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[^{125}I]-GR231118: a high affinity radioligand to investigate neuropeptide Y Y_1 and Y_4 receptors

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1 GR231118 (also known as 1229U91 and GW1229), a purported Y_1 antagonist and Y_4 agonist was radiolabelled using the chloramine T method.

2 [^{125}I]-GR231118 binding reached equilibrium within 10 min at room temperature and remained stable for at least 4 h.

3 Saturation binding experiments showed that [^{125}I]-GR231118 binds with very high affinity (K_d of 0.09–0.24 nM) in transfected HEK293 cells with the rat Y_1 and Y_4 receptor cDNA and in rat brain membrane homogenates. No specific binding sites could be detected in HEK293 cells transfected with the rat Y_2 or Y_5 receptor cDNA demonstrating the absence of significant affinity of GR231118 for these two receptor classes.

4 Competition binding experiments revealed that specific [^{125}I]-GR231118 binding in rat brain homogenates is most similar to that observed in HEK293 cells transfected with the rat Y_1, but not rat Y_4, receptor cDNA.

5 Autoradiographic studies demonstrated that [^{125}I]-GR231118 binding sites were fully inhibited by the Y_1 antagonist BIBO3304 in most areas of the rat brain. Interestingly, high percentage of [^{125}I]-GR231118/BIBO3304-insensitive binding sites were detected in few areas. These [^{125}I]-GR231118/BIBO3304-insensitive binding sites likely represent labelling to the Y_4 receptor subtype.

6 In summary, [^{125}I]-GR231118 is a new radiolabelled probe to investigate the Y_1 and Y_4 receptors; its major advantage being its high affinity. Using highly selective Y_1 antagonists such as BIBO3304 or BIBP3226 it is possible to block the binding of [^{125}I]-GR231118 to the Y_1 receptor allowing for the characterization and visualization of the purported Y_4 subtype.

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Abbreviations: BIBO3304, (R)-N-[[4-(aminocarbonylaminomethyl)-pheyl]methyl]-N2-(diphenylacetyl)-argininamide trifluoroacetate; BIBP3226, R-N^2-(Diphenylacetyl)-N-(4-hydroxyphenyl)-methyl argininamide; GR231118, homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-CONH$_2$; h, human; HEK293, human embryonic kidney cells; KRP, Krebs Ringer phosphate buffer; NPY, neuropeptide Y; p, porcine; PP, pancreatic polypeptide; PYY, peptide YY; r, rat

Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide which shares high sequence homology with peptide YY (PYY) and the pancreatic polypeptides (PP) (Tatemoto *et al*., 1992). NPY is one of the most abundant peptides found in the mammalian brain (de Quidt & Emson, 1986a,b). Several biological effects have been observed following intracerebroventricular injections of NPY and its congeners including increased feeding, decreased anxiety related-behaviours, modulation of LH-RH and ACTH secretions, and the regulation of various neuronal, activities (for reviews see Colmers & Bleakman, 1994; Dumont *et al*., 1992; Gehlert, 1998; Inui, 1999; Kalra & Crowley, 1992; Vezzani *et al*., 1999; Wahlestedt & Reis, 1993). These various effects of NPY are mediated by the activation of at least five receptor subtypes expressed in mammalian brain (Dumont *et al*., 1996a; 1997; 1998a, b; Gehlert & Gackenheimer, 1997; Gehlert *et al*., 1997; Jacques *et al*., 1997; Statnick *et al*., 1997; Trinh *et al*., 1996; Whitcomb *et al*., 1997).

Several studies using [^{125}I]-NPY or [^{125}I]-PYY as radioligands have demonstrated that the rat brain contained high amounts of NPY receptors distributed in various brain structures including the cortex, the hippocampus, multiple thalamic, hypothalamic and brainstem nuclei, and the cerebellum and the spinal cord (Dumont *et al*., 1992; Lynch *et al*., 1989; Martel *et al*., 1990; Kar & Quirion, 1992; Quirion & Martel, 1992). Subsequent autoradiograph studies using [^{125}I]-PYY in the presence of [Pro^{34}]-PYY or C-terminal NPY/PYY fragments revealed the existence of at least two classes (Y_1-like and Y_2-like) of receptors in the rat brain (Aicher *et al*., 1991; Dumont *et al*., 1990; 1993; Gehlert *et al*., 1992; Larsen *et al*., 1993). The differential distribution of these receptor subtypes was confirmed using more selective radioligands such as [^{125}I]-Leu^{31},Pro^{34}]-PYY (Y_1-like) and [^{125}I]-PYY$_{3-36}$ (Y_2-like) (Dumont *et al*., 1995; 1996a; Gehlert & Gackenheimer, 1997). However, further competition binding experiments revealed that both radioligands also recognized additional receptor sites suggesting further NPY receptor heterogeneity in the CNS (Dumont *et al*., 1995; 1998a; Statnick *et al*., 1997).

The cloning of additional NPY receptors confirmed this hypothesis. In addition to the well established Y_1 and Y_2 subtypes proposed by Wahlestedt *et al*. (1986) on the basis of the differential potencies of C-terminal fragments in various bioassays, three additional NPY receptors have been cloned namely by Y_4 (Bard *et al*., 1995; Gregor *et al*., 1996a; Lundell *et al*., 1995; 1996), Y_5 (Gerald *et al*., 1996; Hu *et al*., 1996) and y_6 (Gregor *et al*., 1996b; Matsumoto *et al*., 1996; Weinberg *et al*., 1996) subtypes. They are all expressed in various mammalian species except for the y_6 subtype which has not been found in the rat (Burkhoff *et al*., 1998) while in human and primates, its cDNA contains a single base deletion

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resulting in the expression of a non-functional receptor protein (Gregor *et al*., 1996b; Matsumoto *et al*., 1996). Structure-activity studies revealed that each receptor subtype has a unique pharmacological profile (Michel *et al*., 1998). However, none of the synthetic agonists used thus far demonstrated high selectivity for a single receptor subtype. In fact, [^{125}I]-[Leu^{31},Pro^{34}]-PYY, first developed as a Y_1 agonist radioligand (Dumont *et al*., 1995), was later demonstrated to bind with high affinity to the Y_4 (Gehlert *et al*., 1996a, b; 1997) and Y_5 (Dumont *et al*., 1998a; Gerald *et al*., 1996) receptors. Similarly, [^{125}I]-PYY_{3-36} originally proposed as a Y_2 receptor probe (Dumont *et al*., 1995) was found to also bind to the Y_5 (Gerald *et al*., 1996) and possibly y_6 (Matsumoto *et al*., 1996) subtypes. Additionally, [^{125}I]-human (h) PP and [^{125}I]-rat (r) PP (Trinh *et al*., 1996) as well as [^{125}I]-bovine (b) PP (Gehlert *et al*., 1997; Whitcomb *et al*., 1997) used to characterize the Y_4 subtype, also recognize the Y_5 (Dumont *et al*., 1998a; Gerald *et al*., 1996) and likely y_6 (Gregor *et al*., 1996b) receptor subtypes.

