Comparative Characterization and Autoradiographic Distribution of Neuropeptide Y Receptor Subtypes in the Rat Brain

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Some evidence has suggested the existence and differential distribution of neuropeptide Y (NPY) receptor subtypes in the mammalian brain (Dumont et al., 1990; Aicher et al., 1991). We now report on the extensive characterization and visualization of at least two classes of NPY receptor sites using a highly selective Y₁ analog, [Leu³¹,Pro³⁴]-NPY or [Pro³⁴]-NPY, and a relatively specific Y₂ competitor, NPY_{13–36}. Autoradiographic studies using ¹²⁵I-peptide YY (¹²⁵I-PYY) clearly reveal that the Y₁ receptor subtype is most abundant in various cortical areas, the dentate gyrus of the hippocampal formation, the claustrum, and the reuniens nucleus of the thalamus. In most other regions, ¹²⁵I-PYY binding is potently inhibited by increasing concentrations of either NPY_{2–36} or NPY_{13–36}, suggesting a Y₂-like profile. Furthermore, binding assays using homogenates from discrete brain regions clearly demonstrate that various NPY fragments and analogs compete for ¹²⁵I-PYY labeling with profiles indicative of heterogeneity of NPY receptor subtypes, even in the presence of a selective Y₁ blocker. Thus, it is likely that, in addition to the Y₁ receptor, which is particularly concentrated in cortical areas, the rat brain is enriched with a receptor class (Y₂) that can exist under high- or low-affinity states or with additional receptor subtypes that are recognized by ¹²⁵I-PYY. These findings cannot be explained by the existence of the very recently reported Y₃ receptor subtype, since PYY does not possess significant affinity to this site (Grundemar et al., 1991). Further experiments are currently in progress to determine the nature and functional significance of each of these NPY/PYY receptor sites.

[Key words: neuropeptide Y, peptide YY, receptor subtypes, autoradiography, distribution, rat brain]

Neuropeptide Y (NPY), a 36 amino acid polypeptide isolated from porcine brain, shares important sequence homology with peptide YY (PYY) and various pancreatic polypeptides (PP) (Tatemoto, 1982; Tatemoto et al., 1982). Therefore, it has been included as a member of the PP family (Tatemoto, 1982; Tatemoto et al., 1982; Emson and DeQuidt, 1984). Interestingly, NPY is one of the most abundant peptides found in the CNS

(Chronwall et al., 1985; O'Donohue et al., 1985; DeQuidt and Emson, 1986a,b). NPY-like immunoreactivity is especially concentrated in various regions of the limbic system including certain cortical areas, the hippocampal formation, the amygdaloid nuclei, and the hypothalamus (Chronwall et al., 1985; O'Donohue et al., 1985; DeQuidt and Emson, 1986a,b). Moreover, *in situ* hybridization studies have revealed that the distribution of NPY mRNA correlates rather well with that of NPY-like-immunoreactive material (Terenghi et al., 1987; Chan-Palay et al., 1988).

Many studies have demonstrated the existence of specific receptors for NPY and related peptides in mammalian brain (Chang et al., 1985, 1986, 1988; Saria et al., 1985; Martel et al., 1986, 1987, 1988, 1990a,b; Inui et al., 1988, 1989; Walker and Miller, 1988; Busch-Sorensen et al., 1989; Lynch et al., 1989; Sheikh et al., 1989a,b; Ohkubo et al., 1990; Quirion et al., 1990; Rosier et al., 1990; Sheikh and Williams, 1990; Dumont et al., 1992; Quirion and Martel, 1992). Using anatomical methods, it was shown that NPY binding sites are concentrated mostly in the hippocampal formation, various cortical areas, the olfactory bulb, and the lateral septum. Lower but still highly significant densities of NPY sites are also present in the striatum, in various thalamic, hypothalamic, and brainstem nuclei, and in the cerebellum (Martel et al., 1986, 1987, 1988, 1990a; Inui et al., 1989; Lynch et al., 1989; Ohkubo et al., 1990; Quirion et al., 1990; Rosier et al., 1990). The overall anatomical distribution observed using a variety of radioligands including ³H-NPY, ¹²⁵I-Bolton-Hunter-NPY, or ¹²⁵I-PYY is similar. However, some differences were detected in certain hypothalamic and brainstem nuclei as well as in the cerebellum (Lynch et al., 1989; Martel et al., 1990a; Quirion et al., 1990), suggesting the possible heterogeneity of NPY receptors in the mammalian brain.

This concept is also supported by functional studies. As early as 1986, Wahlestedt and coworkers suggested the existence of two classes of NPY receptors (Y₁ and Y₂) in peripheral tissues. They observed that while the full NPY/PYY sequence was required to act upon the Y₁ receptor, C-terminal fragments such as NPY_{13–36} and PYY_{13–36} were able to activate the Y₂ subtype fully, albeit with lower potencies than the native peptides (Wahlestedt et al., 1986). In the CNS, NPY acts to stimulate food and water intake, probably via Y₁ receptors since various C-terminal fragments were inactive in this paradigm (Danho et al., 1988; Flood and Morley, 1989; Jolicoeur et al., 1991a,b; Leibowitz and Alexander, 1991). In contrast, the facilitation of memory retention induced by NPY is most likely related to the activation of Y₂ receptors (Flood and Morley, 1989). The effects of these peptides on locomotor behaviors and various cardio-

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vascular parameters are either inhibitory or stimulatory depending upon the activation of one of these two receptor types (Heilig and Murison, 1987; Heilig et al., 1988; Aguirre et al., 1990). There is a possibility that these two receptor classes are coupled to different transduction mechanisms, the Y_1 sites being coupled to phosphoinositol turnover (Wahlestedt, 1987; Hinson et al., 1988; Widdowson and Halaris, 1990a) and the Y_2 receptor being linked to the activity of adenylate cyclase (Härfstrand et al., 1987; Petrenko et al., 1987; Wahlestedt, 1987; Westlind-Danielsson et al., 1987, 1988; Widdowson and Halaris, 1990b). More recently, the existence of yet another receptor class (Y_3) was proposed on the basis that certain effects induced by NPY are shared by both Y_1 and Y_2 agonists but not by the homolog PYY (Grundemar et al., 1991; Wahlestedt et al., 1992). Rimland et al. (1991) have just reported the cloning of a G-protein-coupled receptor that could represent that new NPY receptor class, although this remains to be fully established.

