Protein-protein interactions monitored in cells from transgenic mice using bioluminescence resonance energy transfer

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ABSTRACT **Monitoring the dynamics of protein-protein interactions in their natural environment remains a challenge. Resonance energy transfer approaches represent a promising avenue to directly probe these interactions in real time. The present study aims at establishing a proof of principle that bioluminescence resonance energy transfer (BRET) can be used to study the regulation of protein-protein interaction in cells from transgenic animals. A transgenic mouse line coexpressing the β_2-adrenergic receptor fused to *Renilla* luciferase (β_2AR-*R*luc) and βarrestin-2 fused to a green fluorescent protein (GFP2-βarr2) was generated. The fusion proteins were found to be functional in the transgenic animals and the β_2AR-*R*luc maintained pharmacological properties, comparable to that of the native receptor. Sufficiently high luminescence signal was generated to allow detection of BRET in testis cells where the β_2AR-*R*luc transgene was expressed at levels significantly higher than that of the endogenous receptor in this tissue but remain within physiological range when compared with other β_2AR-expressing tissues. Stimulation with a β-adrenergic agonist led to a significant dose- and time-dependent increase in BRET, which reflected ligand-promoted recruitment of βarr2 to the receptor. Our study demonstrates that BRET can be used to monitor the dynamic regulation of protein-protein interactions in cells derived from transgenic mice.—Audet, M., Lagacé, M., Silversides, D. W., Bouvier, M. Protein-protein interactions monitored in cells from transgenic mice using bioluminescence resonance energy transfer. *FASEB J.* 24, 2829–2838 (2010). www.fasebj.org**

Key Words: β-arrestin2 · β2-adrenergic receptor · Renilla *luciferase · green fluorescent protein*

In recent years, a large variety of assays were designed to allow the identification and monitoring of protein-protein interactions. However, most of these assays rely on *in vitro* or heterologous expression systems in cultured cells. Yet the ability to identify interacting partners and to monitor their interaction dynamics in native environments will be essential to build a realistic view of the molecular events underlying many physiological and pathophysiological processes. Proximity assays based on resonance energy transfer (RET) offer an avenue to monitor protein-protein interaction in the living organism. RET is a phenomenon occurring between two or more chromophores close to each other when the emission spectrum of one (the donor) overlaps with the excitation spectrum of the other (the acceptor). Following donor excitation, part of the electronic relaxation energy of the donor molecule is transferred to the acceptor molecule as a function of the distance between the donor and the acceptor, and the relative orientation of their dipole moments. RET can be determined by monitoring the light emission from the acceptor on donor excitation. The strict dependence of RET on the interchromophoric distance (RET efficacy being inversely proportional to the 6th power of the distance between the energy donor and acceptor) makes it an assay of choice to monitor protein complexes. This is particularly true when considering that the average radius of a protein is ~50 Å and that the maximal distance allowing RET for most chromophores is ~100 Å. The occurrence of RET between chromophores attached to two proteins can, therefore, be taken as a result of the interaction of these two proteins within a macromolecular complex.

Intramolecular fluorescence resonance energy transfer (FRET) has already been used in living animals to monitor the conformational rearrangements of biosensors designed to detect changes in second messengers or kinase activities. For this purpose, transgenic animals [mice (1–6), fruit fly (7), and zebrafish (8)] expressing unimolecular protein sensors fused to FRET-pairs were generated and the intramolecular FRET signals recorded by fluorescence microscopy imaging in tissues derived from these animals. To our knowledge, however, FRET in transgenic animal tissues has not as yet been used to monitor protein-protein interactions in a bimolecular FRET configuration.

A different form of RET that relies on a biolumines-

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cent enzyme (generally *Renilla* luciferase, *R*luc) as the energy donor has also been used to monitor conformational rearrangements and protein-protein interactions (9, 10). Since the transfer of energy is initiated by the addition of the bioluminescent enzyme substrate (generally coelenterazine), one of the main advantages of bioluminescence resonance energy transfer (BRET) over FRET is the complete absence of background emission coming from nonspecific cell excitation by an external light beam, as is the case in FRET, where both donor and acceptor are photoexcitable chromophores. In recent years, BRET has become increasingly popular to monitor different types of protein-protein interactions and their dynamic regulation in cultured cells, with a special emphasis on G-protein-coupled receptor (GPCR) signaling regulation (11–18). A few studies aimed at adapting BRET to the detection of protein-protein interaction in animals. In one such study, human embryonic kidney (HEK) 293T cells expressing FKBP-*R*luc and a green fluorescent protein (GFP) fused to FRB (GFP2-FRB) as BRET partners were injected subcutaneously in mice, and the BRET generated by the rapamycin-promoted interaction between FKBP and FRB was monitored in the entire animal by imaging (19). Using a similar imaging approach, Xia and Rao (20) showed that quantum dots can be used as BRET acceptors and can be visualized in mice injected with C6 glioma cells labeled with quantum dots chemically fused to *R*luc. Although useful steps toward the establishment of a proof of principle that BRET can be monitored in the tissues of living animals, these studies used engineered systems that relied on exogenous cells injected in the animals. Thus, the present study was initiated to determine whether protein-protein interactions could be monitored by BRET in cells from animals transgenically expressing energy donor and acceptor fused to proteins of interest. For this purpose, we used the well-characterized interaction between the β_2-adrenergic receptor (β_2AR), and the regulatory protein βarrestin (βarr) that had previously been detected by BRET in cultured cell systems (11, 21, 22).

The β_2AR is a member of the GPCR family that is characterized by 7 transmembrane domains and transduces signals from a vast diversity of hormones and neurotransmitters. Aside from promoting the activation of heterotrimeric G proteins, stimulated GPCRs rapidly become phosphorylated by G-protein-coupled receptor kinase (GRK), an event that triggers the translocation of βarr to the receptor, causing its uncoupling from the G protein and its endocytosis (23). Thus, we used this dynamic recruitment event to propose a proof of principle that BRET can be used to monitor real-time protein-protein interactions in a native transgenic mouse environment.

