British Journal of Pharmacology (2003) 139, 1360–1368
www.nature.com/bjp

Development and characterization of a highly selective neuropeptide Y Y5 receptor agonist radioligand: [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY

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1 The existence of multiple classes of neuropeptide Y (NPY) receptors (Y_1, Y_2, Y_4, Y_5 and y_6) is now well established. However, one of the major difficulties in the study of these various receptor subtypes is the current lack of highly selective probes to investigate a single receptor class. Up to most recently, this was particularly true for the Y_4 and Y_5 subtypes.

2 [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY, the first highly selective Y_5 agonist, was iodinated using the chloramine T method and purified by high-pressure liquid chromatography.

3 Binding performed in rat brain homogenates revealed that equilibrium was reached after 120 min ($t_{1/2}=21$ min) and 60 min ($t_{1/2}=12$ min) at 25 and 100 pM [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY, respectively.

4 Isotherm saturation binding experiments demonstrated that [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binds to an apparent single population with high-affinity (K_D of 1.2 and 1.7 nM) and low-capacity (B_{max} of 14 ± 3 fmol/100,000 cells and 20 ± 5 fmol/mg protein) sites in Y_5 receptor HEK293-transfected cells and rat brain membrane homogenates, respectively. No specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding sites could be detected in Y_1, Y_2 or Y_4 receptors transfected HEK293 cells, demonstrating the high selectivity of this ligand for the Y_5 subtype.

5 Competition binding experiments performed in rat brain membrane homogenates and Y_5-receptor transfected HEK293 cells demonstrated that specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding was competed with high affinity by Y_5 agonists and antagonists such as [Ala^{31}, Aib^{32}]NPY, [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY, hPP, CGP71683A and JCF109, but not by Y_1 (BIBP3226), Y_2 (BIIE0246) and Y_1/Y_4 (GR231118) preferential ligands.

6 Taken together, these data demonstrate that [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY is the first highly selective Y_5 radioligand to be developed. This new probe should prove most useful for further detailed studies of the molecular and pharmacological properties of this receptor subtype in brain and peripheral tissues.

British Journal of Pharmacology (2003) **139,** 1360–1368. doi:10.1038/sj.bjp.0705376

Keywords: Y_5 radioligand; NPY receptors; rat brain; Y_5 agonist; receptor binding assays

Abbreviations: BIBP3226, *R*-*N*^{2}-(diphenylacetyl)-*N*-(4-hydroxyphenyl)-methylargininamide; BIIE0246, (*S*)-*N*^{2}-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6 h)-oxodibenz[*b*,*e*]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[1,2-dihydro-3,5(4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamid; BSA, bovine serum albumin; CGP71683A, N1-[(4-{[(4-amino-2-quinazolinyl)amino]methyl}cyclohexyl)methyl]-1-naphtale-nesulphonamide; GR231118, homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-CONH$_2$; HEK293, human embryonic kidney cells; hPP, human pancreatic polypeptide; JCF109, *N*-(4-trans-{[(Naphtalen-2-ylmethyl)-amino]-methyl}-2-nitro-benze-nesulphonamide); NPY, neuropeptide Y; PYY, peptide YY

Introduction

Neuropeptide Y (NPY) is a 36 amino-acid residue polypeptide that was first isolated from porcine brain (Tatemoto, 1982). It shares high sequence homology with two other peptides, namely peptide YY (PYY) and the pancreatic polypeptides (PP) (Tatemoto *et al*., 1982). This peptide family is involved in several physiological functions including feeding, anxiety-related behaviour, memory retention, regulation of the hypothalamo-pituitary axis, as well as of circadian rhythms and sexual behaviours (for recent reviews see Gehlert, 1998; Inui, 1999; Redrobe *et al*., 1999, 2002; Vezzani *et al*., 1999; Dumont *et al*., 2000c; Kask *et al*., 2002).

The effects of NPY and related peptides are mediated by the activation of at least five receptor subtypes designated as Y_1, Y_2, Y_4, Y_5 and y_6 (Michel *et al*., 1998). All five receptor subtypes have been cloned and belong to the seven transmembrane G-protein coupled receptor superfamily (Dumont *et al*., 2002a). They are expressed as functional receptors in most species including rat and human, except for the y_6 subtype (Dumont *et al*., 2000c). Each receptor subtype possesses a

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Advance online publication: 30 June 2003

distinctive pharmacological profile (Michel *et al*., 1998). The Y_1 subtype is preferentially activated by NPY, PYY and Leu^{31}, Pro^{34} substituted analogues, while BIBP3226, BIBO3304 and GR231118 behave as antagonists for this receptor subtype (Larhammar *et al*., 1992; Daniels *et al*., 1995; Doods *et al*., 1996; Bitran *et al*., 1997; Michel *et al*., 1998; Wieland *et al*., 1998). As for the Y_1 subtype, NPY and PYY are potent agonists on the Y_2 subtype. Additionally and in contrast to Y_1 receptors, C-terminal fragments such as NPY_{3-36}, PYY_{3-36}, NPY_{13-36} and PYY_{13-36} act as potent agonists on the Y_2 subtype (Gerald *et al*., 1995; Michel *et al*., 1998). BIIE0246 is a highly selective antagonist on the Y_2 receptors (Doods *et al*., 1999; Dumont *et al*., 2000b). The main characteristic of the Y_4 receptor protein is its very high affinity for the PPs and GR231118 (Lundell *et al*., 1995; Schober *et al*., 1998); the later being an agonist in contrast to its antagonistic activity on the Y_1 subtype (Parker *et al*., 1998; Schober *et al*., 1998). The Y_5 receptor is activated by NPY, PYY, [Leu^{31}, Pro^{34}]-NPY or PYY substituted analogues, human PP and long C-terminal fragments such as NPY_{3-36} and PYY_{3-36} (Gerald *et al*., 1996; Hu *et al*., 1996; Michel *et al*., 1998). Most recently, the development of highly selective Y_5 receptor agonists has been reported including [Ala^{31}, Aib^{32}]NPY, [hPP_{1-17}, Ala^{31}, Aib^{32}]NPY and [cPP_{1-7}, NPY_{19-23}, Ala^{31}, Aib^{32}, Gly^{34}]hPP (Cabrele *et al*., 2000, 2001). These agonists are likely to represent ideal tools in an attempt to develop selective Y_5 radioligands.