Few antagonists have also been used as radioligands to study NPY receptors. Among them, (R-N^2-(Diphenylacetyl)-N-(4-hydroxyphenyl)-methyl argininamide), known as BIBP3226, a non-peptide Y_1 receptor antagonist (Rudolf *et al*., 1994) which demonstrated no activity at the Y_2, Y_4 and Y_5 subtypes (Gerald *et al*., 1996; Gehlert *et al*., 1996b, c; Jacques *et al*., 1995; Rudolf *et al*., 1994) was used as radioligand (Entzeroth *et al*., 1995). However, autoradiographic studies revealed that [^{3}H]-BIBP3226 lacked adequate affinity leading to high non-specific labelling and low resolution compared to [^{125}I]-[Leu^{31},Pro^{34}]-PYY (Dumont *et al*., 1996b). More recently, the same group of investigators reported the development of a second non-peptide Y_1 receptor antagonist ((R)-N-[[4-(aminocarbonylaminomethyl) - phenyl]methyl] - N2 - (diphenylacetyl)-argininamide trifluoroacetate) or BIBO3304 having a 10 fold higher affinity than BIBP3226 for the Y_1 receptor with a similar selectivity profile (Dumont *et al*., 1999; Wieland *et al*., 1998). However, BIBO3304 is not available in radiolabelled form. In that context, the peptidergic Y_1 antagonist, homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$ known as GR231118 (or 1229U91, Daniels *et al*., 1995; and GW1229, Bitran *et al*., 1997) could prove most useful taking into account its very high affinity for the Y_1 (Daniels *et al*., 1995; Dumont *et al*., 1998a) and Y_4 (Parker *et al*., 1998; Schober *et al*., 1998) receptor subtypes. GR231118, first reported as a potent Y_1 antagonist (Daniels *et al*., 1995) was recently shown to be a potent agonist at the Y_4 (Parker *et al*., 1998; Schober *et al*., 1998) and possibly y_6 (Parker *et al*., 1998) receptors; the later being absent in rat and primate tissues (Burkhoff *et al*., 1998; Gregor *et al*., 1996b; Matsumoto *et al*., 1996). We report here on the development of [^{125}I]-GR231118 as a new radioligand having very high affinity for the Y_1 (Kanatani *et al*., 1996) and Y_4 receptors expressed in HEK293 cells and endogenously in rat brain tissues.

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 07.00 h) 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 and hPP were synthesized as previously described (Forest *et al*., 1990) while rPP was purchased from Bachem California (Torrance, CA, U.S.A.). R-N^2-(Diphenylacetyl)-N-(4-hydroxyphenyl)-methyl argininamide, known as BIBP3226 and ((R)-N-[[4-(aminocarbonylaminomethyl) - phenyl]methyl] - N2 - (diphenylacetyl)-argininamide trifluoracetate), code name BIBO3304 were generously provided by Boehringer Ingelheim (Germany) while homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$, (firstly known as 1229U91, GW1229 and now GR231118) was a gift from Glaxo Wellcome (Research Triangle Park NC, U.S.A.). Human embryonic kidney cells (HEK293) were obtained from Drs S.H. Shen and Y. Tong, Biotechnology Research institute (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 Xymotech (Montréal, QC, Canada). [^{3}H]-Hyperfilms and ^{125}I-microscale standards were purchased from Amersham (Mississauga, ON, Canada). All tissue culture media, antibiotics and reagents were obtained from Gibco-BRL (Burnington, ON, Canada). The expressing vector, pcDNA3, was purchased from Invitrogen (San Diego, CA, U.S.A.). All other chemicals were of analytical grade and obtained from Fisher Scientific (Montréal, QC, Canada) or Sigma Chemical (St-Louis, MI, U.S.A.).

Iodine-125 was incorporated into the tyrosine residue of GR231118 using the chloramine T method as previously described (Dumont *et al*., 1995) and the specific activity was assumed to be of the theoretical value (2000 Cimmol^{-1}).

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 (mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, dextrose 5.5 and $NaHCO_3$ 25, 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 *et al*., 1976).

Transfected cells

HEK 293 cells were maintained in Dulbecco's modified Eagle medium (DMEM) 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 vectors and was slowly mixed with 1.25 ml 2$\times$ 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. Forty-eight h later, 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]-GR231118 and unlabelled peptide or competitor as needed. Time dependency was established using 25 pM [^{125}I]-GR231118 at both 4°C and 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]-GR231118 whereas competition binding experiments were performed in the presence of 25–30 pM [^{125}I]-GR231118 and various competitors (pNPY, hPYY, [Leu^{31},-Pro^{34}]-pNPY, [Leu^{31},Pro^{34}]-pPYY, $pNPY_{2-36}$, $pNPY_{13-36}$, $hPYY_{3-36}$, $hPYY_{13-36}$, rPP, hPP, GR231118, BIBO3304 and BIBP3226) at concentrations ranging from 10^{-13} M to 10^{-6} M. Non-specific binding was determined in the presence of 1 μM GR231118. After a 1 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 (Camberra Packard Instruments, Meriden CT, U.S.A.).