To do so, heterozygous transgenic mouse lines expressing the β_2AR fused to *R*luc (β_2AR-*R*luc) and βarr2 fused to GFP2 (GFP2-βarr2) were generated and crossed to generate transgenic animals expressing the two transgenes. The ability of a β_2-adrenergic agonist to promote βarr recruitment to the β_2AR was tested in tissues from these mice.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, glutamine, and phosphate-buffered saline (PBS) were from Wisent, Inc. (St. Jean-Baptiste, QC, Canada) Cell culture plates and dishes were from Corning (Corning, NY, USA). Nitrocellulose membranes were from Whatman (Dassel, Germany). Hybond-N+ Southern blot membranes were form GE Healthcare (Baie d'Urfe, QC, Canada). ULTRAhyb hybridation buffer was from Ambion (Austin, TX, USA). The Bio-Rad D_c protein assay kit and the 10DG desalting column were from Bio-Rad Laboratories (Mississauga, ON, Canada), while the nickel-NTA beads were from IBA (Göttingen, Germany). [^{3}H]cAMP, [^{32}P]ATP, [^{32}P]CTP, and [^{125}I] were from PerkinElmer Life Sciences (Waltham, MA, USA). SuperSignal West Dura Luminol for Western blot detection was from Pierce Biotechnology (Rockford, IL, USA). (−)-Isoproterenol, alprenolol, bovine serum albumin (BSA), soybean trypsin inhibitor, leupeptin, benzamidine, PMSF, 1,10-phenanthroline, and lysozyme were from Sigma (St. Louis, MO, USA). Polyethylenimine (PEI) was from Polysciences, Inc. (Warrington, PA, USA). Collagenase type IV was from Worthington Biochemical Corp. (Lakewood, NJ, USA). All DNA restriction enzymes were from New England Biolabs (Ipswich, MA, USA). DeepBlueC coelenterazine was purchased from PerkinElmer. Coelenterazine h was purchased from Prolume (Pinetop, AZ, USA). The rabbit anti-βarr2 antibody (C16D9) was from Cell Signaling Technology (Danvers, MA, USA), and the horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibody (NA934V) was from GE Healthcare. The optimal cutting temperature (OCT) embedding medium was from Sakura Tissue Tek (Torrance, CA, USA).

Vectors

The vectors used for transgenic mice were generated as follows: a previously described 0.8-kb DNA fragment located 5′ to the associated transcript's exon 1 of the ROSA26 gene promoter (24, 25) was PCR amplified, inserted into the cloning pGemT vector (Promega) between *Age1/Sal*I, and subcloned into the modified pEGFP-1 plasmid using *Pst*I and S*acII* restriction sites. The eGFP gene was then removed from the modified pEGFP-1 vector using *Sac*II/X*baI*. For the GFP2-βarr2 fusion protein transgenic expression vector, a DNA adaptor encoding an *Nhe*I restriction site was inserted in the *Sac*II/*Xba*I site of the modified pEGFP-1. The fusion GFP2-βarr2 sequence was PCR amplified and inserted between N*heI/Xba*I in the modified pEGFP-1. For the β_2AR-h*R*luc fusion protein transgenic expression vector, the β_2AR-h*R*luc sequence was PCR amplified and inserted between S*acII/Xba*I in the modified pEGFP-1 vector. The amino acid sequence of the linker between GFP2-βarr2 and β_2AR-h*R*luc is GSGTGS. Finally, these vectors were linearized using E*coRI/Ssp*I prior to microinjection. The His-tagged GFP2 vector (pQE80GFP2) for GFP2 bacterial expression and purification was constructed as follows: the DNA sequence of the last 29 amino acid residues (343–371) of the soluble C-tail region of the arginine vasopressin receptor 2 (V2R), as well as the GFP2 DNA sequence, were PCR amplified and then fused by overlapping PCR. The DNA fusion product was inserted between *Bam*HI/*Hind*III in pQE80 vector (Qiagen, Mississauga, ON, Canada). The amino acid sequence of the linker between the C-tail of V2R and GFP2 is GGKGDGN.

Animals

Transgenic mice were generated *via* conventional pronuclear microinjection using embryos derived from FVB/N inbred mice (Jackson Laboratory, Bar Harbor, ME, USA) (26). For each of the lines created, a tyrosinase minigene was coinjected to provide a visible marker for transgenesis, thus reducing the need for genetic validation during routine expansion and maintenance of the mouse lines (27). For pigmented lines generated, the integration of the β_2AR-*R*luc or GFP2-βarr2 transgenes was confirmed by polymerase chain reaction (PCR), and lines displaying cointegration (β_2AR-*R*luc transgene and tyrosinase minigene, GFP2-βarr2 transgene and tyrosinase minigene) were selected for further biological studies. All animal manipulations were carried out in accordance with institutional guidelines and were approved by the University of Montreal Faculty of Veterinary Medicine ethics committee [le comité d'éthique pour l'utilisation des animaux (CEUA)].

Southern blot and integrated copy number determination

The number of integrated copies of the transgenes in the β_2AR-1 and the βarr2-1 lines was determined by Southern blot analysis. Genomic DNA from mouse tail was isolated with the E.Z.N.A. tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA). DNAs were digested with *Nhe*I restriction enzyme, separated by agarose gel electrophoresis, and then transferred to a Hybond-N+ membrane. Membranes were then hybridized in ULTRAhyb hybridization buffer with [^{32}P]dCTP-labeled probes targeting the *R*luc or the GFP2 sequences of the β_2AR-*R*luc and GFP2-βarr2 transgenes in the β_2AR-1 and βarr2-1 lines, respectively. Signals were collected on a phosphorimager screen using a FLA-5000 detection device (Fujifilm, Mississauga, ON, Canada). The integrated copy number of each of the transgenes was determined by comparing the hybridization signal of the transgenes with a standard curve consisting of increasing amounts of *Nhe*I linearized ph*R*luc-GFP2 plasmid (22), as described previously (www.phenogenomics.ca/transgenics/protocols_forms.html). Densitometric analysis of the bands was performed using Multi Gauge software (Fujifilm).

Transfection and cell culture

HEK293T cells were cultured in DMEM supplemented with 5% FBS, 2 mM glutamine, 0.1 U/ml penicillin, and 0.1 mg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. Cells were transfected using a PEI transfection procedure. Briefly, 1 ml of DMEM was mixed with 30 μg of PEI and 10 μg of DNA, vortexed, and added to HEK293 cells plated onto 100-mm Petri dishes. The transfected cells were then cultured for an additional 48 h before further experimental procedures.

Testis cell dispersion

Freshly excised testes were decapsulated on ice and dispersed by 3 rounds of 37°C incubation in 0.5 mg/ml collagenase type IV alternated with 3 rounds of washing with PBS. Supernatants from these dispersion steps were pooled, and the cells were pelleted by centrifugation at 300 *g* for 10 min. Cells were then resuspended in 900 μl of PBS, counted, and resuspended to a final concentration of 10^7 cells/ml.

BRET assay

Freshly dispersed testis cells (10^6 cells/well) were distributed on 96-well white Optiplate microplates (PerkinElmer). Following the indicated pharmacological treatment, 10 μM of DeepBlueC coelenterazine was added to the cells, and the signals were collected using a modified TopCount-NXT reader (Packard Instrument Co., Meriden, CT, USA) that is able to sequentially integrate light signals detected in the 370- to 450- and 500- to 530-nm windows using appropriate filters. The BRET is determined as the ratio of the signal detected in the 500- to 530-nm bandpass divided by the signal detected in the 370- to 450-nm bandpass multiplied by 1000 and expressed as mBRET units.

