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Characterization of *N*-(1-Acetyl-2,3-dihydro-1*H*-indol-6-yl)-3-(3-cyano-phenyl)-*N*-[1-(2-cyclopentyl-ethyl)-piperidin-4yl]-acrylamide (JNJ-5207787), a Small Molecule Antagonist of the Neuropeptide Y Y_2 Receptor

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ABSTRACT

The in vitro pharmacological properties of *N*-(1-Acetyl-2,3-dihydro-1*H*-indol-6-yl)-3-(3-cyano-phenyl)-*N*-[1-(2-cyclopentyl-ethyl)-piperidin-4yl]-acrylamide (JNJ-5207787), a novel neuropeptide Y Y_2 receptor (Y_2) antagonist, were evaluated. JNJ-5207787 inhibited the binding of peptide YY (PYY) to human Y_2 receptor in KAN-Ts cells (pIC_{50} = 7.00 ± 0.10) and to rat Y_2 receptors in rat hippocampus (pIC_{50} = 7.10 ± 0.20). The compound was >100-fold selective versus human Y_1, Y_4, and Y_5 receptors as evaluated by radioligand binding. In vitro receptor autoradiography data in rat brain tissue sections confirmed the selectivity of JNJ-5207787. [^{125}I]PYY binding sites sensitive to JNJ-5207787 were found in rat brain regions known to express Y_2 receptor (septum, hypothalamus, hippocampus, substantia nigra, and cerebellum), whereas insensitive binding sites were observed in regions known to express Y_1 receptor (cortex and thalamus). JNJ-5207787 was demonstrated to be an antagonist via inhibition of PYY-stimulated guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding ([^{35}S]GTPγS) in KAN-Ts cells (pIC_{50} corrected = 7.20 ± 0.12). This was confirmed autoradiographically in rat brain sections where PYY-stimulated guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding was inhibited by JNJ-5207787 (10 μM) in hypothalamus, hippocampus, and substantia nigra. After intraperitoneal administration in rats (30 mg/kg), JNJ-5207787 penetrated into the brain (C_{max} = 1351 ± 153 ng/ml at 30 min) and occupied Y_2 receptor binding sites as revealed by ex vivo receptor autoradiography. Hence, JNJ-5207787 is a potent and selective pharmacological tool available to establish the potential role of central and peripheral Y_2 receptors in vivo.

Neuropeptide Y (NPY) is a 36-amino acid peptide discovered in the early 1980s that belongs to a family of peptides that includes pancreatic polypeptides (PP) and peptide YY (PYY) (Tatemoto and Mutt, 1980). NPY is widely distributed in the central and peripheral nervous systems (for reviews, see Blomqvist and Herzog, 1997; Gehlert, 1999; Wieland et al., 2000; Kask et al., 2002; Malmstrom, 2002; Thorsell and Heilig, 2002). In the brain, NPY is the most abundant neuropeptide yet identified and its localization suggests an involvement in a variety of physiological processes, including anxiety, food intake, water consumption, circadian rhythms, hormone release, learning, and memory.

The use of various cloning techniques has resulted in the identification of five receptors to date (Y_1, Y_2, Y_4, Y_5, and y_6) (Herzog et al., 1992; Larhammar et al., 1992; Bard et al., 1995; Gerald et al., 1995, 1996; Gregor et al., 1996; Hu et al., 1996; Matsumoto et al., 1996; Weinberg et al., 1996), all of them belong to the superfamily of G protein-coupled receptors. All NPY receptor subtypes are expressed in several species, including human, except the y_6, which is absent in rat and not functional in the human and primates (Blomqvist and Herzog, 1997). Y_1 and Y_2 receptors are the most abun-

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ABBREVIATIONS: NPY, neuropeptide Y; PP, pancreatic polypeptide; PYY, peptide YY; CHO, Chinese hamster ovary; JNJ-5207787, *N*-(1-acetyl-2,3-dihydro-1*H*-indol-6-yl)-3-(3-cyano-phenyl)-*N*-[1-(2-cyclopentyl-ethyl)-piperidin-4-yl]-acrylamide); HEK-293, human embryonic kidney; PBS, phosphate-buffered saline; 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] acetyl]-*N*-[2-[1,2-di-hydro-3,5(4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamide; BIBP-3226, *R*-*N*^{2}-(diphenylacetyl)-*N*-(4-hydroxyphenyl)-methyl argininamide; BSA, bovine serum albumin; GTPγS, guanosine-5′-*O*-(3-thio)-triphosphate; L-152804, 5,5-dimethyl-2-(2,3,4,9-tetrahydro-3,3-dimethyl-1oxo-*1H*-xanthene-9-yl)-1,3-cyclohexanedione; BNST, bed nucleus of the stria terminalis.

dantly expressed NPY receptor subtypes in the brain (Dumont et al., 1998b). In rat, the Y_1 receptor is found primarily in the cerebral cortex and thalamic regions, whereas the Y_2 receptor is found in a variety of areas, including the septum, hypothalamus, hippocampus, substantia nigra, and cerebellum (Dumont et al., 1996; Gehlert and Gackenheimer, 1997). The presence of a high level of Y_5 receptor mRNA has been demonstrated in hypothalamus but the radioligand binding data has not confirmed such a high abundance (Dumont et al., 1998a).

Among the NPY receptor subtypes known today, mainly the Y_1 and Y_5 have been investigated as drug targets in the obesity field, due to the observation that these subtypes mediate the orexigenic action of NPY (Parker et al., 2002). Small molecule receptor antagonists for the Y_1 and Y_5 receptor subtypes have been described (for reviews, see Ling, 1999; Wieland et al., 2000). Recently, the Y_2 receptor subtype has attracted particular interest because of its possible implication in control of food intake (Naveilhan et al., 1999; Kaga et al., 2001; Batterham et al., 2002; Sainsbury et al., 2002) and bone formation (Baldock et al., 2002; Herzog, 2002). Pharmacologically, the Y_2 receptor is characterized by high affinity for NPY and PYY, but unlike the Y_1 receptor, is relatively resistant to the effect of the N-terminal deletions and retains a high binding affinity for C-terminal fragments such as $NPY_{13–36}$ (Gerald et al., 1995; Rose et al., 1995). Activation of the Y_2 receptor results in the inhibition of adenylyl cyclase (Gehlert et al., 1996). In contrast to other NPY receptor subtypes, Y_2 receptor has been shown to exist as a pre- and a postsynaptic receptor (Gehlert et al., 1996). Recently, BIIE0246, the first nonpeptide Y_2 receptor antagonist has been described (Doods et al., 1999). However, its complex structure and high molecular weight limit its usefulness as an in vivo pharmacological tool. In a program directed toward the discovery of novel Y_2 receptor ligands, we have discovered JNJ-5207787, a nonpeptidic, low-molecular-weight, selective Y_2 ligand (Jablonowski et al., submitted).