All binding experiments were repeated three to six times (each in triplicate), and results (mean $\pm$ s.e.mean) expressed as percentage of specific binding or fmol mg^{-1} protein. All data obtained from the saturation isotherm experiments were subtracted for [^{125}I]-GR231118 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.). IC_{50} values (i.e. concentration of unlabelled competitor required to compete for 50% of specific binding of the radioligand) for the various competitors were calculated using the GraphPad Prism.

Quantitative receptor autoradiography

Receptor autoradiography was performed as described in details elsewhere (Dumont *et al*., 1996a; 1998a). Briefly, rats were sacrificed by decapitation, and their brains rapidly removed from the skull, frozen in 2-methylbutane at −40°C for 15 s, and then kept at −80°C until needed. Sections (20 μM) were obtained using a cryomicrotome at −17°C, mounted on gelatin-chrome-alum-coated slides, dried overnight in a desiccator at 4°C, and then kept at −80°C until use.

On the days of the experiments, adjacent coronal sections were preincubated for 60 min at room temperature in KRP buffer at pH 7.4 and then incubated for 60 min in a fresh preparation of KRP buffer containing 0.1% BSA, 0.05% bacitracin, 25 pM [^{125}I]-GR231118 in the presence and absence of 100 nM BIBO3304 (Y_1 antagonist), hPP(Y_4/Y_5) or GR231118. Following a 1 h incubation, sections were washed four times, 2 min each in ice-cold KRP buffer then dipped in deionized water to remove salts and rapidly dried. Non-specific binding was determined using 100 nM GR231118. Incubated sections were apposed against ^{3}H-Hyperfilms for 4 days alongside radioactive standards. Films were developed and quantified as described in details elsewhere (Dumont *et al*., 1996a; 1998a).

Results

GR231118, a Y_1 receptor antagonist/Y_4 agonist was iodinated using the chloramine T method (Hunter & Greenwood, 1962) and purified by HPLC. All binding experiments were performed with enriched fractions of the iodinated peptide. We tested first if [^{125}I]-GR231118 could bind to the glass fibre filters used to terminate incubation. Various concentrations (5–2000 pM) of [^{125}I]-GR231118 were incubated in 0.5 ml of KRP at room temperature for 1 h in the presence or absence of 1 μM GR231118 but without membrane homogenates. [^{125}I]-GR231118 binding increased linearly with increasing concentrations of radioligands and no difference was observed between [^{125}I]-GR231118 bound to filters in the presence or absence of 1 μM GR231118 (not shown) indicating that [^{125}]-GR231118 did not bind specifically to filters. Amounts of [^{125}I]-GR231118 found on filters represented less than 1% of the total radioactivity of the incubation buffer.

As shown in Figure 1, [^{125}I]-GR231118 binding reached equilibrium in a time- and temperature-dependent manner in rat brain membrane homogenates. Specific [^{125}I]-GR231118 binding reached equilibrium after 45 min at 4°C and remained stable for at least 3 h (Figure 1). Similarly, specific [^{125}I]-GR231118 binding reached equilibrium within 10 min at room temperature and remained stable for up to 4 h (Figure 1). Half time association ($t_{½}$) was 2.4 min at room temperature and 9.8 min at 4°C. All subsequent experiments were performed at room temperature using 60 min incubation periods.

Saturation parameters of [^{125}I]-GR231118 binding in rat brain membrane homogenates and HEK293 cells transfected with either of the rat Y_1, Y_2, Y_4 or Y_5 receptor cDNA were established next. In rat brain membrane preparations, specific [^{125}I]-GR231118 binding (25 pM) represented 95% of totally bound ligand (Figure 2). Binding parameters derived from saturation isotherms (Figure 2) demonstrated that [^{125}I]-GR231118 binds with very high affinity (K_d of 0.11 $\pm$ 0.02 nM) to an apparent single class of saturable sites (B_{max} of 39 $\pm$ 4 fmol mg^{-1} protein) in rat brain homogenates (Table 1). The nature and specificity of [^{125}I]-GR231118 binding was demonstrated further using HEK293 cells transfected with either of the rat Y_1, Y_2, Y_4 or Y_5 receptor cDNA. Using concentrations up to 1 nM, we could not detect significant amounts of specific [^{125}I]-GR231118 binding sites in HEK293 cells expressing the rat Y_2 and Y_5 receptors. In contrast, saturation isotherms demonstrated that [^{125}I]-GR231118 bound with very high affinity (K_d of 0.09 $\pm$ 0.01 nM for Y_1 and 0.24 $\pm$ 0.03 nM for Y_4) to saturable population of sites in HEK293 cells transfected with the rat Y_1 or Y_4 receptor cDNA (Table 1).

The ligand binding profile of sites targeted by [^{125}I]-GR231118 was investigated next in HEK293 cells transfected with the rat Y_1 or Y_4 receptor cDNA, as well as in rat brain homogenates. In HEK293 cells expressing the rat Y_1 receptor cDNA, the observed competition binding profile was as follows: BIBO3304 (Y_1 antagonist) = GR231118 > BIBP3226 (Y_1 antagonist) = pNPY, [Leu^{31},Pro^{34}]-pNPY > hPYY, [Leu^{31},-Pro^{34}]-pPYY > > $pNPY_{2-36}$, $hPYY_{3-36}$ > $pNPY_{13-36}$, $hPYY_{13-36}$, hPP and rPP (Table 2). As expected, a completely different profile was obtained in HEK293 cells transfected with the rat Y_4 receptor. While hPP and rPP were basically unable to compete for [^{125}I]-GR231118 binding sites in Y_1-transfected

HEK293 cells, both peptides potently inhibited binding in HEK293 cells transfected with the rat Y_4 receptor cDNA (Table 2). In contrast, BIBP3226 and BIBO3304 were inactive on Y_4 transfected cells (Table 2). In rat brain homogenates, competition binding experiments revealed that GR231118, BIBO3304 and BIBP3226 competed with high affinities for specific [^{125}I]-GR231118 sites (Figure 3; Table 2). Interestingly, while GR231118 was able to fully inhibit specific [^{125}I]-GR231118 binding, the non-peptide Y_1 receptor antagonists, BIBP3226 and BIBO3304 competed for approximately 95% of specific [^{125}I]-GR321118 binding sites. These data may indicate that [^{125}I]-GR321118 is labelling at least two population of sites in the rat brain, the major one being the Y_1 subtype.

