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[LEU^{31}-PRO^{34}] NEUROPEPTIDE Y IDENTIFIES A SUBTYPE OF ^{125}I-LABELED PEPTIDE YY BINDING SITES IN THE RAT BRAIN

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Abstract—Subtypes of the neuropeptide Y (NPY) receptor in the rat brain were identified by the use of the selective Y-1 analog, [Leu^{31}-Pro^{34}] NPY. In rat brain homogenate binding studies, [Leu^{31}-Pro^{34}] NPY was found to produce a partial inhibition of 100 pM ^{125}I-labeled peptide YY (PYY) binding with a plateau at 50–1000 nM [Leu^{31}-Pro^{34}] NPY resulting in a 70% inhibition of binding. The C-terminal fragment NPY 13–36, a putative Y-2 agonist, exhibited very little selectivity in rat brain homogenates. Scatchard analysis of ^{125}I-labeled PYY binding to rat brain homogenate yielded biphasic plots with K_d values of 40 and 610 pM. Inclusion of 100 nM [Leu^{31}-Pro^{34}] NPY was found to eliminate the low affinity component of ^{125}I-labeled PYY binding leaving a single, high affinity binding site with a K_d of 68 pM. In autoradiographic studies, displacement curves indicated that [Leu^{31}-Pro^{34}] NPY completely inhibited binding in the cerebral cortex with little effect on the binding in the hypothalamus. On the other hand NPY 13–36 inhibited binding in the hypothalamus at low concentrations but required higher concentrations to inhibit binding in the cerebral cortex. Other brain regions such as the hippocampus, appeared to contain both subtypes. Subsequent to these studies, a quantitative autoradiographic map was conducted using 50–100 pM ^{125}I-labeled PYY in the presence and absence of [Leu^{31}-Pro^{34}] NPY which produced a selective displacement of binding in certain distinct brain regions. These areas included the cerebral cortex, certain thalamic nuclei and brainstem while ligand binding was retained in other brain regions including the zona lateralis of the substantia nigra, lateral septum, nucleus of the solitary tract and the hippocampus. Numerous brain regions appeared to contain both receptor subtypes. Therefore, the Y-1 and Y-2 receptor subtypes exhibited a somewhat distinct distribution in the brain. In addition, ^{125}I-labeled PYY appears to label the Y-2 receptor with relatively higher affinity when compared to the Y-1 receptor.

Neuropeptide Y (NPY), a 36 amino acid peptide amide, was originally isolated from porcine brain in 1982 using a chemical assay for detecting peptides which contain a C-terminal amide (Tatemoto, 1982; Tatemoto *et al.*, 1982). Neuropeptide Y is a member of a family of conserved pancreatic polypeptides which includes NPY, peptide YY (PYY) and pancreatic polypeptide (PP). NPY exhibits a broad distribution in the brain, adrenal and peripheral nervous system while PYY is generally considered to be a gut hormone produced in endocrine cells of the lower intestine (Allen *et al.*, 1986; Lukinius *et al.*, 1986; Lundberg

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Abbreviations: ac, anterior commissure; Acb, accumbens nucleus; AON, anterior olfactory nucleus; Arc, arcuate hypothalamic nucleus; AV, anteroventral thalamic nucleus; CA1, field CA1 of Ammon's horn; CA2, field CA2 of Ammon's horn; CA3, field CA3 of Ammon's horn; Cg, cingulate cortex; Cl, claustrum; CM, central medial nucleus of the thalamus; CPu, caudate-putamen; DG, dentate gyrus; DLG, dorsal lateral geniculate nucleus; DM, dorsal medial hypothalamus; E/OV, ependyma and olfactory ventricle; EPl, external plexiform layer of the olfactory bulb; fi, fimbria of the hippocampus; Fr, frontal cortex; GP, globus pallidus; gr, granule cell layer of the cerebellum; IAM, interanterodorsal thalamic nucleus; IGr, internal granular layer of the olfactory bulb; IM, intermedial nucleus of the thalamus; LD, laterodorsal thalamic nucleus; [Leu^{31}-Pro^{34}] NPY, Leucine^{31}-Proline^{34}-Neuropeptide Y; LH, lateral hypothalamic area; LI–III, laminae 1–3 of the parietal cerebral cortex; LIV, lamina 4 of the parietal cerebral cortex; LM, lateral mammillary nucleus; LPC, lateral posterior thalamic nucleus, central part; LPO, lateral preoptic area; LS, lateral septal nucleus; LSD, lateral septal nucleus, dorsal part; LSI, lateral septal nucleus, intermediate; MD, mediodorsal thalamic nucleus; MGD, medial geniculate nucleus, medial part; MGV, medial geniculate nucleus, ventral; MM, medial mammillary nucleus, medial part; Mol, molecular layer of the dentate gyrus; NPY, Neuropeptide Y; ON, olfactory nerve layer; Or, oriens layer of the hippocampus; Pir, piriform cortex; PMCo, posteromedial cortical amygdaloid nucleus; Po, posterior thalamic group; PT, paratenial thalamic nucleus; PYY, Peptide YY; Rad, stratum radiatum of the hippocampus; Re, reuniens thalamic nucleus; SFi, septofimbrial nucleus; SFO, subfornical organ; SL, lateral septum; SNC, substantia nigra, compact part; SNL, substantia nigra, lateral part; Sol, nucleus of the solitary tract; st, stria terminalis; SuM, supramammillary nucleus; TT, tenia tecta; Tu, olfactory tubercle; VMH, ventromedial hypothalamic nucleus; VPM, ventral posterior medial thalamic nucleus; VTA, ventral tegmental area.

et al., 1984; Miyachi *et al.*, 1986; O'Donohue *et al.*, 1985; Tatemoto, 1982) though some PYY positive neurons have been found in some brain regions (Broome *et al.*, 1985; Ekman *et al.*, 1986). Neuropeptide Y containing cell bodies and fibers can be seen in a variety of brain regions (Chronwall *et al.*, 1985; de Quidt and Emson, 1986; Gray and Morley, 1986; Yamazoe *et al.*, 1985) being most concentrated in hypothalamic and brainstem regions. In the CNS, NPY and PYY produce a number of effects including regulation of food intake, pituitary secretion and blood pressure (O'Donohue *et al.*, 1985; Scott *et al.*, 1989; Wahlestedt *et al.*, 1989). Immunocytochemical studies have provided additional support for a role for NPY as a neurotransmitter. In neurons, NPY is often found to be colocalized with norepinephrine (NE) in both central and peripheral neurons (Blessing *et al.*, 1986; Everitt *et al.*, 1984; Gray and Morley, 1986; Härfstrand *et al.*, 1987b; Hökfelt *et al.*, 1983a,b). Neuropeptide Y mRNA can be found in neurons in the rat, mouse (Gehlert *et al.*, 1987; Morris, 1989) and human (Brene *et al.*, 1989) brain indicating that the NPY seen immunocytochemically is of neuronal origin. Neuropeptide Y also appears to potentiate the postsynaptic actions of NE in both the peripheral and central nervous systems (Clark *et al.*, 1988; Heilig *et al.*, 1988; Lundberg *et al.*, 1988). Central injection of NPY mimics many of the effects of NE including regulation of feeding (Clark *et al.*, 1987; Kalra *et al.*, 1988a,b; Kuenzel *et al.*, 1987; Morley *et al.*, 1987; Stanley and Leibowitz, 1984), blood pressure (Boublik *et al.*, 1989; Chalmers *et al.*, 1989; Chen *et al.*, 1988; Clarke *et al.*, 1991; Härfstrand, 1987; Louis *et al.*, 1987; McCloskey and Potter, 1991), sexual behavior (Clark *et al.*, 1985; Frederiksen *et al.*, 1991; Kalra *et al.*, 1988a,b; Wahlestedt *et al.*, 1989) and neuroendocrine function (Kalra and Crowley, 1984; Kaynard and Spies, 1991; Leibowitz *et al.*, 1988; O'Donohue *et al.*, 1985). Central injections of PYY appear to produce similar effects to those seen with NPY (Clark *et al.*, 1987; Kuenzel *et al.*, 1987; Morley *et al.*, 1985; O'Donohue *et al.*, 1985; Stanley *et al.*, 1985) suggesting that these two peptides interact with common receptors or receptor populations.

The receptor distribution in the brain is generally in agreement with the distribution of the NPY immunoreactive terminals, but there were some important areas containing mismatches (Martel *et al.*, 1988). These include the hypothalamic regions which contain a dense immunoreactive terminal field, but relatively few receptors. Certain C-terminal fragments of NPY and PYY were originally described to have some selectivity for a receptor subtype that has been termed a prejunctional or Y-2 receptor (Jolicoeur *et al.*, 1991; Sheikh *et al.*, 1989a; Wahlestedt *et al.*, 1986; Walker and Miller, 1988). Recently a NPY analog ([Leu^{31}-Pro^{34}] NPY) has been described as a highly selective agonist for the Y-1 receptor in cell lines (Fuhlendorff *et al.*, 1990) with a good separation between Y-1 and Y-2 activity. Prior to the discovery of this peptide, subtypes of NPY receptors in the brain have been postulated on the basis of the differential distribution of the binding of low concentrations of ^{125}I-labeled PYY and ^{125}I-labeled NPY in biochemical (Walker and Miller, 1988) and autoradiographic studies (Lynch *et al.*, 1989; Martel *et al.*, 1990). Other investigators have not seen this difference (Ohkubo *et al.*, 1990) and this discrepancy may be due to the lower levels of nonspecific binding seen when using ^{125}I-labeled PYY as a ligand which could have made interpretation difficult (Martel *et al.*, 1990). Initial results have indicated that [Leu^{31}-Pro^{34}] NPY can be used to reveal a differential distribution of the Y-1 and Y-2 receptor subtypes in rat brain (Aicher *et al.*, 1991; Dumont *et al.*, 1990). In the present study, we evaluate the selectivity of [Leu^{31}-Pro^{34}] NPY and NPY 13–36 for subtypes of ^{125}I-labeled PYY binding sites in the rat brain and provide a quantitative autoradiographic map of the distribution of Y-1 and Y-2 receptors.