In the present study, we report the receptor binding pharmacology of JNJ-5207787 as well as its functional in vitro properties and in vivo selectivity. We demonstrate that JNJ-5207787 selectively binds Y_2 receptors in vivo and will thus be a useful tool for the in vivo pharmacological evaluation of the role of Y_2 receptors in a variety of physiological conditions.

Materials and Methods

All the experiments described in this study have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Radioligand Binding Assays. Cells used in the radioligand binding experiments with NPY receptor subtypes were SK-N-MC endogenously expressing Y_1 receptors (Larhammar et al., 1992), KAN-Ts endogenously expressing Y_2 receptors (Rimland et al., 1996), CHO cells transfected with human Y_4 cDNA for Y_4 receptors (Bard et al., 1995), and HEK-293 transfected with human Y_5 cDNA for Y_5 receptors (Gerald et al., 1996; Hu et al., 1996). Cells were grown to confluence on 150-cm^2 tissue culture plates, washed with phosphate-buffered saline (PBS), and scraped into 50-ml tubes. After centrifugation, the supernatant was aspirated, and the pellets frozen and stored at −80°C. Thawed pellets were homogenized with a Polytron tissue grinder for 15 s in 20 mM Tris-HCl, 5 mM EDTA. The homogenate was centrifuged at 800*g* for 5 min and the collected supernatant recentrifuged at 25,000*g* for 25 min. The resulting pellet was resuspended in binding buffer (20 mM HEPES, 120 mM NaCl, 0.22 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$). Membranes were incubated with [^{125}I]PYY (80 pM) for Y_1, Y_2, and Y_5 or [^{125}I]PP (100 pM) for Y_4 in the presence or absence of test compound for 1 h at room temperature. The reaction was stopped by filtration through GF/C filter plates presoaked in 0.3% polyethylenimine and subsequently washed with Tris 50 mM, 5 mM EDTA buffer. Plates were dried for 1 h in a 55°C oven, scintillation fluid was added, and the radioactivity was counted in a PerkinElmer TopCount. Specific binding to the NPY receptor subtypes was determined by radioactivity that was bound in the presence of 1 μM NPY for Y_1, Y_2, Y_5, and 100 nM PP for Y_4. Membranes from rat hippocampus were prepared and assayed for [^{125}I]PYY binding following the same procedure. Binding experiments were repeated three to eight times, each in duplicate. IC_{50} values (i.e., concentration of unlabeled peptide or antagonist required to compete for 50% of specific binding to the radioligand) were calculated using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA) with a fit to a sigmoidal dose-response curve. Data were expressed as pIC_{50} values where $pIC_{50} = -\log IC_{50}$.

In addition, the selectivity of JNJ-5207787 was evaluated in a large variety of ion channels, transporters, and receptor binding assays. These assays were performed by CEREP (Celles L'Evescault, France).

In Vitro Receptor Autoradiography. Adult male rats (Sprague-Dawley) were euthanized using carbon dioxide and decapitated. Brains were immediately removed from the skull and rapidly frozen in dry ice. Twenty-micrometer-thick horizontal, sagittal, and coronal sections were cut using a Cryostat-microtome (Microm HM505E) and thaw-mounted on adhesive microscope slides (Superfrost$^+$ Plus; VWR, West Chester, PA). The sections were kept at −70°C until use. The procedure for autoradiography was performed according to Dumont et al. (1993). Briefly, brain sections were preincubated for 30 min at room temperature in a Krebs-Ringer phosphate buffer at pH 7.4 and then incubated for 120 min in a fresh preparation of Krebs-Ringer phosphate buffer supplemented with 0.1% bovine serum albumin, 0.05% bacitracin, and 25 pM [^{125}I]PYY in the presence or absence of either JNJ-5207787, or BIBP-3226. Nonspecific binding was determined using adjacent sections incubated in the presence of 1 μM unlabeled hNPY. At the end of the incubation, sections were washed 4 times (4 min each) in ice-cold buffer, dipped in deionized water and rapidly dried under a stream of cold air. Sections were exposed to a Fujifilm Imaging Plate (BAS-MS2025) for 12 h. The Phosphor Imaging Plate was scanned using a Fuji Bio-Imaging Analyzer System (BAS-5000). The digitized computer images generated by the scanner were visualized and quantified using ImageGauge V3.12 software (Fujifilm). Adobe Photoshop 7.0 and Microsoft Power Point were used for preparation of the figures.

[^{35}S]GTPγS Binding Assay in KAN-Ts Cells. Membranes from KAN-Ts cells were prepared as described above. Membranes were thawed on ice and diluted in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 5 μM GDP, 0.25% bovine serum albumin. Assay mixtures (150 μl) were preincubated with compounds for 30 min at ambient temperature. Then, 50 μl of [^{35}S]GTPγS in assay buffer was added to a final concentration of 200 pM, and the assay mixtures were incubated for 1 h at ambient temperature. Reactions were terminated by rapid filtration thought GF/C filters. Filters were washed twice with ice-cold 50 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$. Basal [^{35}S]GTPγS was measured in the absence of compounds. In initial experiments, nonspecific binding was measured in the presence of 100 μM GTPγS. This nonspecific binding never exceeded 10% of basal binding and was thus not subtracted from experimental data. Stimulation of [^{35}S]GTPγS is presented as percentage over basal and was calculated as one hundred times the difference between stimulated and basal binding (in cpm). Agonist concentration-response curves for in-

creases in [^{35}S]GTPγS binding and antagonist inhibition curves for inhibition of PYY (300 nM)-stimulated [^{35}S]GTPγS binding were analyzed by nonlinear regression using GraphPad Prism software (GraphPad Software Inc.). EC_{50} (concentration of compound at which 50% of its own maximal stimulation is obtained) and IC_{50} (concentration of its own maximal inhibition of PYY-stimulated [^{35}S]GTPγS binding is obtained) were derived from the curves. IC_{50} values were corrected as follows: corrected IC_{50} (IC_{50} corr) = IC_{50}/ (1 + [PYY]/ EC_{50} (PYY)) and pIC_{50} corr = −log IC_{50} corr.