Further evidence for the existence of [^{125}]-GR231118 binding sites which are resistant to BIBO3304 was provided using adjacent coronal rat brain sections incubated with [^{125}I]-GR231118 (25–30 pM) in the presence of either 100 nM BIBO3304 (to occlude Y_1 sites), hPP (to block Y_4 sites) or GR231118 (total population of labelled sites). The concentration of competitors used in autoradiographic studies was chosen on the basis of data obtained in membrane binding assays which demonstrated that 100 nM of GR231118 and BIBO3304 generated the maximal inhibition of [^{125}I]-GR231118 binding obtainable by each molecule (Figure 3). As shown in Figure 4, specific [^{125}I]-GR231118 binding sites are fully competed by the Y_1 antagonist BIBO3304 in the anterior olfactory nuclei, tenia tecta, claustrum, olfactory tubercle, islands of Calleja, lateral septum, various cortical areas, the dentate gyrus of the hippocampus, various thalamic, hypothalamic and brainstem nuclei, the nucleus tractus solitarius and the cerebellum, revealing the Y_1 nature of specific [^{125}I]-GR231118 labelling in these structures. Interestingly, relatively high levels of [^{125}I]-GR231118/BIBO3304-insensitive binding sites were detected in the area postrema (Figure 4). Quantitative autoradiographic data confirmed that most, if not all, specific [^{125}I]-GR231118 binding sites seen in most areas of the rat brain are fully competed by 100 nM BIBO3304 (Table 3). However, significant amounts of specific

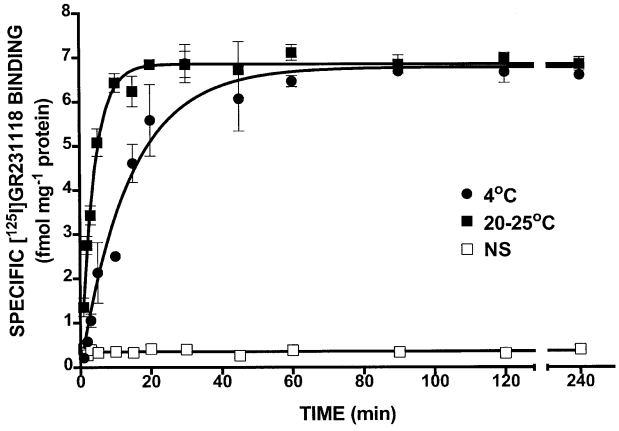


Figure 1 Typical profiles of the time course association of [^{125}I]-GR231118 binding in rat brain membrane homogenates at 4°C and room temperature. Data represent the mean ± s.e.mean of a prototypical experiment performed in triplicate. This experiment was repeated three times with similar results.

Table 1 Data derived from saturation isotherms of [^{125}I]-GR231118 binding in rat brain membrane homogenates and in HEK293 cells transfected with either of the rat Y_1, Y_2, Y_4 or Y_5 receptor cDNA

	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Rat brain homogenates	0.11 ± 0.02	39 ± 4
Y_1 transfected in HEK293 cells	0.09 ± 0.01	87 ± 14
Y_2 transfected in HEK293 cells	NO BINDING	NO BINDING
Y_4 transfected in HEK293 cells	0.24 ± 0.03	30 ± 3
Y_5 transfected in HEK293 cells	NO BINDING	NO BINDING

Data represent the mean ± s.e.mean of three to six individual determinations, each performed in triplicate. NO BINDING means no specific binding detected at 1 nM [^{125}I]-GR231118. K_d represents the apparent affinity of [^{125}I]-GR231118 and B_{macx} represents the maximal binding capacity expressed in fmol mg^{-1} protein. These values were calculated using nonlinear regression with the GraphPad Prism program.

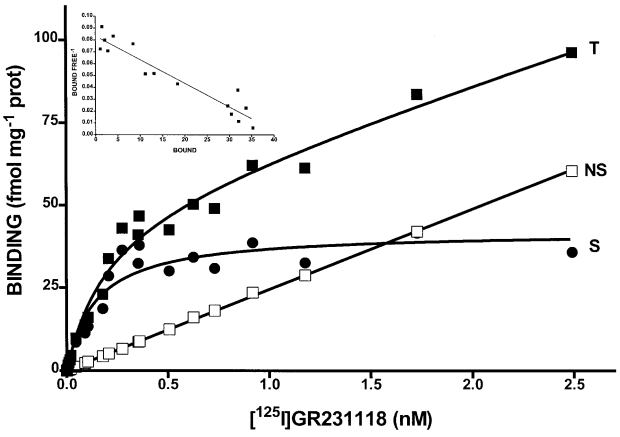


Figure 2 Typical profiles of saturation binding isotherms of [^{125}I]-GR231118 binding in rat brain membrane homogenates. Membranes were incubated with increasing concentrations of [^{125}I]-GR231118 (T; total binding) in the presence of 1 μM GR231118 (NS; non-specific binding). Specific (S) binding represents the difference between total and non-specific binding. Insert is a Scatchard transformation of the isotherm saturation binding experiment. Data represent the mean ± s.e.mean of a prototypical experiment performed in triplicate.

