

BODIPY[®]-conjugated neuropeptide Y ligands: new fluorescent tools to tag Y₁, Y₂, Y₄ and Y₅ receptor subtypes

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1 N-terminal labelled fluorescent BODIPY[®]-NPY peptide analogues were tested in Y₁, Y₂, Y₄ and Y₅ receptor-binding assays performed in rat brain membrane preparations and HEK293 cells expressing the rat Y₁, Y₂, Y₄ and Y₅ receptors.

2 BODIPY[®]TMR/FL-[Leu³¹, Pro³⁴]NPY/PYY were able to compete for specific [¹²⁵I][Leu³¹, Pro³⁴]PYY-binding sites with an affinity similar to that observed for the native peptide at the Y₁ (K_i = 1–6 nM), Y₂ (K_i > 1000 nM), Y₄ (K_i = 10 nM) and Y₅ (K_i = 1–4 nM) receptor subtypes.

3 BODIPY[®]FL-PYY(3–36) was able to compete for specific Y₂ (K_i = 10 nM) and Y₅ (K_i = 30 nM) binding sites, but had almost no affinity in Y₁ and Y₄ assays.

4 BODIPY[®]FL-hPP was able to compete with high affinity (K_i; 1 and 15 nM) only in Y₄ and Y₅ receptor-binding assays.

5 BODIPY[®]TMR-[cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP and BODIPY[®]TMR-[hPP(1–17), Ala³¹, Aib³²]NPY were potent competitors only on specific Y₅-binding sites (K_i = 0.1–0.6 nM).

6 As expected, these fluorescent peptides inhibited forskolin-induced cAMP accumulation, demonstrating that they retained their agonist properties.

7 When tested in confocal microscopy imaging, fluorescent Y₁ and Y₅ agonists internalized in a time-dependent manner in Y₁ and Y₅ transfected cells, respectively.

8 These results demonstrate that BODIPY[®]-conjugated NPY analogues retain their selectivity, affinity and agonist properties for the Y₁, Y₂, Y₄ and Y₅ receptor subtypes, respectively. Thus, they represent novel tools to study and visualize NPY receptors in living cells.

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Abbreviations: BIBO3304, (*R*)-*N*-[[4-(aminocarbonylaminoethyl)-phenyl]methyl]-*N*2-(diphenylacetyl)-argininamide trifluoroacetate; BSA, bovine serum albumin; GR231118, homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-CONH₂; HEK293, human embryonic kidney cells; hPP, human pancreatic polypeptide; NPY, neuropeptide Y; PYY, peptide YY

Introduction

Neuropeptide Y (NPY), a 36-amino-acid peptide isolated from porcine brain (Tatemoto, 1982), shares high sequence homology with peptide YY (PYY) and the pancreatic polypeptides (PPs) (Tatemoto *et al.*, 1982). NPY is one of the most abundant peptides found in the central nervous system (CNS) (Chronwall *et al.*, 1985; De Quidt & Emson, 1986) and is believed to be involved in several physiological functions such as circadian rhythms, feeding and sexual behaviours, moods and memory, as well as neuroendocrine and cardiovascular controls (for reviews, see Inui, 1999; Vezzani *et al.*, 1999; Dumont *et al.*, 2000b; 2002; Kask *et al.*, 2002; Redrobe *et al.*, 2002; Kalra & Kalra, 2004; Carvajal *et al.*, 2005).

The biological effects of NPY and related peptides are mediated by the activation of specific plasma membrane receptors. Five NPY receptor subtypes have been cloned and designated as Y₁, Y₂, Y₄, Y₅ and y₆ (Michel *et al.*, 1998). They all belong to the type 1 G-protein-coupled receptors superfamily, and each one possesses a distinct pharmacological profile (Dumont *et al.*, 2002). The Y₁ subtype is preferentially activated by NPY, PYY and [Leu³¹, Pro³⁴]-substituted analogues, while C-terminal fragments bind with much lower affinities (Herzog *et al.*, 1992; Larhammar *et al.*, 1992). In contrast, the Y₂ subtype exhibits high affinity for NPY, PYY and C-terminal fragments such as NPY(2–36), NPY(3–36), PYY(3–36), NPY(13–36) and PYY(13–36), whereas [Leu³¹, Pro³⁴]-substituted analogues are less potent (Gerald *et al.*, 1995; Gehlert *et al.*, 1996a). The Y₄ subtype displays very high affinity for PPs (Bard *et al.*, 1995; Lundell *et al.*, 1995) and

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[Leu³¹, Pro³⁴]PYY (Gehlert *et al.*, 1996b), while the Y₅ subtype is characterized by its ability to bind NPY, PYY, [Leu³¹, Pro³⁴]-substituted analogues, human PP and long C-terminal fragments such as NPY(3–36) and PYY(3–36) (Gerald *et al.*, 1996). Recently, a number of antagonists have been developed for the Y₁ (BIBO3304, J-104870, G1264879A, LY357897 and J-115814), Y₂ (BIIE0246 and JNJ-5207787) and Y₅ (CGP71683A, NPY5RA, GW438014A and L-152,804) receptors (for reviews, see Berglund *et al.*, 2003a; Dumont *et al.*, 2004).

NPY receptor subtypes have been characterized and their differential distribution investigated in the brain using various radioligands, including [¹²⁵I][Leu³¹,Pro³⁴]PYY, [¹²⁵I]PYY(3–36), [¹²⁵I]hPP, [¹²⁵I]GR231118 and [¹²⁵I][cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP (Dumont *et al.*, 2000b; 2002; 2004). However, the use of radioligands does not easily allow for discrete cellular localization and, most importantly, for investigation in live cells of processes triggered by ligand–receptor interaction (internalization, trafficking, sequestration and recycling). While receptor antibodies represent important tools for detailed cellular localization, their application is limited in living cells (necessity of developing extracellular site-directed antibodies, assessment that the ligand–receptor–antibody and ligand–receptor complexes use identical internalization and trafficking pathways, etc.). Fluorescent peptide ligands represent an attractive alternative. However, a critical issue relates to the preservation of ligand affinity, selectivity and agonist properties. Fluorescent ligands have been developed for a variety of peptide receptors, including the opioids (Gaudriault *et al.*, 1997; Arttamangkul *et al.*, 2000), neurotensin (Faure *et al.*, 1994; 1995), substance P (Bennett & Simmons, 2001), somatostatin (Nouel *et al.*, 1997) and growth hormone-releasing hormone (Veyrat-Durebex *et al.*, 2005) using a variety of chromophores. The development of BODIPY[®] fluorophores has generated much interest mainly because (a) their spectral characteristics are often superior to those of conventional dyes, (b) they are relatively nonpolar, (c) the chromophore lacks ionic charge, (d) they are insensitive to solvent polarity and pH changes and (e) they exist in numerous wavelength versions (McGrath *et al.*, 1996).

Cyamin and fluorescein have been used to label various peptides, including NPY, [Pro³⁴]NPY and [Ahx(5–24)]NPY (Ingenhoven & Beck-Sickinger, 1997; Fabry *et al.*, 2000). However, high background was observed with these fluorescent NPY analogues in addition to the high pH sensitivity of these dyes and rapid photobleaching. To circumscribe these problems, NPY and PYY analogues bearing a single BODIPY[®] molecule at the N-terminus were developed here and characterized in Y₁, Y₂, Y₄ and Y₅ receptor preparations. We have already reported that BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY can be internalized in a time-dependent manner as do radiolabelled probes in human embryonic kidney (HEK293) cells transfected with the rat Y₁ receptor cDNA (Pheng *et al.*, 2003). In the present study, using competition-binding experiments and cAMP assays, we demonstrate that BODIPY[®] derivatives of NPY, [Leu³¹, Pro³⁴]NPY, [Leu³¹, Pro³⁴]PYY, PYY(3–36), hPP and the Y₅ receptor agonists, [hPP(1–17), Ala³¹, Aib³²]NPY and [cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP (Cabrele *et al.*, 2000; 2001) maintain their selectivity, high affinity and agonist properties for the targeted NPY receptor subtype. In addition, cell imaging using confocal microscopy reveals that these fluorescent probes can be used to visualize NPY receptors in living cells.

Methods

Materials

Male (200–250 g) Sprague–Dawley CD rats were obtained from Charles River Canada (St-Constant, QC, Canada) and kept on a 12-h light–dark cycle (light on at 0700 h) in temperature- and humidity-controlled rooms. Animals were fed standard laboratory chow and had free access to food and tap water. Animal protocols were approved by the McGill University Animal Care Committee in compliance with guidelines of the Canadian Council of Animal Care. All animals were killed between 0900 and 1030 h.

NPY receptor-transfected cells

HEK293 cells were obtained from Dr S.H. Shen Biotechnology Research Institute (Montreal, QC, Canada) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS) with antibiotics–antimycotics (penicillin G sodium, 100 U ml⁻¹; streptomycin sulphate, 100 µg ml⁻¹; amphotericin B, 0.25 µg ml⁻¹). Cultured cells were transfected with either of the rat Y₁, Y₂, Y₄ or Y₅ receptor cDNA using a calcium phosphate method (Tong *et al.*, 1995). Briefly, 125 µl of 2.5 M calcium phosphate was added to 1.125 ml water containing 50 µg of either rat Y₁, Y₂, Y₄ or Y₅ receptor cDNA, which was previously inserted in expressing pcDNA3 (Y₂ and Y₄) and pTR5-DC-GFP/TK/hygro (Y₁ and Y₅) vectors, and was slowly mixed with 1.25 ml 2 × HEPES buffer at pH 7.05 and left at room temperature for 20 min. The mixture was added to a 150 mm dish containing HEK293 cells at 30% confluence and returned to the incubator. The medium was changed the next morning. Transiently transfected HEK293 cells (Y₂ and Y₄) were used 48–72 h after transfection, while stably transfected HEK293 cells (Y₁ and Y₅) were kept at –80°C (10% DMSO in FCS) until use. Stably transfected HEK293 cells expressing Y₁ and Y₅ subtypes cells were cultured in a 150-mm dish containing DMEM supplemented with 10% FCS, antibiotics–antimycotics, doxycycline (25 µg ml⁻¹) and hygromycin (125 µg ml⁻¹). Cells transfected with this vector are GFP-positive and levels of GFP protein expression were used as an indicator of Y₁ and Y₅ receptor expression levels. In these cells, both proteins (GFP and NPY receptor) are transcribed in opposite directions under the control of a bidirectional promoter stimulated in the presence of doxycycline. SK-N-MC cells were purchased from the American Tissue Culture Collection (ATCC) (Manassa, VA, U.S.A.) and plated in a 150-mm dish containing DMEM, 10% FCS containing antibiotic–antimycotic substances. Those cells are endogenously expressing Y₁ receptors.