EXPERIMENTAL PROCEDURES

Homogenate binding studies

Rat (Male, Sprague–Dawley, 250–300 g) brains (minus the brainstem and cerebellum) were homogenized in 50 mM Tris (pH 7.4) buffer using four 10 s bursts with a Brinkman Polytron (Brinkman Instruments, NJ). After an initial spin at 800 ***g***, the supernatant was transferred to a new centrifuge tube and pelleted at 40,000 ***g*** using a Beckman centrifuge, resuspended in Tris buffer and pelleted again. The homogenate binding was conducted as previously described by Walker and Miller (1988) with slight modification. The pellet was resuspended in 25 mM HEPES (pH 7.4) buffer containing 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$. Incubations were conducted in a final volume of 200 μl containing 0.1 nM ^{125}I-labeled PYY (SA 2200 Ci/mmol, DuPont-NEN, Boston, MA), 0.4% bovine serum albumin (BSA, Fraction 5, Boehringer Manneheim, Indianapolis, IN), 0.1% bacitracin (Sigma, St Louis, MO) and 0.2–0.4 mg brain protein for 2 h at room temperature. Nonspecific binding was defined as the radioactivity remaining bound to the tissue after incubation with 1 μM NPY (Peninsula, CA). In some experiments, various concentrations of NPY, [Leu^{31}-Pro^{34}] NPY or NPY 13–36 (Peninsula) were included in the incubation mixture as described in the figure legends. In other experiments the concentration of ligand in the mixture was varied to examine

the saturation kinetics. Incubations were terminated by rapid filtration through Whatman GF/C filters, which had been presoaked in 1.0% polyethyleneimine (Sigma), using a Brandel (Gaithersburg, MD) cell harvester. The filters were washed 5 times each with 1 ml of 50 mM Tris (pH 7.5 at 4°C) and allowed to dry. Radioactivity retained on the filters was counted using a gamma counter and the results analyzed using the Lundon-1 or Lundon-2 software package (Lundon Inc., Chagrin Falls, OH) running on a VAX computer. Protein concentrations were measured using Commassie Protein Assay Reagent (Pierce, Rockford, IL) (Bradford, 1976) using BSA as the standard.

Autoradiographic studies

In the autoradiographic studies, rats were anesthetized with Halothane®, decapitated and brains rapidly removed and frozen on dry ice. The tissue was then mounted on cryostat chucks and sectioned at 12 μm using a Hacker (Fairfield, NJ) cryostat. Sections were thaw mounted onto gelatin coated slides and stored at −20°C overnight after which the slides were then stored at −70°C. Sections were labeled using a previously described protocol (Martel *et al.*, 1990) with slight modification. Sections were preincubated for 1 h in a Krebs–Ringer buffer containing 0.4% BSA and 0.5% bacitracin (Sigma, St Louis, MO). The slides were then transferred to the same media containing 0.1 nM ^{125}I-labeled PYY with and without the addition of various concentrations of [Leu^{31}-Pro^{34}] NPY, NPY 13–36 or NPY. Following a 2 h incubation, the slides were rinsed in fresh buffer without BSA four times for 5 min each and dried. The labeled sections were placed on Hyperfilm β-max (Amersham, Arlington Hts, IL) for one week and the film was developed in D-19 developer (Kodak, Rochester, NY). Quantitation was facilitated by the use of iodine containing standards (^{125}I-Microscales, Amersham) and an image analysis system (MCID, Imaging Research, Ontario, Canada).

RESULTS

Pharmacological characterization of [Leu^{31}-Pro^{34}] neuropeptide Y in rat forebrain homogenates

The ability of NPY, NPY 13–36 and [Leu^{31}-Pro^{34}] NPY to inhibit ^{125}I-labeled PYY binding to homogenates of rat forebrain was tested. NPY and NPY 13–36 inhibition curves were best fit by a single site analysis while [Leu^{31}-Pro^{34}] NPY produced a displacement curve which was best fit by two site analysis (Fig. 1). NPY was a potent inhibitor with a K_i of 0.82 nM while NPY 13–36 was somewhat less potent with a K_i of 78 nM. [Leu^{31}-Pro^{34}] NPY was also a potent inhibitor of ^{125}I-labeled PYY binding producing a partial inhibition of binding with a K_i of 0.87 nM for the high affinity site and an estimated K_i of 2200 nM for the low affinity site. At a ligand concentration of 100 nM [Leu^{31}-Pro^{34}] NPY produced approx 70% inhibition of binding. A 50–100 nM concentration of [Leu^{31}-Pro^{34}] NPY was used to mask the binding of ^{125}I-labeled PYY to the [Leu^{31}-Pro^{34}] NPY sensitive site while retaining much of the [Leu^{31}-Pro^{34}] NPY insensitive ^{125}I-labeled PYY binding. Subsequently, saturation analysis was then performed both in the presence and absence of 100 nM [Leu^{31}-Pro^{34}] NPY (Fig. 2). Lundon-1 analysis of the binding of ^{125}I-labeled PYY indicated that this ligand recognized two sites in rat brain homogenates with a K_d of 40 pM for the high affinity site and a K_d of 608 pM for the low affinity site. Inclusion of 100 nM [Leu^{31}-Pro^{34}] NPY produced a complete inhibition of ^{125}I-labeled PYY binding to the low affinity site leaving a single site with a K_d of 68 pM.

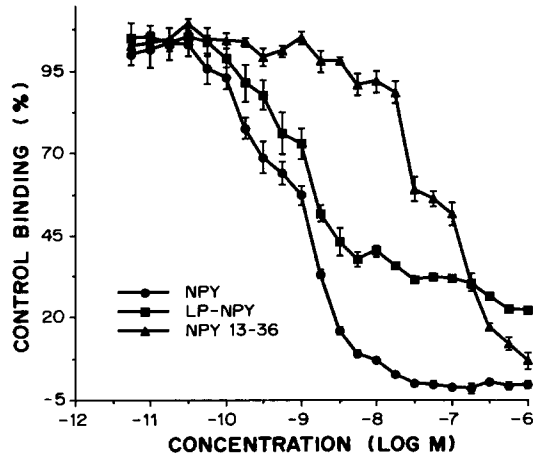


Fig. 1. Inhibition of ^{125}I-labeled PYY Binding to rat brain homogenates by NPY, [Leu^{31}-Pro^{34}] NPY and NPY 13–36. Rat brain homogenates were incubated with various concentrations of the displacing peptide as described in the Experimental Procedures section. K_i values were determined using the Lundon-2 software. The curves for NPY (K_i = 0.82 nM) and NPY 13–36 (K_i = 77.8 nM) modeled to one site while the curve for [Leu^{31}-Pro^{34}]-NPY modeled to two sites with a high affinity component (K_i = 0.87 nM) and a low affinity component (estimated K_i = 2200 nM). The data shown is the result of two experiments performed in duplicate. Similar results were obtained in four additional experiments.

Autoradiographic evaluation of the selectivity of [Leu^{31}-Pro^{34}] neuropeptide Y

Initial experiments were performed to verify the selectivity of NPY, [Leu^{31}-Pro^{34}] NPY and NPY 13–36 in sections of rat brain. A series of sections from different regions of rat brain were incubated in the presence of ^{125}I-labeled PYY alone or in the presence of various concentrations of NPY, [Leu^{31}-Pro^{34}] NPY or NPY 13–36. The binding was then assessed in several regions of the brain. These data are summarized in Figs 3–7. In these studies it was observed that NPY

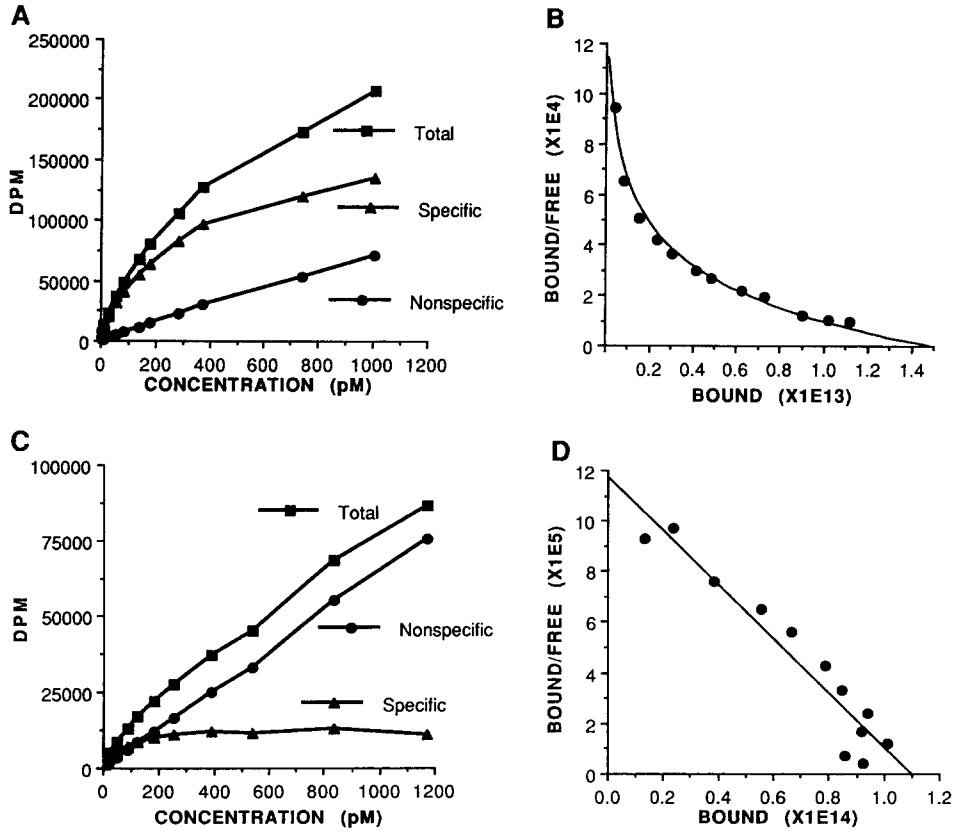


Fig. 2. Scatchard analysis of ^{125}I-labeled PYY binding to rat brain homogenates. (A) Rat brain homogenates were incubated with various concentrations of ^{125}I-labeled PYY as described in the Experimental Procedures section. Nonspecific binding was conducted in a parallel set of incubations by including 1 μM NPY. (B) The resulting saturation isotherms seen in (A) were analyzed using the Lundon-1 software for the best fit to a one or two site model. In this case the best fit was obtained using a two site model with a K_d estimate of 40 pM for the high affinity site and 608 pM for the low affinity site. The estimated β_{max} for the high affinity site was 21.7 fmol/mg protein and for the low affinity site was 133.1 fmol/mg protein. (C) Rat brain homogenates were incubated with various concentrations of ^{125}I-labeled PYY in the presence of 100 nM [Leu^{31}-Pro^{34}] NPY to produce the saturation isotherm seen in this panel. (D) The Scatchard plot of the results seen in (C). These results modeled to a single site which appeared to correlate to the low affinity site seen in (A) having a K_d of 68 pM and a β_{max} of 10.0 fmol/mg protein. The data shown in each panel is the result of two experiments performed in duplicate. Similar results were obtained in four additional experiments.