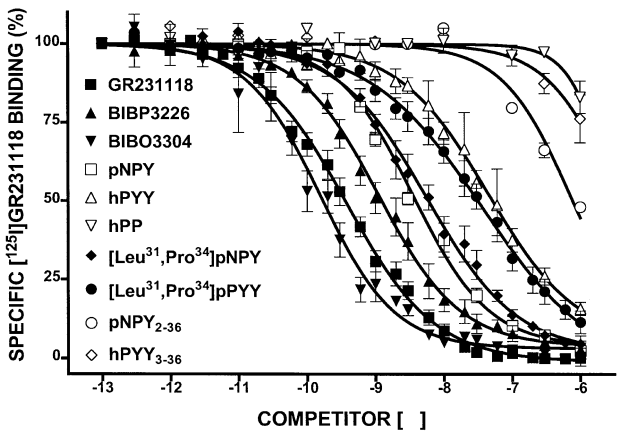


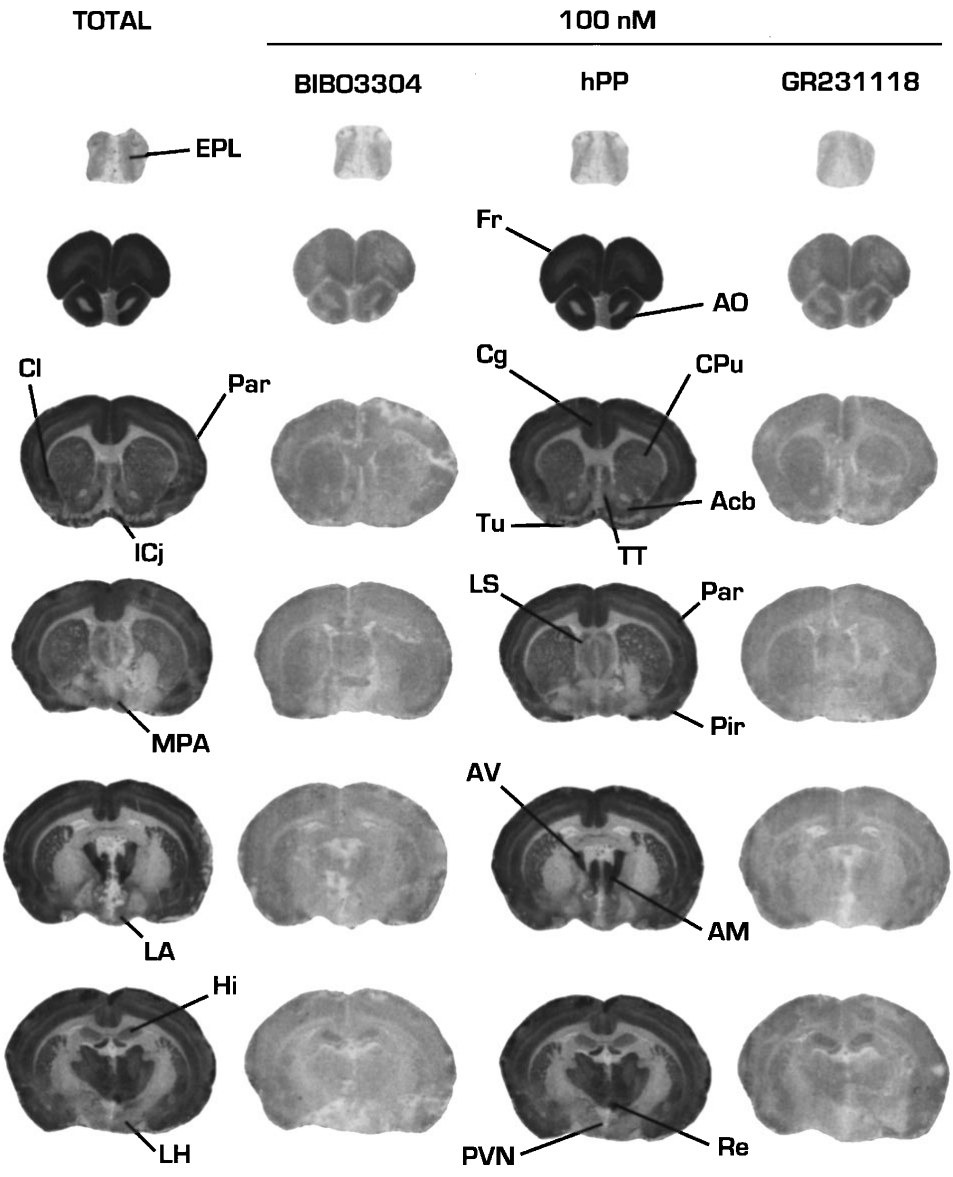
Figure 3 Competition binding profiles of various agonists and antagonists of the NPY family against specific [^{125}I]-GR231118 binding in brain membrane homogenates. Data represent the mean ± s.e.mean of four to six determinations, each performed in triplicate.

[^{125}I]-GR231118/BIBO3304-insensitive sites were observed in the area postrema (Table 3). Additionally, few other areas such as the external plexiform layer of the olfactory bulb and the CA1, CA2 and CA3 subfields of the hippocampus contained low but still significant amounts of [^{125}I]-GR231118 sites that were resistant to BIBO3304 (Table 3). These sites may belong to the Y_4 subtype since hPP was able to compete for a fraction of specific [^{125}I]-GR231118 labelling in these regions (Table 3).

Table 2 Competition binding parameters of various agonists and antagonists of the NPY family against [^{125}I]-GR231118 binding in rat brain homogenates and HEK 293 cells transfected with either of the rat Y_1 or Y_4 receptor cDNA

Competitors	*Rat brain* IC_{50} (nM)	*Y_1 transfected* IC_{50} (nM)	*Y_4 transfected* IC_{50} (nM)
pNPY	3.5±1.0	2.6±0.9	38±14
[Leu^{31}, Pro^{24}]-pNPY	5.6±1.3	3.6±0.5	4.3±1.0
$pNPY_{2-36}$	780±240	520±140	>1000
$pNPY_{13-36}$	>1000	>1000	>1000
hPYY	44±12	27±5	31±10
[Leu^{31},Pro^{34}]-PYY	37±9	36±6	10±4
$hPYY_{3-36}$	2400±850	720±180	>1000
$hPYY_{13-36}$	>1000	>1000	>1000
hPP	>1000	>1000	0.08±0.03
rPP	>1000	>1000	0.2±0.05
BIBP3226	1.2±0.4	1.7±0.3	>1000
BIBO3304	0.2±0.03	0.2±0.05	>1000
GR231118	0.4±0.06	0.3±0.04	0.3±0.05

Data represent the mean±s.e.mean of three to six individual determinations, each performed in triplicate. IC_{50} represents the concentration of competitors needed to inhibit 50% of specific binding.