In a previous study, we provided preliminary evidence for the differential distribution of Y_1 and Y_2 receptors in the rat brain (Dumont et al., 1990), based on the differential competition profile of ^{125}I -PYY binding sites by selective Y_1 agonists such as [Leu³¹,Pro³⁴]-NPY and [Pro³⁴]-NPY (Fuhlendorff et al., 1990a,b; Schwartz et al., 1990) and Y_2 -preferential fragments. However, the precise proportion of each receptor subtype in a given brain region was not established. Moreover, it is still unclear if these two broad classes of NPY receptors constitute a homogeneous population of sites or if they include other putative subtypes.

Consequently, the aim of the present study was to investigate, in detail, the respective autoradiographic distribution of Y_1 and Y_2 receptors in the rat brain. Competition binding profiles using various selective NPY-related peptides clearly suggest further heterogeneity of NPY receptors in the rat brain.

Materials and Methods

Materials. Male Sprague-Dawley CD rats (200–250 gm) obtained from Charles River Canada (St-Constant, Québec, Canada) were maintained on a 12 hr light-dark cycle (light on at 7:00 AM) in temperature- and humidity-controlled rooms. Animals were fed 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 for Animal Care.

Analogues and fragments of porcine NPY (pNPY) were synthesized in our laboratories as previously described (Forest et al., 1990; Martel et al., 1990b). ^{125}I -PYY (2200 Ci/mmol) was obtained from New England Nuclear-DuPont (Boston, MA), and film (Hyperfilm) and ^{125}I standards were purchased from Amersham Canada Ltd. (Oakville, Ontario, Canada). All other chemicals were of analytical grade and were obtained from Fisher Scientific Co. (Montréal, Québec, Canada) or Sigma Chemical Co. (St. Louis, MO).

Receptor autoradiography. Receptor autoradiography was performed as recently described by our group (Martel et al., 1990a). Briefly, rats were killed by decapitation, and their brains were rapidly removed from the skull, frozen in 2-methylbutane at -40°C for 15 sec, and then kept at -80°C until needed. Sections (20 μm) were obtained using a Bright cryomicrotome at -17°C before being mounted on gelatin-chromalum-coated slides, dried overnight in a desiccator at 4°C , and then kept at -80°C until use.

On the day of the experiments, adjacent coronal brain sections were preincubated for 60 min at room temperature in a Krebs-Ringer phosphate buffer (KRP) at pH 7.4 and then incubated for 120 min in a fresh preparation of KRP buffer supplemented with 0.1% (w/v) bovine serum albumin, 0.05% (w/v) bacitracin, and 25 μM ^{125}I -PYY in the presence or absence of either NPY₂₋₃₆, NPY₁₃₋₃₆, or [Pro³⁴]-NPY at concentrations ranging from 10^{-9} to 10^{-6} M. Nonspecific binding was determined using adjacent sections incubated in the presence of 1 μM unlabeled porcine NPY (pNPY). At the end of the incubation period, sections were washed four times (4 min each) in ice-cold buffer, dipped in deionized water to remove salts, and rapidly dried under a stream of cold air. Sections

were then placed in x-ray cassettes and apposed against Hyperfilm for 6 d. Films were developed and analyzed using the standard procedure described by Martel et al. (1990a).

Membrane binding assays. Membrane binding assays were performed as previously described (Martel et al., 1986, 1990b). Briefly, rats were killed by decapitation and their brains rapidly removed; the olfactory bulb, frontoparietal cortex, striatum (including the lateral septum), hippocampus, and cerebellum were rapidly dissected on ice and then homogenized in KRP buffer using a Brinkman polytron (at setting 6 for 15–20 sec) and centrifuged at $49,000 \times g$ for 15 min. Supernatants were discarded and pellets washed, resuspended, and recentrifuged. Final pellets from the olfactory bulb, the frontoparietal cortex, and the hippocampus obtained from four animals and from the striatum and the cerebellum from eight rats were rinsed and resuspended in 50 ml of KRP buffer each.

Binding assays were initiated by adding 100 μl of membrane preparations in a final volume of 500 μl of KRP buffer containing 0.1% bovine serum albumin, 0.05% bacitracin, 20–25 μM ^{125}I -PYY, and various competitors (pNPY, PYY, NPY₂₋₃₆, NPY₁₃₋₃₆, or [Leu³¹,Pro³⁴]-NPY) at concentrations varying from 10^{-12} to 10^{-6} M. Following a 2 hr incubation period at 25°C , the reaction was terminated by rapid filtration through Schleicher and Schuell #32 filters (previously soaked in 1.0% polyethyleneimine) using a cell harvester filtering apparatus (Brandel Instruments, Gaithersburg, MD). Filters were rinsed three times with 3 ml of cold KRP buffer and dried, and the radioactivity remaining on filters was quantified using a gamma counter (LKB Instruments) with 70% efficiency. The percentage of specifically bound ^{125}I -PYY was determined as the difference in binding observed in the presence and absence of 1 μM unlabeled pNPY and represented between 80% and 90% of total binding.

Binding data were analyzed using the LUNDON SOFTWARE competition program. Individual competition binding curves were fitted to both a one- and a two-binding-site model. If the p value was less than 0.05 on the Fisher test, a two-site model was accepted as representing the best fit for the data. All values are expressed as percentage of specific binding and represented as the mean $\pm$ SEM of four to eight individual experiments determinations, with each point done in triplicate.