[^{35}S]GTPγS Autoradiography in Rat Brain Tissue Sections. Rat brain sections were prepared as described above. [^{35}S]GTPγS binding was visualized using the method described by Primus et al. (1998) with slight modifications. Briefly, slides were preincubated in assay buffer (50 mM Tris, pH 7.7, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl) at ambient temperature for 10 min. Slides were then incubated in the presence of 2 mM GDP, 10 μM dipropylcyclopentylxanthine, and either in the absence or presence of 10 μM BIBP-3226 or JNJ-5207787 for an additional 15 min. Slides were then incubated for 2 h at ambient temperature in assay buffer supplemented with 0.04 nM [^{35}S]GTPγS and 1 μM PYY in the presence or absence of 10 μM BIBP-3226 or 10 μM JNJ-5207787. Basal activity was determined in the absence of PPY, but with GDP. Nonspecific binding was assessed by including 10 μM unlabeled GTPγS in the incubation buffer. The reaction was terminated by rinsing twice for 3 min in ice-cold 50 mM Tris buffer. Slides were rinsed once in distilled water and dried under a cool air stream. Sections were exposed to a Fujifilm imaging plate (BAS-SR2025) for 12 h and processed as described in the in vitro receptor autoradiography section. Stimulation of [^{35}S]GTPγS was presented as percentage over basal.

Blood-Brain Barrier Penetration. Two groups of sixteen female Sprague-Dawley Rats were used (approximately 300 g of body weight). Animals received a bolus dose of JNJ-5207787 in the peritoneal cavity (i.p.) at a dose of 30 mg/kg in a volume of 1 ml/kg. The dosing solution was prepared in 40% 2-hydroypropyl-β-cyclodextrin in physiological saline solution. Dosing was followed by blood sampling via cardiac puncture over a time course. Blood samples consisted of 250-μl samples taken from the heart using a 23-gauge needle into 1.5-ml microcentrifuge tubes. Brains were removed from the animals and bisected down the midsagittal plane. One hemisphere was frozen on dry ice for ex vivo receptor binding autoradiography and the other was homogenized for liquid chromatography/ tandem mass spectrometry analysis.

All blood samples were deproteinized by 1:4 dilution of the sample with acetonitrile with vigorous mixing. These samples were incubated for 5 min, and then centrifuged at 14,000 rpm in a microcentrifuge for 4 min. The supernatant was recovered into auto-sampler vials and diluted 1:1 with sterile water. A Vydac SP C18 (2.1 × 50-mm) analytical column was used for separation.

Ex vivo receptor binding autoradiography was performed on brain sections as described by Langlois et al. (2001). Twenty-micrometer-thick sagittal sections at the level of the hypothalamic regions were collected and incubated as described in the in vitro autoradiography section, but with the following modification: the sections were not washed before incubation and were incubated 10 min with 100 pM [^{125}I]PYY in the presence of 1 μM BIBP-3226 for Y_1 receptor occlusion.

Chemicals. JNJ-5207787 was synthesized and prepared as a free base at Johnson & Johnson Pharmaceutical Research and Development. L-152804 was obtained from Tocris Cookson (Ellisville, MO). BIBP-3226 and all peptides were obtained from Bachem (Torrance, CA). All peptides used in this study were human. For in vitro assays, JNJ-5207787, L-152804, and BIBP-3226 were dissolved in dimethyl sulfoxide (stock solution at 10 mM) and further diluted in assay buffer. [^{125}I]PYY (2200 Ci/mmol), [^{125}I]PP (2200 Ci/mmol), and [^{35}S]GTPγS (1053 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA).

Results

Receptor Binding Pharmacology. Receptor binding assays in KAN-Ts cells endogenously expressing human Y_2 receptors demonstrated that JNJ-5207787 (Fig. 1) competed with high affinity (pIC_{50} = 7.00 ± 0.10, S.E.M) against specific [^{125}I]PYY receptor binding sites (Fig. 2A). Shallow curves were observed for JNJ-5207787 with Hill slope values significantly different from unity (0.60). Curves could not be fitted to a two site model.

In contrast to JNJ-5207787, the selective Y_1 antagonist BIBP-3226 and the selective Y_5 antagonist L-152804 at concentrations up to 10 μM failed to significantly compete for specific [^{125}I]PYY receptor binding sites (Fig. 2A). The different peptides competed for specific [^{125}I]PYY receptor binding sites in KAN-Ts with the following order of affinity: PYY > PYY3-36 > NPY > NPY3-36 (Table 1).

At concentrations up to 10 μM, JNJ-5207787 failed to compete for significant amounts of specific [^{125}I]PYY receptor binding sites in SK-N-MC (Y_1) and HEK-293 (Y_5) (Fig. 2, B and C; Table 1), whereas BIBP-3226 and L-152804 competed with high affinity for specific [^{125}I]PYY receptor binding sites in these cells (Fig. 2, B and C; Table 1). The different peptides competed for the specific [^{125}I]PYY receptor binding sites in SK-N-MC (Y_1) and HEK-293 (Y_5) with the following order of affinity: PYY > NPY > PYY3-36 > NY3-36 (Table 1).

JNJ-5207787, BIBP-3226, and L-152804 also failed to compete (up to 10 μM) for significant amount of specific [^{125}I]PP binding sites in CHO cells transfected with cDNA encoding Y_4 receptors (Table 1).

In homogenates from rat brain hippocampus, a brain region known to express Y_2, but not Y_1 receptors (Gehlert and Gackenheimer, 1997), JNJ-5207787 was able to compete for specific [^{125}I]PYY receptor binding sites with high affinity (pIC_{50} = 7.10 ± 0.09, S.E.M.), whereas BIBP-3226 and L-152804 did not compete for specific [^{125}I]PYY receptor binding sites up to 10 μM (Fig. 2D; Table 1). As observed in KAN-Ts cells, shallow curves were obtained for JNJ-5207787. Curves could not be fitted to a two site model. The different peptides competed for the specific [^{125}I]PYY receptor binding sites in rat hippocampus with the following order of affinity: PYY > NPY > PYY3-36 > NY3-36 (Table 1).