Fluorescence measurement

The total fluorescence signal was detected in a 96-well plate fluorescence reader (FluoroCount; Packard Instrument Co.) with excitation and emission filters set at 400 and 510 nm, respectively. Measurements were done using 2.5×10^5 testis cells for screening the single-transgenic (primary) mouse lines and using 100 μg of soluble protein preparation for screening the expression in the various tissues of the β_2AR-1/βarr2-1 double-transgenic mice.

Luciferase activity

Luminescence signal was detected as followed: Coelenterazine h was added to a final concentration of 5 μM, and readings were collected using a luminescence plate reader (Fusion; Packard Instrument Co.) without any emission filter. Measurements were done using 10^6 testis cells for screening single-transgenic (primary) mouse lines and using 100 μg of membrane protein preparation for screening the expression in the various tissues of the β_2AR-1/βarr2-1 double-transgenic mice. To transform the luminescence signal detected into molar quantities of β_2AR-*R*luc, luminescence measurements and radioligand binding using [^{125}I]-cyanopindolol ([^{125}I]CYP) were performed in the same testis membrane preparation derived from the β_2AR-1/βarr2-1 transgenic line. The net number of β_2AR-*R*luc sites was obtained by subtracting the number of receptors detected in the wild-type (WT) mouse testis membrane fraction, and the luminescence signal was transformed into relative luminescence units (RFU) per picomole of receptor.

Tissue section preparation

Frozen tissue sections from testes of transgenic and nontransgenic mice were generated using standard procedures. Briefly, rapidly after excision, testes were fixed overnight at room temperature in a 10% buffered formalin solution and immerged for 2 h in a 25% sucrose aqueous solution. Testes were then snap-frozen at −20°C in OCT, and 5-μm-thick sections were obtained using a Leica Reichert-Jun Cryocut 1800 tissue cryostat (Leica Microsystems, Richmond Hill, ON, Canada).

Fluorescence microscopy

All fluorescence microscopy images were generated on an Axiovert LSM510 META confocal microscope (Carl Zeiss, Jena, Germany) using an oil ×40 objective with 1.3 numerical aperture, 405-nm diode laser, 505- to 530-nm bandpass emission filter, 158-μm pinhole, and 1.6-μs aquisition time.

Western blot analysis

Organs from transgenic mouse or HEK293T cells transiently expressing GFP2-βarr2 were lysed with a potter in hypotonic

buffer (25 mM Tris, 2 mM EDTA, and protease inhibitor, pH 7.4) and centrifuged at 40,000 *g* for 20 min at 4°C, and the protein concentration in the supernatant was measured. Aliquots from the supernatant were then heated in Laemmli buffer containing 50 mM dithiothreitol for 5 min at 95°C before separation of the protein on SDS-PAGE (100 μg protein/lane for tissue extracts and 25 μg protein/lane for HEK293T cell extracts). GFP-βarr2 was detected by immunoautoradiography using the rabbit anti-βarr2 specific antibody (C16D9) and HRP-conjugated anti-rabbit IgG secondary antibody followed by chemiluminescence reaction. Light emission was detected using a LAS-3000 CCD camera device (Fujifilm), and densitometric analysis of the bands were performed using Multi Gauge software (Fujifilm).

Membrane preparation and radioligand binding assay

Tissues were excised from the mice and lysed with a potter in hypotonic buffer (25 mM Tris, 2 mM EDTA, and protease inhibitor, pH 7.4) and centrifuged at 800 *g* for 10 min at 4°C to get rid of the nuclei and cell debris. Supernatants were then centrifuged at 40,000 *g* for 20 min at 4°C, and the pelleted membranes were washed twice in the same buffer. Fifty micrograms of membrane proteins (25 μg for lung) was then incubated with a saturating concentration (250 pM) of the β-adrenergic antagonist [^{125}I]CYP for 60 min at room temperature. The binding reaction was stopped by rapid filtration of the membranes on GF/C fiberglass filters separating free from bound [^{125}I]CYP. Nonspecific binding was defined in the presence of 10 μM alprenolol.

Adenylyl cyclase activity assay

Adenylyl cyclase activity was determined according to the method of Salomon *et al.* (28). Briefly, 20 μg of membrane preparation was incubated in the presence or absence of various concentrations of the β-adrenergic ligands isoproterenol and/or alprenolol or AlF4 with 10 mM theophyllin, 1 mM $MgSO_4$, 1 mM EGTA, 50 mM NaCl, 0.5 mM ATP, 50 μM GTP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase in a total volume of 50 μl in the presence of 0.5 μCi [^{32}P]ATP and 0.02 μCi [^{3}H]cAMP. Samples were incubated for 20 min at 37°C. The reaction was stopped as previously described (29). [^{32}P]cAMP was separated from [^{32}P]ATP by sequential chromatography over Dowex and alumina resins, and [^{3}H]cAMP was used to calibrate chromatography efficacy.

Statistics

Analysis of variance (ANOVA) followed by *post hoc* Dunnett or Tukey tests were used for multiple comparisons; Dunnett tests being used to compare multiple conditions to a single control, whereas Tukey tests were used to identify the difference within a group. Student *t* tests were performed for pairwise comparison. Nonlinear regression analyses of the dose-response curves, as well as the linear regression on the standard curve, were done using GraphPad Prism software (Graph Pad, La Jolla, CA, USA). The EC_{50} ± SD and slope ± SD values were derived from the respective curves.

RESULTS

Production and selection of the double-transgenic mouse line

Heterozygous transgenic mouse lines were generated expressing either the β_2AR fused to *R*luc at its C terminus or βarr2 fused to GFP2 at its N terminus (**Fig. 1*A***). Since the ROSA26 promoter was reported to direct moderate ubiquitous expression of transgenes in mice (24, 25),