Results

Comparative autoradiographic distribution of NPY receptor subtypes

As reported earlier (Lynch et al., 1989; Martel et al., 1990a), the total population of ^{125}I -PYY receptor binding sites is widely but discretely distributed in the rat brain (Figs. 1–4). Autoradiographic data obtained at the level of the olfactory bulb (Fig. 1), striatum (Fig. 2), hippocampal formation (Fig. 3), and cerebellum (Fig. 4) show that ^{125}I -PYY bound with high specificity to NPY/PYY receptor sites as indicated by the absence of labeling in the presence of a saturating concentration of pNPY. High levels of specific ^{125}I -PYY binding are present in all parts of the anterior olfactory nuclei (Fig. 1, Table 1), the superficial layers of the cortex (Figs. 1–3, Table 1), the lateral septum (Fig. 2, Table 1), the piriform cortex, and the oriens layer and stratum radiatum of the hippocampus (Fig. 3, Table 1). Areas such as the striatum (Fig. 2, Table 1), the cerebellum (Fig. 4, Table 1), and most brainstem nuclei (Fig. 4, Table 1) contained relatively moderate to low amounts of ^{125}I -PYY binding.

The fragment NPY₂₋₃₆ inhibited, in a concentration-dependent manner, ^{125}I -PYY binding without obvious specificity for a given brain region or nucleus (Figs. 1–4). At a concentration of 10^{-6} M, NPY₂₋₃₆ fully competed for ^{125}I -PYY binding sites in all areas including the cingulum, frontal, and orbital cortices and the anterior olfactory nuclei (Fig. 1); the frontoparietal cortex, claustrum, striatum, and lateral septum (Fig. 2); the hippocampus, piriform cortex, and various thalamic and hypothalamic nuclei (Fig. 3); as well as the cerebellum and various brainstem nuclei (Fig. 4).

In contrast, the specific Y_1 agonist [Pro³⁴]-NPY and the Y_2 fragment NPY₁₃₋₃₆ showed different competition profiles in var-

Figure 1. Photomicrographs of the autoradiographic distribution of ^{125}I -PYY binding sites at the level of the frontal cortex of the rat brain. Adjacent coronal sections were incubated with ^{125}I -PYY alone (20–25 pM) (*TOT*) or in the presence of either 1 μM pNPY (nonspecific binding, *NS*) or increasing concentrations (molar) of competitors such as NPY_{2-36} , NPY_{13-36} , or $[\text{Pro}^{34}]\text{-NPY}$. Note that cortical labeling is highly sensitive to $[\text{Pro}^{34}]\text{-NPY}$ but not to NPY_{13-36} . *AOD*, anterior olfactory nucleus, dorsal part; *AOV*, anterior olfactory nucleus, ventral part; *Cg*, cingulate cortex; *Fr*, frontal cortex; *OC*, orbital cortex.

ious brain regions. In the frontal cortex, the labeling of ^{125}I -PYY is almost completely inhibited by a low (10 nM) concentration of $[\text{Pro}^{34}]\text{-NPY}$, whereas NPY_{13-36} is only weakly active in this area, even at micromolar concentrations (Fig. 1, Table 1). This suggests that the frontal cortex is especially enriched with the

Y_1 receptor subtype. Interestingly, neither NPY_{13-36} nor $[\text{Pro}^{34}]\text{-NPY}$ acted as potent competitors in the olfactory bulb while NPY_{2-36} almost completely inhibited labeling in this structure (Fig. 1, Table 1). At the level of the striatum, ^{125}I -PYY binding is potently inhibited by increasing concentrations of $[\text{Pro}^{34}]\text{-}$

Figure 2. Photomicrographs of the autoradiographic distribution of ^{125}I -PYY binding sites at the level of the striatum of the rat brain. Adjacent coronal sections were incubated with ^{125}I -PYY alone (20–25 pM) (TOT) or in the presence of either 1 μM pNPY (nonspecific binding, NS) or increasing concentrations (molar) of competitors such as NPY_{2-36} , NPY_{13-36} , or $[\text{Pro}^{34}]\text{-NPY}$. Note that cortical labeling is sensitive to $[\text{Pro}^{34}]\text{-NPY}$ while subcortical binding is more sensitive to NPY_{13-36} . cc, corpus callosum; Cl, claustrum; CPu, caudate putamen; Fr, frontal cortex; LS, lateral septum; Par, parietal cortex.

Figure 3. Photomicrographs of the autoradiographic distribution of ^{125}I -PYY binding sites at the level of the hippocampal formation of the rat brain. Adjacent coronal sections were incubated with ^{125}I -PYY alone (20–25 pM) (**TOT**) or in the presence of either 1 μM pNPY (nonspecific binding, **NS**) or increasing concentrations (molar) of competitors such as NPY_{2-36} , NPY_{13-36} , or $[\text{Pro}^{34}]\text{-NPY}$. Note that cortical labeling is highly sensitive to $[\text{Pro}^{34}]\text{-NPY}$ while hippocampal binding is competed by NPY_{2-36} and NPY_{13-36} . **CA1** and **CA3**, fields CA1 and CA3 of Ammon's horn of the hippocampus; **CPu**, caudate putamen; **Hb**, habenular nucleus; **IAM**, interanteromedial thalamic nucleus; **LH**, lateral hypothalamic area; **Re**, reuniens thalamic nuclei; **st**, stria terminalis; **Th**, thalamus; **I–VI**, cortical layers I–VI.

Table 1. Quantitative autoradiographic distribution of ^{125}I -PYY binding sites in rat brain