Up to date, NPY receptor binding sites have been characterized and their differential distribution investigated using various radioligands including [^{125}I]NPY (Martel *et al*., 1990), [^{125}I]PYY (Dumont *et al*., 1990; Gehlert *et al*., 1992), [^{125}I][Leu^{31}, Pro^{34}]NPY (Larsen *et al*., 1993), [^{125}I][Leu^{31}, Pro^{34}]PYY (Dumont *et al*., 1996a; Gehlert & Gackenheimer, 1997), [^{125}I]NPY_{2-36} (Schober *et al*., 1996), [^{125}I]PYY_{3-36} (Dumont *et al*., 1996a; Gehlert & Gackenheimer, 1997), [^{125}I]bPP (Gehlert *et al*., 1997), [^{125}I]hPP (Trinh *et al*., 1996), [^{125}I]GR231118 (Dumont & Quirion, 2000; Schober *et al*., 2000) and [^{3}H]BIBP3226 (Dumont *et al*., 1996b). However, these probes are known to recognize more than one receptor subtype and/or to possess signal/noise ratio that are too high for detailed receptor binding studies. For example, the only means to access directly the differential distribution of an NPY receptor such as the Y_5 subtype was by including a blocking concentration of an analogue in order to visualize and characterize the receptor subtype of interest. In that regard, using [^{125}I][Leu^{31}, Pro^{34}]PYY in the presence of a selective nonpeptide Y_1 receptor antagonist, we were able to demonstrate that [^{125}I][Leu^{31}, Pro^{34}]PYY/BIBP3226-insensitive sites have a ligand selectivity profile similar to that of the Y_5 receptor subtype and a unique distribution in the rat CNS (Dumont *et al*., 1998a). However, under these conditions, the possible labelling of the Y_4 receptor could not be fully excluded, as [^{125}I][Leu^{31}, Pro^{34}]PYY also possesses a rather high affinity for this subtype (Gehlert *et al*., 1996).

In order to develop highly selective Y_5 receptor radioligand, we have iodinated the newly reported selective Y_5 receptor agonist [hPP_{1-17}, Ala^{31}, Aib^{32}]NPY (Cabrele *et al*., 2000) and characterized its binding properties in HEK293 cells transfected with the rat Y_1, Y_2, Y_4 or Y_5 receptor cDNA as well as in rat brain membrane homogenates. Our results demonstrate that [^{125}I][hPP_{1-17}, Ala^{31}, Aib^{32}]NPY binds with high affinity to the Y_5 receptor protein, while being devoid of affinity for the Y_1, Y_2 and Y_4 subtypes. Furthermore, specific [^{125}I][hPP_{1-17}, Ala^{31}, Aib^{32}]NPY binding sites are competed with high affinity by Y_5 but not by Y_1, Y_2 and Y_4 receptor agonists and antagonists. Thus, [^{125}I][hPP_{1-17}, Ala^{31}, Aib^{32}]NPY represents the first selective radiolabelled probe allowing for detailed studies of the Y_5 receptor subtype.

Methods

Materials

Male Sprague–Dawley CD rats (200–250 g) obtained from Charles River Canada (St-Constant, Québec, Canada) were kept on a 12 h light–dark cycle (light on at 0700) in temperature- and humidity-controlled rooms. Animals were fed with standard laboratory chow and had access to tap water *ad libitum*. Animal care was according to protocols and guidelines approved by McGill University and the Canadian Council of Animal Care.