was the most potent of the three peptides in displacing ^{125}I-labeled PYY binding, producing a uniform inhibition of binding in the brain regions examined. On the other hand, [Leu^{31}-Pro^{34}] NPY and NPY 13–36 produced selective displacement of ^{125}I-labeled PYY binding in certain brain regions while leaving other regions relatively unaffected. This was apparent in the cerebral cortex (Figs 3–5) where [Leu^{31}-Pro^{34}] NPY inhibited almost all the ligand binding at a concentration of 100 nM while in regions such as the hypothalamus (Fig. 4) little inhibition could be detected at similar concentrations (Figs 3–7). Other regions such as the hippocampus, [Leu^{31}-Pro^{34}] NPY produced a partial inhibition of specific binding which plateaued at concentrations higher than 10 nM (Figs 4 and 5). NPY 13–36 produced a different pattern of inhibition displacing areas which were resistant to [Leu^{31}-Pro^{34}] NPY at lower concentrations than those areas which were inhibited by [Leu^{31}-Pro^{34}] NPY. NPY 13–36 inhibited binding in regions such as the lateral septum, hippocampus and hypothalamus (Figs 4, 5 and 7) at lower concentrations while binding in the cerebral cortex was inhibited at higher concentrations (Figs 3–7). In the brainstem and cerebellum, little [Leu^{31}-

Pro^{34}] NPY displaceable binding was observed while the binding was completely inhibited by NPY 13-36. Since [Leu^{31}-Pro^{34}] NPY produced a better separation in both autoradiographic and homogenate binding assays; this analog was utilized in subsequent autoradiographic mapping studies.

Autoradiographic localization of subtypes of ^{125}I-peptide PYY binding sites

From the data obtained in these studies and the homogenate binding studies, an autoradiographic map was performed to determine the regional distribution of [Leu^{31}-Pro^{34}] NPY displaceable and non-displaceable binding sites in the rat brain. Serial sections were incubated either with 100 pM ^{125}I-labeled PYY alone, with the addition of 50 or 100 nM [Leu^{31}-Pro^{34}] NPY or with the inclusion of 1 μM NPY to determine nonspecific binding. The results of these experiments are summarized in Table 1 and Figs 8–10. In general, the subtypes of ^{125}I-labeled PYY binding exhibited a broad distribution being found in a variety of brain regions. A few brain regions such as the cerebral cortex, hypothalamus and lateral septum had a single subtype of the receptor, but in most brain regions both receptor subtypes were present. From the inhibition studies [Leu^{31}-Pro^{34}] NPY displaceable binding was found in brain regions that contained NPY 13–36 resistant binding. Therefore, binding that was displaced by 50 nM [Leu^{31}-Pro^{34}] NPY will be referred to as Y-1 while that remaining will be collectively called Y-2. In this context, Y-1 binding was found in regions containing high densities of ^{125}I-labeled PYY binding sites such as the cerebral cortex, as well as regions containing low densities of binding such as the dentate gyrus. Similarly, Y-2 binding was found in areas of high ^{125}I-labeled PYY binding (substantia nigra zona lateralis) as well as low ligand binding (paraventricular nucleus of the hypothalamus).

Rhinencephalon

In the olfactory bulb, a relatively low level of binding was found to be localized to the ependymal cells, external plexiform layer and internal granule cell layer (Fig. 8). A majority of binding to both layers was inhibited by 50 nM [Leu^{31}-Pro^{34}] NPY though some residual binding was seen in the internal granule and ependymal cell layers. A high level of binding was observed in the anterior olfactory nucleus with binding to the lateral aspects being inhibited by [Leu^{31}-Pro^{34}] NPY leaving a uniform lower level of specific Y-2 binding.

Telencephalon

High levels of ^{125}I-labeled PYY binding were seen in the hippocampus which were localized primarily to the stratum oriens and radiatum of CA1-3, with relatively little specific binding to the molecular layer and pyramidal cell layer (Figs 9 and 10). The binding was significantly higher in these regions in more caudal sections of the hippocampus. In more anterior sections through the hippocampus, high levels of binding were also seen in the stria terminalis. In general, receptor binding in the hippocampus was modestly inhibited by a 50 nM concentration of [Leu^{31}-Pro^{34}] NPY indicating the presence of Y-2 binding with some Y-1 binding. In the dentate gyrus, a low level of binding was seen and this appeared to be exclusively Y-1 in nature. The cerebral cortex contained a relatively high level of binding particularly in the superficial (I–III) laminae with somewhat lower levels seen in laminae IV, V and VI (Figs 8 and 9). All the specific binding of ^{125}I-labeled PYY seen in the cortex was inhibited by [Leu^{31}-Pro^{34}] NPY indicating that the Y-1 subtype was primarily found in this region. The low level of labeling in the piriform cortex appeared to be Y-2 while the higher binding in the claustrum was predominantly Y-1 (Fig. 8). In the amygdala, significant levels of binding were seen in the centrolateral and basolateral nuclei (Table 1). In the central nucleus a small amount of binding was inhibited by the presence of the Y-1 selective peptide. In contrast, a majority of the binding was inhibited in the basolateral nucleus. The septal nuclei, high levels of binding were seen in the lateral septum and the tenia tecta which appeared to be exclusively Y-2 in nature. Low levels of binding were seen in the nucleus accumbens, olfactory tubercle and globus pallidus (Figs 8 and 9). Binding in the caudate putamen appeared to be Y-1 while the globus pallidus contained mostly Y-2. The nucleus accumbens had a low level of binding with approx 50% of each subtype.

Diencephalon

Significant levels of ^{125}I-labeled PYY binding were seen in a variety of thalamic nuclei. The binding to subtypes in the thalamus appeared to be heterogeneous in the various nuclei (Figs 9 and 10). The anterodorsal, central medial, lateral posterior and reuniens contained primarily Y-1 binding while the reticular nucleus contained mostly Y-2. The moderate levels of binding in the medial and dorsal lateral geniculate were inhibited by the addition of [Leu^{31}-Pro^{34}] NPY indicating the presence of the Y-1 receptor. Low levels of binding were observed in the hypothalamus

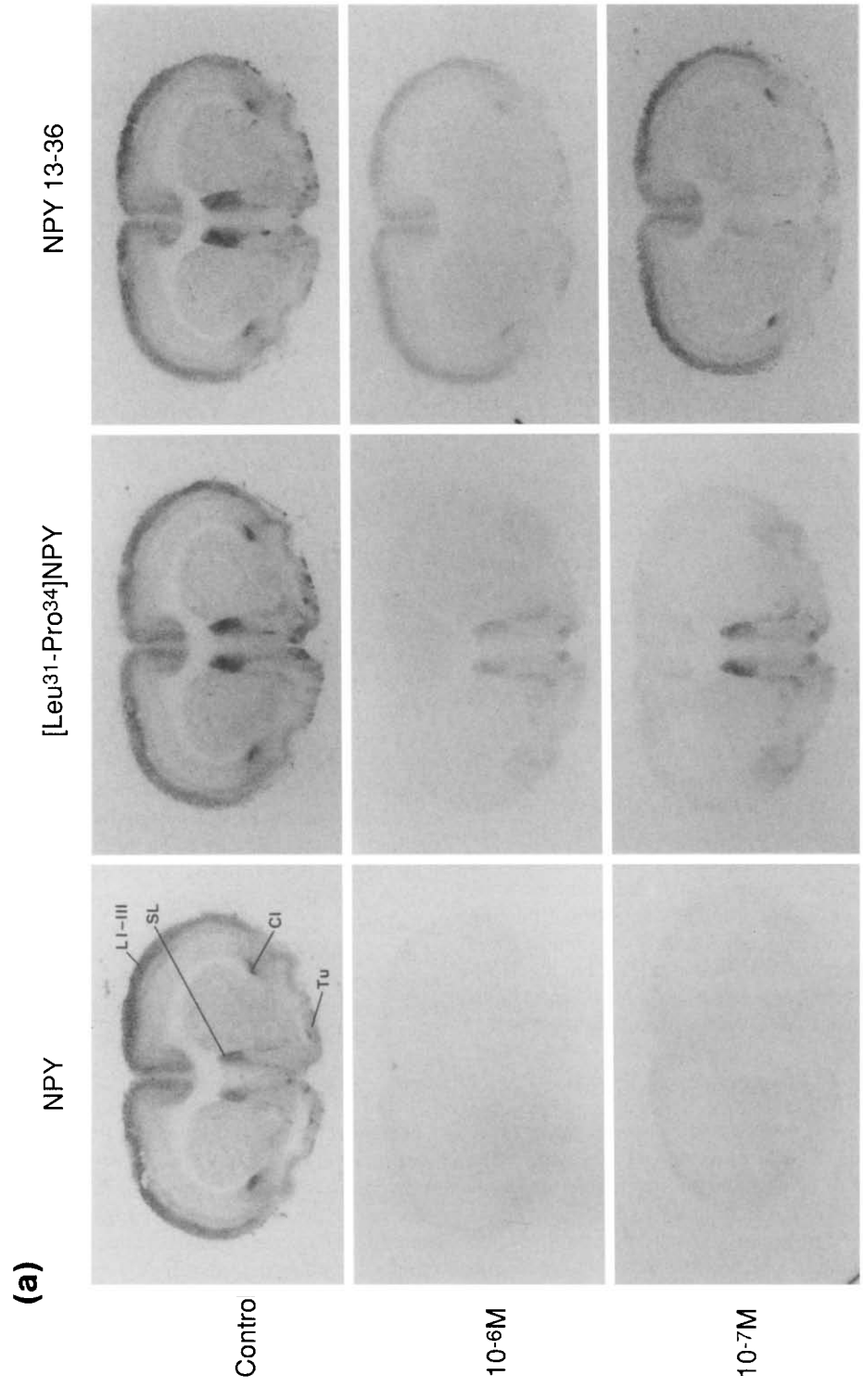


Fig. 3(a).