Discussion

Our results demonstrated that [^{125}I]-GR231118 has very high affinity (K_d in the sub-nM range) for the Y_1 and Y_4 receptor subtypes while being mostly inactive on the Y_2 and Y_5 receptors. In fact, [^{125}I]-GR231118 is one of the highest affinity radioligand developed thus far to target the Y_1 receptor subtype including [^{125}I]-[Leu^{31},Pro^{34}]-PYY (Dumont *et al*., 1995) and [^{3}H]-BIBP3226 (Entzeroth *et al*., 1995). These two radioligands have at least a 10 fold lower affinity for the Y_1 receptor compared to [^{125}I]-GR231118. This new radioligand also has very low non-specific binding, this being a major advantage for both homogenate binding assays and receptor autoradiography. Moreover, despite its relatively poor selectivity for the Y_1 vs Y_4 receptor subtypes, [^{125}I]-GR231118 in the presence of a saturating concentration of a highly selective Y_1 antagonist such as BIBO3304, allows for the investigation of the Y_4 receptor without interference from the Y_5 subtype, in contrast to PP-related radioligands (Gerald *et al*., 1996; Hu *et al*., 1996). Hence, [^{125}I]-GR231118 should become a most useful probe to investigate both the Y_1 (as a peptide antagonist ligand) and Y_4 (as a peptide agonist ligand) receptors in mammalian tissues.

Receptor binding assays (Dumont *et al*., 1995; 1998a), autoradiographic studies (Dumont *et al*., 1996a, b; 1998a, b; Gehlert & Gackenheimer, 1997; Gehlert *et al*., 1997; Trinh *et al*., 1996; Whitcomb *et al*., 1997) and *in vivo* assays (Colmers & Bleakman, 1994; Dumont *et al*., 1992; Inui, 1999; Kalra &