Membrane preparations from transfected cells and brain tissues

At confluence, the transient (Y₂ and Y₄) or stably (Y₁ and Y₅) transfected HEK293 cells were washed with Krebs Ringer phosphate (KRP) buffer, pH 7.4, and scraped. Detached cells were then centrifuged at 400 g for 10 min and the pellet was washed with KRP buffer (pH 7.4) of the following composition: NaCl (120 mM), KCl (4.7 mM), CaCl₂ (2.2 mM), KH₂PO₄ (1.2 mM), MgSO₄ (1.2 mM), dextrose (5.5 mM) and NaHCO₃

(25 mM), recentrifuged twice, and resuspended in 8 ml of KRP buffer, pH 7.4 ($1-2 \times 10^6$ cells ml⁻¹) and used for receptor-binding assays.

Rat brain membranes were prepared as described previously (Dumont *et al.*, 1995). Briefly, rats were killed by decapitation and their brains rapidly removed and homogenized in KRP buffer at pH 7.4 using a Brinkman polytron (at setting 6 for 15–20 s). Homogenates were centrifuged at $49,000 \times g$ for 20 min, supernatants discarded and pellets washed, resuspended and recentrifuged twice. Pellets were then resuspended in KRP to get a final protein concentration of 2–6 mg ml⁻¹.

Radioligand receptor-binding assays

All binding assays were initiated by adding 100 μ l of membrane or cell preparations in a final volume of 500 μ l of KRP containing 0.1% (w v⁻¹) bovine serum albumin (BSA), 0.05% (w v⁻¹) bacitracin, 30–35 pM of radiolabelled peptides in the presence and absence of various competitors at concentrations ranging from 10^{-12} to 10^{-6} M. In rat brain membrane preparations, NPY receptors were investigated using [¹²⁵I][Leu³¹, Pro³⁴]PYY for the Y₁, Y₄ and Y₅ subtypes and [¹²⁵I]GR231118 for Y₁-like receptors, [¹²⁵I]PYY(3–36) for the Y₂-like subtype, [¹²⁵I]hPP for Y₄- and Y₅-like sites (Dumont *et al.*, 1995; 2005; Dumont & Quirion, 2000) while Y₅-like binding was also performed using [¹²⁵I][Leu³¹, Pro³⁴]PYY in the presence of 1 μ M BIBO3304 (Dumont *et al.*, 1998). [¹²⁵I]GR231118 and [¹²⁵I][Leu³¹, Pro³⁴]PYY were also used in HEK293 cells expressing the rat Y₁ or Y₅ receptor, respectively. Binding in HEK293 cells expressing the rat Y₂ or Y₄ receptor was performed using [¹²⁵I]PYY(3–36) and [¹²⁵I]hPP, respectively. Nonspecific binding was determined in the presence of 1 μ M of the same unlabeled peptide, that is, [Leu³¹, Pro³⁴]PYY, PYY(3–36), GR231118 or hPP (specific binding representing 75–90% of total binding). Following a 2-h incubation, the binding reaction was terminated by rapid filtration through Schleicher and Schuell #32 glass filters (previously soaked in 1.0% polyethyleneimine) using a cell harvester filtering apparatus (Brandel Instruments, Gaithersburg, MD, U.S.A.). Filters were rinsed three times with 3 ml of cold KRP and the radioactivity remaining on filters was quantified using a gamma counter with 85% efficiency (Packard Instruments).

Cyclic AMP assay

Cyclic AMP accumulation was determined in HEK293 cells expressing either the rat Y₁, Y₂, Y₄ or Y₅ receptor. At confluence, cells were washed with Krebs buffer and resuspended in 5 ml Krebs buffer (pH 7.4). In all, 100 μ l of cell suspension (150,000 cells) was preincubated in a final volume of 0.5 ml of Krebs containing 0.1% BSA and 0.1 mM 3-isobutyl-1-methylxanthine at 37°C for 30 min, and then incubated in the presence of 10 μ M forskolin and increasing concentrations of peptides (0.1–1000 nM) for 30 min at 37°C (Gerald *et al.*, 1996; Cabrele *et al.*, 2000). Incubation was stopped by adding 100 μ l of 1 M HCl. Cell lysis was performed by incubation for 3 min at 100°C, followed by centrifugation. Then, 0.5 ml of the supernatant was lyophilized and cAMP determined using a cAMP radioimmunoassay (Amersham, Mississauga, ON, Canada) performed according to the manufacturer's instructions.

Visualization of fluorescent probes

Transfected HEK293 cells and SK-N-MC cells (endogenously expressing Y₁ receptors) were plated as described above, except that they were cultured in a 150-mm petri dish on polylysine-coated glass coverslips. At 80% confluence, cells were washed twice with KRP buffer and incubated in fresh KRP supplemented with 0.1% BSA, 0.05% bacitracin and 1, 5 or 20 nM fluorescent peptides at 37°C for 5, 15 and 45 min. Nonspecific binding was determined in the presence of 1 μ M nonfluorescent parent peptide. At the end of the incubation period, cells were rapidly washed twice in cold KRP and air-dried (Pheng *et al.*, 2003). Preliminary experiments using cells fixed with 4% paraformaldehyde give similar results and cellular integrity (data not shown). Labelled cells were examined under a Nikon confocal microscope system (PCM200) equipped with argon (488 nm) and HeNe (543 nm) lasers. For BODIPY[®]FL peptides and GFP the argon laser (488/510 nm) was used, while for BODIPY[®]TMR, we used the HeNe laser (543/580 nm). Specificity of the fluorescent signal detected was confirmed with the disappearance of the fluorescent signal with the other laser. All pictures were captured using identical parameters in order to prevent any false-positive results, and were saved as tiff files.

Data analysis

All binding experiments were repeated two to five times, each in triplicate, and results expressed as percentage of specific binding. Cyclic AMP assays were performed three times, each in duplicate. Confocal microscope visualization was performed on three different experiments. IC₅₀ values (i.e. concentration of unlabelled peptide required to compete for 50% of specific binding of the radioligand) of the various analogues used in our study were calculated from the competition-binding assays data using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, U.S.A.) with a fit to one- to two-site competition curves. K_i values were determined according to the equation of Cheng and Prusoff ($K_i = IC_{50}/(1 + C/K_D)$) (Cheng & Prusoff, 1973). As all binding experiments were performed at concentrations of radioligand equal to or below 0.1 K_D of the radiolabelled probe, this usually generates K_i values similar to IC₅₀. EC₅₀ values represent the concentrations of the agonist that were able to inhibit 50% of 10 μ M forskolin-induced cyclic AMP accumulation, calculated from a concentration–response curve and analysed using GraphPad Prism sigmoid curve function. Student's *t*-test was used to assess statistical differences between native and BODIPY[®]-conjugated NPY analogues ($P < 0.05$ being considered significant).

Reagents

NPY, [Leu³¹, Pro³⁴]NPY, [Leu³¹, Pro³⁴]PYY, PYY(3–36) and hPP were synthesized by one of us (AF) as described previously (Forest *et al.*, 1990), while [hPP(1–17), Ala³¹, Aib³²]NPY and [cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP were generously provided by Dr Annette Beck-Sickenger (University of Leipzig, Germany). High-quality N-terminus-labelled BODIPY[®]TMR and BODIPY[®]FL-peptides were used. BODIPY[®]TMR-NPY, BODIPY[®]TMR-PYY(3–36), BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY, BODIPY[®]TMR-[hPP(1–17), Ala³¹, Aib³²]NPY and BODI-

PYY[®]TMR-[cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP were synthesized by Perkin-Elmer (Advanced BioConcept, Montreal, QC, Canada). BODIPY[®]FL-[Leu³¹, Pro³⁴]PYY, BODIPY[®]FL-PYY(3–36) and BODIPY[®]FL-hPP were synthesized by one of us (PG) (Veyrat-Durebex *et al.*, 2005). ((*R*)-*N*-[[4-(aminocarbonylaminoethyl)-phenyl]methyl]-*N*-(diphenylacetyl)-argininamide trifluoro-acetate), known as BIBO3304, was provided by Boehringer Ingelheim (Germany), while homodimeric Ile–Glu–Pro–Dpr–Tyr–Arg–Leu–Arg–Tyr–CONH₂ (firstly known as 1229U91, GW1229 and now GR231118) was a gift from Glaxo Wellcome (Research Triangle Park, NC, U.S.A.). BSA and Iodine-125 were obtained from MP Biochemical/ICN (Montreal, QC, Canada) and bacitracin was purchased from Sigma/Aldrich (St-Louis, MI, U.S.A.). Schleicher and Schuell #32 glass filters were obtained from VWR International (Montréal, QC, Canada). All tissue culture media, antibiotics and reagents were obtained from Gibco-BRL (Burlington, ON, Canada). The rat Y₁, Y₂, Y₄ and Y₅ receptor cDNA were generously provided by Dr H. Herzog (Sidney, Australia), The expression vectors, pcDNA3 and pTR5-DC-GFP/TK/hygro were obtained from Invitrogen (San Diego, CA, U.S.A.) and Dr Dick D. Moose (Biotechnology Institute, Montreal, QC, Canada), respectively. All other chemicals were of analytical grade and obtained from Fisher Scientific (Montreal, QC, Canada) or Sigma/Aldrich (St-Louis, MI, U.S.A.).