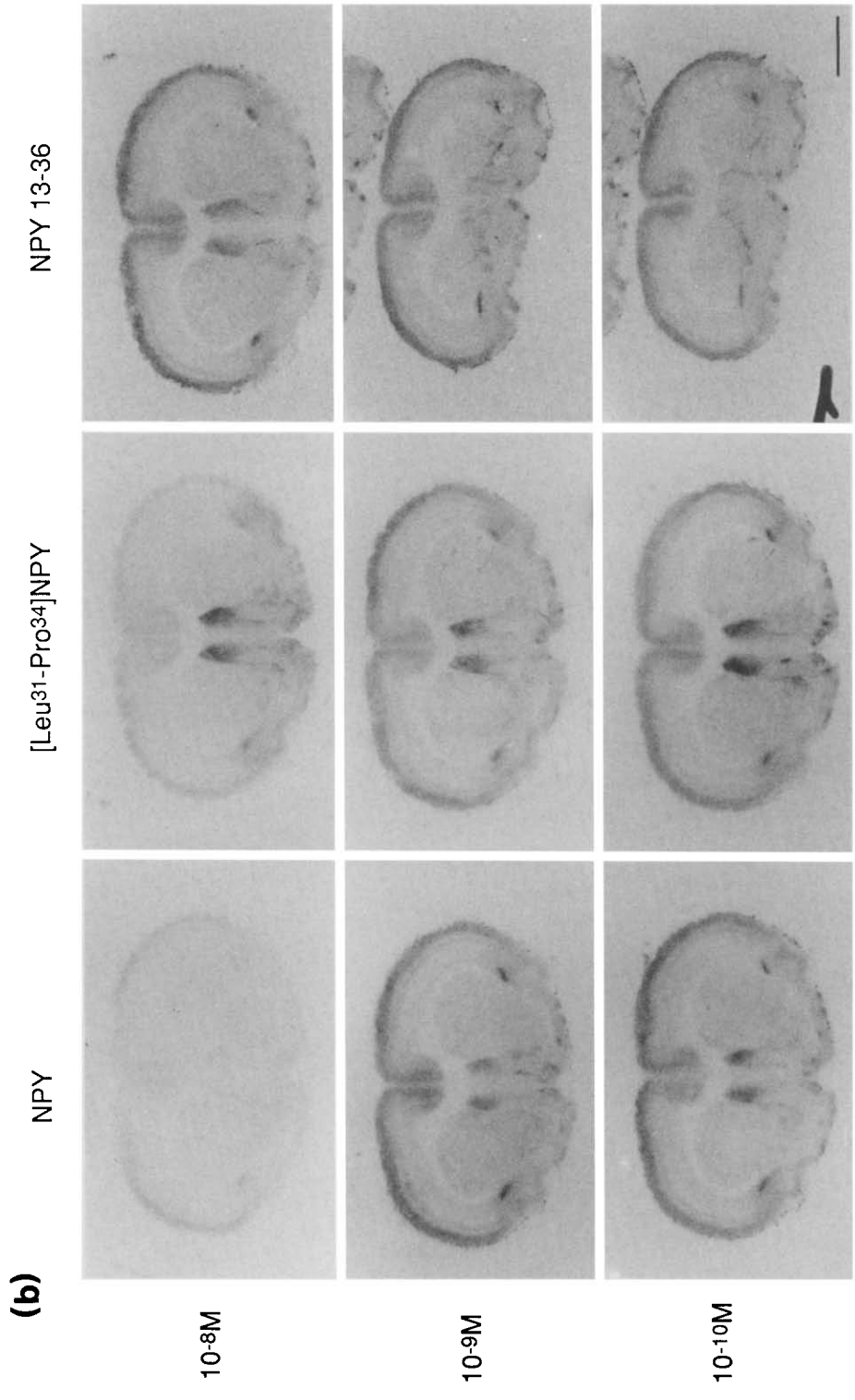


Fig. 3(b).

(*See p. 57 for legend to Fig. 3*).

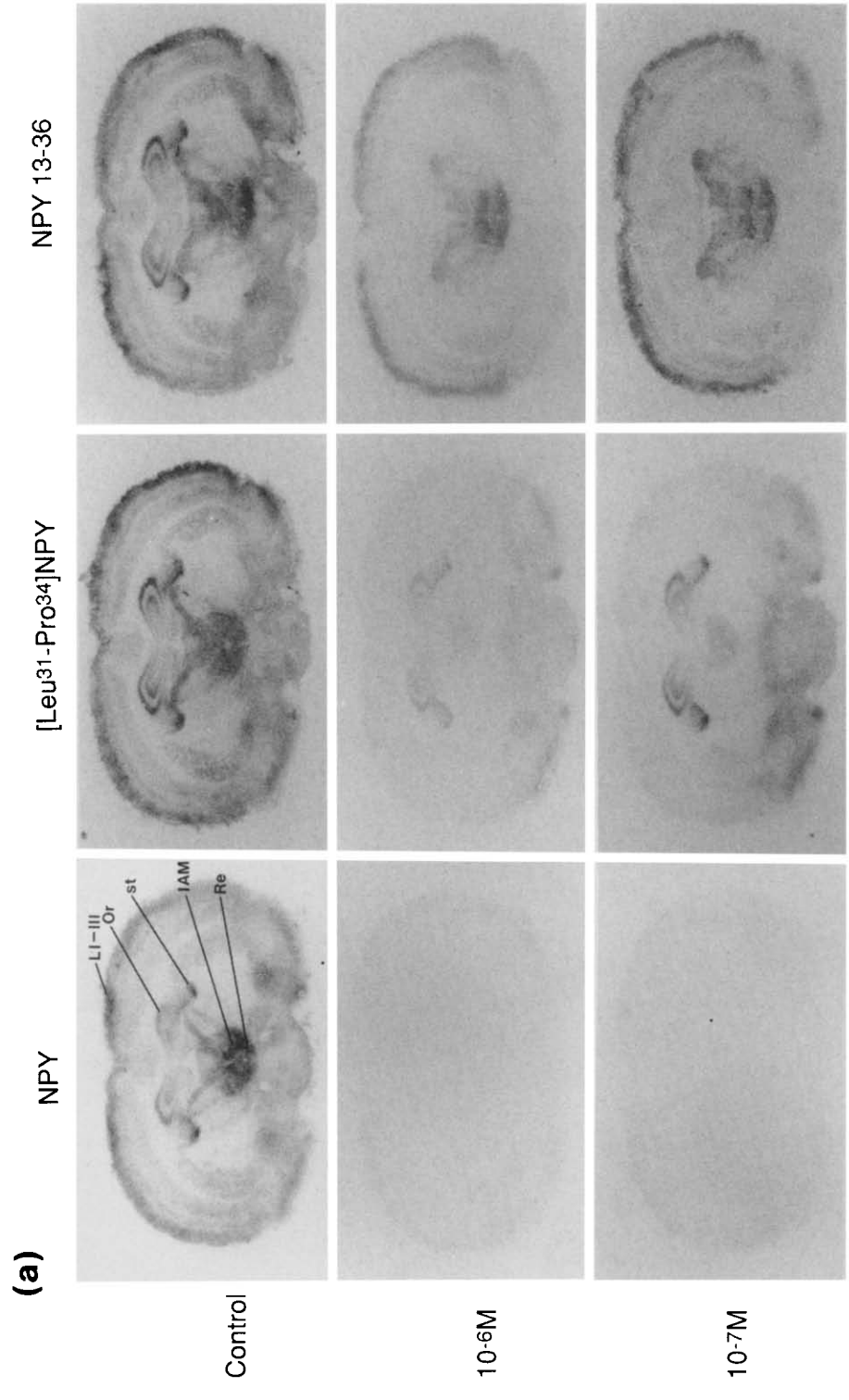

(a)
NPY
[Leu31-Pro34]NPY
NPY 13-36
LI–III
Or
st
IAM
Re
Control
10-6M
10-7M

Fig. 4(a).

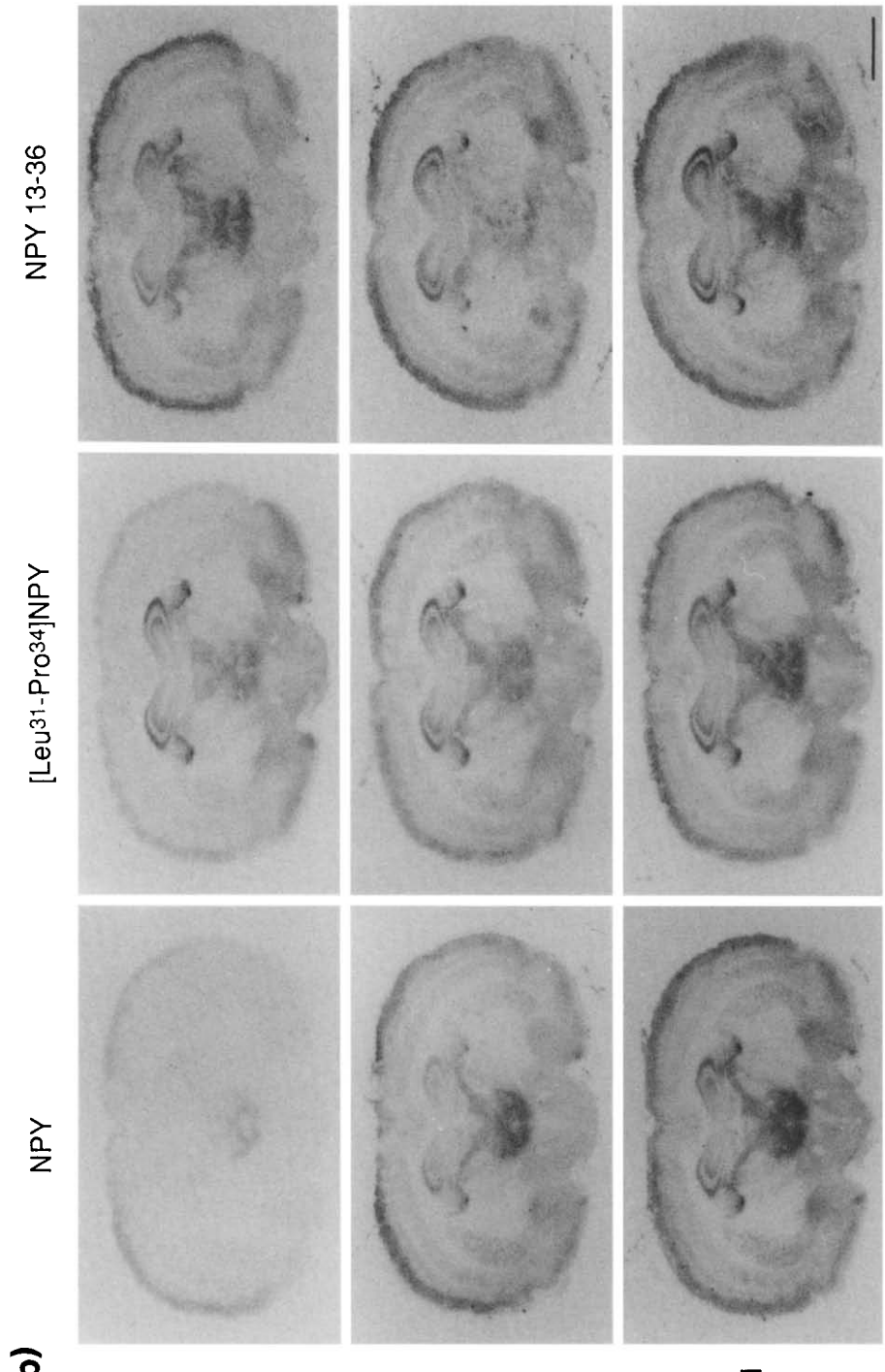


Fig. 4(b).