Analogues and fragments of hPYY, porcine (p) NPY, hPP, [Ala^{31}, Aib^{32}]NPY and [hPP_{1-17}, Ala^{31}, Aib^{32}]NPY were synthesized as previously described (Forest *et al*., 1990; Cabrele *et al*., 2000). *R*-N^2-(diphenylacetyl)-*N*-(4-hydroxyphenyl)-methylargininamide (BIBP3226) and (*S*)-N^2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6 h)-oxodibenz[*b*,*e*]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[1,2-dihydro-3,5(4 *H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamid (BIIE0246) were generously provided by Boerhinger Ingelheim (Germany), while *N*-(4-*trans*-{[(naphtalen-2-ylmethyl)-amino]-methyl}-2-nitro-benze-nesulphonamide) (JCF109 also named compound 34 as described in patent #97/319425 by Synaptic Pharmaceut. Corp.) and N1-[(4-{[(4-amino-2-quinazolinyl)amino]methyl}cyclohexyl)methyl]-1-naphtale-nesulphonamide (CGP71683A) were graciously obtained from Servier (Paris, France). Homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$ (GR231118) was a gift from Glaxo Wellcome (Research Triangle Park, NC, U.S.A.). Human embryonic kidney cells (HEK293) were a donation of Drs S.H. Shen and Y. Tong, Biotechnology Research Institute (BRI, Montréal, QC, Canada). Bovine serum albumin (BSA) and Iodine-125 were obtained from ICN Pharm. Canada Ltd. (Montréal, QC, Canada) and bacitracin was purchased from Sigma Chemical (St Louis, MI, U.S.A.). Schleicher and Schuell #32 glass filters were obtained from VWR-Canlab (Montréal, QC, Canada). All tissue culture media, antibiotics and reagents were purchased from Gibco-BRL (Burnington, ON, Canada). The expressing vector, pcDNA3 and pTR5-DC-GFP/TK/hygro were obtained from Invitrogen (San Diego, CA, U.S.A.) and Dr Dick D. Moose (BRI., Montreal, QC, Canada), respectively. All other chemicals were of analytical grade and obtained from Fisher Scientific (Montreal, QC, Canada) or Sigma Chemical (St Louis, MI, U.S.A.).

Iodine-125 was incorporated into the tyrosine residue of [Ala^{31}, Aib^{32}]NPY and [hPP_{1-17}, Ala^{31}, Aib^{32}]NPY using the chloramine T method as previously described (Dumont *et al*., 1995) except that the column used for the HPLC purification of iodinated peptide was a C18 Guard-Pak (Waters, Mississauga, ON, Canada). Specific activity was assumed to be of the theoretical value (2000 Ci/mmol).

Membrane preparations

Membranes were prepared as previously described (Dumont *et al*., 1995). Briefly, rats were killed by decapitation and their brains rapidly removed and homogenized in a Krebs Ringer phosphate (KRP) buffer at pH 7.4 of the following composition: NaCl (120 mM), KCl (4.7 mM), $CaCl_2$ (2.2 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), dextrose (5.5 mM) and $NaHCO_3$ (25 mM) 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. Protein concentration was determined with BSA as the standard (Bradford, 1976).

Transfected cells

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% foetal calf serum and antibiotics (penicillin G sodium, streptomycin sulphate and amphotericin B). Cultured cells were transfected with either of the rat Y_1, Y_2, Y_4 or Y_5 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 Y_1, Y_2, Y_4 or Y_5 receptor cDNA, which was previously inserted in expressing pcDNA3 (Y_2 and Y_4) and pTR5-DC-GFP/TK/hygro (Y_1 and Y_5) 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% confluent and returned to the incubator. The medium was changed the next morning. At confluence, the transient (Y_2 and Y_4) or stable (Y_1 and Y_5) transfected HEK293 cells were washed with KRP buffer pH 7.4 and scratched. Detached cells were then centrifuged at $400 \times g$ for 10 min and the pellet washed with KRP buffer (pH 7.4), recentrifuged twice, and resuspended in 8 ml of KRP buffer pH 7.4 and used for receptor binding assay.

Binding assays

All binding assays were initiated by adding 100 μl of membrane preparations in a final volume of 500 μl of KRP containing 0.1% (w v^{-1}) BSA, 0.05% (w v^{-1}) bacitracin, [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY and unlabelled peptide or competitor as needed. Time dependency was established using 25 and 100 pM [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY at room temperature. Isotherm saturations and competition binding assays were performed at room temperature. Saturation experiments were performed in the presence of increasing concentrations of [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY, whereas competition binding experiments were performed in the presence of 50 pM [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY and various competitors at concentrations ranging from 10^{-13} to 10^{-6} M. Nonspecific binding was determined in the presence of 1 μM [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY. After 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 cold KRP and the radioactivity remaining on filters was quantified using a gamma counter with 85% efficiency (Cambera Packard Instruments, Meriden CT, U.S.A.).

All binding experiments were repeated three to six times (each in triplicate), and results (mean ± s.e.m.) were expressed as percentage of specific binding or fmol mg^{-1} protein. All data obtained from the saturation isotherm experiments were subtracted for [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY values found on filters in absence of membrane homogenates. K_D, B_{max} and half-time association values were calculated from data using the GraphPad Prism (GraphPad Software Inc., San Diego, CA, U.S.A.). K_i values (Cheng & Prusoff, 1973) were determined from IC_{50} values (i.e. concentration of unlabelled competitor required to compete for 50% of specific binding of the radioligand) for the various competitors calculated using the GraphPad Prism.

Results

The newly developed Y_5 receptor agonists, [Ala^{31}, Aib^{32}]NPY and [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY (Cabrele *et al*., 2000), were iodinated using the chloramine T method (Hunter & Greenwood, 1962) and purified by high-pressure liquid chromatography at a flow rate of 1 ml min^{-1}. Fractions (1 ml) were collected and amounts of radioactive material were determined for each fraction. Prototypical purification profile obtained for [^{125}I][Ala^{31}, Aib^{32}]NPY and [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY are presented in Figure 1. Fractions containing the highest levels of radioactive materials were then tested for binding to Schleicher and Schuell #32 glass filters. Various concentrations of [^{125}I][Ala^{31}, Aib^{32}]NPY and [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY were incubated in 0.5 ml of KRP buffer at room temperature for 2 h in the presence or absence of 1 μM [Ala^{31}, Aib^{32}]NPY or [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY, but without biological membrane preparations. [^{125}I][Ala^{31}, Aib^{32}]NPY (fraction 18; Figure 1a) and [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY (fractions 16 and 18; Figure 1b) binding increased linearly with increasing concentrations of radioligands and no difference was observed in the presence or absence of 1 μM [Ala^{31}, Aib^{32}]NPY or [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY indicating that [^{125}I][Ala^{31}, Aib^{32}]NPY and [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY did not bind specifically to filters (data not shown). Since preliminary studies revealed better signal/noise ratio for specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY fraction 18 to bind to Y_5-receptor transfected HEK293 cells and rat brain membrane homogenates as compared to [^{125}I][Ala^{31}, Aib^{32}]NPY and [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY fraction 16 (Dumont *et al*., unpublished results), all subsequent experiments were performed using [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY fraction 18.