Finally, JNJ-5207787 was assayed by binding in a panel of 50 receptors, ion channels, and transporters assays including adenosine (A_1, A_{2A}, and A_3), adrenergic (α_1, α_2, and β_1),

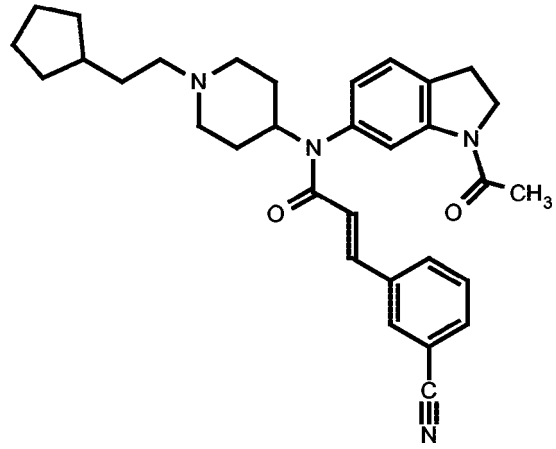

Fig. 1. Structure of JNJ-5207787.

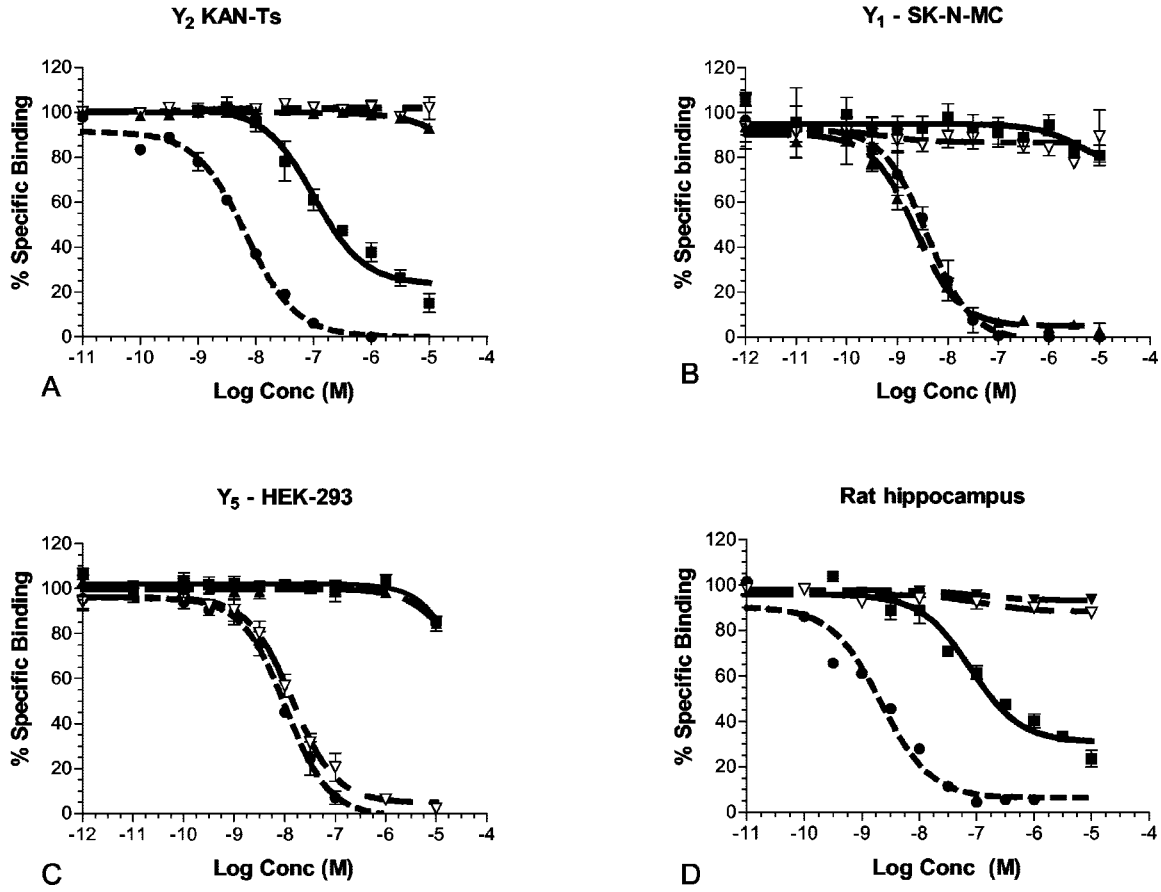

Fig. 2. Inhibition of [^{125}I]PYY binding to human Y_2 (A), Y_1 (B), and Y_5 (C) receptors cell membrane homogenates and rat hippocampus homogenates (D) by various concentrations of JNJ-5207787, BIBP-3226, L-152804, and hNPY. Data represent the mean ± S.E.M. of three to eight determinations, each performed in duplicate. Nonspecific binding was defined as the binding remaining in the presence of 1 μM NPY. Mean pIC_{50} values are summarized in Table 1. ■, JNJ-5207787; ▲, BIBP-3226; ▽, L-152804; and ●, NPY.

TABLE 1

Comparative binding parameters (pIC_{50}) of JNJ-5207787, BIBP-3226, L-152804, and various peptides against selective [^{125}I]PYY binding sites in SK-N-MC (human Y_1 receptor), KAN-Ts (human Y_2 receptor), HEK-293 (human Y_5 receptor), and rat brain hippocampal homogenates or selective [^{125}I]PP binding sites in CHO (human Y_4 receptor)

Data represent the mean ± S.E.M. of three to eight determinations, each performed in duplicate. $pIC_{50} = -\log IC_{50}$, and IC_{50} represents the concentration of competitors needed to inhibit 50% of the specific binding.