(See p. 57 for legend to Fig. 4).

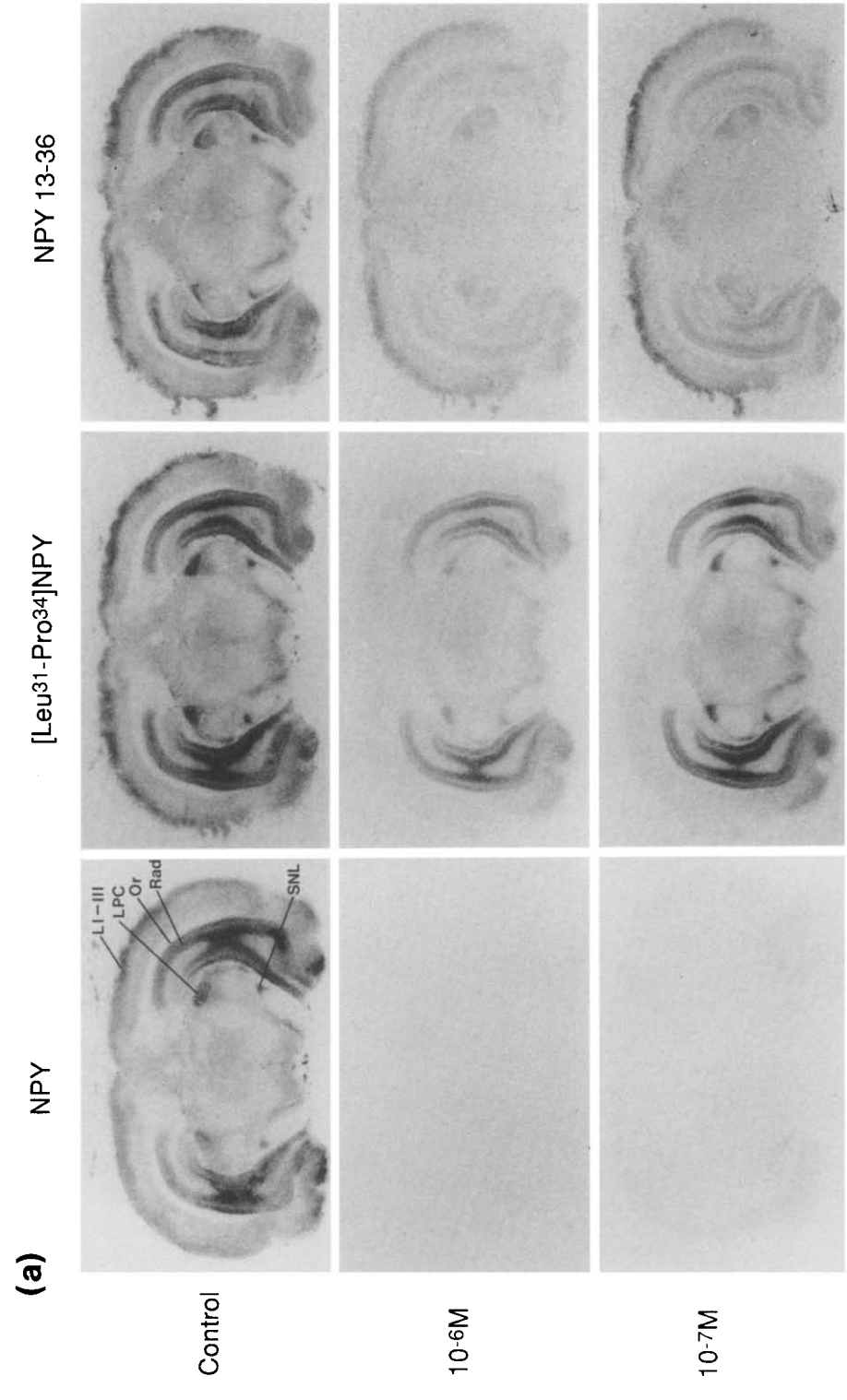

(a)
NPY
[Leu31-Pro34]NPY
NPY 13-36
Control
10-6M
10-7M
LI–III
LPC
Or
Rad
SNL

Fig. 5(a).

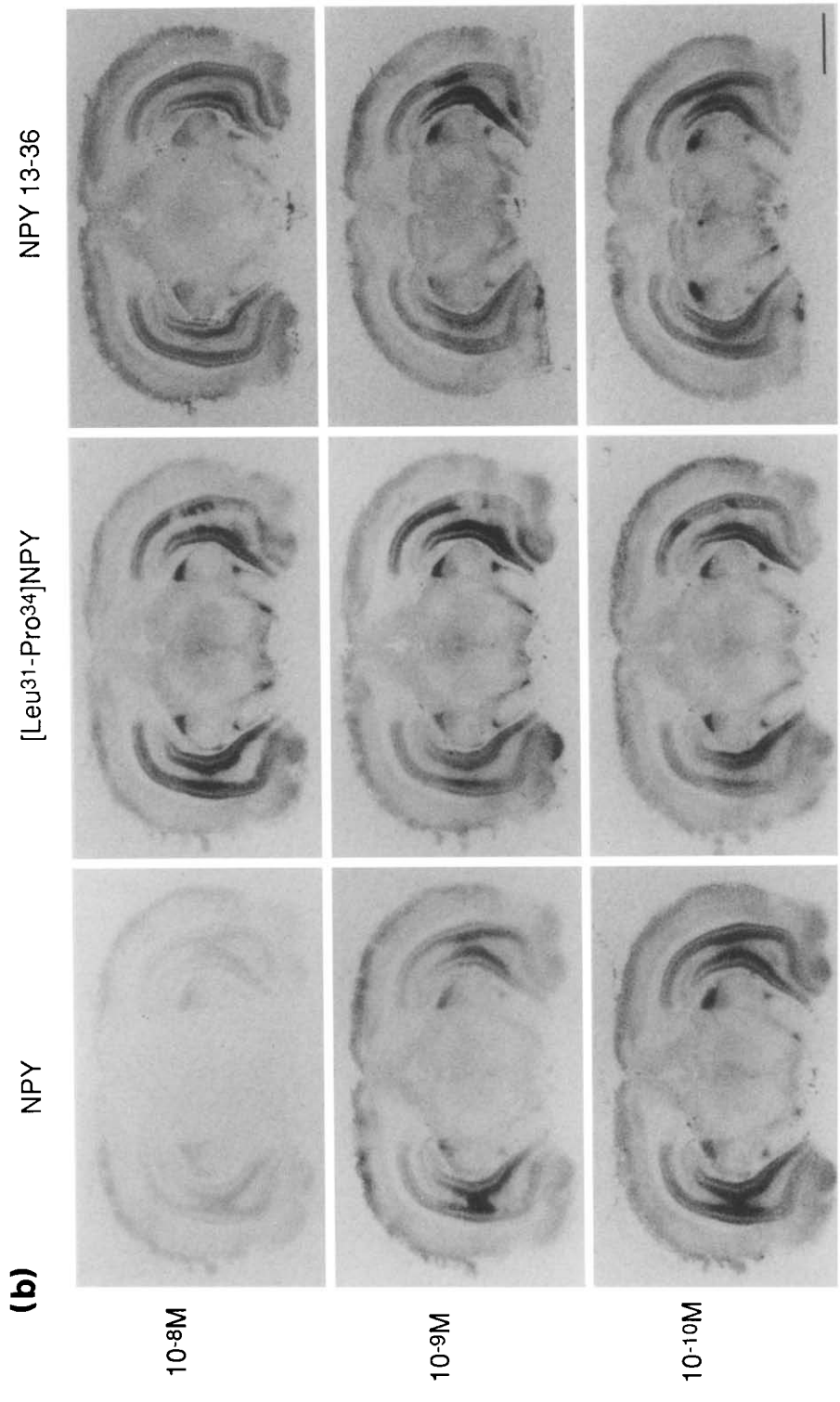


Fig. 5(b).

(See p. 57 for legend to Fig. 5).