As shown in Figure 2, [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding performed at room temperature reached equilibrium in a time-dependent manner in rat brain membrane homogenates. Specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding reached equilibrium after 120 min at 25 pM (Figure 2a) and 60 min at 100 pM (Figure 2b). Furthermore, [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding remained stable for at least 3 h after reaching equilibrium. The half-time association ($t_{1/2}$) was of 21 min at 25 pM and of 12 min at 100 pM [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY, at room temperature. Accordingly, all subsequent experiments were performed at room temperature using a 2 h incubation period.

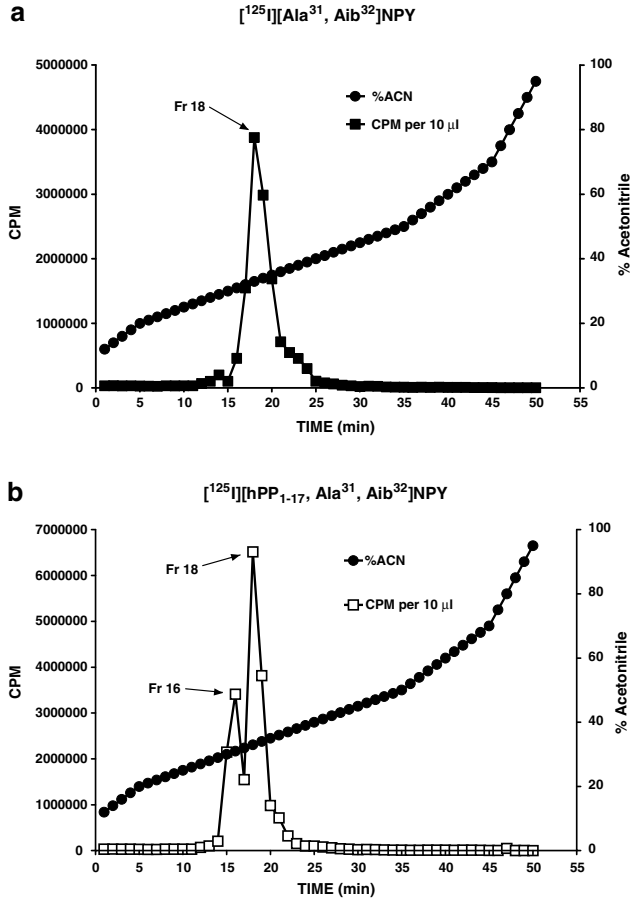

Figure 1 Typical HPLC purification profile of radiolabelled [^{125}I][Ala^{31}, Aib^{32}]NPY (a) and [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY (b).

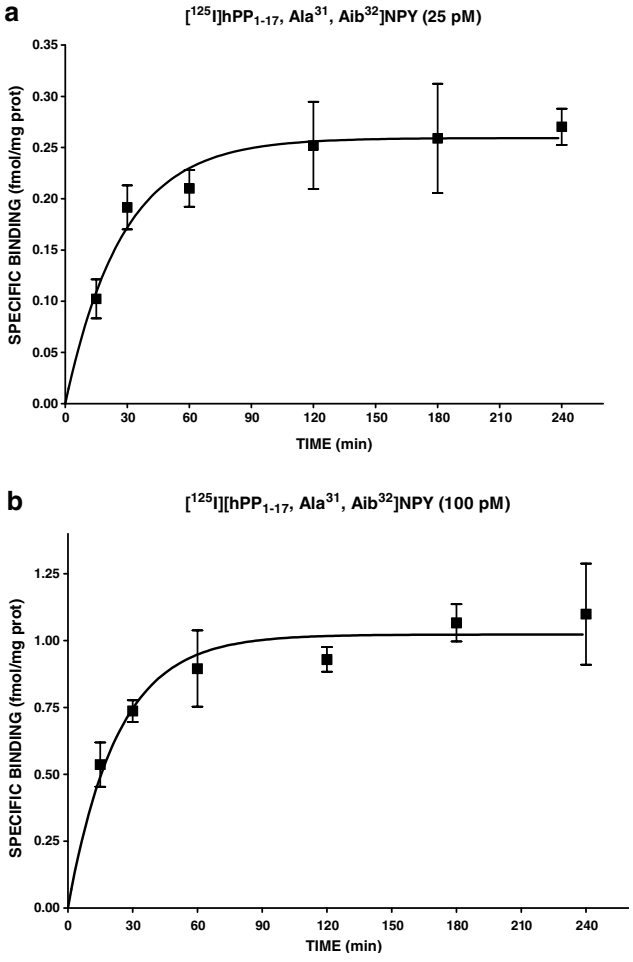

Figure 2 Typical profiles of the time course association of [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding in rat brain membrane homogenates at room temperature. Data represent the mean $\pm$ s.e.m. of a prototypical experiment performed in triplicate.