	hY_1 SK-N-MC [^{125}I]PYY	hY_2 KAN-Ts [^{125}I]PYY	hY_4 CHO [^{125}I]PP	hY_5 HEK293 [^{125}I]PYY	rY_2 Rat Hippocampus [^{125}I]PYY
JNJ-5207787	<5.00	7.00 ± 0.10	<5.00	<5.00	7.10 ± 0.20
BIBP-3226	8.62 ± 0.04	<5.00	<5.00	<5.00	<5.00
L-152804	<5.00	<5.00	<5.00	7.76 ± 0.04	<5.00
NPY	8.39 ± 0.05	8.43 ± 0.01	N.D.	8.01 ± 0.04	8.29 ± 0.04
PYY	8.64 ± 0.08	9.52 ± 0.04	N.D.	8.17 ± 0.07	8.68 ± 0.02
NPY_{3-36}	6.92 ± 0.07	7.89 ± 0.08	N.D.	7.21 ± 0.08	7.80 ± 0.01
PYY_{3-36}	7.21 ± 0.05	8.92 ± 0.10	N.D.	7.43 ± 0.04	7.39 ± 0.03
PP	<5.00	<5.00	9.10 ± 0.10	<5.00	<5.00

N.D., not determined.

angiotensin (AT1), dopamine (D_1 and D_2), bradykinin (B_2), cholecystokinin (CCKA), galanin (GAL_2), melatonin ML_1), muscarinic (M_1, M_2, and M_3), neurotensin (NT_1), neurokinin (NK_2 and NK_3), opiate (μ, κ, and δ), serotonin (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_3, 5-HT_{5A}, 5-HT_6, and 5-HT_7), somatostatin, vasopressin (V_{1a}), norepinephrine transporter, dopamine transporter, and ion channels (sodium, calcium, potassium, and chloride). JNJ-5207787 at concentrations up to 1 μM was inactive (inhibition of less than 50%) except in sodium channel site 2 (IC_{50} = 10 μM; data not shown).

In Vitro Receptor Autoradiography Studies. The distribution of specific [^{125}I]PYY receptor binding sites that are sensitive to JNJ-5207787 and BIBP-3226 in different rat brain regions was established next using in vitro receptor autoradiography. Representative digitized computer images from sagittal, horizontal, and coronal sections are shown in Fig. 3.

As reported previously (Dumont et al., 1993), the total population of [^{125}I]PYY receptor binding sites is widely but discretely distributed in rat brain (Fig. 3, A–A″). High densities of

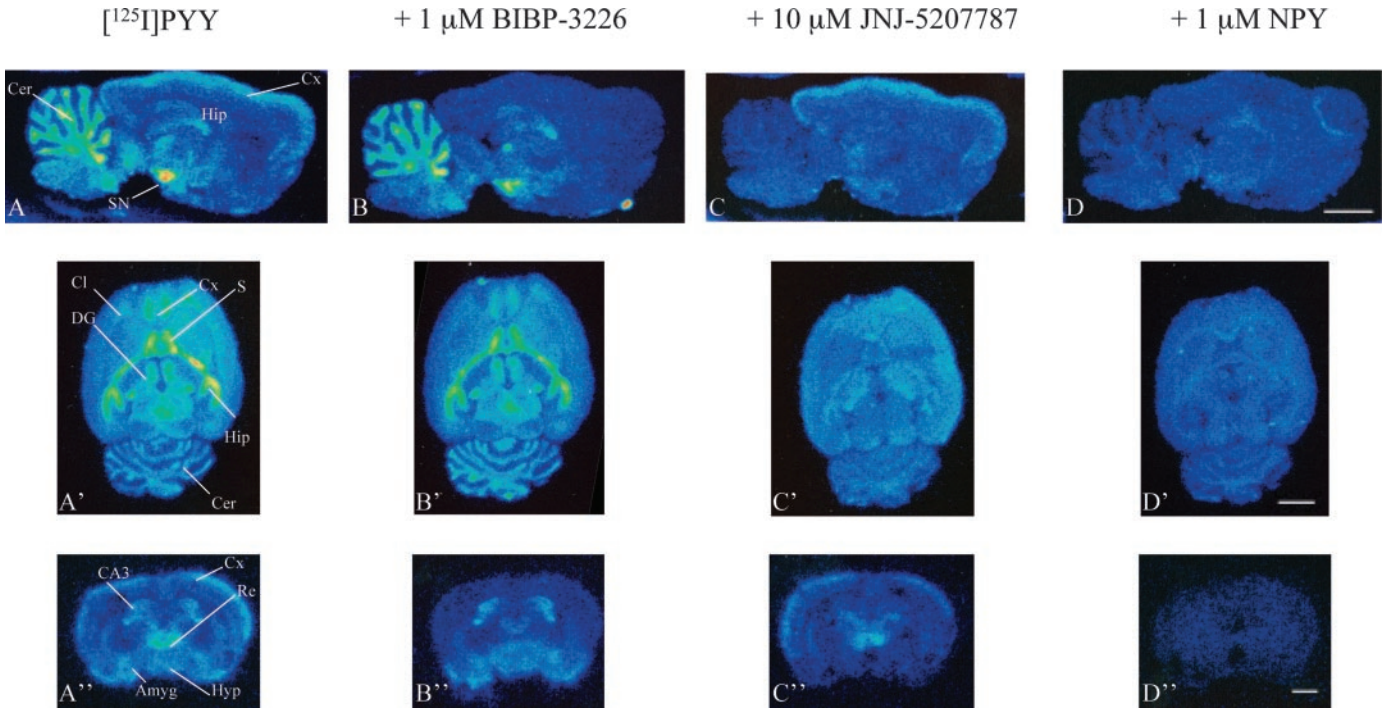

Fig. 3. Digitized computer images of the distribution of [^{125}I]PYY binding sites in sagittal (A), horizontal (A′), and coronal sections (A″), in the presence of 1 μM BIBP-3226 for Y_1 receptor subtype occlusion (B–B″), 10 μM JNJ-5207787 for Y_2 receptor occlusion (C–C″). Nonspecific binding was determined in the presence of 1 μM NPY (D–D″). Note in B, B′, and B″ the displacement of [^{125}I]PYY binding sites in cortex and in B″ in the reuniens nucleus of the thalamus. Specific [^{125}I]PYY binding sites sensitive to JNJ-5207787 were found cerebellum (C and C′), substantia nigra (C), septum, (C′), hippocampus (C–C″), hypothalamus (C″), and amygdala (C″). Colors represent relative levels of optical density, ranging from red > yellow > green > blue > black. Scale bar, 0.25 cm. Amyg, amygdala; Cer, cerebellum; Cl, claustrum; Cx, cortex; CA3, field CA3 of the hippocampus; DG, dentate gyrus; Hip, hippocampus; Hyp, hypothalamus; Re, reuniens nucleus of the thalamus; S, septum; SN, substantia nigra.