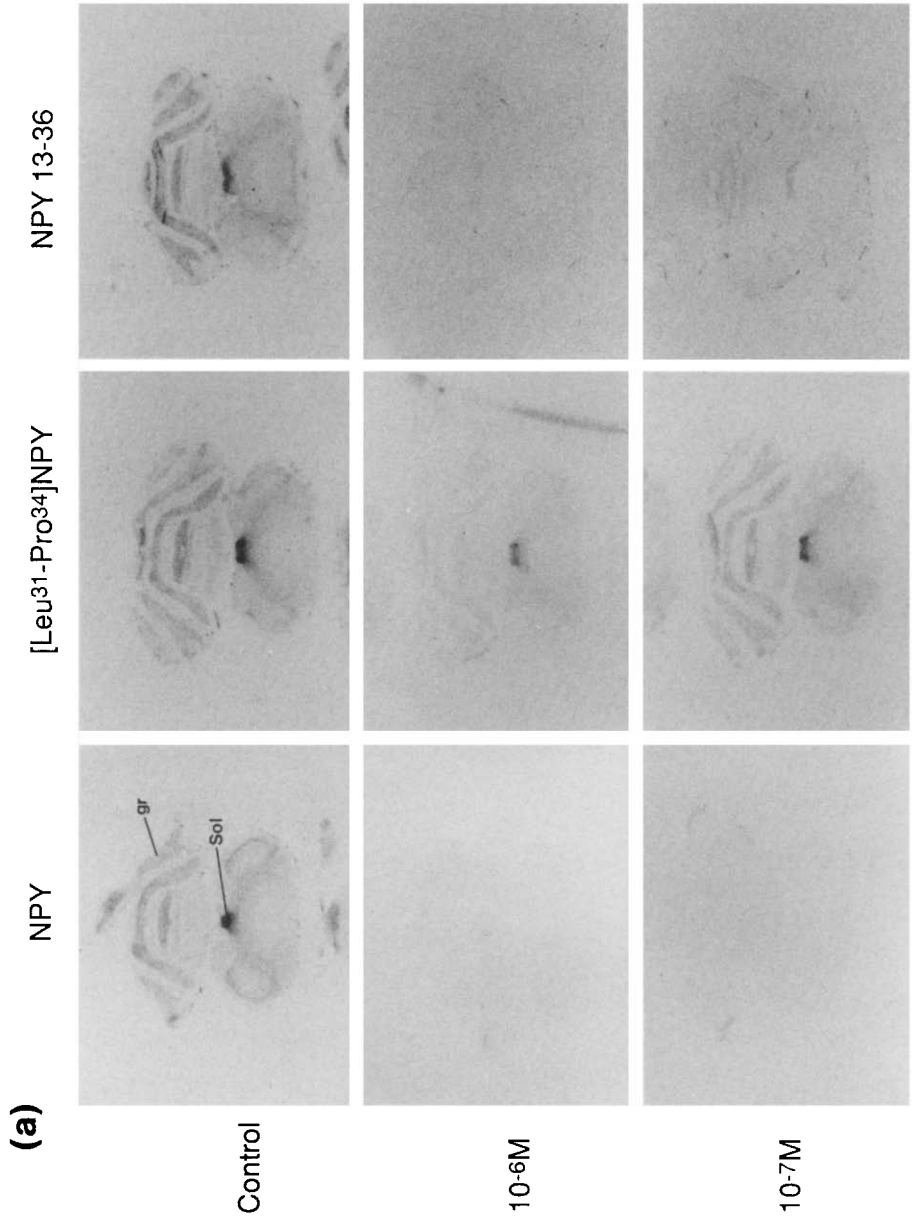


Fig. 6(a).

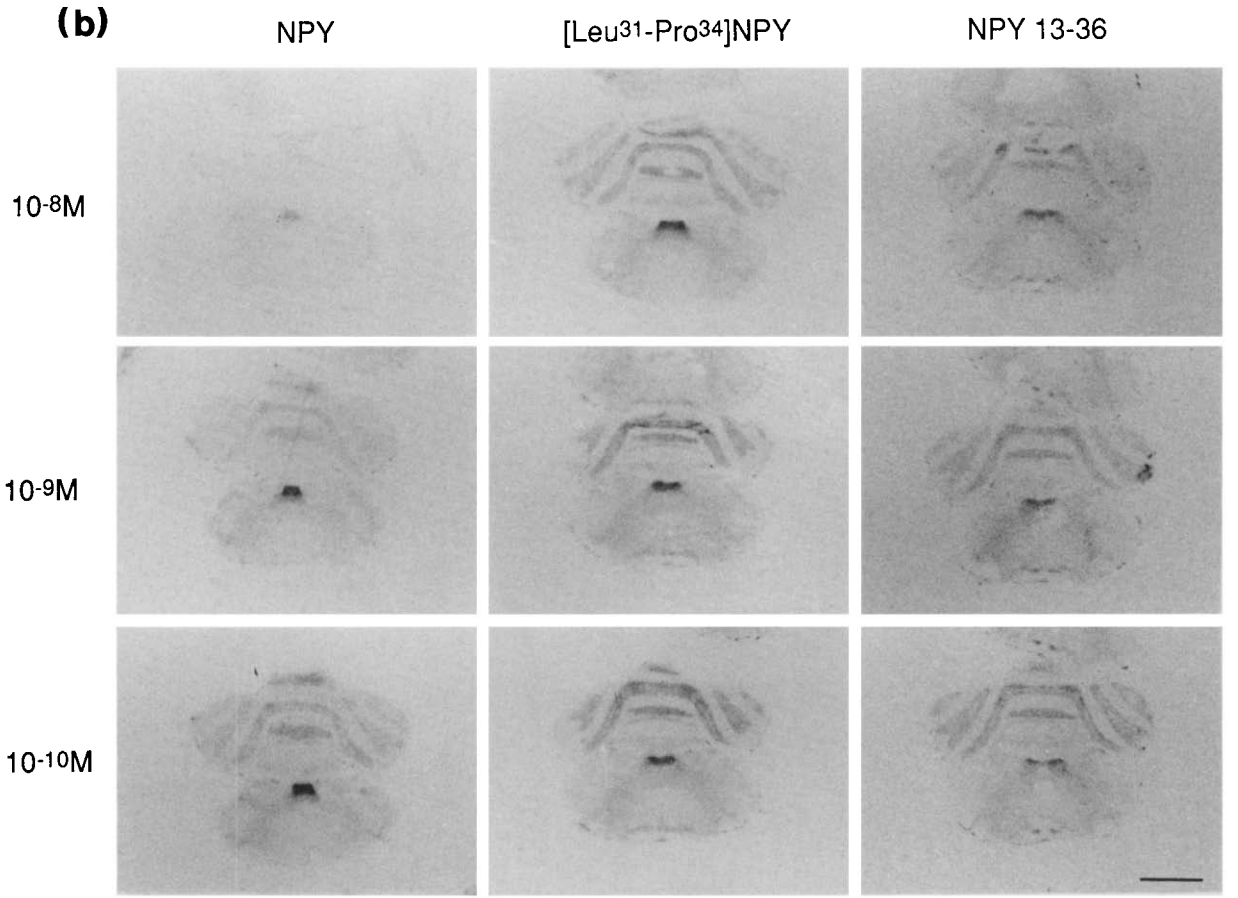


Fig. 6(b).

Figs 3–6. Displacement of ^{125}I-labeled PYY binding to sections of rat brain by various concentrations of NPY, [Leu^{31}-Pro^{34}] NPY and NPY 13–36. Sections of rat brain were incubated with 0.1 nM ^{125}I-labeled PYY alone or in the additional presence of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M NPY or [Leu^{31}-Pro^{34}] NPY. Autoradiograms of these sections are seen in the figures with areas exhibiting a high density of grains and, consequently, a high density of ^{125}I-labeled PYY binding, appearing as dark regions on a light background. Note the selective displacement of several brain regions such as the cerebral cortex by Leu^{31}-Pro^{34} NPY while a uniform inhibition of binding is seen when NPY is used. NPY 13–36 produced an inhibition of binding in regions such as the hippocampus and brainstem while leaving binding in the cerebral cortex relatively less affected. Bar = 2 mm.

Table 1. Quantitative autoradiography of NPY receptor subtypes

Brain region	Specific binding of ^{125}I-PYY (fmol/mg protein)		
	Total	[Leu^{31}-Pro^{34}] NPY	Inhibition (%)
Amygdala			
Central, lateral	15.6 ± 0.2	12.5 ± 0.5	20
Basolateral, anterior	11.6 ± 0.2	3.8 ± 0.6	68
Anterior olfactory nucleus	26.4 ± 1.4	6.9 ± 0.2	74
Caudate putamen	6.0 ± 0.2	1.9 ± 0.2	68
Central gray	10.9 ± 0.2	9.0 ± 0.1	18
Cerebellum			
Granular layer	11.2 ± 0.5	7.6 ± 0.5	32
Molecular layer	3.9 ± 0.2	4.0 ± 0.1	−3
Cerebral cortex—parietal			
Laminae I–III	21.4 ± 1.1	0.7 ± 0.2	97
Lamina IV	10.5 ± 0.3	0.7 ± 0.3	93
Lamina V–VI	5.9 ± 0.3	0.8 ± 0.2	87
Claustrum	15.7 ± 1.3	4.1 ± 0.2	74
Dentate gyrus	8.3 ± 0.1	1.5 ± 0.1	82
Dorsal raphe	11.7 ± 0.4	5.3 ± 0.7	54
Geniculate nucleus			
Dorsolateral	13.3 ± 0.7	2.0 ± 0.2	85
Medial	15.2 ± 1.1	1.6 ± 0.1	90
Gracile nucleus	17.5 ± 1.0	9.8 ± 0.8	44
Hippocampus			
CA 1-Oriens	10.2 ± 0.2	6.2 ± 0.1	40
Radiatum	8.7 ± 0.3	4.9 ± 0.2	43
CA 3-Oriens	21.7 ± 1.1	13.2 ± 0.3	39
Radiatum	18.5 ± 0.8	11.4 ± 0.2	38
Hypothalamus			
Paraventricular nucleus*	3.2 ± 0.1	3.5 ± 0.1	−9
Ventromedial nucleus*	6.7 ± 0.3	4.8 ± 0.6	28
Inferior olive	13.9 ± 0.3	5.7 ± 0.3	59
Islands of calleja	11.7 ± 0.2	3.5 ± 0.3	71
Lateral septum*	16.9 ± 0.1	16.5 ± 0.8	2
Medial mammillary nucleus	17.3 ± 1.4	3.0 ± 0.2	83
Nucleus accumbens*	6.4 ± 0.1	3.4 ± 0.2	47
Olfactory bulb			
External plexiform	7.6 ± 0.2	3.5 ± 0.1	54
Internal granular	10.5 ± 0.1	7.0 ± 0.1	34
Olfactory tubercle	8.8 ± 0.6	3.0 ± 0.3	65
Piriform cortex	9.4 ± 0.6	5.6 ± 0.3	40
Stria terminalis	24.3 ± 1.3	16.4 ± 0.5	32
Subfornical organ	40.0 ± 0.5	23.0 ± 2.4	42
Substantia nigra			
Compacta	13.0 ± 0.4	16.1 ± 0.6	−24
Lateralis	27.4 ± 0.7	23.5 ± 2.5	14
Reticulata	3.2 ± 0.3	2.3 ± 0.3	29
Superior colliculus	9.4 ± 0.3	5.9 ± 0.2	37
Supramammillary nucleus	10.7 ± 0.3	7.8 ± 0.5	27
Tenia tecta*	10.5 ± 0.4	6.6 ± 0.2	37
Thalamus			
Anterodorsal	17.8 ± 0.5	6.2 ± 0.5	65
Central medial	23.7 ± 1.0	9.5 ± 0.5	60
Lateral posterior*	14.4 ± 0.8	10.5 ± 0.9	27
Mediorostral	28.5 ± 1.6	4.4 ± 0.3	85
Mediodorsal, central*	12.6 ± 0.3	5.9 ± 0.5	53
Reticular	28.6 ± 1.7	17.9 ± 1.9	38
Reuniens	29.1 ± 3.0	3.6 ± 0.4	88
Stria medullaris	21.7 ± 1.5	3.5 ± 0.2	84
Ventrolateral*	13.4 ± 0.3	2.1 ± 0.1	84

Sections of rat brain were incubated in the presence of 100 pM ^{125}I-PYY with or without the addition of 50 nM [Leu^{31}-Pro^{34}] NPY. Values represent the mean (±SEM) of 8 determinations. The asterisks denote values obtained from experiments where 100 nM [Leu^{31}-Pro^{34}] NPY was used to differentiate the receptor subtypes. Nonspecific binding was determined in a set of adjacent sections and was subtracted from the values seen in the table.