Radiolabelling of [Leu³¹, Pro³⁴]PYY, PYY(3–36), hPP and GR231118 was performed using the chloramine T method as described previously (Dumont *et al.*, 1995; Dumont & Quirion, 2000). The specific activity was assumed to be of theoretical value (2000 Ci mmol⁻¹).

Results

The ability of various BODIPY[®]-conjugated NPY-related peptides to compete against NPY Y₁, Y₂, Y₄ and Y₅ receptors was evaluated in HEK293 cells transfected with the Y₁, Y₂, Y₄ or Y₅ receptor cDNA and in rat brain homogenates. As shown

in Table 1, the addition of the chromophore BODIPY[®]TMR at the N-terminus of NPY and [Leu³¹, Pro³⁴]NPY had little effect on the ability of BODIPY[®]TMR-NPY and BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY to compete against specific [¹²⁵I]GR231118 binding in a Y₁ receptor-binding assay. In fact, these two fluorescent peptides competed against specific [¹²⁵I]GR231118 binding with K_i values slightly higher or lower to those observed for the nonfluorescent probe in HEK293 cells transfected with the Y₁ receptor cDNA: NPY (K_i = 4.8 nM), BODIPY[®]TMR-NPY (K_i = 15 nM), [Leu³¹, Pro³⁴]NPY (K_i = 2.4 nM) and BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY (K_i = 0.8 nM) (Table 1). The addition of BODIPY[®]FL at the N-terminus of [Leu³¹, Pro³⁴]PYY resulted in a lower K_i value in this receptor-binding assay (K_i of 0.4 vs 6.1 nM for the fluorescent peptide) (Table 1). Additionally and as seen for the nonfluorescent corresponding peptides, BODIPY[®]FL-PYY(3–36), BODIPY[®]FL-hPP, BODIPY[®]TMR-[hPP(1–17), Ala³¹, Aib³²]NPY and BODIPY[®]TMR-[cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP were much less potent or inactive in competing against specific [¹²⁵I]GR231118 binding in HEK293 cells transfected with the rat Y₁ receptor cDNA (Table 1).

The respective profiles of BODIPY[®]TMR-NPY, BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY, BODIPY[®]FL-[Leu³¹, Pro³⁴]PYY, BODIPY[®]FL-PYY(3–36), BODIPY[®]FL-hPP, BODIPY[®]TMR-[hPP(1–17), Ala³¹, Aib³²]NPY and BODIPY[®]TMR-[cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP in a Y₂ receptor-binding assay using transfected HEK293 cells is shown in Table 2. The addition of BODIPY[®] in the N-terminal region of NPY and PYY(3–36) resulted in some changes in apparent affinities. NPY and PYY(3–36) competed against specific [¹²⁵I]PYY(3–36) binding with K_i's of 3.8 and 0.6 nM, respectively, while BODIPY[®]TMR-NPY and BODIPY[®]FL-PYY(3–36) had a K_i of 10 nM (Table 2). As expected, fluorescent [Leu³¹, Pro³⁴]NPY, [Leu³¹, Pro³⁴]PYY, hPP, [hPP(1–17), Ala³¹, Aib³²]NPY and [cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP were much less potent in competing against specific [¹²⁵I]PYY(3–36) binding, as also seen for the nonfluorescent homologues (Table 2).

Table 1 Competition-binding parameters of BODIPY[®]-labelled and native peptides of the NPY family in various NPY Y₁ receptor assays

Competitors	Y ₁ HEK293-transfected cells		Rat brain K _i (nM)	
	[¹²⁵ I]GR231118 K _i (nM)	[¹²⁵ I][Leu ³¹ , Pro ³⁴]PYY	[¹²⁵ I]GR231118	[¹²⁵ I]GR231118
NPY	4.8 ± 1 (5) ^a		2.1 ± 0.8 (5)	48 ± 10 (5)
BODIPY [®] TMR-NPY	15 ± 4 (4) [*]		5.3 ± 2.4 (4)	85 ± 25 (5)
[Leu ³¹ , Pro ³⁴]NPY	2.4 ± 0.6 (5)		2.4 ± 1.1 (5)	37 ± 6 (5)
BODIPY [®] TMR-[Leu ³¹ , Pro ³⁴]NPY	0.8 ± 0.3 (4) [*]		2.9 ± 0.7 (4)	55 ± 9 (5)
[Leu ³¹ , Pro ³⁴]PYY	0.4 ± 0.1 (5)		2.3 ± 1.1 (5)	24 ± 5 (5)
BODIPY [®] FL-[Leu ³¹ , Pro ³⁴]PYY	6.1 ± 1.5 (4) [*]		35 ± 5 (5) [*]	73 ± 15 (5) [*]
PYY(3–36)	150 ± 35 (3)		140 ± 40 (4)	> 1000 (3)
BODIPY [®] FL-PYY(3–36)	380 ± 70 (3) [*]		100 ± 25 (3)	> 1000 (2)
hPP	180 ± 65 (3)		32 ± 10 (5)	> 1000 (3)
BODIPY [®] FL-hPP	> 500 (3) [*]		> 500 (3) [*]	> 1000 (2)
[hPP(1–17), Ala ³¹ , Aib ³²]NPY	> 1000 (2)		> 1000 (4)	> 1000 (3)
BODIPY [®] TMR-[hPP(1–17), Ala ³¹ , Aib ³²]NPY	> 1000 (2)		> 1000 (3)	> 1000 (2)
[cPP(1–7), NPY(19–23), Ala ³¹ , Aib ³² , Gln ³⁴]hPP	> 1000 (2)		> 1000 (4)	> 1000 (3)
BODIPY [®] TMR-[cPP(1–7), NPY(19–23), Ala ³¹ , Aib ³² , Gln ³⁴]hPP	> 1000 (2)		> 1000 (3)	> 1000 (2)

Data are the mean ± s.e.m. of two to five determinations, each performed in triplicate.

^aNumber in parenthesis represents the number of determinations. K_i represents the concentration of competitor needed to inhibit 50% specifically bound [¹²⁵I][Leu³¹, Pro³⁴]PYY or [¹²⁵I]GR231118. *P < 0.05, fluorescent vs nonfluorescent peptide.

Table 2 Competition-binding parameters of BODIPY[®]-labelled and native peptides of the NPY family in various NPY Y₂ receptor assays

Competitors	Y ₂ HEK293-transfected cells K _i (nM)	Rat brain [¹²⁵ I]PYY(3–36) K _i (nM)
NPY	3.8 ± 1.6 (3) ^a	2.9 ± 1.0 (5)
BODIPY [®] TMR-NPY	10 ± 4 (3)*	16 ± 6 (4)*
[Leu ³¹ , Pro ³⁴]NPY	> 1000 (3)	200 ± 45 (5)
BODIPY [®] TMR-[Leu ³¹ , Pro ³⁴]NPY	> 1000 (3)	> 1000 (3)
[Leu ³¹ , Pro ³⁴]PYY	650 ± 110 (3)	165 ± 30 (5)
BODIPY [®] FL-[Leu ³¹ , Pro ³⁴]PYY	> 1000 (3)	> 1000 (3)
PYY(3–36)	0.6 ± 0.2 (3)	1.2 ± 0.3 (5)
BODIPY [®] FL-PYY(3–36)	10 ± 3 (3)*	17 ± 5 (4)*
hPP	> 1000 (3)	> 1000 (3)
BODIPY [®] FL-hPP	> 1000 (3)	> 1000 (2)
[hPP(1–17), Ala ³¹ , Aib ³²]NPY	> 1000 (3)	> 1000 (3)
BODIPY [®] TMR-[hPP(1–17), Ala ³¹ , Aib ³²]NPY	> 1000 (3)	> 1000 (2)
[cPP(1–7), NPY(19–23), Ala ³¹ , Aib ³² , Gln ³⁴]hPP	> 1000 (3)	> 1000 (3)
BODIPY [®] TMR-[cPP(1–7), NPY(19–23), Ala ³¹ , Aib ³² , Gln ³⁴]hPP	> 1000 (3)	> 1000 (2)

Data are the mean ± s.e.m. of two to five determinations, each performed in triplicate.

^aNumber in parenthesis represents the number of determinations. K_i represents the concentration of competitor needed to inhibit 50% specifically bound [¹²⁵I]PYY(3–36). *P < 0.05, fluorescent vs nonfluorescent peptide.

BODIPY[®]FL-[Leu³¹, Pro³⁴]PYY, BODIPY[®]FL-PYY(3–36) and BODIPY[®]FL-hPP were also tested in a Y₄ receptor-binding assay. In Y₄ receptor-transfected HEK293 cells, only [Leu³¹, Pro³⁴]PYY, BODIPY[®]FL-[Leu³¹, Pro³⁴]PYY, hPP and BODIPY[®]FL-hPP were able to compete against specific [¹²⁵I]hPP binding with K_i values in the nM range, while NPY, PYY(3–36), BODIPY[®]TMR-NPY and BODIPY[®]FL-PYY(3–36) were much less potent (Table 3).

The ability of native and BODIPY[®]-conjugated peptide analogues to compete against specific [¹²⁵I][Leu³¹, Pro³⁴]PYY binding in HEK293 cells transfected with the rat Y₅ receptor cDNA was evaluated next. [hPP(1–17), Ala³¹, Aib³²]NPY, [cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP and their BODIPY[®]-conjugated analogues were the most potent analogues to compete against specific [¹²⁵I][Leu³¹, Pro³⁴]PYY binding with K_i values in the low nM range (Table 4). Additionally, and as previously reported on the Y₅ receptor subtype, NPY, [Leu³¹, Pro³⁴]NPY, [Leu³¹, Pro³⁴]PYY, PYY(3–36) and hPP were able to compete against specific [¹²⁵I][Leu³¹, Pro³⁴]PYY binding with K_i values between 1 and 25 nM (Table 4). The addition of BODIPY[®] at the N-terminus of these peptides had no or little effect on their ability to compete against specific [¹²⁵I][Leu³¹, Pro³⁴]PYY/Y₅ binding, except for BODIPY[®]FL-hPP (Table 4).