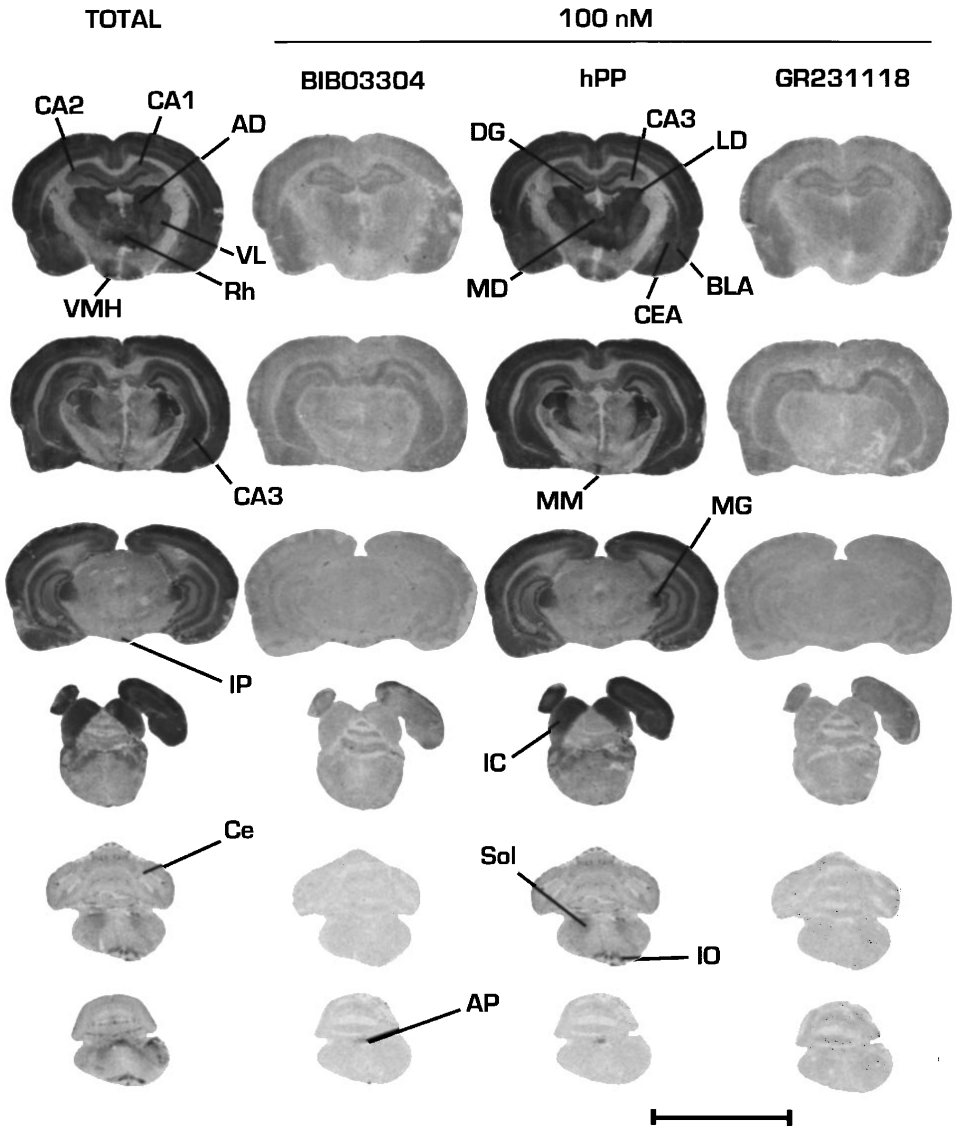


Figure 4 Photomicrographs of the autoradiographic distribution of [^{125}I]-GR231118 binding sites in the rat brain. Adjacent coronal rat brain sections were incubated in the presence of 25 pM [^{125}I]-GR231118 (total binding, Total) and either 100 nM BIBO3304 (to block Y_1 sites), 100 nM hPP (to block Y_4 sites), 100 nM hPP (to block Y_4 sites) or 100 nM GR231118 (non-specific binding). Abbreviations: Acb, Accumbens; AD, anterodorsal thalamic nuclei; AM, anteromedial thalamic nuclei; AO, anterior olfactory nuclei; AP, area postrema; AV, anteroventral thalamic nuclei; BLA, basolateral amygdaloid nuclei; CA1, CA1 subfield of the hippocampus; CA2, CA2 subfield of the hippocampus; CA3, CA3 subfield of the hippocampus; Ce, cerebellum; CEA, Central amygdaloid nucleus; Cg, cingulate cortex; Cl, claustrum; CPu, caudate putamen; DG, dentate gurus; EPL, external plexiform layer of the olfactory bulb; Fr, frontal cortex; Hi, hippocampus; IC, inferior colliculus; ICj, Island of Calleja; IO, inferior olive; IP, interpeduncular nucleus; LA, lateroanterior hypothalamic nucleus; LD, laterodorsal thalamic nuclei; LH, lateral hypothalamic nucleus; LS, lateral septum; MD, mediodorsal thalamic nuclei, MG, medial geniculate nuclei, MM, mamilary nucleus; MPA, medial preoptic area; Par, parietal cortex; Pir, piriform cortex; PVN, paraventricular hypothalamic nucleus; Re, reuniens thalamic nucleus; Rh, Rhomboid thalamic nucleus; Sol, nucleus tractus solitarius; TT, tenia tecta; Tu, olfactory tubercle; VL, ventrolateral thalamic nuclei; VMH, ventromedial hypothalamic nucleus. Scale bar represents 10 mm.

Table 3 Quantitative autoradiographic distribution of specific [^{125}I]-GR231118 binding sites in the rat brain

	Specific [^{125}I]-GR231118 binding			
		Per cent of inhibition in the presence of		
Regions	*nCi g^{-1} tissue wet weight*	*100 nM BIB03304*	*100 nM hPP*	*Per cent of putative Y_4 sites*
External plexiform layer of the olfactory bulb	108±25	60±9	45±6	40–45
Anterior olfactory nuclei	1358±143	92±8	6±4	–
Frontal cortex				
Superficial layers	1304±92	95±6	0	–
Mid layers	918±126	97±4	0	–
Deep layers	685±30	103±5	0	–
Cingulate cortex	893±79	100±6	0	–
Parietal cortex				
Superficial layers	1173±114	100±5	0	–
Mid layers	855±73	100±6	0	–
Deep layers	643±48	100±4	1	–
Piriform cortex	617±70	102±5	3	–
Tenia tecta	969±106	102±4	0	–
Caudate putamen	388±34	95±6	3	–
Olfactory tubercle	824±57	99±4	2	–
Island of Calleja	1194±128	99±5	1	–
Claustrum	1453±137	101±7	0	–
Lateral septum	241±33	99±6	2	–
Medial preoptic area	230±44	97±5	0	–
Anteroventral thalamic nuclei	979±126	95±7	0	–
Anterodorsal thalamic nuclei	886±58	96±5	1	–
Anteromedial thalamic nuclei	762±84	97±6	0	–
Mediodorsal thalamic nuclei	840±64	101±7	0	–
Rhomboid nucleus	786±81	103±4	1	–
Reuniens thalamic nucleus	1187±168	102±5	0	–
Laterodorsal thalamic nuclei	901±67	99±6	0	–
Ventrolateral thalamic nuclei	478±48	98±6	4	–
Medial geniculate nuclei	1030±125	96±7	0	–
Lateroanterior hypothalamic nucleus	346±49	98±5	0	–
Lateral hypothalamic nucleus	143±28	97±7	1	–
Paraventricular hypothalamic nucleus	234±30	100±5	0	–
Ventromedial hypothalamic nucleus	535±65	104±6	3	–
Mammillary nucleus	1027±85	96±5	0	–
Hippocampal formation				
Dentate gyrus	727±69	96±7	0	–
Dorsal hippocampus				
CA1 subfield	499±36	93±4	10±5	5–10
CA2 subfield	307±37	87±8	18±7	13–18
CA3 subfield	225±18	91±6	6±3	6–9
Ventral hippocampus				
CA3 subfield	860±59	83±7	9±5	9–17
Basolateral amygdaloid nuclei	598±86	100±6	0	–
Central amygdaloid nuclei	355±56	101±4	0	–
Interpenduncular nucleus	115±21	100±5	0	–
Central gray	227±34	100±7	0	–
Inferior colliculus	1160±107	102±5	1	–
Cerebellum	90±20	100±4	0	–
Vestibular nucleus	329±95	95±6	0	–
Nucleus tractus solitarius	190±31	100±4	0	–
Inferior olive	600±57	100±4	0	–
Area postrema	512±71	69±8	40±5	30–40

Values represent the mean±s.e.mean of three determinations using a concentration of 30 pM [^{125}I]-GR231118. Nonspecific labelling in the presence of 100 nM GR231118 was digitally subtracted from all readings. These values should not be regarded as reflecting maximal binding capacities since full saturation experiments on sections have not been performed. The values given here are hence relative. The percentage of putative Y_4 sites was defined as the percentage of specific [^{125}I]-GR231118 binding resistant to 100 nM BIBO3304 and/or inhibited by 100 nM hPP.

Crowley, 1992; Quirion *et al*., 1990; Vezzani *et al*., 1999) have demonstrated the existence of heterogeneous populations of NPY receptors in the rat brain. Similarly, mouse, guinea-pig, monkey and human brains are also enriched with multiple NPY receptors (Caberlotto *et al*., 1997; Dumont *et al*., 1997; 1998b; Gehlert & Gackenheimer, 1997; Jacques *et al*., 1997; Statnick *et al*., 1997; Widdowson, 1993). Furthermore, *in situ* hybridization studies demonstrated that Y_1, Y_2, Y_4 and Y_5 mRNA are expressed in various structures of the rat (Gerald *et al*., 1996; Gustafson *et al*., 1997; Larsen *et al*., 1993; Larsen & Kristensen, 1997; 1998; Parker & Herzog, 1998; 1999; Tong *et al*., 1997) and human (Caberlotto *et al*., 1997; 1998; Jacques *et al*., 1996; 1998; Statnick *et al*., 1998) brains. Accordingly, the development of optimal radioreceptor assay conditions to investigate each NPY receptor subtype is still a most significant objective.