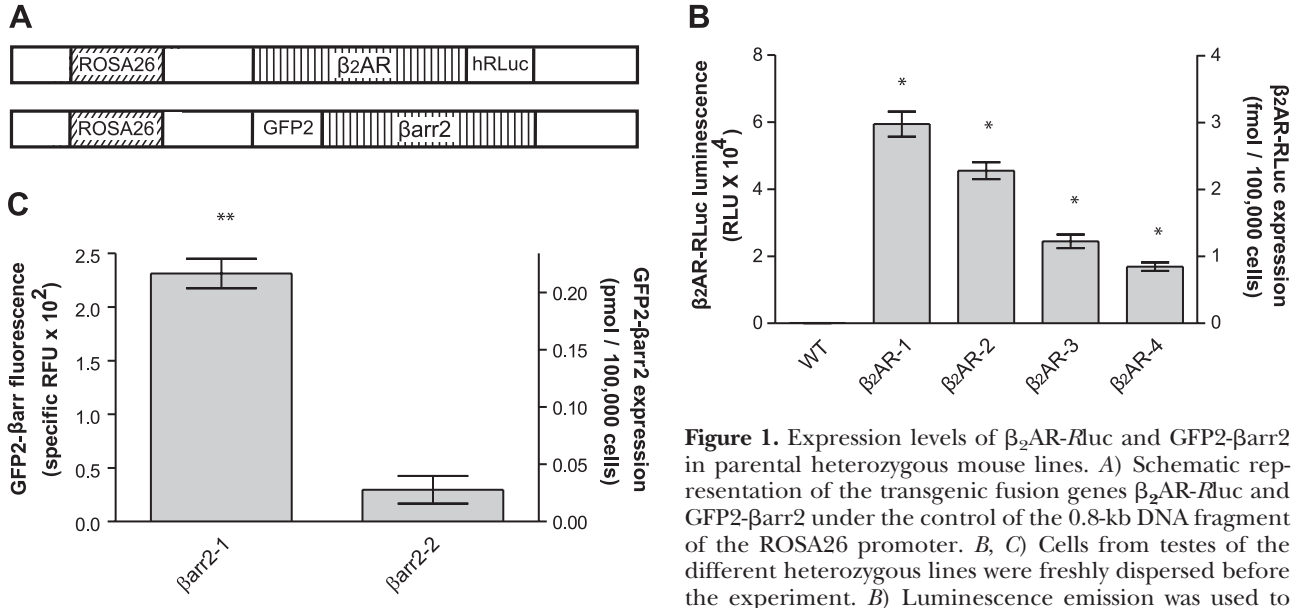

Figure 1. Expression levels of β_2AR-*R*luc and GFP2-βarr2 in parental heterozygous mouse lines. *A*) Schematic representation of the transgenic fusion genes β_2AR-*R*luc and GFP2-βarr2 under the control of the 0.8-kb DNA fragment of the ROSA26 promoter. *B, C*) Cells from testes of the different heterozygous lines were freshly dispersed before the experiment. *B*) Luminescence emission was used to monitor expression levels of the β_2AR-*R*luc transgene in the different mouse lines (10^6 cells) and is expressed as RLU (left axis). Relative luminescence was also correlated to number of specific binding sites, as described in Materials and Methods (fmol/10^5cells, right axis). *C*) Fluorescence emission was used to monitor the GFP2-βarr2 transgene in the 2 parental mouse lines obtained (2.5×10^5 cells) and is expressed as specific RFU, (left axis), where the signal from WT mouse was subtracted. Specific relative fluorescence was also correlated to the amount of GFP2-βarr2 protein expression using a standard curve of purified GFP2 proteins, as described in Supplemental Fig. 1 (pmol/10^5 cells, right axis). Error bars = means ± SE of measurements performed in ≥3 different experiments. *$P < 0.05$, **$P < 0.01$ *vs.* WT; ANOVA with Dunnett *post hoc* analysis.

we selected the 0.8-kb DNA fragment located 5′ to the associated transcript's exon 1 of the ROSA26 gene promoter with the intention of driving the expression of the two BRET partners in many tissues, while avoiding exaggerated overexpression.

Lines that integrated the β_2AR (4 lines) and βarr2 (2 lines), as detected by PCR analysis of genomic DNA, were analyzed for construct expression. The expression of β_2AR-*R*luc and GFP2-βarr2 in each of the generated lines was determined by measuring the luminescence and the fluorescence signal, respectively, from freshly dispersed testis cells (Fig. 1*B*, *C*). β_2AR-*R*luc was found to be significantly expressed, albeit at different levels, in all of the lines selected. The luminescence level varied by a factor of 3 between the lowest (β_2AR-4) and the highest expressing (β_2AR-1) lines (Fig. 1*B*), reflecting different receptor expression levels in the different mouse lines. The relationship between the luminescence signal obtained for β_2AR-*R*luc and the number of receptor sites determined by radioligand binding was established and indicates expression levels between 0.8 and 3 fmol/10^5 cells (Fig. 1*B*, right axis). Of the two GFP2-βarr2 lines, only one, βarr2-1, was found to express detectable level of GFP2-βarr2 in the testis, as shown by the significant fluorescence signal above the autofluorescence detected in the WT mouse cells (Fig. 1*C*). To transform the relative fluorescence values into molar quantities of GFP2-βarr2, we established a standard curve using purified GFP2 (Supplemental Fig. 1). From that curve, the expression level of the GFP2-βarr2 in the βarr2-1 line was estimated to be ~0.2 pmol/10^5 cells (Fig. 1*C*, right axis). Despite the lack of significant detectable fluorescence in the βarr2-2 line, individuals from the two GFP2-βarr2 lines were crossed with mice from each of the β_2AR-*R*luc lines so as to generate eight crosses of heterozygous double-transgenic animals, whose genome contained both β_2AR-*R*luc and GFP2-βarr2 transgenes. The expression of the two transgenic proteins was then analyzed in freshly dispersed cells from the testes of the double-transgenic animals (**Fig. 2**). The luminescence signals observed in these mice were equivalent to those obtained in the respective parental lines. Similarly, as was observed in the single GFP2-βarr2 mouse lines, only the double-transgenic lines derived from the βarr2-1 mouse line displayed a fluorescence signal above the WT autofluorescence. Thus, the double-transgenic mice retain parental line expression, confirming proper germ line transmission for both β_2AR-*R*luc and GFP2-βarr2. We selected the double-transgenic mice yielding the highest luciferase and GFP activities, β_2AR-1/βarr2-1, for further characterization. Using quantitative Southern blot analysis, we estimated that 1 copy of the β_2AR-*R*luc and 2 copies of GFP2-βarr2 transgenes were integrated per diploid genome of mice (data not shown).

Expression analysis of the transgenes

The expression levels of β_2AR-*R*luc and GFP2-βarr2 were assessed in extracts of kidney, testis, liver, spleen, heart, lung, and brain tissues derived from the β_2AR-1/βarr2-1 line. As shown in **Fig. 3*A***, significant luminescence signals above background were observed to different extents in the extract of all tissues tested. The highest luminescence level was detected in the testes (~350-fold over background), whereas the lowest was found in the kidney (~4-fold over background). Radioligand-binding experiments carried out in membrane preparations using [^{125}I]CYP as the tracer confirmed the high expression level of a binding competent β_2AR in testes (Fig. 3*B*). Indeed, the receptor level achieved in the testes of the transgenic animals (105±13 fmol/mg) represents ~8-fold increase over the endogenous level (12±3 fmol/mg). In all other tissues, the small increase in [^{125}I]CYP binding observed did not reach statistical significance. This is consistent with the low luminescence signal observed in all tissues, except the