Brain region	Total specific binding	¹²⁵ I-PYY binding (fmol/mg tissue, wet weight)				% Putative Y ₁ site
		+NPY ₁₃₋₃₆ (Y ₂)		+[Pro ³⁴]-NPY (Y ₁)		
		10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁸ M	10 ⁻⁷ M	
Anterior olfactory nucleus						
Ventral part	15.3 ± 3.5	12.9 ± 2.6	9.7 ± 1.0	6.3 ± 0.6	3.9 ± 0.2	59
Dorsal part	14.1 ± 3.2	10.0 ± 2.8	6.6 ± 0.4	5.9 ± 0.3	4.7 ± 0.5	59
Medial part	12.6 ± 1.9	9.2 ± 1.6	7.0 ± 0.5	7.2 ± 0.7	6.3 ± 0.7	43
Lateral part	14.8 ± 3.1	8.8 ± 1.4	6.1 ± 0.4	7.8 ± 1.4	7.6 ± 0.5	48
Cortical area						
Frontal cortex						
Superficial layers	8.9 ± 1.7	10.0 ± 0.5	8.1 ± 0.3	1.1 ± 0.2	0.1 ± 0.1	88
Mid layers	4.0 ± 0.3	4.1 ± 0.7	3.1 ± 1.5	1.2 ± 0.3	0.4 ± 0.2	70
Deep layers	3.9 ± 0.5	3.3 ± 0.5	2.4 ± 0.9	1.7 ± 1.6	1.2 ± 0.4	57
Parietal cortex						
Superficial layers	8.9 ± 1.6	9.5 ± 1.4	9.0 ± 0.5	1.2 ± 0.4	0.2 ± 0.1	87
Mid layers	3.2 ± 0.1	3.6 ± 0.6	2.9 ± 0.5	0.6 ± 1.3	0.3 ± 0.1	82
Deep layers	2.9 ± 0.4	2.6 ± 0.3	2.0 ± 0.6	1.4 ± 0.5	0.9 ± 0.2	52
Orbital cortex	6.5 ± 1.2	7.2 ± 0.9	5.8 ± 0.6	1.5 ± 0.4	0.7 ± 0.2	77
Primary olfactory cortex	6.8 ± 1.9	3.2 ± 1.3	1.6 ± 0.7	5.3 ± 0.8	6.0 ± 0.6	23
Piriform cortex	10.2 ± 2.4	4.8 ± 0.5	1.4 ± 0.2	7.6 ± 2.8	6.6 ± 0.7	26
Basal ganglia						
Lateral septum	29.9 ± 5.7	8.6 ± 3.4	2.8 ± 1.0	26.8 ± 5.8	24.6 ± 3.8	11
Caudate putamen	2.7 ± 0.6	1.8 ± 0.6	1.0 ± 0.2	2.0 ± 0.6	1.9 ± 0.2	26
Clastrum	13.0 ± 2.5	12.9 ± 1.9	9.9 ± 2.4	4.6 ± 1.6	3.1 ± 0.5	65
Nucleus accumbens	4.9 ± 0.6	2.6 ± 0.7	1.2 ± 0.4	4.6 ± 1.2	4.3 ± 0.3	7
Olfactory tubercle	6.5 ± 1.0	4.5 ± 0.7	2.8 ± 0.6	5.4 ± 1.4	5.0 ± 1.3	17
Hippocampal formation						
Oriens layer						
CA1	22.2 ± 4.6	3.8 ± 0.6	1.6 ± 0.4	17.4 ± 4.6	16.1 ± 1.8	22
CA2	31.1 ± 5.7	5.5 ± 1.5	2.1 ± 0.7	25.6 ± 5.8	23.4 ± 2.1	18
CA3	34.8 ± 6.2	6.5 ± 0.8	1.6 ± 0.8	32.3 ± 4.5	29.7 ± 3.4	7
Stratum radiatum						
CA1	17.0 ± 3.4	4.4 ± 0.8	2.0 ± 0.6	14.1 ± 2.9	13.2 ± 1.6	17
CA2	22.7 ± 5.2	4.9 ± 1.3	2.4 ± 1.4	20.8 ± 3.5	18.5 ± 2.1	8
CA3	29.4 ± 4.9	6.1 ± 0.7	2.9 ± 1.1	25.1 ± 3.9	23.5 ± 2.2	15
Dentate gyrus						
Molecular layer	4.2 ± 1.9	4.3 ± 1.2	3.1 ± 1.1	1.2 ± 0.5	0.9 ± 0.3	72
Granular layer	3.1 ± 1.5	1.9 ± 0.7	1.3 ± 0.4	1.5 ± 0.6	1.1 ± 0.5	52
Stria terminalis	25.8 ± 4.6	4.4 ± 0.6	1.0 ± 0.4	24.5 ± 3.4	23.1 ± 2.9	5
Thalamic nuclei						
Habenular	9.6 ± 1.5	2.9 ± 0.7	1.1 ± 0.4	9.2 ± 1.2	8.5 ± 0.3	4
Ventroposterior	4.0 ± 1.3	1.9 ± 0.3	1.0 ± 0.4	2.6 ± 0.7	2.3 ± 0.2	35
Reuniens	12.4 ± 3.8	11.6 ± 1.1	8.7 ± 1.0	4.2 ± 0.9	3.2 ± 1.7	66
Posterior	4.1 ± 1.1	4.0 ± 0.9	2.2 ± 0.3	1.2 ± 0.2	0.8 ± 0.3	70
Interanteromedial	8.6 ± 2.0	4.1 ± 0.4	2.9 ± 0.3	5.7 ± 0.5	4.9 ± 0.6	34
Laterodorsal	6.1 ± 2.0	5.1 ± 1.1	3.7 ± 0.4	2.3 ± 0.3	1.9 ± 0.8	63
Mediodorsal	10.1 ± 2.5	3.6 ± 0.5	1.5 ± 0.8	8.1 ± 0.9	7.4 ± 0.8	20
Hypothalamic nuclei						
Dorsomedial	5.2 ± 0.5	4.2 ± 1.3	2.4 ± 0.7	4.8 ± 1.1	4.6 ± 0.9	8
Ventromedial	5.5 ± 0.5	2.6 ± 0.4	0.9 ± 0.2	4.7 ± 0.6	4.1 ± 0.7	15
Lateral	7.1 ± 0.8	3.2 ± 0.7	1.1 ± 0.4	5.6 ± 1.2	5.8 ± 0.5	22
Amygdaloid complex	6.9 ± 2.2	3.2 ± 0.5	1.2 ± 0.3	5.3 ± 0.6	3.4 ± 0.4	24
Brainstem nuclei						
Posterodorsal tegmental	9.4 ± 2.1	1.6 ± 0.8	1.2 ± 0.3	10.1 ± 2.1	6.2 ± 1.4	—
Principal sensory trigeminal	4.8 ± 0.6	1.4 ± 0.3	0.1 ± 0.1	4.7 ± 0.5	3.7 ± 0.4	3