Using HEK293 cells transfected with the rat Y₁, Y₂ Y₄ or Y₅ receptor cDNA, the agonist property of BODIPY[®]-conjugated analogues was investigated next by measuring their ability to inhibit forskolin-induced cAMP accumulation. NPY inhibited forskolin-stimulated cAMP accumulation with EC₅₀ values of 5, 10 and 19 nM in cells transfected with the rat Y₁, Y₂ or Y₅ receptor cDNA, respectively (Table 5). The BODIPY[®]-

Table 3 Competition-binding parameters of BODIPY[®]-labelled and native peptides of the NPY family in various NPY Y₄ receptor assays

Competitors	Y ₄ HEK293-transfected cells K _i (nM)	Rat brain [¹²⁵ I]hPP K _i (nM)
NPY	140 ± 30 (3) ^a	10.0 ± 1.5 (5)
BODIPY [®] TMR-NPY	875 ± 180 (3)*	56.0 ± 12.0 (4)*
[Leu ³¹ , Pro ³⁴]PYY	5.0 ± 1 (3)	2.0 ± 0.6 (5)
BODIPY [®] FL-[Leu ³¹ , Pro ³⁴]PYY	10 ± 3 (3)	1.0 ± 0.4 (4)
PYY(3–36)	350 ± 120 (3)	2.0 ± 0.5 (5)
BODIPY [®] FL-PYY(3–36)	> 1000 (3)	2.0 ± 0.3 (4)
hPP	5.0 ± 1 (3)	0.6 ± 0.2 (5)
BODIPY [®] FL-hPP	10 ± 3 (3)	3.0 ± 0.6 (4)*

Data are the mean ± s.e.m. of three to five determinations, each performed in triplicate.

^aNumber in parenthesis represents the number of determinations. K_i represents the concentration of competitor needed to inhibit 50% specifically bound [¹²⁵I]hPP. *P < 0.05, fluorescent vs nonfluorescent peptide.

Table 4 Competition-binding parameters of BODIPY[®]-labelled and native peptides of the NPY family in various NPY Y₅-like receptor assays

Competitors	Y ₅ HEK293-transfected cells K _i (nM)	Rat brain K _i (nM)
NPY	1.3 ± 0.4 (3) ^a	4.0 ± 1.9 (5)
BODIPY [®] TMR-NPY	2.0 ± 0.8 (3)	2.9 ± 1.5 (4)
[Leu ³¹ , Pro ³⁴]NPY	5.2 ± 0.3 (3)	7.7 ± 2.8 (5)
BODIPY [®] TMR-[Leu ³¹ , Pro ³⁴]NPY	1.3 ± 0.4 (3)*	2.7 ± 0.3 (4)
[Leu ³¹ , Pro ³⁴]PYY	3.2 ± 0.8 (3)	3.5 ± 0.9 (5)
BODIPY [®] FL-[Leu ³¹ , Pro ³⁴]PYY	3.0 ± 0.7 (3)	4.0 ± 1.0 (4)
PYY(3–36)	25 ± 6 (3)	14 ± 2 (5)
BODIPY [®] FL-PYY(3–36)	30 ± 5 (3)	12.0 ± 1.0 (4)
hPP	1.6 ± 0.4 (3)	2.0 ± 0.7 (5)
BODIPY [®] FL-hPP	15 ± 3 (3)*	26 ± 10 (4)*
[hPP(1–17), Ala ³¹ , Aib ³²]NPY	0.6 ± 0.2 (3)	0.7 ± 0.2 (5)
BODIPY [®] TMR-[hPP(1–17), Ala ³¹ , Aib ³²]NPY	0.4 ± 0.1 (3)	0.6 ± 0.1 (4)
[cPP(1–7), NPY(19–23), Ala ³¹ , Aib ³² , Gln ³⁴]hPP	0.1 ± 0.04 (3)	0.15 ± 0.03 (5)
BODIPY [®] TMR-[cPP(1–7), NPY(19–23), Ala ³¹ , Aib ³² , Gln ³⁴]hPP	0.1 ± 0.03 (3)	0.2 ± 0.1 (4)

Data are the mean ± s.e.m. of three to five determinations, each performed in triplicate.

^aNumber in parenthesis represents the number of determinations. K_i represents the concentration of competitor needed to inhibit 50% specifically bound [¹²⁵I][Leu³¹, Pro³⁴]PYY in the presence of BIBO3304 in order to mask Y₁ receptors. *P < 0.05, fluorescent vs nonfluorescent peptide.

conjugated analogue, BODIPY[®]TMR-NPY, was able to inhibit forskolin-stimulated cAMP accumulation with EC₅₀ values of 40, 65 and 25 nM in Y₁, Y₂ and Y₅ assays, respectively (Table 5). BODIPY[®]FL-[Leu³¹, Pro³⁴]PYY displayed similar potency as the nonconjugated peptide to inhibit cAMP accumulation in Y₅-transfected cells (Table 5), while being somewhat less potent in Y₄ and Y₁ assays (Table 5). BODIPY[®]FL-PYY(3–36) was less potent than PYY(3–36) in cells transfected with the rat Y₂ receptor cDNA to inhibit forskolin-induced cAMP production (Table 5), while it had a

Table 5 Potencies of various BODIPY[®]-labelled and native peptides of the NPY family in HEK293 cells transfected with either of the rat Y₁, Y₂, Y₄ or Y₅ receptor cDNA to inhibit forskolin-induced cAMP accumulation

Peptides	cAMP assay in HEK293-transfected cells (EC ₅₀) (nM)			
	Y ₁	Y ₂	Y ₄	Y ₅
NPY	5 ± 1 (3) ^a	10 ± 2 (3)	ND	19 ± 2 (3)
BODIPY [®] TMR-NPY	40 ± 5 (3)*	65 ± 15 (3)*	ND	25 ± 3 (3)
[Leu ³¹ , Pro ³⁴]NPY	ND	ND	ND	30 ± 5 (3)
BODIPY [®] TMR-[Leu ³¹ , Pro ³⁴]NPY	ND	ND	ND	20 ± 4 (3)
[Leu ³¹ , Pro ³⁴]PYY	3.0 ± 0.6 (3)	ND	35 ± 4 (3)	15 ± 4 (3)
BODIPY [®] FL-[Leu ³¹ , Pro ³⁴]PYY	32 ± 8 (3)*	ND	80 ± 16 (3)*	12 ± 3 (3)
PYY(3–36)	ND	7 ± 2 (3)	ND	50 ± 6 (3)
BODIPY [®] FL-PYY(3–36)	ND	45 ± 9 (3)*	ND	60 ± 8 (3)
hPP	ND	ND	7 ± 2 (3)	9 ± 3 (3)
BODIPY [®] FL-hPP	ND	ND	15 ± 3 (3)	60 ± 15 (3)*
[hPP(1–17), Ala ³¹ , Aib ³²]NPY	ND	ND	ND	35 ± 4 (3)
BODIPY [®] TMR-[hPP(1–17), Ala ³¹ , Aib ³²]NPY	ND	ND	ND	40 ± 5 (3)
[cPP(1–7), NPY(19–23), Ala ³¹ , Aib ³² , Gln ³⁴]hPP	ND	ND	ND	22 ± 3 (3)
BODIPY [®] TMR-[cPP(1–7), NPY(19–23), Ala ³¹ , Aib ³² , Gln ³⁴]hPP	ND	ND	ND	30 ± 5 (3)

Data are the mean ± s.e.m. of three determinations, each performed in duplicate.

^aNumber in parenthesis represents the number of determinations. EC₅₀ represents the concentration of agonist needed to inhibit 50% of cAMP accumulation induced by forskolin. ND, not determined. *P < 0.05, fluorescent vs nonfluorescent peptide.

similar potency in a Y₅ assay (Table 5). BODIPY[®]-hPP was somewhat less potent than hPP to inhibit forskolin-induced cAMP accumulation in Y₄- and Y₅-transfected cells, while conjugated BODIPY[®]TMR Y₅ agonists such as BODIPY[®]TMR-[hPP(1–17), Ala³¹, Aib³²]NPY and BODIPY[®]TMR-[cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP were as potent as their homologues in cells transfected with the rat Y₅ receptor cDNA (Table 5). All fluorescent peptides tested in HEK293 cells transfected with either the rat Y₁, Y₂, Y₄ or Y₅ receptor cDNA were able to inhibit forskolin-induced cAMP accumulation at maximal levels similar to those observed for the nonfluorescent homologues (data not shown). In fact, all BODIPY[®]TMR and BODIPY[®]FL peptides maintained their full agonist properties.