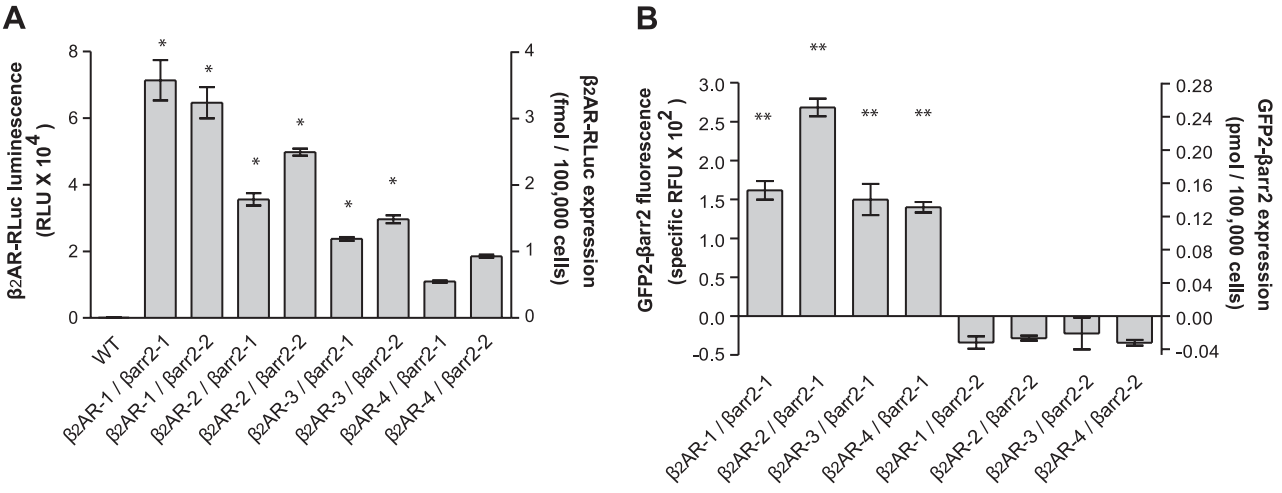

Figure 2. Expression levels of β_2AR-*R*luc and GFP2-βarr2 in double-transgenic heterozygous mice. Double-transgenic mice were generated by crossing the parental lines presented in Fig. 1. Expression levels in dispersed testis cells of different crosses were monitored by luminescence emission from β_2AR-*R*luc (*A*) and fluorescence emission from GFP2-βarr2 (*B*) (left axes), and transformed into femtomoles and picomoles per 10^5 cells, respectively, (right axes) as described in Fig. 1. Error bars = mean ± SE of measurements performed in ≥3 different experiments. $^*P < 0.05$, $^{**}P < 0.01$ *vs.* WT values; ANOVA with Dunnett *post hoc* analysis.

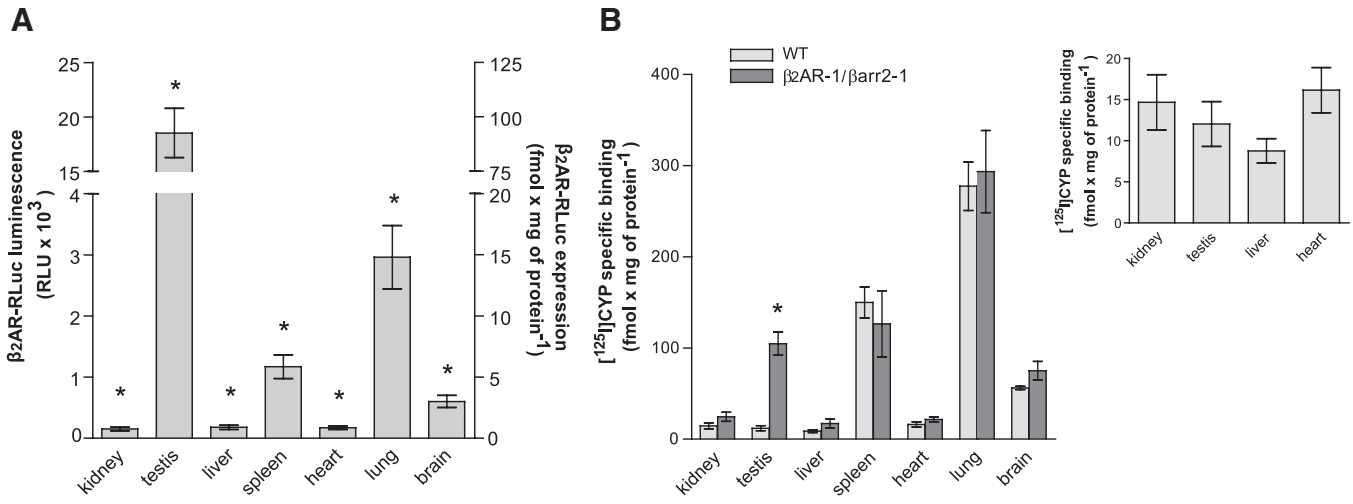

Figure 3. Expression levels of β_2AR-*R*luc in tissue membrane preparation from double-transgenic β_2AR-1/βarr2-1 mice. *A*) Expression levels of β_2AR-*R*luc transgene were measured in 100 μg of membrane preparation from kidney, testis, liver, spleen, heart, lung, and brain using luminescence emission and correlated to femtomoles per milligram of protein, as described in Materials and Methods. *B*) Total number of β_2AR sites was measured by [^{125}I]CYP radioligand binding assay. Inset: βAR density on kidney, testes, liver, and heart of the WT mice is magnified. Results are expressed as means ± SE of measurements performed in ≥5 (luminescence) and 3 ([^{125}I]CYP radioligand binding) different experiments.*$P < 0.005$ *vs.* background in WT; Student's *t* test.

testes (Fig. 3*A*), and indicates that the resulting small increase in the number of receptor sites above endogenous levels fell below the radioligand binding assay sensitivity and/or within the error margins of the signals. Consistent with this interpretation, transforming the luminescence signal into receptor number, using the correspondence between luminescence and the [^{125}I]CYP binding observed in the testes, predicts increase in expression levels varying between 0.8 fmol/mg protein for the kidney and 14.8 fmol/mg for the lung (Fig. 3*A*, right axis).