Table 1. Continued

Brain region	Total specific binding	¹²⁵ I-PYY binding (fmol/mg tissue, wet weight)				% Putative Y ₁ site
		+NPY ₁₃₋₃₆ (Y ₂)		+[Pro ³⁴]-NPY (Y ₁)		
		10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁸ M	10 ⁻⁷ M	
Cerebellum						
Granular layer	13.5 ± 3.5	1.0 ± 0.3	0.1 ± 0.1	12.7 ± 3.4	7.5 ± 0.7	6
Molecular layer	2.1 ± 0.6	0.6 ± 0.3	0.4 ± 0.2	1.2 ± 0.4	0.8 ± 0.3	43

Values represent the mean ± SEM of three to four determinations using a concentration of 25 pM ¹²⁵I-PYY. Nonspecific labeling in the presence of 1 μM PYY 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 therefore a relative assessment of the amount of binding sites being competed by increasing concentration of a specific Y₁ agonist, [Pro³⁴]-NPY, or a relatively selective Y₂ agonist NPY₁₃₋₃₆. Percentage of putative Y₁ site was defined as the percentage of specific ¹²⁵I-PYY binding inhibited by 10⁻⁸ M [Pro³⁴]-NPY.

NPY in the frontoparietal cortex and the claustrum but not in the caudate putamen, the lateral septum, and cortical areas below the rhinal sulcus (Fig. 2, Table 1). Almost the opposite situation is observed with the fragment NPY₁₃₋₃₆, with ¹²⁵I-PYY labeling being displaced by low concentrations of this competitor in the striatum and the septum but not in cortical areas (Fig. 2, Table 1).

At the level of the hippocampal formation (Fig. 3, Table 1), ¹²⁵I-PYY labeling is again inhibited by the Y₁ agonist in cortical areas, while binding in the amygdaloid body and most subcortical structures is resistant to inhibition by [Pro³⁴]-NPY. In contrast, ¹²⁵I-PYY labeling in the oriens layer and the stratum radiatum of the hippocampus as well as in the stria terminalis is highly sensitive to increasing concentrations of the Y₂ fragment (Fig. 3, Table 1). However, ¹²⁵I-PYY binding in the dentate gyrus is not effected by NPY₁₃₋₃₆ but very sensitive to the Y₁ agonist, even at low nanomolar concentrations (Fig. 3, Table 1). Thus, ¹²⁵I-PYY sites in the dentate gyrus are most likely of a different subtype to those present in other sectors of the hippocampal formation. Most ¹²⁵I-PYY binding sites in thalamic and hypothalamic nuclei are apparently more sensitive to the Y₂ than the Y₁ competitor (Fig. 3, Table 1). An exception is seen in the reuniens, posterior and laterodorsal thalamic nuclei, which are apparently enriched with [Pro³⁴]-NPY/Y₁ sites, whereas the habenular, interanteromedial, ventroposterior, and mediodorsal thalamic nuclei, and the lateral, dorsomedial and ventromedial hypothalamic nuclei are enriched with the Y₂ subtype (Fig. 3, Table 1).

As in the olfactory bulb (Fig. 1), ¹²⁵I-PYY binding in the cerebellum and various brainstem nuclei (Fig. 4) was found to be more susceptible to inhibition by NPY₂₋₃₆ than by the shorter

fragment NPY₁₃₋₃₆. The Y₁ agonist was much weaker than the two NPY fragments in these same areas.

Characterization of ¹²⁵I-PYY binding sites in membranes from rat brain homogenates

Membranes prepared from various regions of the rat brain revealed that the homologs PYY and NPY, the fragments NPY₂₋₃₆ and NPY₁₃₋₃₆, and the analog [Leu³¹,Pro³⁴]-NPY inhibited specific ¹²⁵I-PYY binding in a concentration-dependent manner (Figs. 5, 6). The rank order of potency in olfactory bulb, striatal, hippocampal, and cerebellar homogenates revealed PYY > NPY > NPY₂₋₃₆ ≥ NPY₁₃₋₃₆ >> [Leu³¹,Pro³⁴]-NPY (Fig. 5). A different profile of competition was detected in the frontoparietal cortex (Fig. 6). In this preparation, a clearly biphasic profile is seen with the Y₁ agonist [Leu³¹,Pro³⁴]-NPY. At low concentrations (nanomolar), the ligand selectivity pattern showed PYY > NPY = [Leu³¹,Pro³⁴]-NPY > NPY₂₋₃₆ > NPY₁₃₋₃₆, while at higher concentrations (micromolar), the Y₁ agonist was only weakly active, with PYY > pNPY > NPY₂₋₃₆ ≥ NPY₁₃₋₃₆ > [Leu³¹,Pro³⁴]-NPY (Fig. 6). This is also clearly shown in Figure 7. For example, while the competition profile of the fragment NPY₁₃₋₃₆ appears to be identical in membranes prepared from either the olfactory bulb, striatum, hippocampus, or cerebellum, it was much less potent in cortical preparations (Fig. 7A). In contrast, the Y₁ agonist [Leu³¹,Pro³⁴]-NPY inhibited ¹²⁵I-PYY binding with a clearly biphasic profile, especially in the frontoparietal cortex area, and to a lesser extent in the olfactory bulb (Fig. 7B). This suggests the existence of different classes or affinity states of ¹²⁵I-PYY binding sites in these brain regions. The low Hill coefficient values observed for all competitors also supports this hypothesis (Tables 2–6).

Table 2. Binding parameters of the homolog PYY derived from competition curves against ¹²⁵I-PYY in various regions of the rat brain

Regions	n _H	K _{D1} (pM)	K _{D2} (pM)	K _{D1} /K _{D2}	% R1
Olfactory bulb	0.58 ± 0.05	24 ± 13	900 ± 200	0.026	59 ± 8
Frontoparietal cortex	0.57 ± 0.03	18 ± 10	1300 ± 400	0.014	30 ± 6
Striatum	0.61 ± 0.04	4 ± 4	400 ± 200	0.010	32 ± 5
Hippocampus	0.61 ± 0.02	10 ± 7	300 ± 200	0.033	49 ± 6
Cerebellum	0.54 ± 0.05	12 ± 9	600 ± 200	0.020	59 ± 9

Data (means ± SEM of four to six determinations) were fitted to a one- or a two-binding-site model by a nonlinear method of analysis using the LUNDON Software COMPETITION data analysis program. The goodness of fit between the two models was tested by *F* test (*p* < 0.05). n_H represents the Hill coefficient determined by a linear method of analysis. K_{D1} and K_{D2} represent the affinity of PYY for the high- and low-affinity site, respectively. K_{D1}/K_{D2} is the ratio of the affinities of the two binding sites. % R1 represents the percentage of sites in the high-affinity mode.

