport machinery of *Xenopus* rods and transports the expressed fusion proteins efficiently to the ROS. Importantly, this tag contains the epitope for the 1D4 antibody and can be effectively used for chromatographic purification. The lack of cross reactivity of the 1D4 antibody with *Xenopus* rhodopsin allows great enrichment of GPCRs by a single-step affinity chromatography.

The study presented here is limited to just two receptor families, 5HTRs and EDGRs. Although the members of these two families display a high level of similarity among each family, differences in the location and level of the transgene expression were observed, indicating the sensitivity of this expression system and variability within these families. However, this expression system is not limited to these two families. We have also expressed more than 10 other GPCRs, including cannabinoid CB<sub>2</sub> receptor, melanocortin-4 receptor, and *N*-formyl peptide receptor, using this system, and have

obtained results similar to those described here. These results suggest that this expression system could be universal for GPCRs, and extended to other membrane proteins. We have developed an automated injection system for producing large numbers of transgenic tadpoles. These amounts of receptor samples could be the start point for crystallization, using recently developed and commercially available low-volume nanocrystallization systems.

**Glycosylation of GPCRs in *X. laevis* Tadpoles.** Previous studies have shown that GPCR purified from HEK293 cells displays a diffuse pattern, which can be converted to a tighter pattern by deglycosylation, suggesting that there is a high degree of heterogeneous glycosylation. We have found the same heterogeneity in the 20 receptors studied in this work when expressed in HEK293 cells (not shown). In contrast, rhodopsin extracted from native rods shows a tight electrophoretic band, suggesting it is homogeneously glycosylated. This lack of contamination from unglycosylated protein is due to the fact that in rods, only properly folded and homogeneously glycosylated rhodopsin is transported to the ROS, where it forms 90% of all the membrane protein content (1). This means that the rods themselves purify rhodopsin intracellularly within the ROS, with an exquisite selection for homogeneous material. The separation between the folded and fully processed receptor from unprocessed receptor is thus another hallmark of the homogeneity of the purified receptor sample from these rods relative to standard cell lines. Unlike the receptors expressed in HEK293 cells, the 5HT<sub>1A</sub>R-EGFP-RHO<sub>15</sub>, EDG<sub>1</sub>R-EGFP-RHO<sub>15</sub>, and EDG<sub>6</sub>R-EGFP-RHO<sub>15</sub> constructs expressed in ROS of transgenic *Xenopus* tadpoles show a very homogeneous pattern of receptor glycosylation, as shown in Figure 3A.

**Agonist and Antagonist Binding with 5HT<sub>1A</sub>R.** The expressed 5HT<sub>1A</sub>R in transgenic tadpoles was effective in the binding of agonist and antagonist. The low nanomolar affinity and the extent of specific binding indicate that the 5HT<sub>1A</sub>R is properly folded in transgenic tadpoles. However, the 82-fold difference observed in the dissociation constant ( $K_d$ ) of transgenic tadpoles and CHO cells indicates that other factors, such as different lipid composition of their respective membranes, may affect the ligand affinity of the receptor (10–12). Recently, depletion of cholesterol has been implicated in the altered binding properties of 5HT<sub>1A</sub>R (36). In general, the lipid environment is critical in the functioning of GPCRs (37). Similar differences were observed for the mGluR expressed in the eye of *Drosophila melanogaster* (10–12) using a strategy similar to that proposed here, compared with heterologous cultured cell expression. The binding affinity of a selective ligand for the mGluR expressed in *Drosophila* eyes was 10-fold lower than the affinity for the same receptor expressed in insect Sf9 cells, likely due to the low level of cholesterol-like lipids in the membranes of photoreceptors expressing rhodopsin. For the structural studies, these differences will not be an obstacle, as the purification requires a detergent-solubilized form of the receptor stabilized by specific ligands and/or detergents. This is confirmed by the similar binding affinity of [*methoxy*-<sup>3</sup>H]WAY 100635 for the purified 5HT<sub>1A</sub>R from both HEK 293T cells and transgenic *Xenopus* retinas, where different lipid composition of membranes is not involved. It also demonstrates that the proteins expressed by this system can be purified in a properly folded form by taking advantage of the RHO<sub>15</sub>

tag. In these binding assays, the binding affinity of ligand was measured with proteins bound to Sepharose gel coupled with the immobilized 1D4 antibody, which could be set up as an efficient universal ligand binding assay for all the receptors expressed by this expression system, regardless of ligand properties.

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