Using [^{125}I]-[Leu^{31},Pro^{34}]-PYY in the presence of selective non-peptide Y_1 receptor antagonists, we were able to

demonstrate that [^{125}I]-[Leu^{31},Pro^{34}]-PYY/BIBP3226-insensitive sites have a ligand binding profile similar to the Y_5 receptor subtype in the rat CNS (Dumont *et al*., 1998a). However, under these conditions, the possible labelling of putative Y_4 receptor could not be fully excluded as [^{125}I]-[Leu^{31},Pro^{34}]-PYY also possesses some affinity for this subtype (Gehlert *et al*., 1996a). Interestingly, the present study demonstrated that [^{125}I]-GR231118 binds with very high affinity to the Y_1 and Y_4 receptors, but not to the Y_2 and Y_5 subtypes, transfected in HEK293 cells. Moreover, competition binding experiments using various analogues of NPY, PYY and the PPs as well as selective Y_1 antagonists such as BIBP3226 (Rudolf *et al*., 1994) and BIBO3304 (Wieland *et al*., 1998) demonstrated that [^{125}I]-GR231118 binds to the Y_1 and Y_4 receptors with a different ligand binding profile depending upon the receptor subtype expressed in transfected HEK293 cells. The apparent affinity of various agonists and antagonists to compete against [^{125}I]-GR231118 binding in Y_1 or Y_4 receptor transfected in HEK 293 cells is rather similar to those previously reported for these two receptors using other radioligands and/or preparations (Bard *et al*., 1995; Gerald *et al*., 1996; Gehlert *et al*., 1996a, b; Gregor *et al*., 1996a).

In rat brain membrane homogenates, various agonists and antagonists of the Y family competed for [^{125}I]-GR231118 binding with a ligand selectivity profile similar to that observed in HEK293 cells transfected with the rat Y_1 but not with the Y_4 receptor cDNA. These data suggest that most of the sites targeted by [^{125}I]-GR231118 in the rat brain are of the Y_1 subtype. This hypothesis is supported further by the high affinities of BIBP3226 and BIBO3304 to compete for specific [^{125}I]-GR231118 binding sites in the rat CNS. Interestingly however, and in contrast to non-radioactive GR231118, BIBP3226 and BIBO3304 competed for up to a maximum of 95% of specific [^{125}I]-GR231118 binding in the rat brain suggesting that in addition to the Y_1 receptor subtype, another population of sites (possibly the Y_4 receptor) is expressed and recognized by [^{125}I]-GR231118 in this tissue.

Autoradiographic studies revealed that the distribution of [^{125}I]-GR231118 labelling is largely similar to those seen using [^{125}I]-[Leu^{31},Pro^{34}]-PYY as radioligand (Dumont *et al*., 1996a; 1998a, b; Gehlert & Gackenheimer, 1997). Additionally, most specific [^{125}I]-GR231118 binding is competed by 100 nM BIBO3304 (and not by 100 nM hPP) in various brain structures supporting further the recognition of the Y_1 receptor subtype. However, adjacent coronal rat brain sections incubated with 25 pM [^{125}I]-GR231118 in the presence of a saturating concentration (100 nM) of BIBO3304 (to block Y_1 sites) revealed the existence of [^{125}I]-GR231118/BIBO3304-insensitive sites. These specific binding sites are mainly found in the area postrema. Additionally, lower but still significant amounts of [^{125}]-GR231118/BIBO3304-insensitive sites are expressed in the external plexiform layer of the olfactory bulb and in CA1, CA2 and CA3 subfields of the hippocampal formation. Specific [^{125}I]-GR231118 binding sites seen in these regions were also partly sensitive to hPP (100 nM). Considering that [^{125}I]-GR231118 failed to recognize the Y_2 and Y_5 receptors, it may be taken as an indication that [^{125}I]-GR231118/BIBO3304-insensitive sites represent a Y_4 subtype. This is in accordance with data reported here in Y_4-transfected HEK293 cells that demonstrated the high affinity (K_d = 0.24 nM) of [^{125}I]-GR231118 for this receptor. Moreover, other purported Y_4 ligands including [^{125}I]-hPP, [^{125}I]-rPP and [^{125}I]-oPP (Gehlert *et al*., 1997; Trinh *et al*., 1996; Whitcomb *et al*., 1990; 1997) and Y_4 receptor mRNA studies (Larsen & Kristensen, 1997) highlighted the area postrema as a targeted structure. However, moderate to very high levels of specific [^{125}I]-PP binding sites were also found in the medial preoptic area, paraventricular nucleus of the hypothalamus and interpeduncular nucleus (Dumont *et al*., 1998b; Gehlert *et al*., 1997; Trinh *et al*., 1996; Whitcomb *et al*., 1997). These structures contained much lower amounts of specific [^{125}I]-Leu^{31},Pro^{34}]-PYY (Dumont *et al*., 1996a; 1998a, b; Gehlert *et al*., 1997) and [^{125}I]-GR231118 (this study) binding sites even if these two radioligands possess high affinities for the Y_4 receptor subtype (Gehlert *et al*., 1996a; this study). This may be taken as evidence for the existence in these regions of yet another receptor that is preferentially recognized by PP-related molecules, in addition to the Y_4 and Y_5 subtypes (Gehlert *et al*., 1996a, b; Gerald *et al*., 1996). Naturally, molecular information is currently lacking to support this hypothesis. On the other hand, as reported by Walker *et al*. (1997) for [^{125}I]-PYY, it could be that [^{125}I]-[Leu^{31},Pro^{34}]-PYY and [^{125}I]-GR231118 have a different sensitivity for guanine nucleotides as compared to [^{125}I]-rPP or [^{125}I]-hPP at the Y_4 receptor subtype. This could explain differences in labelling intensity observed between these radioligands. Further studies will be required to clarify this issue.

In summary, our results demonstrated that [^{125}I]-GR231118 binds with very high affinity to rat brain homogenates, rapidly reaching equilibrium at room temperature. Isotherm saturation experiments revealed that [^{125}I]-GR231118 binds with very high affinity to the Y_1 and Y_4 receptors while it is basically inactive at the Y_2 and Y_5 subtypes transfected and expressed in HEK293 cells. Additionally, in the presence of a selective Y_1 antagonist, it is possible to discriminate between the Y_1 and Y_4 subtypes in tissues expressing both receptors such as the rat brain. Hence, [^{125}I]-GR231118 should prove most useful to investigate in detail the respective characteristics of these two NPY receptor subtypes in a variety of tissues.

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