Figure 4. Photomicrographs of the autoradiographic distribution of ^{125}I -PYY binding sites at the level of the cerebellum of the rat brain. Adjacent coronal sections were incubated with ^{125}I -PYY alone (20–25 μM) (*TOT*) or in the presence of either 1 μM pNPY (nonspecific binding, *NS*) or increasing concentrations (molar) of competitors such as NPY_{2-36} , NPY_{13-36} , or $[\text{Pro}^{34}]\text{-NPY}$. Note that the cerebellum labeling is most sensitive to both NPY_{2-36} and NPY_{13-36} but not to $[\text{Pro}^{34}]\text{-NPY}$.

Figure 5. Competition binding profiles of NPY-related peptides against specific ^{125}I -PYY labeling in rat brain membrane homogenates of the olfactory bulb (A), striatum (B), hippocampus (C), and cerebellum (D). Similar competition profiles were obtained in each of these four regions. Each point represents the mean $\pm$ SEM of data obtained from four to seven experiments, each performed in triplicate and expressed as the percentage of specific binding.

In fact, binding data were all best fitted using a two-site model ($p < 0.05$). The apparent affinities (K_D) of the high- and low-affinity components detected using PYY itself varied between 4–24 μM and 340–1350 μM , depending upon the regions studied (Table 2). Similar results were obtained with NPY (Table 3). The deletion of the tyrosine residue in position 1 (NPY₂₋₃₆) decreased the affinity by three- to fivefold for the high- as well

as for the low-affinity binding components in all membrane preparations except the frontoparietal cortex; in this tissue, a greater (15-fold) decrease is observed for the low-affinity site (Table 4). Interestingly, the shorter C-terminal fragment NPY₁₃₋₃₆ demonstrated different affinities for the two binding components depending upon the brain region studied. In striatal and hippocampal membrane homogenates, NPY₁₃₋₃₆ possessed rela-

Table 3. Binding parameters of the homolog pNPY derived from competition curves against ^{125}I -PYY in various regions of the rat brain

Regions	n_H	$K_{D1}(\mu\text{M})$	$K_{D2}(\mu\text{M})$	K_{D1}/K_{D2}	% R1
Olfactory bulb	0.61 ± 0.07	31 ± 12	1500 ± 500	0.021	44 ± 5
Frontoparietal cortex	0.67 ± 0.08	46 ± 10	1100 ± 300	0.042	17 ± 8
Striatum	0.70 ± 0.03	70 ± 40	1500 ± 600	0.047	35 ± 7
Hippocampus	0.64 ± 0.05	52 ± 15	1900 ± 500	0.027	50 ± 4
Cerebellum	0.62 ± 0.04	35 ± 11	1300 ± 400	0.027	47 ± 6

Data (means $\pm$ SEM of four to seven determinations) were fitted to a one- or a two-binding-site model by a nonlinear method of analysis using the LUNDON Software COMPETITION data analysis program. The goodness of fit between the two models was tested by F test ($p < 0.05$). n_H represents the Hill coefficient determined by a linear method of analysis. K_{D1} and K_{D2} represent the affinity of pNPY for the high- and low-affinity site, respectively. K_{D1}/K_{D2} is the ratio of the affinities of the two binding sites. % R1 represents the percentage of sites in the high-affinity mode.

Figure 6. Competition binding profile of NPY-related peptides against specific ^{125}I -PYY labeling in rat frontoparietal cortex membrane homogenates. In contrast to other tissues, $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY potently competed for binding in this area. Each point represents the mean $\pm$ SEM of data obtained from four to eight experiments, each performed in triplicate and expressed as the percentage of specific binding.

tively similar affinities for the two binding components (Table 5), while important differences were detected in the olfactory bulb (30-fold) and the cerebellum (over 60-fold) (Table 5). An even greater dissociation (almost 100-fold) between the high- and low-affinity binding components is observed for the frontoparietal cortex (Table 5). Moreover, the apparent affinities of the Y_1 agonist $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY for the high- and low-affinity binding components are dramatically different with values in the sub- to low nanomolar range on the first binding site but only in the micromolar range for the second portion of the competition curves (Table 6). This is especially evident in the frontoparietal cortex, a region enriched with the putative Y_1 receptor subtype (Table 6). In the other regions, the proportion of Y_1 sites is apparently smaller, the lowest amount being detected in the hippocampal formation (Table 6).

In order to investigate further the possible heterogeneity of ^{125}I -PYY-labeled binding sites, we performed a series of experiments in the presence of 10 nM $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY, a concentration known to be sufficient to block most, if not all, binding to the Y_1 site (Figs. 5, 7B; Table 6). Under such assay conditions, the competition profile is still best fitted to a two-site model ($p < 0.05$) in regions such as the hippocampus and the striatum

Figure 7. Comparative competition binding profiles of NPY_{13-36} (A) and $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY (B) against specific ^{125}I -PYY labeling in membrane homogenates derived from various regions of the rat brain. Note that while NPY_{13-36} is less potent in the cortical area as compared to all other regions, the opposite situation is observed for the Y_1 analog $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY. Each point represents the mean $\pm$ SEM of data obtained from four to eight experiments, each performed in triplicate and expressed as the percentage of specific binding.

(Table 7). Similarly, Hill coefficients remained below unity, suggesting further heterogeneity of ^{125}I -PYY-labeled sites in these regions, even in the presence of a saturating concentration of the Y_1 blocker.