Saturation binding parameters of [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY were established next. Isotherm saturation binding experiments performed in HEK293 cells transfected with the rat Y_5 receptor cDNA (Figure 3a) demonstrated that [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY specifically binds with high affinity (K_D of 1.2 ± 0.4 nM) to a saturable amount of sites (B_{max} of 14 ± 3 fmol/100,000 cells) (Table 1). Similar results were obtained in rat brain membrane preparations (Figure 3b) with a K_D of 1.7 ± 0.5 nM and maximal binding capacity of 20 ± 5 fmol mg^{-1} protein (Table 1). However, while low levels of nonspecific binding was seen in HEK293 cells transfected with the rat Y_5 receptor cDNA (Figure 3a), relatively high amount of nonspecific binding was detected in rat brain homogenates (Figure 3b). In fact, at 50 pM [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY over 90% of the total binding was specific in Y_5 HEK293 transfected cells, whereas it represented only 25% in rat brain homogenates. No significant amounts of specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding was detected in HEK293 cells transfected with the rat Y_1, Y_2 or Y_4 receptor cDNA even at ligand concentrations up to 500 pM (Table 1), demonstrating the selectivity of [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY for the Y_5 subtype.

In order to characterize pharmacologically specific binding sites recognized by [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY, competition binding curves were performed in Y_5-receptor transfected HEK293 cells and in rat brain membrane homogenates using various agonists and antagonists of the Y_1, Y_2, Y_4 and Y_5 receptor subtypes such as [Leu^{31}, Pro^{34}]pNPY and [Leu^{31}, Pro^{34}]PYY (Y_1, Y_4 and Y_5 agonists) (Fuhlendorff *et al*., 1990; Eva *et al*., 1992; Krause *et al*., 1992; Dumont *et al*., 1995; 1998a; Gehlert *et al*., 1996; 1997; Gerald *et al*., 1996), GR231118 (Y_1 antagonist and Y_4 agonist) (Parker *et al*., 1998), hPP (Y_4 and Y_5 agonists) (Lundell *et al*., 1995; Gerald *et al*., 1996; Dumont *et al*., 1998a), [Ala^{31}, Aib^{32}]NPY and [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY (Y_5 agonists) (Cabrele *et al*., 2000), CGP71683A and JCF109 (Y_5 antagonists) (Criscione *et al*., 1998; Feletou *et al*., 1999; Dumont *et al*., 2000a), BIBP3226 (Y_1 antagonist) (Doods *et al*., 1995; Jacques *et al*., 1995) and BIIE0246 (Y_2 antagonist) (Doods *et al*., 1999; Dumont *et al*., 2000b).

In Y_5-receptor transfected HEK293 cells, specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding was competed with a ligand selectivity profile congruent to that reported for the Y_5 receptor subtype with CGP71683A, hPP > [Leu^{31}, Pro^{34}]-PYY, [Leu^{31}, Pro^{34}]NPY, [Ala^{31}, Aib^{32}]NPY, [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY, JCF109 > GR231118 $\gg$ BIBP3226, BIIE0246 (Figure 4a). In fact, specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding was competed by all Y_5 agonists and antagonists in the nM range, while Y_1 (BIBP3226) and Y_2 (BIIE0246) antagonists