The apparent affinity of native and BODIPY[®]-conjugated peptides for specific [¹²⁵I][Leu³¹, Pro³⁴]PYY (labelling Y₁, Y₄ and Y₅ subtypes), [¹²⁵I]GR231118 (Y₁ and Y₄ subtypes), [¹²⁵I]PYY(3–36) (Y₂ and Y₅ subtypes), [¹²⁵I]hPP (Y₄ and Y₅ subtypes) and [¹²⁵I][Leu³¹, Pro³⁴]PYY in the presence of the Y₁ blocker BIBO3304 (Y₄ and Y₅ subtypes) binding in rat brain homogenates was investigated next. As shown in Figure 1, BODIPY[®]TMR-NPY and BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY are the most potent competitors against specific [¹²⁵I][Leu³¹, Pro³⁴]PYY binding in rat brain homogenates. On the other hand, and as previously reported for Y₅-related molecules such as CGP71683A (Dumont *et al.*, 2000a) and the Y₅ peptide agonists [hPP(1–17), Ala³¹, Aib³²]NPY and [cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP (Dumont *et al.*, 2005), BODIPY[®]TMR-conjugated Y₅ peptides were able to compete for only 30% of specific sites recognized by [¹²⁵I][Leu³¹, Pro³⁴]PYY in rat brain membrane homogenates (Figure 1). We also evaluated the competition-binding profile of these peptides against specific [¹²⁵I]GR231118 binding, another Y₁ receptor-binding assay (Dumont & Quirion, 2000). GR231118 having Y₁ antagonist properties, it can recognize Y₁ receptors in various affinity states, contrasting with the radiolabelled agonist probe [¹²⁵I][Leu³¹, Pro³⁴]PYY, which mostly binds to the high, affinity state (Dumont & Quirion, 2000). Under these binding assay conditions, BODIPY[®]TMR-NPY, BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY and BODIPY[®]FL-[Leu³¹,

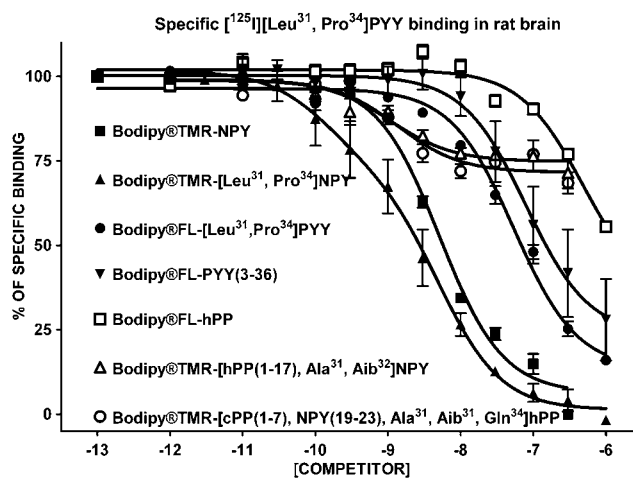


Figure 1 Competition-binding profiles of BODIPY[®]-labelled peptides against specific [¹²⁵I][Leu³¹, Pro³⁴]PYY-binding sites (Y₁, Y₄ and Y₅-like). Data are the mean ± s.e.m. of two to five determinations, each performed in triplicate.

Pro³⁴]PYY were the only analogues that were able to compete against specific [¹²⁵I]GR231118 binding in rat brain (Figure 2) but with K_i values 3–20 times lower than those observed when using [¹²⁵I][Leu³¹, Pro³⁴]PYY (Table 1). All other peptides, including native and conjugated BODIPY[®] peptides such as PYY(3–36), hPP, [hPP(1–17), Ala³¹, Aib³²]NPY and [cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP, were basically inactive in this assay (Figure 2 and Table 1).

In a Y₂ receptor-binding assay using rat brain membrane homogenates, adding a BODIPY[®] in the N-terminal region of NPY and PYY(3–36) resulted in lower K_i values (Figure 1 and Table 2) in accordance with data obtained in HEK293-Y₂ receptor-transfected cells (Table 2). NPY and PYY(3–36) competed against specific [¹²⁵I]PYY(3–36) binding with K_i's of 2.9 and 1.2 nM, respectively, while BODIPY[®]TMR-NPY and BODIPY[®]FL-PYY(3–36) had values of 16 and 17 nM, in rat brain homogenates (Table 2). Fluorescent [Leu³¹, Pro³⁴]NPY, [Leu³¹, Pro³⁴]PYY, hPP, [hPP(1–17), Ala³¹, Aib³²]NPY and

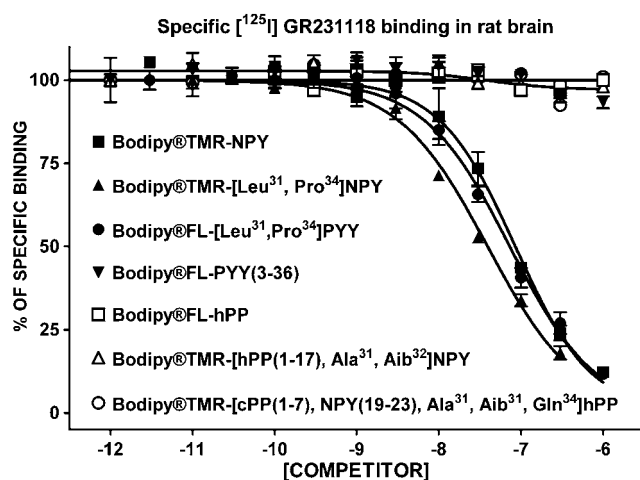


Figure 2 Competition-binding profiles of BODIPY[®]-labelled peptides against specific $[^{125}\text{I}]\text{GR231118}$ -binding sites (Y_1 -like). Data are the mean \pm s.e.m. of two to five determinations, each performed in triplicate.

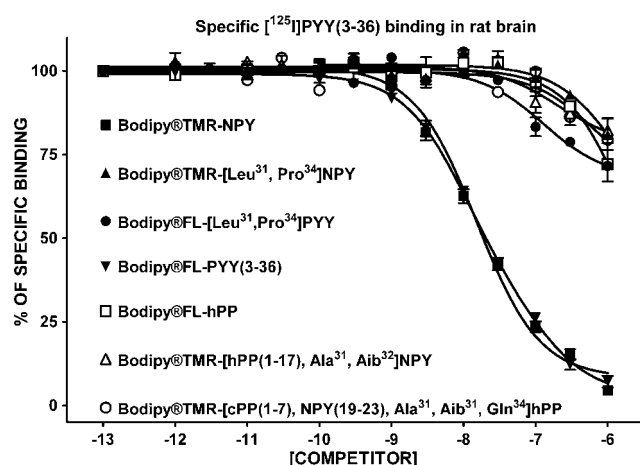


Figure 3 Competition-binding profiles of BODIPY[®]-labelled peptides against specific $[^{125}\text{I}]\text{PYY}(3-36)$ -binding sites (Y_2 -like). Data are the mean \pm s.e.m. of two to five determinations, each performed in triplicate.

[cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP were mostly inactive, as well as the nonfluorescent homologues in competing against specific $[^{125}\text{I}]\text{PYY}(3-36)$ binding in rat brain homogenates (Figure 3 and Table 2).

The addition of BODIPY[®] in the N-terminal region did not induce major changes in the affinity of various analogues to compete against specific $[^{125}\text{I}]\text{hPP}$ binding in rat brain membrane homogenates (Figure 4 and Table 3). Native and fluorescent peptides including BODIPY[®]FL-[Leu³¹, Pro³⁴]PYY, BODIPY[®]FL-PYY(3-36) and BODIPY[®]FL-hPP at concentrations of 1–3 nM inhibited 50% of specific $[^{125}\text{I}]\text{hPP}$ binding in this preparation (Table 3) while NPY and BODIPY[®]TMR-NPY were able to compete against specific $[^{125}\text{I}]\text{hPP}$ binding with K_i values of 10 and 56 nM, respectively (Table 3). The competition-binding curves clearly revealed that $[^{125}\text{I}]\text{hPP}$ recognizes a heterogeneous population of sites in the rat brain (Figure 4), in accordance with earlier results (Dumont *et al.*, 2005).

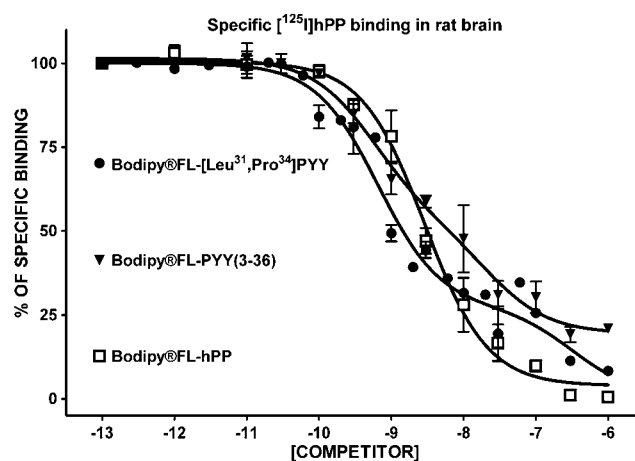


Figure 4 Competition-binding profiles of BODIPY[®]-labelled peptides against specific $[^{125}\text{I}]\text{hPP}$ -binding sites (Y_4 - and Y_5 -like). Data are the mean \pm s.e.m. of two to five determinations, each performed in triplicate.

The ability of native and BODIPY[®]-conjugated peptide analogues to compete against specific $[^{125}\text{I}][\text{Leu}^{31}, \text{Pro}^{34}]\text{PYY}$ binding resistant to a saturating concentration of BIBO3304 was determined next. This assay condition was reported to represent binding to the Y_5 receptor subtype in rat brain membrane homogenates (Dumont *et al.*, 1998). [hPP(1-17), Ala³¹, Aib³²]NPY, [cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP and their BODIPY[®]-conjugated analogues were the most potent analogues competing against specific $[^{125}\text{I}][\text{Leu}^{31}, \text{Pro}^{34}]\text{PYY}/\text{BIBO3304}$ -insensitive sites in rat brain membrane homogenates, with K_i values of 0.15–0.7 nM (Table 4). Additionally, all analogues having significant Y_5 receptor affinity, such as NPY, [Leu³¹, Pro³⁴]NPY, [Leu³¹, Pro³⁴]PYY, PYY(3-36) and hPP, were able to compete against specific $[^{125}\text{I}][\text{Leu}^{31}, \text{Pro}^{34}]\text{PYY}/\text{BIBO3304}$ -insensitive sites, with K_i values between 2 and 12 nM (Figure 5 and Table 4). The addition of BODIPY[®] at the N-terminus of these peptides had no or little effect on their apparent affinity, except for BODIPY[®]FL-hPP (Table 4).