[^{125}I]PYY receptor binding sites were observed in septum (Fig. 3A′), cortical area (superficial layers) (Fig. 3, A–A″), claustrum (Fig. 3A′), hippocampus (oriens layer and stratum radum; Fig. 3A′), reuniens nucleus of the thalamus (Fig. 3A″), ventral tegmental area, substantia nigra (Fig. 3A), and cerebellum (granular layer, Fig. 3, A and A″). Moderate densities were observed in hypothalamus and amygdala (Fig. 3A″).

The selective Y_1 receptor antagonist BIBP-3226 (1 μM) almost completely inhibited the labeling of [^{125}I]PYY in cortical area (Fig. 3, B–B″), claustrum (Fig. 3B′) and also competed for almost all the labeling in the reuniens nucleus of the thalamus (Fig. 3B″).

JNJ-5207787 (10 μM) inhibited [^{125}I]PYY labeling in lateral septum (Fig. 3C′), cerebellum (Fig. 3 C′), ventral tegmental area (data not shown), substantia nigra (Fig. 3B′), hippocampus (CA1 and CA3; Fig. 3, C–C″), septum (Fig. 3C′), amygdala (Fig. 3C″), and hypothalamus (Fig. 3C″).

As determined by quantitative autoradiography in rat hippocampus, JNJ-5207787 was able to compete for specific [^{125}I]PYY binding sites with high affinity (pIC_{50} = 6.89 ± 0.25, S.E.M; data not shown). Nonspecific binding determined on adjacent sections in the presence of 1 μM NPY was very low (Fig. 3, D–D″).

[^{35}S]GTPγS Binding Study in KAN-Ts Cells. The antagonistic properties of JNJ-5207787 were then evaluated in a [^{35}S]GTPγS binding assay in KAN-Ts cells endogenously expressing human Y_2 receptors (Fig. 4).

PYY stimulated binding of [^{35}S]GTPγS to membranes of KAN-Ts cells with a maximal response of about 110% over the basal level (pEC_{50} = 7.50 ± 0.20) (Fig. 4A). JNJ-5207787, by itself, did not affect [^{35}S]GTPγS binding up to 10 μM (Fig. 4A).

JNJ-5207787 was examined for its ability to inhibit PYY (300 nM)-stimulated [^{35}S]GTPγS binding to membranes of Y_2-KAN-Ts cells. JNJ-5207787 had antagonistic properties and inhibited the PYY-stimulated [^{35}S]GTPγS binding to basal level with a pIC_{50} corr of 7.20 ± 0.12 (Fig. 4B).

[^{35}S]GTPγS Binding in Rat Brain Sections. PYY (1 μM) was used to stimulate [^{35}S]GTPγS binding at NPY receptor subtypes in rat brain sections. Representative digi-

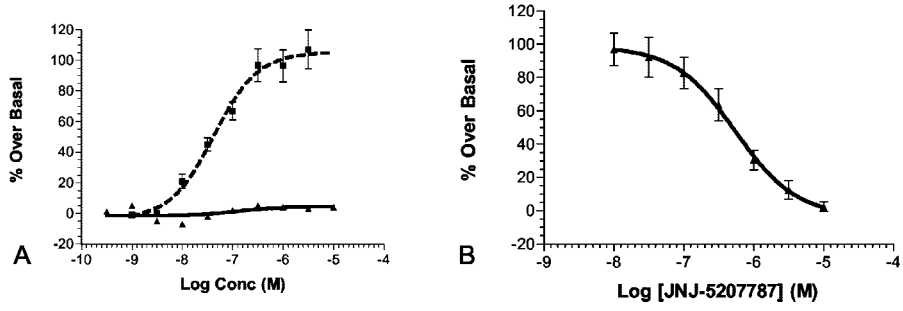

Fig. 4. [^{35}S]GTPγS binding to membranes of hY_2-KAN-Ts cell membranes. A, stimulation of [^{35}S]GTPγS binding by PYY (■) or JNJ-5207787 (▲). B, antagonism of PYY (300 nM)-stimulated [^{35}S]GTPγS binding by JNJ-5207787. Data represent the mean ± S.E.M. of three determinations, each performed in triplicate. Mean pEC_{50} and corrected pIC_{50} (pIC_{50}) corr are given under *Results*.

tized computer images from sagittal rat brain sections are shown in Fig. 5. The percentage of increase in [^{35}S]GTPγS binding over basal levels after stimulation with 1 μM PYY in the absence or presence of BIBP-3226 and JNJ-5207787 for several representative brain regions (cortex, hippocampus, hypothalamus, and substantia nigra) are presented in Table 2.

In the absence of agonist, [^{35}S]GTPγS binding densities corresponding to basal levels were observed in bed nucleus of the stria terminalis (BNST), substantia nigra, and in several hypothalamic nuclei (Fig. 5A). In the presence of PYY (1 μM) [^{35}S]GTPγS binding densities increased in cortex, hippocampus, hypothalamus, thalamus, substantia nigra, septum, and BNST (Fig. 5B). For the representative regions where quantification was performed, the highest increase compared with basal level was observed in cortex > hypothalamus > substantia nigra > hippocampus (Table 2). No increase in [^{35}S]GTPγS binding densities was observed in cerebellum (Fig. 5B).

In the presence of BIBP-3226 (10 μM), PYY (1 μM) increased [^{35}S]GTPγS binding densities in hippocampus, hypothalamus, substantia nigra, and BNST but not in cortex and thalamus (Fig. 5C; Table 2).