For the GFP2-βarr2 construct, significant levels of fluorescence above the autofluorescence of the WT mice were detected only in the testes of the β_2AR-1/βarr2-1 line (**Fig. 4*A***). This testis-specific expression pattern was confirmed by Western blot analysis using an anti-βarr2 antibody (Fig. 4*B*). Indeed, the ~77-kDa immunoreactive band corresponding to the molecular

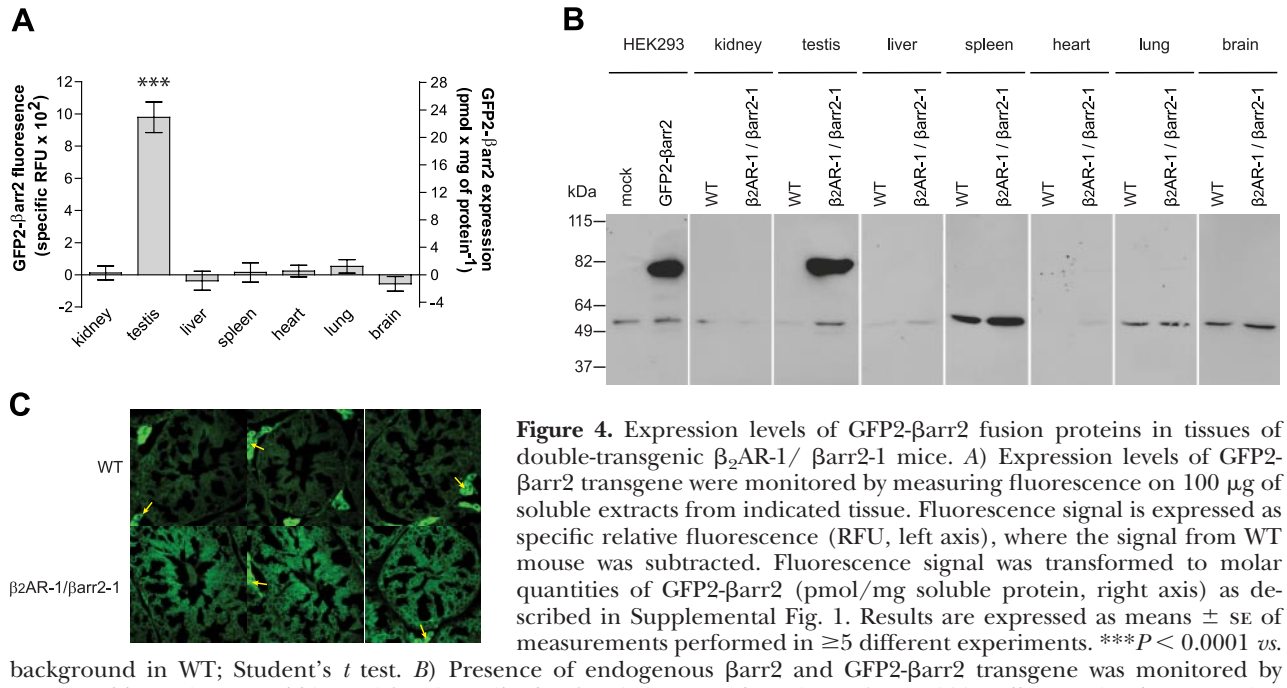

Figure 4. Expression levels of GFP2-βarr2 fusion proteins in tissues of double-transgenic β_2AR-1/ βarr2-1 mice. *A*) Expression levels of GFP2-βarr2 transgene were monitored by measuring fluorescence on 100 μg of soluble extracts from indicated tissue. Fluorescence signal is expressed as specific relative fluorescence (RFU, left axis), where the signal from WT mouse was subtracted. Fluorescence signal was transformed to molar quantities of GFP2-βarr2 (pmol/mg soluble protein, right axis) as described in Supplemental Fig. 1. Results are expressed as means ± SE of measurements performed in ≥5 different experiments. ***$P < 0.0001$ *vs.* background in WT; Student's *t* test. *B*) Presence of endogenous βarr2 and GFP2-βarr2 transgene was monitored by Western blot using a rabbit anti-βarr2 antibody. βarr2 immunodetection of HEK293 cells transiently expressing GFP2-βarr2 was used as control. Western blots are representative of 3 different experiments. *C*) Expression level of GFP2-βarr2 in a 5-μm testes seminiferous tubule cross section from β_2AR-1/βarr2-1 mice was confirmed by fluorescence microscopy. Yellow arrows indicate Leydig cells. Fluorescence microscopy images are representative of 4 different experiments.

mass of GFP2-βarr2 was observed only in extracts of testes and HEK293 cells transfected with a vector coding for the fusion protein. The immunoreactive band observed at 53 kDa in all tissue extracts corresponds to the endogenous βarr2. Assuming that the antibody (raised against human βarr-2) recognized the mouse βarr with the same affinity, the relative intensity of the bands suggests that the GFP2-βarr2 transgene was expressed at a level ~60-fold over that of the endogenous protein in the testes. This is consistent with the expression level of 20 pmol/mg of protein estimated using purified GFP2 as a standard (Fig. 4*A*, right axis), when considering that endogenous βarr2 levels range from undetectable in H9c2 cells to ~0.8 pmol/mg in Jurkat cells (J. Benovic, Thomas Jefferson University, Philadelphia, PA, USA; personal communication, December 2009).

The expression of GFP2-βarr2 in testes was also documented by confocal fluorescence microscopy of 5-μm cross sections of the testes seminiferous tubules obtained from β_2AR-1/βarr2-1 double-transgenic and WT mice. As shown in Fig. 4*C*, the fluorescence signal from the β_2AR-1/βarr2-1 mice was spread over Leydig, Sertoli, and germ cells. In contrast, high autofluorescence signal from the WT mice was limited to interstitial Leydig cells, as expected (30).

Because the two transgenes were simultaneously expressed at detectable levels in testes, this tissue was chosen for subsequent characterization. First, the functionality of the β_2AR-*R*luc was assessed by monitoring the β_2AR-promoted adenylyl cyclase activity in testis membrane preparations derived from either WT or β_2AR-1/βarr2-1 mice. As shown in **Fig. 5*A***, the ability of the β-adrenergic agonist isoproterenol to stimulate the production of cAMP was significantly greater (3.3-fold) in the double-transgenic mice, whereas the ability of a direct activator of the stimulatory G protein, aluminum fluoride, was only marginally different (1.2-fold), indicating that the β_2AR-*R*luc fusion construct was functional and conferred increased β-adrenergic signaling efficacy. The selectivity of the activation was confirmed by the ability of alprenolol, a β-adrenergic antagonist, to block the isoproterenol-stimulated response. Also, a dose-response curve of the isoproterenol-stimulated cAMP production (Fig. 5*B*) revealed an EC_{50} of 117 ± 31 nM, which is similar to values previously reported for β_2AR-stimulated adenylyl cyclase activity (31). The receptor transgene expressed in the testes thus preserved functional properties comparable to that of the endogenous receptor. The barely detectable isoproterenol-stimulated cAMP production observed in testis membrane preparations derived from WT mice is consistent with the very low level of endogenous β_2AR detected in this tissue (~12 fmol/mg protein; Fig. 3*B*).