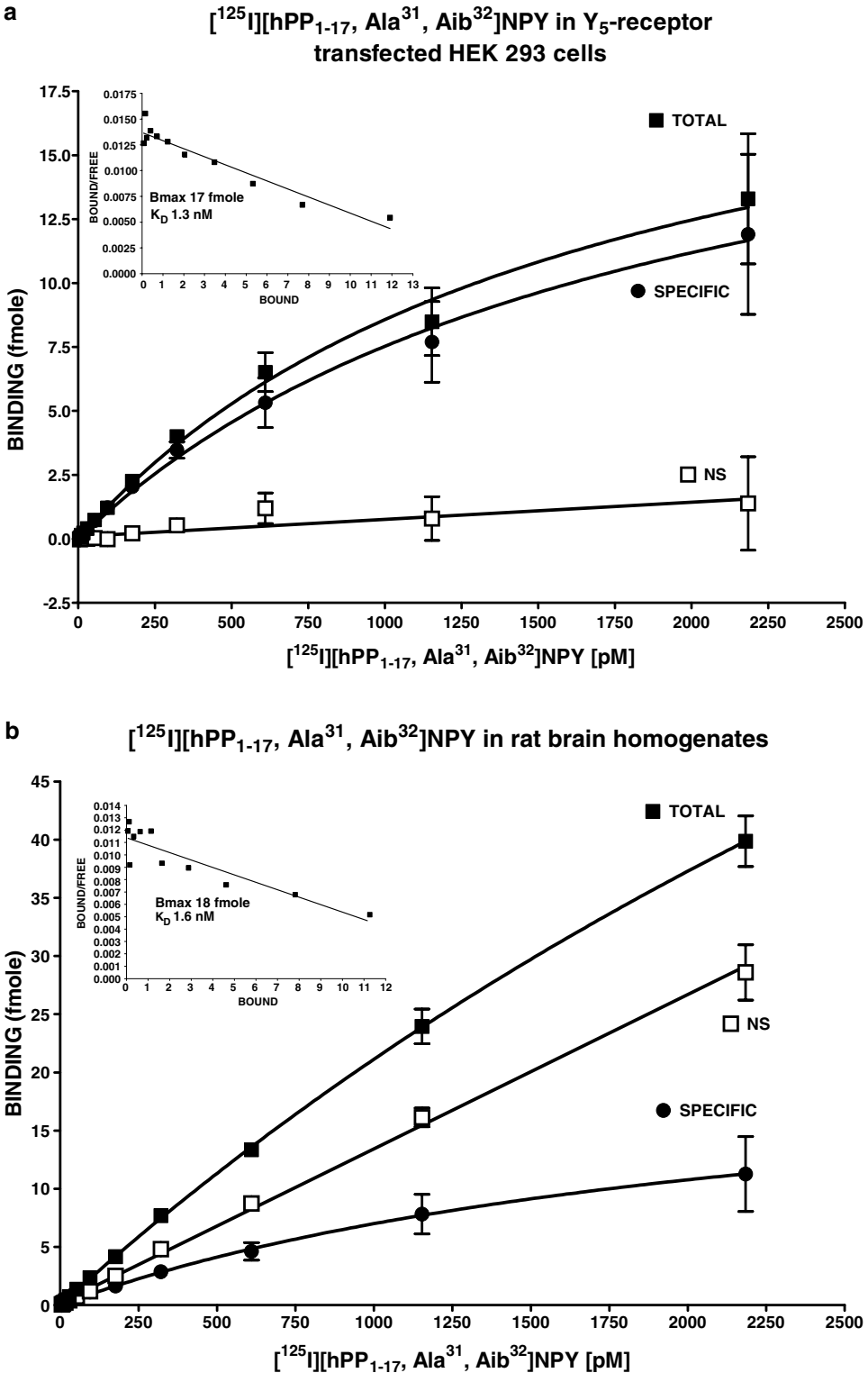

Figure 3 Typical profiles of saturation binding isotherms of [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding in HEK293 cells transfected with the rat Y_5 receptor cDNA (a) and rat brain membrane homogenates (b). Inset is a Scatchard transformation of the isotherm saturation binding experiment. Data represent the mean ± s.e.m. of a prototypical experiment performed in triplicate.

were inactive. The Y_1/Y_4 ligand, GR231118 had some affinity but only in the high nM range (Table 2). An identical ligand selectivity profile was observed in rat brain membrane homogenates (Figure 4b; Table 2). Furthermore, similar levels of specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding (defined as the difference obtained in the presence and absence of 1 μM [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY) were inhibited by all Y_5 agonists and antagonists, suggesting that sites labelled by [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY represent a single population of binding sites. Additionally, Figure 5 shows that a highly positive

Table 1 Data derived from saturation isotherms of [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding in rat brain membrane homogenates and in HEK293 cells transfected with either the rat Y_1, Y_2, Y_4 or Y_5 receptor cDNA

	K_D (nM)	B_{max}
Rat brain homogenates	1.7±0.5	20±5 fmol mg^{-1} protein
Y_1-receptor transfected HEK293 cells	No binding	
Y_2-receptor transfected HEK293 cells	No binding	
Y_4-receptor transfected HEK293 cells	No binding	
Y_5-receptor transfected HEK293 cells	1.2±0.4	14±3 fmol per 100,000 cells

Data represent the mean±s.e.m. of three individual determinations, each performed in triplicate. No binding means no specific binding detected at 1 nM [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY. K_D represents the apparent affinity of [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY and B_{max} represents the maximal binding capacity expressed in fmol mg^{-1} protein or 100,000 cells. These values were calculated using nonlinear regression with the GraphPad Prism program.

correlation ($r=0.992$; $P<0.0001$) was observed between affinities of various NPY-related molecules to compete against specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding sites in Y_5-receptor transfected HEK293 cells and rat brain membrane homogenates, suggesting the labelling of an identical or highly similar receptor protein.

Discussion

Our study revealed that the Y_5 receptor agonist [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY (Cabrele *et al*., 2000) can be iodinated using the chloramine T method (Hunter & Greenwood, 1962). Receptor binding assays demonstrated that [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binds to the Y_5 receptor protein in a time-dependent manner, reaching equilibrium within 2 h at room temperature and at a concentration of 25 pM. Isotherm saturation binding experiments demonstrated that [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binds with high affinity (K_D of 1.2 nM) to a saturable number of sites in HEK293 cells transfected with the rat Y_5 receptor cDNA, but was devoid of specific labelling in rat Y_1, Y_2 or Y_4-transfected HEK293 cells. This result demonstrates the selectivity of this radioligand for the Y_5 receptor subtype. Similar binding parameters were observed in rat brain membrane homogenates (K_D of 1.7 nM and B_{max} of 20 fmol mg^{-1} protein). Most importantly, competition binding profiles against specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding in Y_5-transfected HEK293 cells and rat brain membrane homogenates revealed identical ligand selectivity pattern. Specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding was competed with high affinity by Y_5 agonists and antagonists, while Y_1 and Y_2 receptor antagonists were inactive and GR231118, a potent Y_1 antagonist/Y_4 agonist, only displayed low affinity.