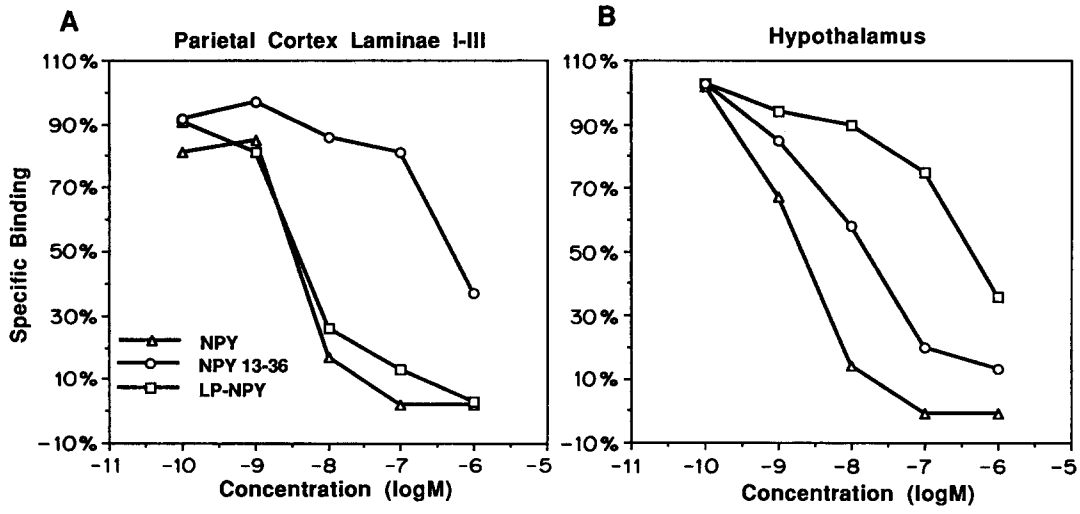


Fig. 7. Displacement curves of the inhibition of ^{125}I-labeled PYY binding by NPY, [Leu^{31}-Pro^{34}] NPY or NPY 13–36 in selected regions of rat brain. (A) Inhibition of binding in laminae I–III of the parietal cerebral cortex. Note that NPY and [Leu^{31}-Pro^{34}] NPY have similar potencies in the inhibition of binding in these regions while NPY 13–36 is relatively less potent. (B) Inhibition of binding in the hypothalamus. Readings were taken from the dorsal medial regions of the hypothalamus in these sections. Note that [Leu^{31}-Pro^{34}] NPY is relatively less potent in this region while NPY 13–36 inhibits binding at much lower concentrations than that seen in (A).

particularly in the lateral nucleus, paraventricular nucleus, ventromedial nucleus and the suprachiasmatic nucleus (Fig. 9). Binding in these regions appeared to be predominantly Y-2. The high density of binding of ^{125}I-labeled PYY to the subfornical organ (Fig. 8) was partially inhibited by [Leu^{31}-Pro^{34}] NPY indicating that both subtypes of the receptor can be found in this region. In the mammillary nucleus, a moderate level of binding was observed which was localized to two distinct subregions (Fig. 10). Binding in the medial mammillary nucleus was completely inhibited by 50 mM [Leu^{31}-Pro^{34}] NPY while this concentration left a significant amount of binding remaining in the lateral mammillary nucleus.

Mesencephalon and metencephalon

Binding in the substantia nigra was found in high levels in the zona lateralis, moderate levels in the zona compacta and low levels in the zona reticulata (Fig. 10). Binding in all these subregions appeared to be resistant to 50 nM [Leu^{31}-Pro^{34}] NPY and indicated the presence of the Y-2 receptor. A moderate level of binding seen in the ventral tegmental area also appeared to be the Y-2 subtype. A low level of binding in the central grey was not inhibited by 50 nM [Leu^{31}-Pro^{34}] NPY while binding in the superior colliculus was partially inhibited indicating the presence of both subtypes in this region.

Moderate levels of binding were localized to the area postrema, nucleus of the solitary tract and the inferior olive (Fig. 6). Binding in all these regions appeared to be predominantly the Y-2 subtype.

DISCUSSION

Several investigators using homogenate binding assays and receptor autoradiography have suggested the presence of NPY receptor subtypes in the brain (Aicher *et al.*, 1991; Dumont *et al.*, 1990). The evidence for this has relied on the development of selective peptide analogs, such as the C-terminal analog NPY 13–36, which recognize the individual receptor subtypes. In the brain membrane binding assay, NPY 13–36 did not appear to display any selectivity, producing displacement curves which modeled to a single site. In autoradiographic studies, this analog showed some regionally specific displacement of binding with a higher potency for inhibiting ^{125}I-labeled PYY binding in the hypothalamus than that seen in the cerebral cortex. More recently, Fuhlendorff and co-workers introduced a peptide analog, [Leu^{31}-Pro^{34}] NPY, which had high selectivity for the Y-1 receptor subtype

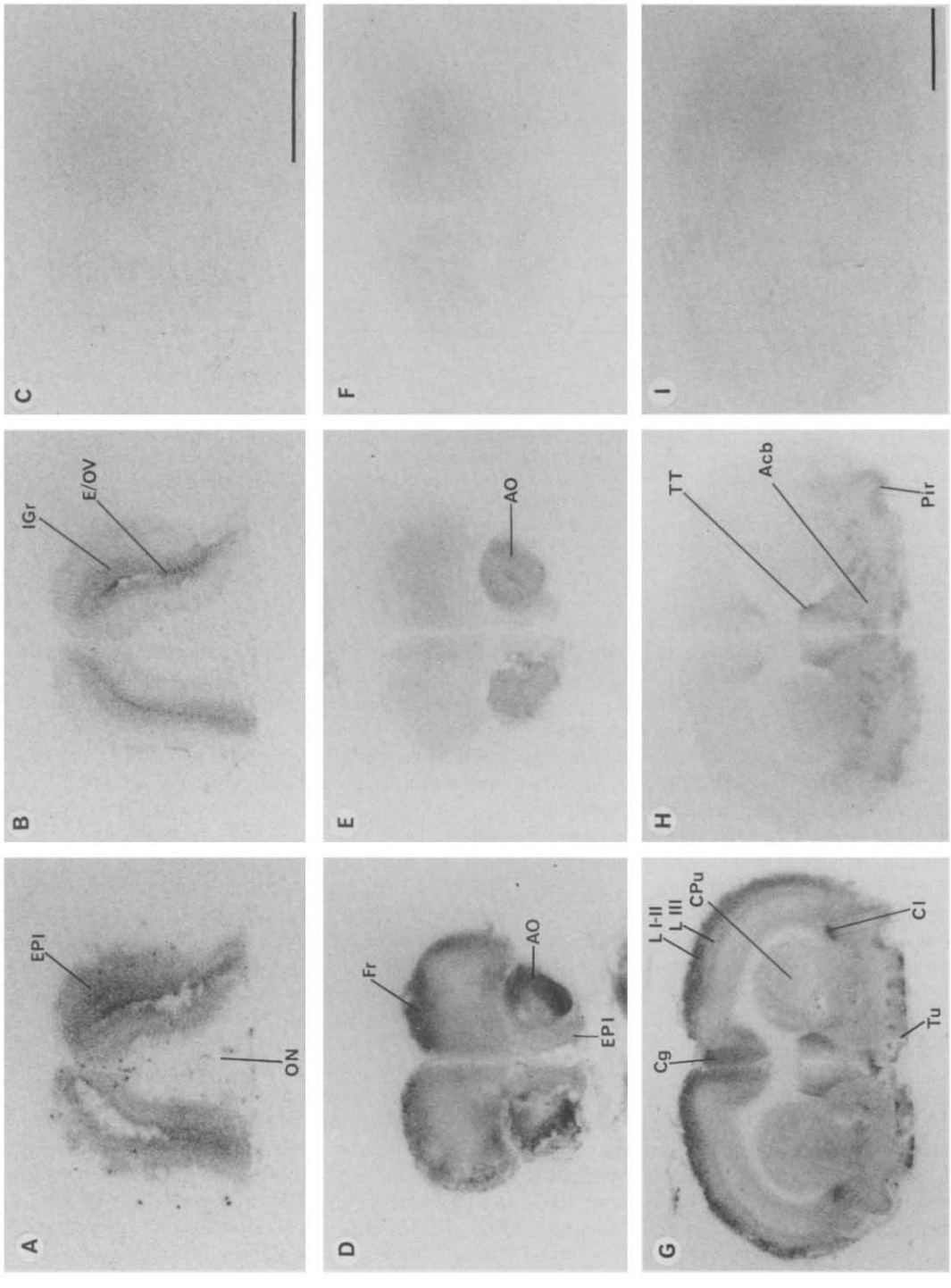


Fig. 8. Autoradiographic localization of subtypes of ^{125}I-labeled PYY binding sites in rat brain. Sections of rat brain were incubated with 100 pM ^{125}I-labeled PYY alone (A, D, G), with the addition of 50 nM [Leu^{31}-Pro^{34}] NPY (B, E, H) or 1 μM NPY (C, F, I). Autoradiograms were obtained by exposing the sections to film for 4 days. Note the different pattern of binding when the sections are incubated with [Leu^{31}-Pro^{34}]NPY indicating the selective displacement of ^{125}I-labeled PYY from brain regions such as laminae I–II (L1–II) of the parietal cerebral cortex while other regions such as the hippocampus (Figs 9 and 10) exhibit a small inhibition of binding. Bar = 2 mm.