BRET monitoring of βarrestin recruitment to the β_2AR

To establish that BRET can be used to study protein-protein interactions in cells derived from transgenic animals expressing the proper biosensors, we monitored the BRET signal between β_2AR-*R*luc and GFP2-βarr in freshly dispersed testis cells from β_2AR-1/βarr2-1 double-transgenic mice. The BRET signal was determined as the ratio of light emitted at 515 ± 15 nm (GFP2 channel) over that emitted at 410 ± 40 nm (*R*luc channel) 2 min following the addition of the *R*luc substrate DeepBlueC coelenterazine. The background BRET signal (originating from the overflow of luciferase signal detected in the GFP2 emission channel) was determined by measuring BRET in testis cells derived from transgenic mice expressing only the energy donor β_2AR-*R*luc (β_2AR-1). As shown in **Fig. 6*A***, the basal BRET signal observed in the β_2AR-1/βarr2-1 mouse cells was not significantly different from the background BRET detected in the β_2AR-1 derived cells, indicating that, as expected, no constitutive recruitment of GFP2-βarr2 to β_2AR-*R*luc was observed. However, stimulation of the receptor with isoproterenol led to a significant increase in BRET in the β_2AR-1/βarr2-1 mouse cells without affecting the background BRET,

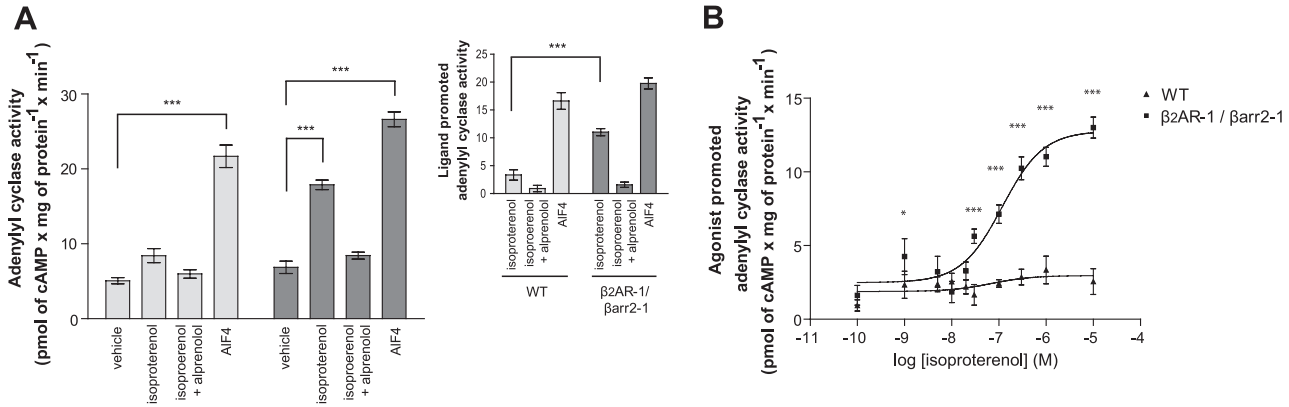

Figure 5. Receptor-promoted adenylyl cyclase activity in β_2AR-1/βarr2-1 and WT mice. *A*) Membrane preparations from testes of β_2AR-1/βarr2-1 and WT mice were incubated for 20 min with 1 μM isoproterenol, 100 μM alprenolol + 1 μM isoproterenol, or ALF4 (10 mM NaF and 10 μM AlCl3). Inset: adenylyl cyclase activity represented as ligand-promoted stimulation. *B*) Concentration-dependent isoproterenol-stimulated adenylyl cyclase activity in testes membrane preparations from β_2AR-1/βarr2-1 and WT mice. EC_{50} for isoproterenol stimulation is 117 ± 31 nM for β_2AR-1/βarr2-1 double-transgenic mice. Basal adenylyl cyclase activities were 6.853 ± 0.824 and 5.074 ± 0.413 pmol·mg^{-1}·min^{-1} for β_2AR-1/βarr2-1 and WT mice, respectively. Results are expressed as means ± SE of measurements performed in 3 independent experiments. $^*P < 0.05$, $^{***}P < 0.001$ *vs.* WT; ANOVA with Tukey *post hoc* analysis.

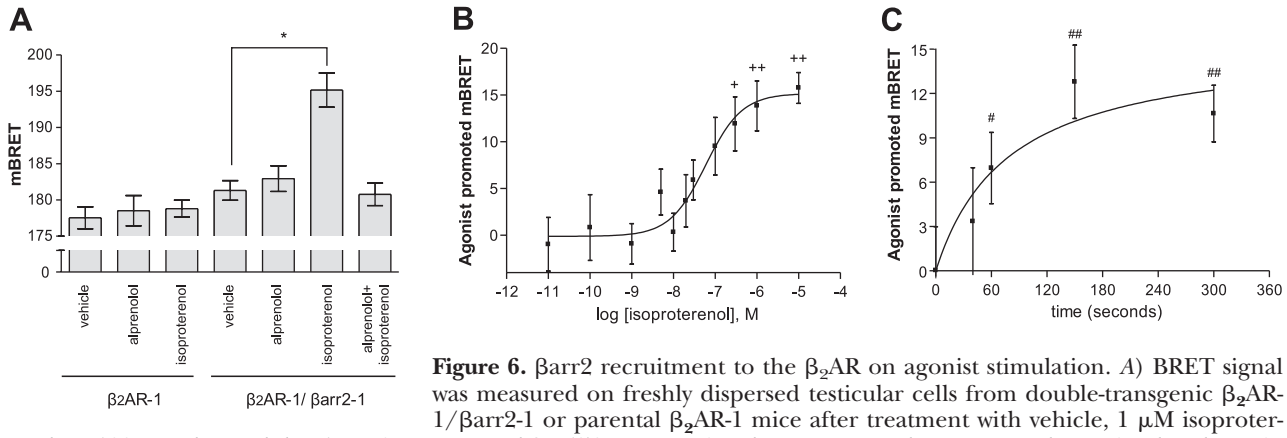

Figure 6. βarr2 recruitment to the β_2AR on agonist stimulation. *A*) BRET signal was measured on freshly dispersed testicular cells from double-transgenic β_2AR-1/βarr2-1 or parental β_2AR-1 mice after treatment with vehicle, 1 μM isoproterenol, or 100 μM alprenolol + 1 μM isoproterenol for 150 s. BRET signals are expressed as mBRET, determined as the ratio of acceptor emission (500–530 nm)/donor emission (370–450 nm) × 1000, and represent means ± SE of 6 independent experiments. $^{*}P < 0.05$; ANOVA with Tukey *post hoc* analysis. *B*) Concentration-response curve of the recruitment of βarr2 to the β_2AR measured by BRET. Data are expressed as means ± SE of 4 to 6 independent experiments. $^{+}P < 0.05$, $^{++}P < 0.01$ *vs.* baseline; ANOVA with Dunnett *post hoc* analysis. *C*) Kinetics of the recruitment of βarr2 to the β_2AR following isoproterenol stimulation, measured by BRET. Data are expressed as means ± SE of 3 independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ *vs.* background in corresponding control; Student's *t* test.

indicating that the agonist-promoted recruitment of βarr2 to the β_2AR could be detected in living cells from the transgenic mice. The isoproterenol-promoted increase in BRET was completely blocked by the antagonist alprenolol, which on its own had no effect on the signal, confirming the pharmacological selectivity of the ligand-promoted BRET increase. A BRET dose-response curve of the isoproterenol-stimulated β_2AR-*R*luc/GFP2-βarr2 mouse cells revealed an EC_{50} of 60 ± 21 nM (Fig. 6*B*), similar to the value observed for the agonist-stimulated cAMP production (Fig. 5*B*). The BRET increase was time-dependent, reaching statistical significance as early as 60 s following the addition of isoproterenol and remaining stable for ≥5 min (Fig. 6*C*), thus giving a kinetic compatible with the recruitment of βarr2 to the β_2AR as documented in heterologous expression systems (22, 32–34).