Various studies using receptor binding assays, receptor autoradiography, *in situ* hybridization, *in vitro* bioassays and *in vivo* experiments have demonstrated the existence of multiple NPY receptor subtypes in the central and peripheral

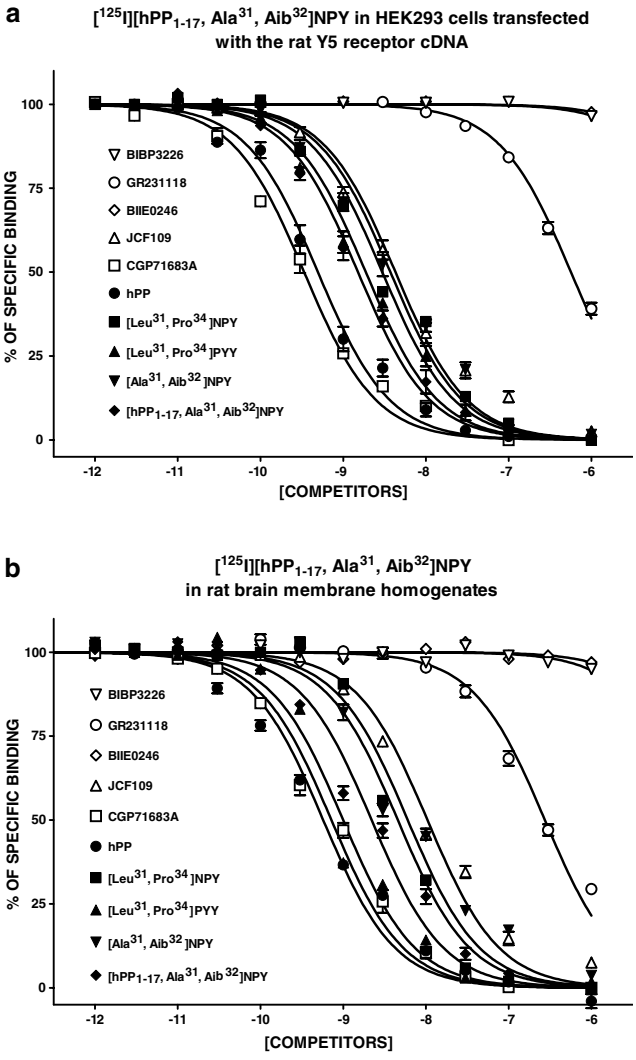

Figure 4 Competition binding profiles of various agonists and antagonists of the NPY family against specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding in HEK293 cells transfected with the rat Y_5 receptor cDNA (a) and rat brain membrane homogenates (b). Data represent the mean±s.e.m. of three to six determinations, each performed in triplicate.

nervous systems (for reviews see Gehlert, 1999; Vezzani *et al*., 1999; Dumont *et al*., 2000c; Kask *et al*., 2002; Redrobe *et al*., 2002). For example, in the rat brain, Y_1, Y_2, Y_4 and Y_5 receptor subtypes are expressed at various levels and differentially distributed in different brain structures (Dumont *et al*., 1997, 2000c). Similarly, the mouse, guinea-pig, monkey and human brains are also enriched with multiple NPY receptors (Gehlert & Gackenheimer, 1997; Jacques *et al*., 1997; Statnick *et al*., 1997; Dumont *et al*., 1998b). Accordingly, in order to target specifically a receptor subtype without possible cross-reactivity with other NPY receptors, the development and characterization of optimal radioreceptor assay conditions and radioligands for each receptor subtype are still key objectives as complementary approaches to the use of transgenic and knockout animal model (Michalkiewicz & Michalkiewicz, 2000; Pedrazzini & Seydoux, 2000; Sainsbury *et al*., 2002a, b).

Most NPY radioligands developed thus far including [^{125}I][Leu^{31}, Pro^{34}]PYY (Dumont *et al*., 1995), [^{125}I]PYY$_{3-36}$

Table 2 Competition binding parameters of various agonists and antagonists of the NPY family against [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding in rat brain homogenates and HEK293 cells transfected with the rat Y_5 receptor cDNA

Competitors	*Rat brain* K_i (nM)	*Y_5 transfected* K_i (nM)
[Leu^{31},Pro^{34}]pNPY	3.5±1.1	2.4±1.0
[Leu^{31},Pro^{34}]PYY	1.0±0.4	1.8±0.5
hPP	0.4±0.08	0.5±0.1
[Ala^{31}, Aib^{32}]NPY	6±1.4	3.7±1.3
[hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY	2.2±0.9	1.5±0.8
GR231118	220±44	360±65
CGP71683A	0.7±0.1	0.3±0.1
JCF109	10±2.5	5±2
BIBP3226	>1000	>1000
BIIE0246	>1000	>1000

Data represent the mean±s.e.m. of two to five individual determinations, each performed in triplicate. K_i represents the concentration of competitors needed to inhibit 50% of specific binding.

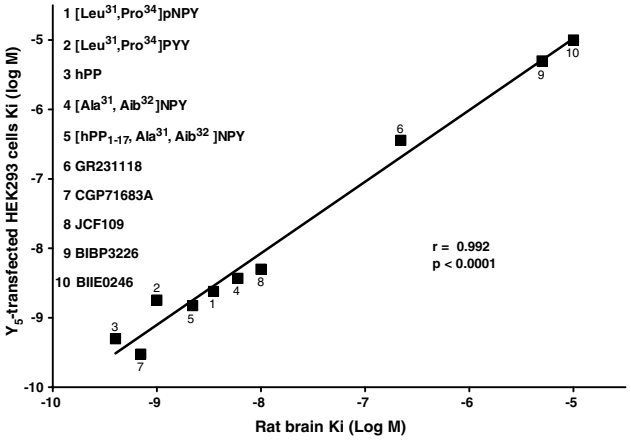

Figure 5 Comparative affinities of various analogues of the NPY family to compete against [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY in HEK293 cells transfected with the rat Y_5 receptor cDNA and rat brain membrane homogenates.