Finally, we investigated the potential usefulness of these fluorescent agonists to visualize NPY receptors in HEK293 cells transfected with the rat Y_1 , Y_2 , Y_4 or Y_5 receptor cDNA, as well as in SK-N-MC cells. HEK293 cells were transfected with the pTR5-DC-GFP/TK/hygro vector bearing the Y_1 or Y_5 receptor cDNA. This construct will then express both GFP and the receptor proteins. As shown in Figures 6 and 7, GFP expression varied from cell to cell. Similarly, HEK293 cells transfected with the Y_1 or Y_5 receptor cDNA in the presence of 5 nM BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY for 45 min at 37°C show that levels of Y_1 (Figure 6) and Y_5 (Figure 7) receptor expression vary between cells. In fact, cells expressing low levels of GFP (green) show lower levels of signal detected with the fluorescent probe (red), while high levels of GFP expression are associated with strong BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY signals (Figures 6 and 7). BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY signal disappeared in the presence of 1 μM [Leu³¹, Pro³⁴]NPY (Figures 6 and 7). Time dependency was investigated next, as previously reported for Y_1 -transfected HEK293 cells (Pheng *et al.*, 2003). HEK293 cells transfected with the Y_5 receptor cDNA and expressing similar

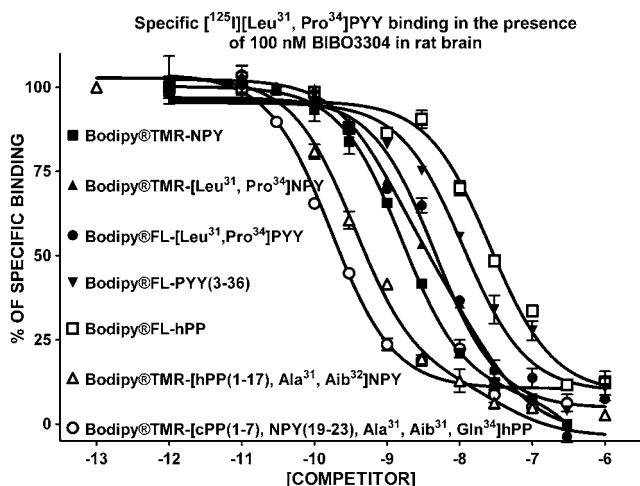


Figure 5 Competition-binding profiles of BODIPY[®]-labelled peptides against specific [¹²⁵I][Leu³¹, Pro³⁴]PYY/BIBO3304-insensitive binding sites (Y₄- and Y₅-like). Data are the mean ± s.e.m. of two to five determinations, each performed in triplicate.

levels of Y₅ receptors (as shown by GFP expression, Figure 8; green signal) revealed that signals obtained with 5 nM BODIPY[®]TMR-[cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP increased over time (Figure 8; red signal). Cells incubated at 37°C in the presence of fluorescent probes for 5 min show that BODIPY[®]TMR-[cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP fluorescent signals are mostly observed at the surface, with limited signal seen intracellularly. Longer incubation time leads to increased fluorescent signals (Figure 8). Signal disappeared in the presence of 1 μM [cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP, demonstrating the specificity of the fluorescent signal observed with this probe (Figure 8). Additionally, in HEK293 cells transfected with the rat Y₁ or Y₅ receptor cDNA, fluorescence is mostly seen intracellularly after a 45-min incubation, suggesting that BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY (Figures 6 and 7) and BODIPY[®]TMR-[cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP (Figure 8) are internalized. In SK-N-MC/Y₁-expressing cells, the fluorescent signal is clearly concentration-dependent. SK-N-MC cells incubated with 5 nM BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY for 45 min at 37°C demonstrated a lower signal than cells incubated with 20 nM BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY (Figure 9). Similar results were obtained using 1 and 5 nM BODIPY[®]TMR-[cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP in HEK293 cells transfected with the rat Y₅ receptor cDNA (Figure 9). Positive fluorescent signals were also observed in HEK293 cells transfected with the rat Y₂, Y₄ or Y₅ receptor cDNA incubated with 20 nM BODIPY[®]FL-PYY(3-36), BODIPY[®]FL-hPP or BODIPY[®]TMR-PYY(3-36) for 45 min at 37°C, respectively (Figure 10). As control, nontransfected HEK293 cells incubated in the presence of fluorescent peptides failed to reveal any significant fluorescent signal (data not shown), confirming the absence of endogenously expressed NPY receptors in those cells and the specificity of the probes under study. In cells expressing Y₁ (Figures 6 and 9) and Y₅ (Figures 7-9) receptors, a punctate pattern of fluorescence was observed and distributed throughout the cytoplasm, the nucleus being negative. This pattern may be different in HEK293 cells transfected with the rat Y₂

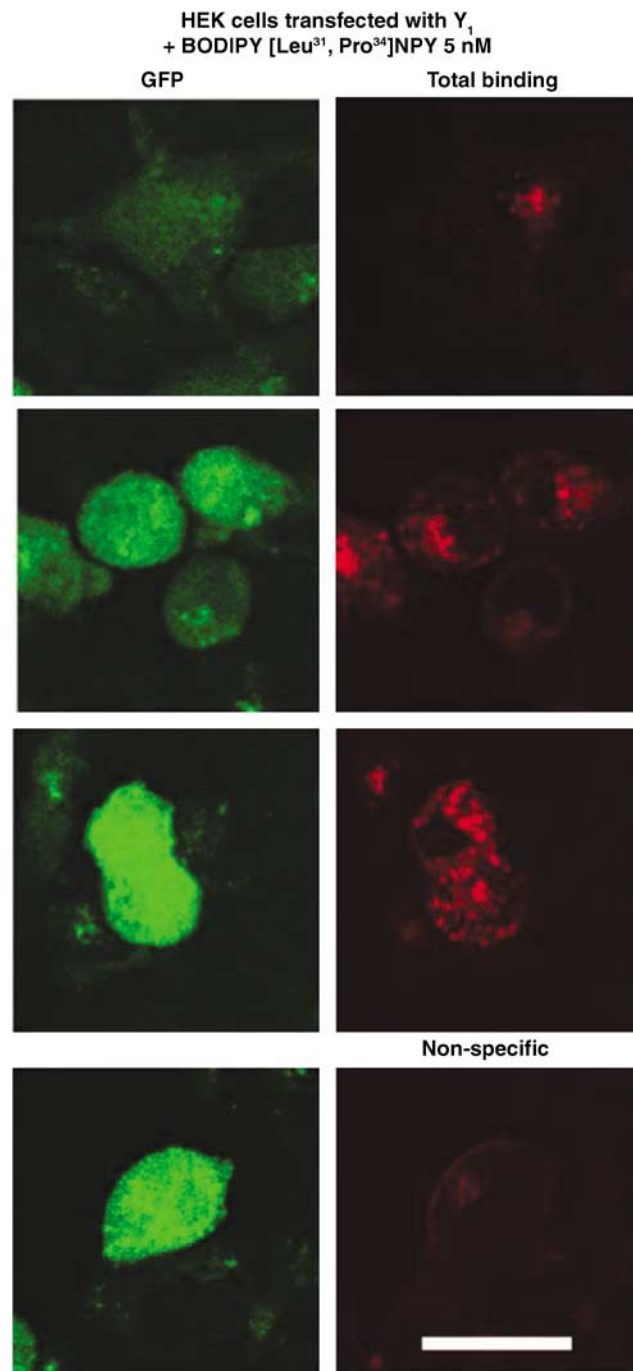


Figure 6 Visualization of BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY in HEK293 cells expressing the rat Y₁ receptor incubated with 5 nM BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY for 45 min at 37°C. Nonspecific binding represents signal obtained in the presence of 1 μM [Leu³¹, Pro³⁴]NPY incubated under the same conditions with the fluorescent probe. The peptide is represented in red following HeNe laser (excitation 543 nm/emission 580 nm), and GFP-positive cells expressing low, moderate and high levels of GFP are represented in green following argon laser (excitation 488 nm/emission 510 nm). Scale bar 10 μm. All images were taken using the same setting.

and Y₄ receptor cDNA (Figure 10). For example, in Y₂-transfected cells most of the fluorescent signal is seen at the level of the plasma membrane (Figure 10), while in Y₄ transfected cells the signal is more diffuse (Figure 10).

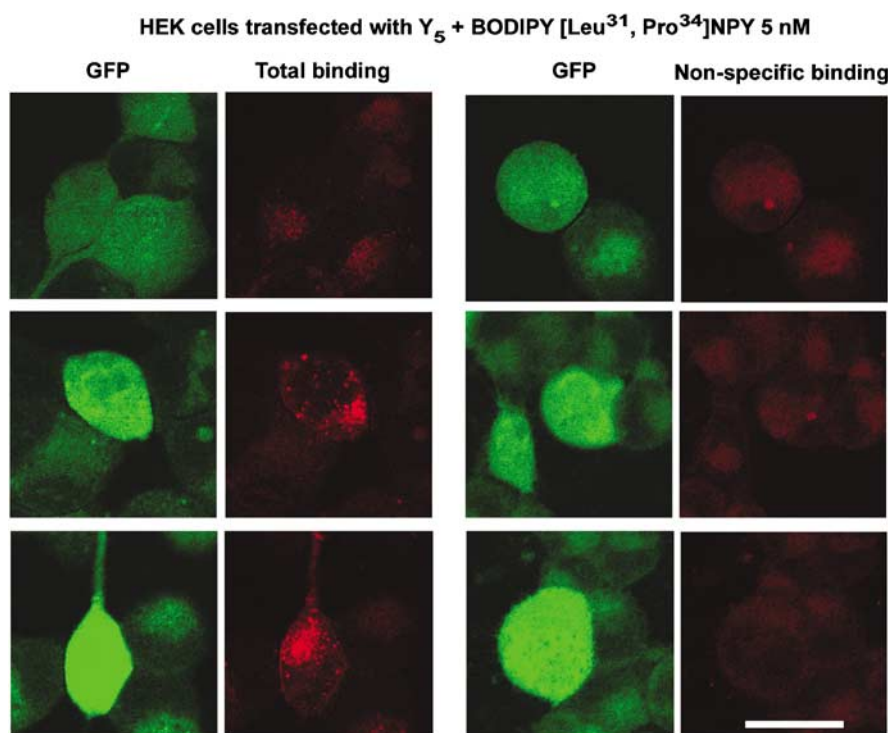


Figure 7 Visualization of BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY in HEK293 cells expressing the rat Y₅ receptor incubated with 5 nM BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY for 45 min at 37°C. Nonspecific binding represents signal obtained in the presence of 1 μM [Leu³¹, Pro³⁴]NPY incubated under the same conditions with the fluorescent probe. The peptide is represented in red following HeNe laser (excitation 543 nm/emission 580 nm), and GFP-positive cells expressing low, moderate and high levels of GFP are represented in green following argon laser (excitation 488 nm/emission 510 nm). Scale bar 10 μm. All images were taken using the same setting.