DISCUSSION

The present study constitutes the first proof of principle that BRET assays can be used to monitor protein-protein interactions in cells derived from transgenic mice. Our approach contrasts with previous studies in which cells heterologously transfected with BRET biosensors were implanted in an animal (19, 35). Although these studies clearly demonstrated that BRET can be detected in living animals, they were not designed to monitor physiologically relevant protein-protein interactions in their normal environment, since either fusion protein directly linking the donor and the acceptor (33) or artificial dimerizing systems (18, 33) were used. The present study demonstrates that regulated interactions between physiologically relevant proteins can be monitored in cells from mice transgenically expressing BRET donor and acceptor. This clearly establishes that the levels of energy donor and acceptor needed to measure BRET can be attained by transgenesis, at least in some tissues. Our study also demonstrates that fusion of GFP2 and *R*luc to the protein of interest did not grossly affect their normal function. Indeed, the concentration-dependence to agonist stimulation and kinetics of the β_2AR/βarr2 interactions observed in the cells from transgenic animals were identical to those previously observed in other systems with different methods (32–34). The functionality of the β_2AR-*R*luc was also confirmed by the potentiated β-adrenergic-stimulated adenylyl cyclase activity observed in the testis membranes from transgenic mice.

Also of interest, agonist-promoted BRET was observed at receptor expression levels that are within the physiological range observed in various tissues. As expected, the β_2AR expression in the testes of the transgenic mice was significantly higher than the endogenous level in this tissue. However, it only reached ~105 fmol/mg of protein, a receptor concentration that is lower than the endogenous levels of βAR observed in spleen (150 fmol/mg) and lung (277 fmol/mg). The expression level of the GFP2-βarr2, for its part, was significantly higher than the endogenous level detected, reaching ~60-fold the native levels in the testes. Whether BRET could have been detected with lower GFP2 expression levels is uncertain. However, it should be mentioned that the selection of the positive mouse lines involved the detection of fluorescence that overcomes the autofluorescence signal. This may favor the selection of highly expressing lines. Since BRET measures do not rely on light excitation, lower level of GFP2 could be amenable to BRET detection. This would, however, require an alternative detection mode (*e.g.*, Western blot analysis) of the GFP2 to identify the positive lines.

The fact that sufficient expression of the two partners permitting BRET was reached only in the testes was unfortunate since it limited the types of tissues where βarr recruitment to the receptor could be monitored. However, the expression of the β_2AR in the testes cannot be considered as physiologically irrelevant since low but detectable levels of endogenous βAR were

measured by radioligand binding in WT mouse testes, reaching levels (12±3 fmol/mg) that are not significantly different from what is detected in the heart (16±3 fmol/mg) where β_1 and β_2AR are known to have physiological roles (36). Consistent with a physiological role of the β_2AR in mouse testis is the report that the positive effect of catecholamine on testosterone production is mediated, in part, by this receptor subtype (37–39).

The reason for which the transgenes had an apparent tissue-dependent distribution clearly favoring the testes, despite the previously described ubiquitous ROSA26-directed expression pattern, is unclear. However, testes were also among the tissues showing the highest expression level of a transgene in a study using the same 0.8-kb ROSA26 promoter fragment, suggesting that it could be an intrinsic property of the promoter (24). Yet, this does not appear to be universal since, in some mouse lines, expression in lung, brain, and kidney was higher than in the testes, indicating that the site of insertion of the transgene in the genome could also be a factor contributing to the differential tissue distribution. The expression pattern of the GFP2-βarr2 appeared even more restricted than that of the β_2AR-*R*luc (Figs. 4 *vs.* 3). This may reflect that the expression achieved in the tissues other than the testes did not reach the levels needed for Western blot detection and were not sufficient to overcome the autofluorescence signal. Alternatively, subtle toxic effect of GFP has previously been suggested as a possible cause for lower expression levels of GFP-fused proteins (40). Future experiments using promoters that drive stronger ubiquitous or tissue-specific expression will be needed to determine whether BRET can be detected in tissues other than the testes.

The proof of principle that BRET can detect protein-protein interactions in cells from transgenic animals opens the possibility of monitoring the influence of both long-term and short-term *in vivo* manipulations (*e.g.*, drug treatment and disease evolution) on such specific protein-protein interactions. It also offers the perspective of monitoring changes in the dynamics of such interactions during animal development in a context where the cells, in which the interactions are monitored, were exposed to the normal physical and hormonal environment.

FRET could also be used to detect protein-protein interactions in living animals. However, the intrinsic autofluorescence of tissues, resulting from the required external excitation of the donor, will present a challenge that may force the expression of supraphysiological concentrations of the fluorophore-fused partners to generate appropriate signal to noise ratio. This drawback is avoided in BRET experiments since the donor energy is provided by a luminescence reaction and does not involve light excitation of the tissue. Because of the absorbance properties of tissues, the excitation and detection of fluorophores in whole animals will also represent technical challenges for *in vivo* FRET experiments. In BRET experiments, the delivery of the substrate to the luminescent donor could also be perceived as a problem. However, several studies have shown that systemic administration of coelenterazine allowed the detection of *R*luc luminescence deep in animal tissues (41), thus suggesting that the penetration of the substrate would not represent a problem for *in vivo* BRET experiments. The detection of the BRET signal *in situ* would nevertheless remain a challenge due to the lack of sensitivity of the current image detection systems.

Obviously, the fact that the BRET detection was performed in freshly dispersed cells does not provide the same advantages as monitoring these changes in whole animals. Yet, it provides significant advantages over heterologous expression in cultured cells, since freshly dispersed cells maintain much more of their native characteristics than immortalized cells or even primary cells that can rapidly undergo phenotypic changes. Although the level of light emitted by the energy donor and acceptor in the present study do not allow the use of BRET to detect protein-protein interactions *in situ*, our study can be seen as a first step toward this goal and represents a proof of principle, establishing that regulated real-time protein-protein interactions should be amenable to BRET analysis in living transgenic animals. The recent development of novel mutant forms of *R*luc with improved light emission intensity (35, 42) coupled to more sensitive detection system should make this goal achievable in a near future, opening the avenue to broad applications of BRET-based biosensors in transgenic mice. FJ

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