(Dumont *et al*., 1995), [^{125}I]hPP (Trinh *et al*., 1996), [^{125}I]bPP (Gehlert *et al*., 1997) and [^{125}I]GR231118 (Dumont & Quirion, 2000; Schober *et al*., 2000) are not selective and recognize more than one NPY receptor subtype. For example, we reported that [^{125}I][Leu^{31}, Pro^{34}]PYY binds to at least two different populations of sites (Y_1 and Y_5) in the rat brain using either BIBP3226 (Dumont *et al*., 1998a) or BIBO3304 (Dumont *et al*., 2000a), two Y_1 receptor antagonists (Doods *et al*., 1996; Wieland *et al*., 1998) as blocking agents. Further studies have also demonstrated that [^{125}I][Leu^{31}, Pro^{34}]PYY possesses significant affinity for the Y_4 subtype (Gehlert *et al*., 1996). Moreover, [^{125}I]PYY$_{3-36}$, a radioligand developed first as a Y_2 selective probe, is likely targeting at least two NPY receptor populations (Dumont *et al*., 2000b). Furthermore, [^{125}I]GR231118 binds with very high affinities to the Y_1 and Y_4 receptor subtypes (Dumont & Quirion, 2000; Schober *et al*., 2000). Most recently, we have also shown that [^{125}I]hPP is labelling at least two NPY receptors, namely the Y_4 and Y_5 subtypes (Dumont *et al*., 2002b). Interestingly, and in contrast to these earlier studies and to the radioligands developed thus far, [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY is apparently highly selective for the Y_5 subtype. Indeed, our results revealed that [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binds to the Y_5 receptor subtype with affinities in the low nM range, while no specific binding could be detected in HEK293 cells transfected with the rat Y_1, Y_2 and Y_4 receptor cDNA, demonstrating further its specificity and selectivity for the Y_5 subtype.

In rat brain membrane homogenates, various agonists and antagonists of the NPY family competed for [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding with a ligand selectivity profile highly similar to that observed in Y_5-receptor transfected HEK293 cells. These data strongly suggest that sites targeted by [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY in the rat brain are of the Y_5 subtype. In fact, in both preparations, specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding is competed fully by nonselective Y_5 agonists such as [Leu^{31}, Pro^{34}]PYY, [Leu^{31}, Pro^{34}]NPY and hPP (Michel *et al*., 1998), and highly selective Y_5 agonists like [Ala^{31}, Aib^{32}]NPY and [hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY (Cabrele & Beck-Sickinger, 2000; Cabrele *et al*., 2000; 2001; 2002). The Y_5 antagonists CGP71683A and JCF109 (Criscione *et al*., 1998; Feletou *et al*., 1999), but not Y_1 (BIBP3226; Doods *et al*., 1996) and Y_2 (BIIE0246; Doods *et al*., 1999) antagonists, potently competed for specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding sites in rat brain membrane homogenates and Y_5-receptor transfected HEK293 cells. GR231118, a Y_1 receptor antagonist (Bitran *et al*., 1997) acting as a potent Y_4 agonist (Parker *et al*., 1998), displayed only low affinity for sites labelled by [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY. This competition profile further demonstrates that specific binding sites targeted by [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY are of the Y_5 receptor subtype (Gerald *et al*., 1996; Michel *et al*., 1998; Dumont *et al*., 2002a).

While specific [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY binding accounted for a very high proportion (over 90% at 50 pM) in Y_5-receptor transfected HEK293 cells, it was significantly lower in rat brain membrane homogenates (25% at 50 pM). One of the limiting features of this new radioligand could relate to its adsorption to proteins and lipids and to the fact that the rat brain is not highly enriched with the Y_5 receptor subtype (Dumont *et al*., 1998a, b). Chimeric peptides using the carboxy-terminal of PYY could generate a radioligand with lower nonspecific binding in rat brain homogenates as it was the case for [^{125}I]PYY compared to [^{125}I]NPY (Martel *et al*., 1990).

In summary, our data have shown that [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY specifically binds with very high affinity to rat brain homogenates and Y_5-receptor-transfected HEK293 cells. Isotherm saturation binding experiments and ligand selectivity profiles revealed that [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY is highly specific for Y_5 receptors expressed in HEK293 cells and in the rat brain. Accordingly, [^{125}I][hPP$_{1-17}$, Ala^{31}, Aib^{32}]NPY should prove useful to investigate in detail the characteristics of the Y_5 receptor subtype in a variety of cell lines and tissues. Furthermore, this radioligand represents the first iodinated peptide of the NPY family that highly specifically binds to a single subtype without any significant crossreactivity for other receptors.

This study was supported by grants from the Canadian Institutes of Health Research (CIHR) to R. Quirion and Deutsche Forschungsgemeinschaft Be1264/3-1 to A. Beck-Sickinger. A. Fournier is a 'Chercheur-Boursier' of the 'Fonds de la Recherche en Santé du Québec (FRSQ)'.

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(Received February 5, 2003
Revised March 27, 2003
Accepted May 12, 2003)