Discussion

In the present study, we investigated the ability of several BODIPY[®]-labelled analogues of the NPY family to bind and activate the Y₁, Y₂, Y₄ or Y₅ receptor subtypes. Receptor-binding assays were performed in HEK293 cells transfected with either the rat Y₁, Y₂, Y₄ or Y₅ receptor cDNA as well as in rat brain membrane homogenates. Agonist properties were evaluated by measuring the capacity of these analogues to inhibit forskolin-induced cAMP production in transfected cells. As expected, BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY and BODIPY[®]FL-[Leu³¹, Pro³⁴]PYY were almost inactive on the Y₂ subtype (as for the nonfluorescent peptides), while they maintained their ability to bind and stimulate the Y₁, Y₄ and Y₅ receptors. BODIPY[®]FL-PYY(3–36) was found to be devoid of activity on the Y₁ and Y₄ receptor subtypes, but retained its affinity and activity for the Y₂ and Y₅ receptors. These data are identical to those reported for [Leu³¹, Pro³⁴]NPY/PYY and PYY(3–36) (Michel *et al.*, 1998). Furthermore and as seen for hPP, BODIPY[®]FL-hPP conserved similar characteristics as those reported for the nonconjugated peptide (Walker *et al.*, 1997; Dumont *et al.*, 1998). BODIPY[®]FL-hPP behaves as a potent agonist on the Y₄ and Y₅ receptors, while having much lower affinities for the Y₁ and Y₂ receptor subtypes. The addition of the fluorochrome BODIPY[®]TMR in the N-terminal region of Y₅ receptor agonists, such as [hPP(1–17), Ala³¹, Aib³²]NPY and [cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP (Cabrele *et al.*, 2000; 2001), generated fluorescent probes that maintained their affinities and agonist activities for the Y₅ subtype. Overall,

conjugated BODIPY[®]FL and BODIPY[®]TMR fluorescent probes had comparatively similar or slightly lower affinities and potencies than parent peptides. They also conserved their ligand selectivity profile for the various NPY receptor subtypes. Moreover, these probes can be used to visualize NPY receptor subtypes as demonstrated here in HEK293 cells transfected with NPY receptors and SK-N-MC cells endogenously expressing Y₁ receptors.

Many attempts have been made to conjugate fluorescent molecules to receptor ligands, in order to develop better tools to investigate ligand–receptor interaction, localization as well as internalization and cellular trafficking processes. One of the major issues is to ensure that fluorescent ligands bound to the receptor will not dissociate or diffuse from the binding site. One approach is to use fluorescent probes that possess very high affinity for the receptor under study (McGrath *et al.*, 1996). Several chromophores are presently available and each one has different properties, including charge, hydrophobicity and size, which might have an impact on the affinity, potency and agonist or antagonist properties (McGrath *et al.*, 1996).

Various studies have demonstrated that fluorescent ligands are useful probes to study peptide ligand–receptor interaction using, for example, *N*-alpha-fluoresceinyl thiocarbonyl (FTC)-[Glu1]neurotensin (Faure *et al.*, 1994); BODIPY[®]FL, BODIPY[®]TR or Alexa Fluor 488 opioid peptides (Gaudriault *et al.*, 1997; Arttamangkul *et al.*, 2000); *N*-hydroxy-succinide-fluorescein, BODIPY[®]FL or BODIPY[®] 576/589 somatostatin (Nouel *et al.*, 1997); Alexa 488, BODIPY[®]FL, fluorescein, Oregon Green 488 or tetramethylrhodamine-conjugated substance P (Bennett & Simmons, 2001) and 5-carboxyfluorescein,

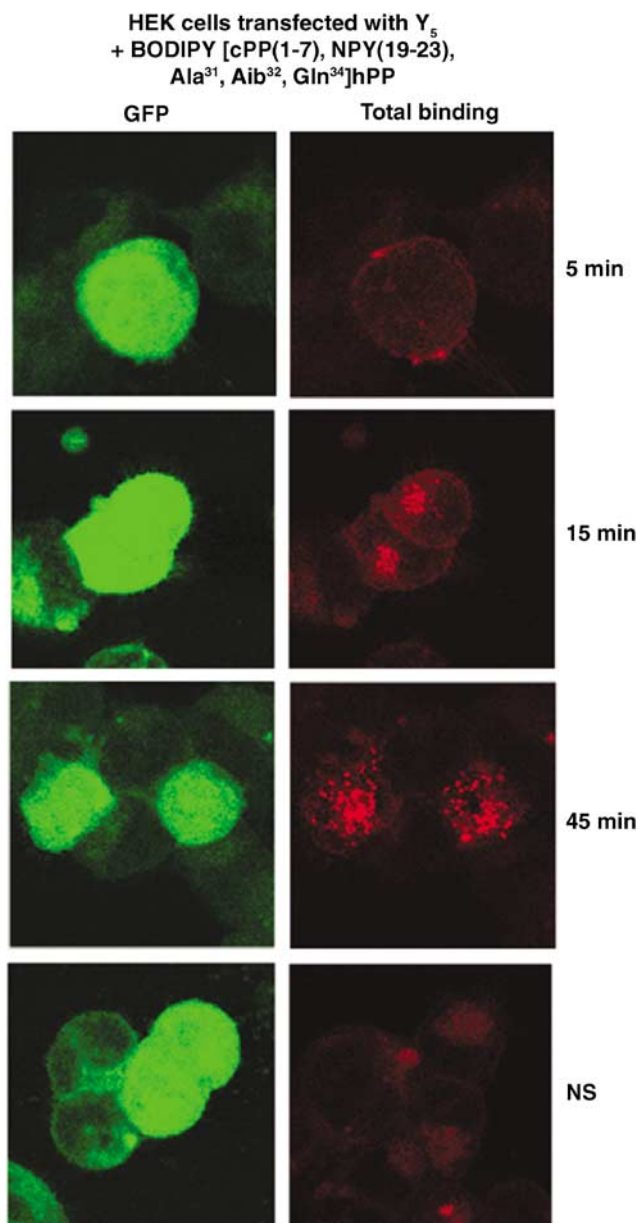


Figure 8 Visualization of BODIPY[®]TMR-[cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP in HEK293 cells expressing the rat Y_5 receptor incubated for 5, 15 and 45 min at 37°C. Nonspecific binding represents signal obtained in the presence of 1 μ M [cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP incubated for 45 min with the fluorescent probe. The peptide is represented in red following HeNe laser (excitation 543 nm/emission 580 nm), and GFP-positive cells are represented in green following argon laser (excitation 488 nm/emission 510 nm). Scale bar 10 μ m. All images were taken using the same setting.

succinimidyl ester growth hormone-releasing hormone (Veyrat-Durebex *et al.*, 2005). Studies that have evaluated different fluorescent dyes have generally shown that the BODIPY[®] dye represents one of the most appropriate fluorescent probes resulting in a fluorescent ligand that usually conserved its pharmacological properties (Arttamangkul *et al.*, 2000; Bennett & Simmons, 2001). Other advantages of BODIPY[®] dyes include very high fluorescent yields, pH insensitivity and long commercially available wavelengths. The conjugation of

BODIPY[®] moieties to peptides generally occurs *via* the formation of an amide bond at either *N*- α - or *N*- ϵ -amino groups or less frequently at a modified C-terminus (Gaudriault *et al.*, 1997). For NPY, it has been reported that the C-terminal region is highly critical for receptor recognition and activation (Schwartz *et al.*, 1990; Beck-Sickingner *et al.*, 1994). Additionally, the C-terminus moiety is identical for NPY, PYY and PPs in all mammals (Larhammar, 1996), suggesting that the C-terminus tyrosine amide moiety may bind in a pocket formed by hydrophobic amino acids of transmembrane domains 1, 2, 6 and 7 at least for the Y_1 receptor subtype (Walker *et al.*, 1994). Therefore, the conjugation of BODIPY[®] dyes was performed under controlled conditions to favour *N*-terminus acylation (Veyrat-Durebex *et al.*, 2005).