Table 4. Binding parameters of the fragment NPY_{2-36} derived from competition curves against ^{125}I -PYY in various regions of the rat brain

Regions	n_H	$K_{D1}(\text{pM})$	$K_{D2}(\text{pM})$	K_{D1}/K_{D2}	% R1
Olfactory bulb	0.56 ± 0.06	130 ± 20	6200 ± 1000	0.021	45 ± 6
Frontoparietal cortex	0.59 ± 0.03	150 ± 50	$23,000 \pm 800$	0.006	31 ± 7
Striatum	0.63 ± 0.03	120 ± 60	4400 ± 2100	0.027	49 ± 5
Hippocampus	0.58 ± 0.05	240 ± 100	5500 ± 1300	0.043	44 ± 5
Cerebellum	0.61 ± 0.06	140 ± 40	6200 ± 2200	0.023	43 ± 6

Data (means $\pm$ SEM of four or five determinations) were fitted to a one- or a two-binding-site model by a nonlinear method of analysis using the LUNDON Software COMPETITION data analysis program. The goodness of fit between the two models was tested by F test ($p < 0.05$). n_H represents the Hill coefficient determined by a linear method of analysis. K_{D1} and K_{D2} represent the affinity of NPY_{2-36} for the high- and low-affinity site, respectively. K_{D1}/K_{D2} is the ratio of the affinities of the two binding sites. % R1 represents the percentage of sites in the high-affinity mode.

Table 5. Binding parameters of the fragment NPY₁₃₋₃₆ derived from competition curves against ¹²⁵I-PYY in various regions of the rat brain

Regions	<i>n_H</i>	<i>K_{D1}</i> (pM)	<i>K_{D2}</i> (pM)	<i>K_{D1}/K_{D2}</i>	% R1
Olfactory bulb	0.59 ± 0.04	840 ± 300	34,000 ± 10,000	0.0025	69 ± 7
Frontoparietal cortex	0.46 ± 0.04	1500 ± 500	175,000 ± 69,000	0.009	41 ± 6
Striatum	0.59 ± 0.03	240 ± 100	7300 ± 2400	0.032	48 ± 7
Hippocampus	0.52 ± 0.05	410 ± 210	6400 ± 1900	0.064	37 ± 6
Cerebellum	0.54 ± 0.06	600 ± 240	39,000 ± 9000	0.015	63 ± 7

Data (means ± SEM of four to six determinations) were fitted to a one- or a two-binding-site model by a nonlinear method of analysis using the LUNDON Software COMPETITION data analysis program. The goodness of fit between the two models was tested by *F* test (*p* < 0.05). *n_H* represents the Hill coefficient determined by a linear method of analysis. *K_{D1}* and *K_{D2}* represent the affinity of NPY₁₃₋₃₆ for the high- and low-affinity site, respectively. *K_{D1}/K_{D2}* is the ratio of the affinities of the two binding sites. % R1 represents the percentage of sites in the high-affinity mode.

Discussion

The major finding of the present study relates to the clear demonstration of the differential distribution of the Y₁ and Y₂ NPY receptor subtypes in the rat brain. For example, Y₁ receptors are especially concentrated in cortical areas while one of the highest proportions of Y₂ sites is seen in the hippocampal formation. Moreover, the apparent resistance of ¹²⁵I-PYY-labeled sites to inhibition by [Pro³⁴]-NPY (Y₁) and NPY₁₃₋₃₆ (Y₂), but not NPY₂₋₃₆, in the olfactory bulb suggests further receptor heterogeneity.

As reported earlier (Inui et al., 1988, 1998; Lynch et al., 1989; Martel et al., 1990a; Quirion et al., 1990; Rosier et al., 1990), the total population of ¹²⁵I-PYY sites is widely, but discretely, distributed in the rat brain, with high densities of labeling present, among other regions, in hippocampal and cortical areas. Using increasing concentrations of the selective Y₁ competitor [Pro³⁴]-NPY, we confirmed and extended earlier findings (Dumont et al., 1990; Aicher et al., 1991) on the preferential localization of Y₁ receptors in the rat CNS. In addition to superficial laminar cortical areas above the rhinal nucleus, comparatively high levels of Y₁ sites are seen in the claustrum, the dentate gyrus of the hippocampal formation, and the reuniens nucleus of the thalamus. Lower but still detectable amounts of Y₁ sites are also present in various thalamic and hypothalamic nuclei.

The functional relevance of the Y₁ receptor class in the brain remains to be fully established. However, their concentrations in cortical areas suggest a modulatory role in the integration of cortical functions, especially since the cortex is enriched with NPY-like-immunoreactive materials (Chronwall et al., 1985;

O'Donohue et al., 1985; DeQuidt and Emson, 1986a,b). In that regard, it is of interest to note that direct injections of NPY-like peptides in cortical areas, through the activation of phosphoinositide turnover (Hinson et al., 1988; Widdowson and Halaris, 1990a), can increase the respiratory quotient and food intake (McGregor et al., 1990). Thus, cortical areas such as the sulcal prefrontal cortex, in association with hypothalamic nuclei (Stanley and Leibowitz, 1984; Stanley et al., 1985; Kalra et al., 1988, 1990; Jolicoeur et al., 1991b; Leibowitz and Alexander, 1991), can, via the stimulation of Y₁ receptors, modulate these behaviors in the rat. The possible functional role of Y₁ sites in other areas such as the dentate gyrus and the reuniens nucleus remains to be established. To date, most studies, especially those related to the hippocampus, have focused on Y₂-enriched areas such as the stratum radiatum of the CA1 (Colmers et al., 1987, 1988, 1991) or the pyramidal cell layer of the CA3 subfield (Monnet et al., 1992). However, the recent cloning of the Y₁ receptor class (Eva et al., 1990; Herzog et al., 1992; Yee et al., 1992) should facilitate further evaluation of its precise role in the CNS, as well as reveal the mechanisms involved in the regulation of its expression.