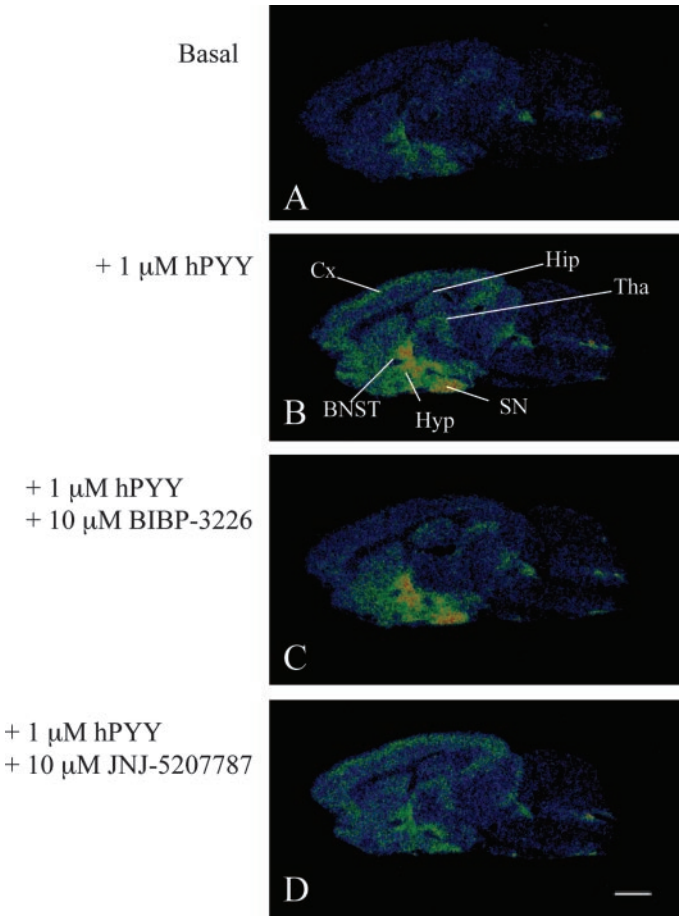

Fig. 5. Digitized computer images showing basal activity in the absence of agonist (A), stimulation of [^{35}S]GTPγS binding by 1 μM hPYY (B), in the presence of 10 μM BIBP-3226 (C) or 10 μM JNJ-5207787 (D) in rat brain sagittal sections. Quantitative values for cortex, hippocampus, hypothalamus, and substantia nigra are summarized in Table 2. Colors represent relative levels of optical density, ranging from red > yellow > green > blue > black. Scale bar, 0.25 cm. Cx, cortex; Hip, hippocampus; Hyp, hypothalamus; SN, substantia nigra; Tha, thalamus.

TABLE 2

Quantitative autoradiography of PYY (1 μM) stimulated [^{35}S]GTPγS binding in the absence or presence of BIBP-3226 (10 μM) or JNJ-5207787 (10 μM) in rat brain sections

Data are expressed as percentage over basal activity (n = 3–4, mean ± S.E.M.).

	PYY	PYY + BIBP-3226	PYY + JNJ-5207787
Cortex	98 ± 15	3 ± 2	92 ± 17
Hippocampus (CA1–CA3)	52 ± 6	43 ± 10	11 ± 4
Hypothalamus	69 ± 5	46 ± 6	9 ± 5
SN	64 ± 9	58 ± 12	9 ± 5

In the presence of JNJ-5207787 (10 μM), PYY (1 μM) increased [^{35}S]GTPγS binding densities in cortex and thalamus but not in hypothalamus, hippocampus, BNST or substantia nigra (Fig. 5D; Table 2).

Blood-Brain Barrier Penetration Study. The blood-brain barrier penetration profile of JNJ-5207787 is shown in Fig. 6. In a preliminary experiment the compound exhibited poor oral bioavailability at 1–3% (data not shown). Intraperitoneal administration of the compound (30 mg/kg) resulted in plasma half-life of 2.03 h (±0.20, S.D.) with a C_{max} of 5859 ng/ml (±140, S.D) at 30 min (Fig. 6A). JNJ-5207787 exhibited fair i.p. bioavailability at 33%. JNJ-5207787 (i.p. 30 mg/kg) crossed the blood brain barrier with a C_{max} of 1351 ng/ml (±153, S.D) at 30 min (Fig. 6A).

The ex vivo receptor occupancy study (i.p. 30 mg/kg) showed that JNJ-5207787 occupied Y_2 receptor binding sites in hypothalamic area. The maximal receptor binding site occupancy (45% ± 10.05, S.D.) was found at 3 h (Fig. 6B).

Discussion

In this article, we report the in vitro pharmacological characterization of JNJ-5207787, a selective nonpeptide antagonist of the Y_2 receptor. JNJ-5207787 was discovered through structure-activity research studies of a piperidinylindoline cinnamide high throughput screening lead (J. A. Jablonowski, W. Chai, X. Li, D. A. Rudolph, W. V. Murray, M. A. Youngman, S. L. Dax, D. Nepomuceno, P. Bonaventure, T. W. Lovenberg, and N. I. Carruthers, manuscript submitted for publication).

JNJ-5207787 was shown to be a moderately potent Y_2 receptor antagonist with similar affinity for human Y_2 receptors endogenously expressed in KAN-Ts cells and rat brain Y_2 receptors (pIC_{50} = 7.00 and 7.10, respectively). The selectivity of JNJ-5207877 was shown by its lack of activity at concentrations up to 10 μM for human Y_1, Y_4, and Y_5 receptor subtypes. In addition, JNJ-5207787 was found to be selective against a wide range of receptors and enzymes (inhibition of less than 50% at 1 μM). In both KAN-Ts cells and rat hippocampus, shallow binding curves were observed for JNJ-5207787 with Hill coefficient significantly different from unity. We have been unable to identify the cause of these shallow binding curves. The presence of high concentration of guanosine 5′-(β,γ-imido)triphosphate (a nonhydrolysable analog of GTP) did not affect the slope of the curve (data not shown), suggesting that G protein coupling or affinity state is not the cause of the shallow curve. In vitro receptor autoradiography confirmed the binding data and further demonstrated the selectivity of JNJ-5207787. JNJ-5207787 competed for specifically bound [^{125}I]PYY in rat brain regions known to express Y_2 receptors (septum, hypothalamus, hippocampus, substantia nigra, and cerebellum). JNJ-5207787-