A critical issue was to establish whether the addition of the BODIPY[®] moiety at the N-terminus region of various NPY analogues will affect their ligand selectivity profile, affinity and agonist properties. Our results demonstrated that BODIPY[®]TMR-NPY was able to compete against specific Y_1 , Y_2 and Y_5 sites with similar or slightly lower affinity as compared to the nonfluorescent peptide. Moreover, BODIPY[®]TMR-NPY was able to inhibit forskolin-stimulated cAMP accumulation, demonstrating that the addition of a BODIPY[®] moiety in the N-terminal region did not affect its agonist properties. Additionally, and as seen for [Leu³¹, Pro³⁴]NPY and [Leu³¹, Pro³⁴]PYY (Michel *et al.*, 1998; Dumont *et al.*, 2002), BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY and BODIPY[®]FL-[Leu³¹, Pro³⁴]PYY were potent competitors in Y_1 , Y_4 and Y_5 receptor-binding assays, but not in Y_2 assays. These analogues were also able to inhibit forskolin-induced cAMP accumulation with similar or slightly lower potency as compared to the nonfluorescent analogues. Competition-binding experiments have also shown that BODIPY[®]FL-PYY(3-36) was able to compete with high affinity for Y_2 and Y_5 , but not for Y_1 and Y_4 sites. This profile is highly similar to that reported for PYY(3-36) itself (Michel *et al.*, 1998; Dumont *et al.*, 2002). BODIPY[®]FL-PYY(3-36) retained its agonist property, as demonstrated by its ability to inhibit forskolin-induced cAMP accumulation. The PP analogue BODIPY[®]FL-hPP displayed similar properties as the nonfluorescent peptide. Indeed, BODIPY[®]FL-hPP was a potent competitor in Y_4 and Y_5 receptor-binding assays, while being almost inactive in Y_1 and Y_2 assays. Finally, the selective Y_5 receptor agonists [hPP(1-17), Ala³¹, Aib³²]NPY, [cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP conjugated with BODIPY[®]TMR were as potent as the nonfluorescent analogues in Y_5 receptor-binding and functional assays, while being much less potent in Y_1 , Y_2 and Y_4 assays (Cabele *et al.*, 2000; 2001). These data clearly demonstrate that BODIPY[®] analogues of NPY, [Leu³¹, Pro³⁴]NPY, [Leu³¹, Pro³⁴]PYY, PYY(3-36), hPP, [hPP(1-17), Ala³¹, Aib³²]NPY and [cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP retained their selectivity profile, affinity and agonist activities for the various classes of NPY receptors under study. Moreover, receptor-binding assays demonstrated that BODIPY[®]-conjugated NPY analogues can be used to tag NPY receptors in rat brain homogenates, a tissue that expresses heterogeneous populations of NPY sites (Dumont *et al.*, 2004; 2005). The fact that the rat brain expresses Y_1 , Y_2 , Y_4 , Y_5 and possibly other not fully characterized NPY receptors (Dumont *et al.*, 2005) could explain apparent differences in K_i values obtained in HEK293-transfected cells as compared with those observed in rat brain homogenates for the Y_1 and Y_4 receptors.

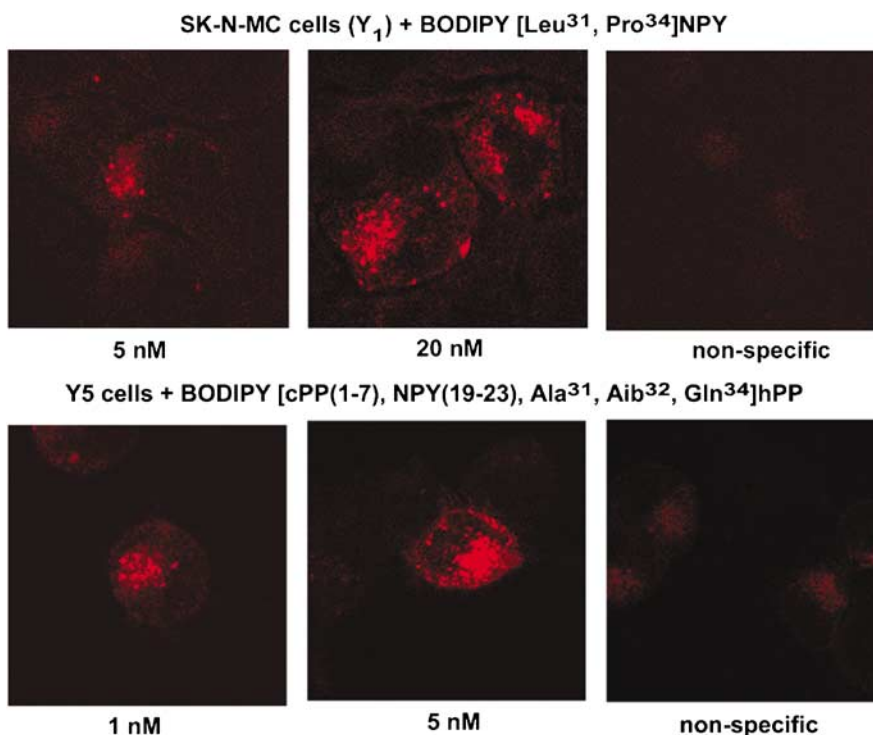


Figure 9 Visualization of 5 and 20 nM BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY in SK-N-MC cells and 1 and 5 nM BODIPY[®]TMR-[cPP(1-7), NPY(19-23), Ala³¹, Aib³², Gln³⁴]hPP in HEK293 cells expressing the rat Y_5 receptor. Cells were incubated with fluorescent probe for 45 min at 37°C. Nonspecific binding represents signal obtained in the presence of 1 μ M native peptide incubated with the highest concentration of the fluorescent probe. Scale bar 10 μ m. All images were taken using the same setting.

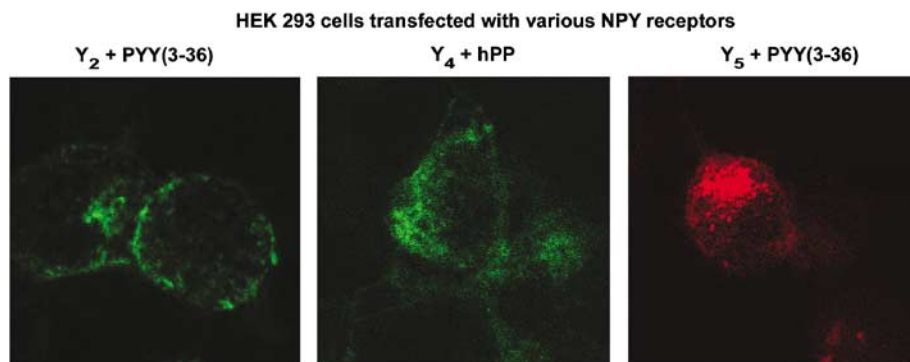


Figure 10 Visualization of BODIPY[®]FL-PYY(3-36), BODIPY[®]FL-hPP and BODIPY[®]TMR-PYY(3-36) in HEK293 cells expressing the rat Y_2 , Y_4 or Y_5 receptor cDNA, respectively. Y_2 -expressing cells were incubated with 20 nM BODIPY[®]FL-PYY(3-36) (excitation 488 nm/emission 510 nm), Y_4 cells were incubated with 20 nM BODIPY[®]FL-hPP (excitation 488 nm/emission 510 nm), and Y_5 cells with 5 nM BODIPY[®]TMR-PYY(3-36) (excitation 543 nm/emission 580 nm) for 45 min at 37°C. Nonspecific binding represents signal obtained in the presence of 1 μ M native peptide incubated under the same conditions with the fluorescent probe. Scale bar 10 μ m. All images were taken using the same setting.

Furthermore, G-protein-coupled receptors, including all NPY receptor subtypes, exist under multiple affinity states (Perez & Karnik, 2005). It is well known that certain agonists are able to distinguish between the affinity states of a given receptor subtype, while antagonists often bind with similar affinity to various receptor conformations. Additionally, the proportion of receptors in a given affinity state may be different from cell to cell, and from tissue to tissue. This could also explain differences observed in K_i values, especially for those obtained using, for example, [¹²⁵I][Leu³¹,Pro³⁴]PYY (agonist) and [¹²⁵I]GR231118 (antagonist or partial agonist), since at

concentrations of radioligands used in the present study, the agonist will mostly bind to the high-affinity state, while a radiolabelled antagonist may target most affinity states.

These various fluorescent probes can be used to visualize NPY receptors in transfected cells or cell lines endogenously expressing a specific receptor subtype. Previous studies in HEK293 cells transfected with the rat Y_1 receptor cDNA have demonstrated that in cells incubated with sucrose or phenylarsine oxide prior to the addition of BODIPY[®], TMR-[Leu³¹, Pro³⁴]NPY resulted in the inhibition of the internalization of the probe, most of the fluorescent signals remaining at the

plasma membrane (Pheng *et al.*, 2003). Furthermore, receptor internalization processes have been investigated for the Y₁, Y₂, Y₄ and Y₅ receptor subtypes using either radiolabelled probes or FRET techniques (Fabry *et al.*, 2000; Parker *et al.*, 2002a, b; 2003; Berglund *et al.*, 2003b; Pheng *et al.*, 2003). Overall, these results have shown that Y₁ and Y₅ receptors are rapidly internalized, while Y₄ receptors are internalized at a slower rate and the Y₂ subtype does not or is only very slowly internalized. Moreover, HEK293 cells transfected with EGFP-Y1 receptors revealed that, at time 0, fluorescent receptors are located at the cell surface with the fluorescent signal increasing in the cytoplasm, in a time-dependent manner, when cells are incubated in the presence of NPY or PYY (Gicquiaux *et al.*, 2002). In the present study, we have shown that levels of NPY receptor protein expression varied from cell to cell in HEK293-transfected cells with the Y₁ or Y₅ receptor cDNA, as observed by GFP expression and BODIPY-peptide signals. These data are in accordance to those reported for HEK293 cells transfected with the rat Y₁ receptor cDNA (Tong *et al.*, 1995). Additionally, signals obtained with fluorescent probes are time- and concentration-dependent. Furthermore, after 45 min in the presence of fluorescent probe, most of the

fluorescent signal was found inside cells incubated with BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY in Y₁-transfected and endogenously expressing (SK-N-MC cells) cells, as well as with BODIPY[®]TMR-[Leu³¹, Pro³⁴]NPY, BODIPY[®]TMR-[cPP(1–7), NPY(19–23), Ala³¹, Aib³², Gln³⁴]hPP and BODIPY[®]TMR-PYY(3–36) in Y₅-transfected cells, suggesting that these fluorescent probes are internalized, in agreement with previous studies on the internalization of Y₁ and Y₅ receptors (Fabry *et al.*, 2000; Parker *et al.*, 2002a, b, 2003; Berglund *et al.*, 2003b; Pheng *et al.*, 2003).

In summary, BODIPY[®] can be conjugated to the N-terminus of various NPY, PYY and PP analogues without inducing significant loss of affinity, agonist properties and receptor selectivity. These tools should prove most useful to investigate in detail NPY receptor pharmacokinetics in living neuronal and non-neuronal cells.

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