In other brain regions, we first assumed that the majority of ¹²⁵I-PYY binding represented labeling of the Y₂ subtype. This was based on the relatively high potencies of C-terminal fragments such as NPY₁₃₋₃₆ to compete for specific ¹²⁵I-PYY or ¹²⁵I-NPY labeling (Dumont et al., 1990; Aicher et al., 1991). However, the more detailed analysis performed here using selected brain region homogenates clearly revealed the biphasic nature of the competition profiles, with Hill coefficients significantly lower than unity, even in the presence of a blocking concentration of the Y₁ agonist [Leu³¹,Pro³⁴]-NPY (see Table 7). The

Table 6. Binding parameters of the analog [Leu³¹,Pro³⁴]-NPY derived from competition curves against ¹²⁵I-PYY in various regions of the rat brain

Regions	<i>n_H</i>	<i>K_{D1}</i> (pM)	<i>K_{D2}</i> (pM)	<i>K_{D1}/K_{D2}</i>	% R1
Olfactory bulb	0.38 ± 0.07	170 ± 100	780,000 ± 150,000	0.0002	24 ± 4
Frontoparietal cortex	0.31 ± 0.03	210 ± 90	660,000 ± 230,000	0.0003	67 ± 5
Striatum	0.32 ± 0.06	1650 ± 500	820,000 ± 380,000	0.002	18 ± 3
Hippocampus	0.33 ± 0.04	3500 ± 1500	980,000 ± 270,000	0.004	13 ± 4
Cerebellum	0.32 ± 0.05	1300 ± 400	710,000 ± 310,000	0.002	25 ± 5

Data (means ± SEM of four to eight determinations) were fitted to a one- or a two-binding-site model by a nonlinear method of analysis using the LUNDON Software COMPETITION data analysis program. The goodness of fit between the two models was tested by *F* test (*p* < 0.05). *n_H* represents the Hill coefficient determined by a linear method of analysis. *K_{D1}* and *K_{D2}* represent the affinity of [Leu³¹,Pro³⁴]-NPY for the high- and low-affinity site, respectively. *K_{D1}/K_{D2}* is the ratio of the affinities of the two binding sites. % R1 represents the percentage of sites in the high-affinity mode.

Table 7. Binding parameters of the homolog PYY derived from competition curves against 125 I-PYY in presence of a blocking concentration of the Y_1 agonist [Leu 31 ,Pro 34]-NPY

Regions	n_H	K_{D1} (pM)	K_{D2} (pM)	% RI
Striatum				
Control	0.61 $\pm$ 0.04	4 $\pm$ 4	400 $\pm$ 200	32 $\pm$ 5
+ [Leu 31 ,Pro 34]-NPY	0.58 $\pm$ 0.06	1 $\pm$ 2	120 $\pm$ 80	14 $\pm$ 6
Hippocampus				
Control	0.61 $\pm$ 0.02	10 $\pm$ 7	300 $\pm$ 200	49 $\pm$ 6
+ [Leu 31 ,Pro 34]-NPY	0.57 $\pm$ 0.03	9 $\pm$ 6	180 $\pm$ 75	45 $\pm$ 7

Data (means $\pm$ SEM of four to eight determinations) were fitted to a one- or a two-binding-site model by a nonlinear method of analysis using the LUNDON Software COMPETITION data analysis program. The goodness of fit between the two models was tested by F test ($p < 0.05$). n_H represents the Hill coefficient determined by a linear method of analysis. K_{D1} and K_{D2} represent the affinity of PYY for the high- and low-affinity site, respectively. % RI represents the percentage of sites in the high-affinity mode observed in presence and absence of a 10 nM blocking concentration of [Leu 31 ,Pro 34]-NPY.

biphasic nature of the competition curves could relate to the existence of a single class of NPY receptors (e.g., Y_2) under different affinity states. This possibility has already been suggested for various preparations including the porcine hippocampus (Inui et al., 1988, 1989), dorsal root ganglia (Walker et al., 1988), whole rat brain (Walker and Miller, 1988), and cervical vagus nerve (Corp and Smith, 1991). However, the fact that we observed markedly different ratios between the high- and low-affinity components (K_{D1}/K_{D2}) for the selective Y_1 agonist [Leu 31 ,Pro 34]-NPY and the Y_2 fragment NPY $_{13-36}$ tends to support an alternate hypothesis, namely, the existence of further NPY receptor heterogeneity.

In support of this concept, some studies have already suggested the existence of either PP (Whitcomb et al., 1990; Adamo and Hazelwood, 1991) or atypical NPY/ σ (Roman et al., 1989; Monnet et al., 1992) receptors for which both PYY and NPY demonstrate high affinities in the mammalian CNS. We are currently investigating these various possibilities using preferential ligands for each additional putative receptor class. Molecular information concerning the presence of Y_2 receptors in various brain regions and tissues should also help to clarify this issue, although the cloning of this receptor class has yet to be reported.

The widespread anatomical distribution of the "so-called" Y_2 receptors reported here suggests its important role(s) in the mediation of the biological effects induced by NPY and related peptides. Various CNS actions of this peptide family have already been characterized as belonging to this receptor class (see Dumont et al., 1992, for a recent review). It remains to be established if, in fact, these effects demonstrate an identical pharmacological profile. This can only be achieved using an extensive series of selective NPY/PYY fragments and analogs. This should reveal the possible functional heterogeneity of the now broadly classified Y_2 receptor class.

It is also of interest that NPY $_{2-36}$ appears to be relatively more potent in competing for 125 I-PYY binding in regions such as the olfactory bulb and the cerebellum. This may relate to the existence in these areas of an atypical site, which possesses a high affinity for the full NPY sequence as well as the NPY $_{2-36}$ fragment, an observation already reported in regard to the effects of NPY and homologs on feeding behaviors (Magdalin et al., 1989; Jolicoeur et al., 1991b).

In summary, Y_1 and Y_2 receptor subtypes are discretely, but differentially, distributed in the rat brain. Y_1 sites are especially concentrated in cortical areas, in the dentate gyrus of the hip-

pocampal formation, and in a few other nuclei, while Y_2 binding sites are more broadly distributed, being enriched in most regions. Moreover, it appears that sites classified as Y_2 receptors may constitute a heterogeneous population of receptors. Experiments are currently in progress to investigate fully this possibility using subtype selective analogs and molecular biological approaches. This work may have an important impact on our understanding of the functional role of NPY and related peptides in the brain.

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