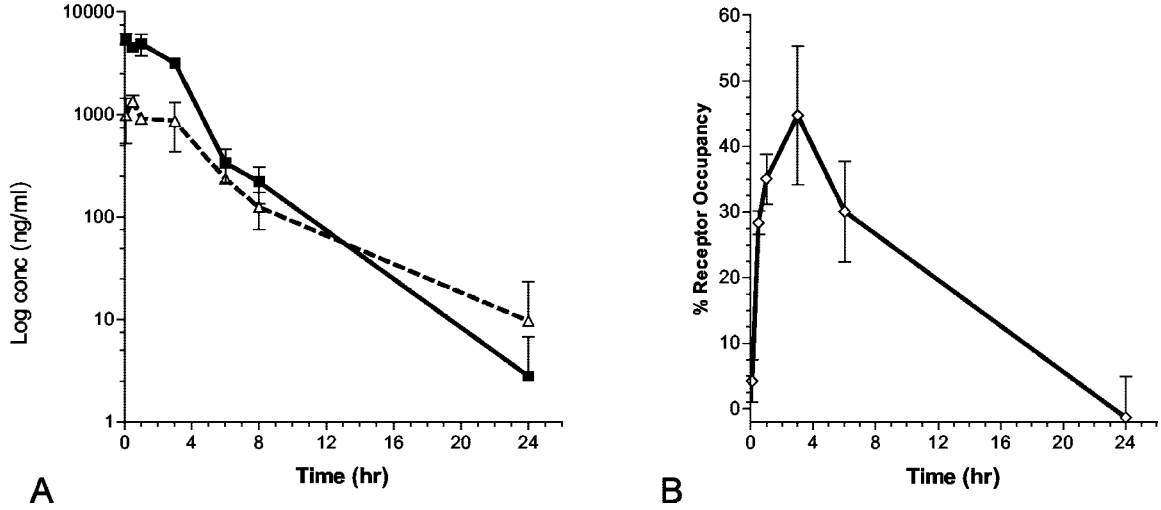

Fig. 6. Blood-brain barrier penetration study of JNJ-5207787 after a single-dose i.p. administration in rat. A, plasma (■) and brain (△) concentration in function of time after i.p. (30-mg/kg) administration of JNJ-5207787. B, percentage of receptor occupancy in rat brain hypothalamus after i.p. (30-mg/kg) administration of JNJ-5207787 as determined by ex vivo receptor autoradiography.

insensitive [^{125}I]PYY binding sites were observed in regions known to express Y_1 receptor (cortex and thalamus).

The antagonistic property of JNJ-5207787 for the Y_2 receptor was demonstrated using [^{35}S]GTPγS binding. JNJ-5207787 inhibits PYY-stimulated [^{35}S]GTPγS binding to basal level in KAN-Ts cells endogenously expressing the human Y_2 receptor, with potency corresponding to its binding affinity for the receptor (pIC_{50} corr = 7.2 and pIC_{50} = 7.0). [^{35}S]GTPγS autoradiography further demonstrated the anatomical selectivity of JNJ-5207787 antagonism. Selective blockade of PYY-stimulated [^{35}S]GTPγS binding was observed in regions known to express Y_2 receptor except in cerebellum where PYY was unable to affect basal activity. In contrast, significant amount of [^{125}I]PYY binding sites sensitive to JNJ-5207787 were detected in cerebellum. As previously reported by Primus et al. (1998), the distribution of Y_1 and Y_2 receptor binding sites using [^{125}I]PYY correlates nicely with the distribution obtained using PYY stimulated [^{35}S]GTPγS binding with one notable exception in cerebellum. Comparison of receptor densities observed using receptor autoradiography and [^{35}S]GTPγS autoradiography are difficult to make because [^{35}S]GTPγS binding tends to favor Gi-linked G protein interaction, and little is known about the composition, turnover, or the function of the endogenous receptor G protein complex. Therefore, regional differences in PYY-stimulated [^{35}S]GTPγS binding may not reflect differences in receptor densities, but instead may be the consequence of region-specific G protein expression and/or different receptor G protein transduction efficacies.

After intraperitoneal administration, JNJ-5207787 was found to penetrate into the brain and occupied Y_2 receptor binding sites. Hence, these functional radioligand binding and pharmacokinetic/blood brain barrier penetration data demonstrated that JNJ-5207787 is the first selective brain penetrant, nonpeptide antagonist of the Y_2 receptor and should be a useful tool to investigate central Y_2 receptor function.

The majority of the Y_2 ligands described to date are peptidic in nature and thus are limited in their use as investigational compounds. Examples include a peptide-based ligand, T4-[NPY 33-36]$_4$, which shows considerable affinity (pIC_{50} = 7.2) for the Y_2 receptor (Grouzmann et al., 1997) and BIIE0246, which also binds to Y_2 receptor with significant affinity (pIC_{50} = 8.5) (Doods et al., 1999). However, both of these ligands have complex structures and high molecular weights, making them unlikely to be useful in vivo probes. JNJ-5207787 is a small molecule that penetrates the brain and therefore has potential as a tool to investigate in vivo Y_2 receptor function in different animal models. JNJ-5207787 is not without its limitations. Maximal central occupancy at high i.p. dose (30 mg/kg) was only 50%. This may be sufficient to address some roles at Y_2 receptor physiology. Higher doses of JNJ-5207787 have been difficult to achieve due to solubility and formulation challenges.

Recently, a key role of presynaptic hypothalamic Y_2 receptor has been suggested in central coordination of energy homeostasis and bone mass regulation (Herzog, 2002). Studies analyzing Y_2 receptor knockout mice have started to unravel some of the individual functions of this receptor subtype. Y_2 receptor knockout mice do show a reduced body weight despite an increase in food intake, which is possibly due to the lack of the feedback inhibition of the postprandially released PYY_{3-36} (Batterham et al., 2002). The Y_2 receptor knockout mice also show a significant increase in bone formation (Baldock et al., 2002). Specific deletion of the Y_2 receptor in the hypothalamus in adult conditional Y_2 receptor knockout mice also causes an increase in bone formation. Conditional Y_2 receptor knockout also causes a significant decrease in body weight, despite an increase in food intake. JNJ-5207787 may provide a new investigational tool to address the role of Y_2 receptor in feeding and energy homeostasis.

In summary, we have demonstrated using several receptor binding assays and [^{35}S]GTPγS binding that JNJ-5207787 is a potent and selective Y_2 receptor antagonist devoid of affinity for Y_1, Y_4, and Y_5 receptors. In addition, JNJ-5207787 is bioavailable after i.p. administration and penetrates into the brain. To the best of our knowledge, JNJ-5207787 is the first potent and selective pharmacological tool to establish the potential role of the Y_2 receptor in vivo.

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