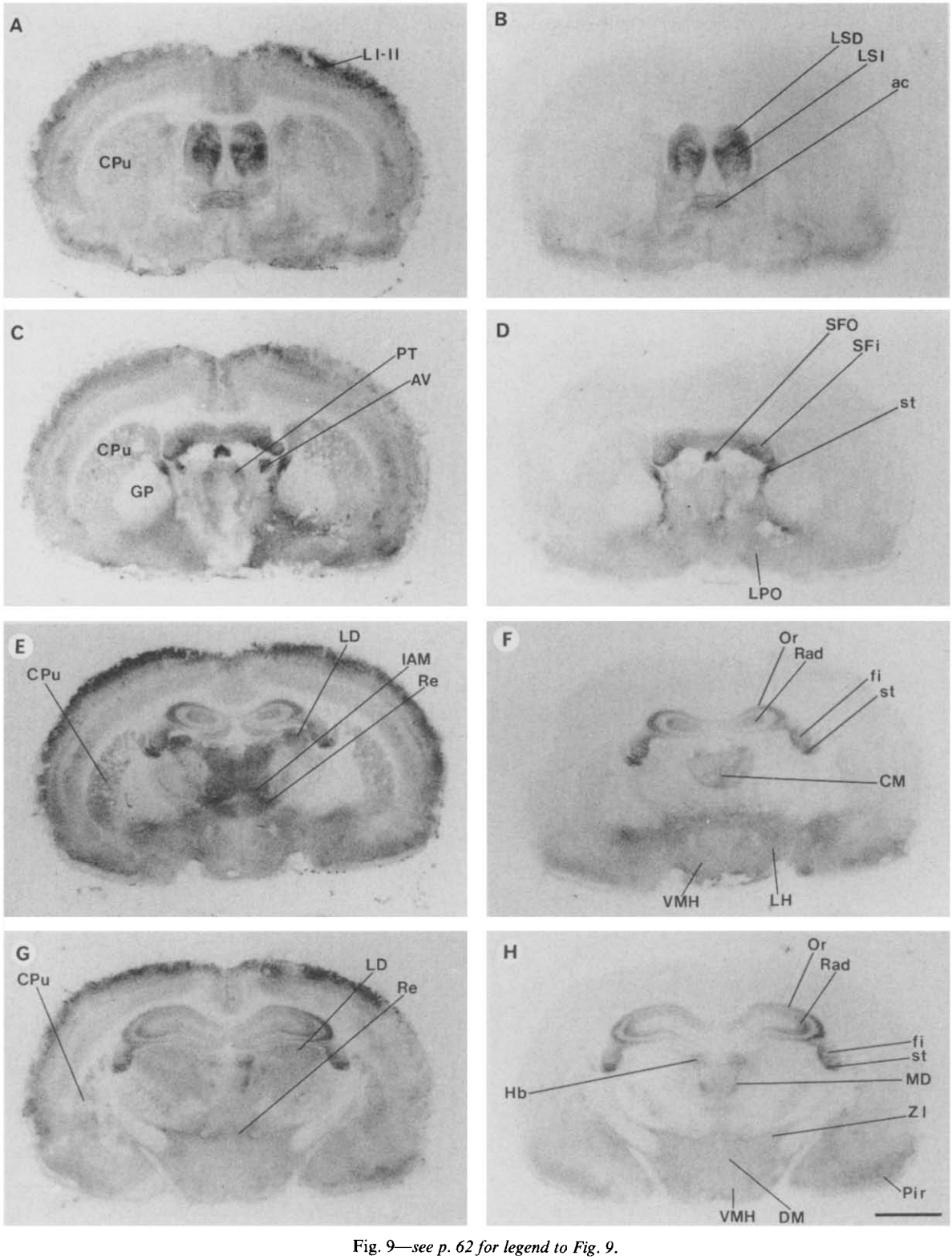
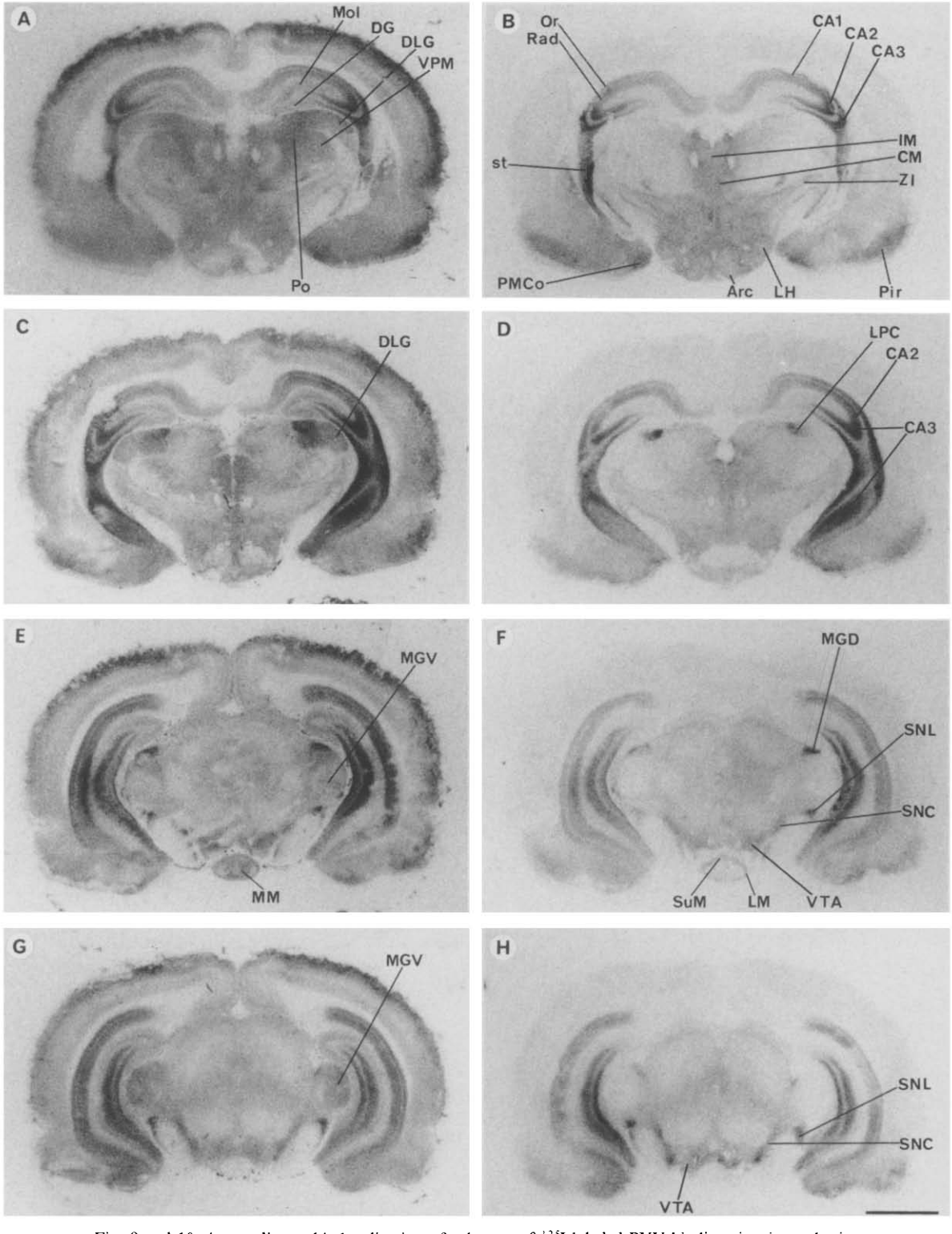


Fig. 9—*see p. 62 for legend to Fig. 9.*



Figs 9 and 10. Autoradiographic localization of subtypes of ^{125}I-labeled PYY binding sites in rat brain. Sections of rat brain were incubated with 100 pM ^{125}I-labeled PYY alone (A, C, E, G) or with the addition of 50 nM [Leu^{31}-Pro^{34}] NPY (B, D, F, H). Bar = 2 mm.

in several cell lines (Fuhlendorff *et al.*, 1990). In the present study, we demonstrate that [Leu^{31}-Pro^{34}] NPY is capable of discriminating subtypes of ^{125}I-labeled PYY binding sites in the rat brain. This analog produced biphasic displacement curves which had a large separation of affinities for the high and low affinity sites. In quantitative autoradiographic studies, this peptide was more potent in displacing ^{125}I-labeled PYY binding from the cortex (Y-1) than the hypothalamus (Y-2) confirming the data obtained in the homogenate studies. The distribution of [Leu^{31}-Pro^{34}] NPY preferring sites correlated well with the distribution of NPY 13–36 resistant sites indicating that the two peptides inhibited distinct subtypes which were localized in different brain regions. The high potency and selectivity of [Leu^{31}-Pro^{34}] NPY made this peptide analog more useful in obtaining a clear separation of the two receptor sites. Scatchard analysis of ^{125}I-labeled PYY saturation isotherms indicated that ^{125}I-labeled PYY identified high and low affinity binding sites in rat forebrain homogenates. This observation has also been reported by other investigators (Walker and Miller, 1988). ^{125}I-labeled PYY binding to the low affinity site was eliminated by including 100 nM [Leu^{31}-Pro^{34}] NPY in the incubation indicating that this site represents the Y-1 receptor and that ^{125}I-labeled PYY binds to the Y-2 receptor with higher affinity than the Y-1. In autoradiographic experiments, ^{125}I-labeled PYY binding to the cerebral cortex (Y-1) was inhibited at a much lower concentration of the Y-1 selective peptide than in the hypothalamus (Y-2) which indicated a reversal of the displacement pattern observed when using NPY 13–36. The distribution of the Y-2 receptor subtype seen in the present study agrees well with the reported distribution of ^{125}I-labeled PYY prefering receptors in the rat brain (Lynch *et al.*, 1989). In that study, areas prefering ^{125}I-labeled PYY included regions such as the lateral septum, hippocampus, piriform cortex, substantia nigra and nucleus reuniens of the thalamus which correlates well with the distribution of [Leu^{31}-Pro^{34}] NPY resistant Y-2 receptors in the brain seen in the present study. However, the use of [Leu^{31}-Pro^{34}] NPY has provided a clearer separation of the binding sites than does the use of low concentrations of ^{125}I-labeled PYY and ^{125}I-labeled NPY.

Subtypes of NPY receptors have been proposed on the basis of experiments on peripheral neuro-effector junctions (Wahlestedt and Håkanson, 1986; Wahlestedt *et al.*, 1986). In these experiments the Y-1 receptor was proposed to be located postsynaptically and mediate the postjunctional effects of NPY such as smooth muscle contraction. On the other hand, the Y-2 subtype was thought to be presynaptic and mediate the inhibition of neurotransmitter release via the inhibition of voltage sensitive calcium channels (Colmers *et al.*, 1988). The effects could be differentiated using long C-terminal fragment of NPY such as NPY 13–36 (Wahlestedt *et al.*, 1986). More recently, these receptor subtypes could be found individually on various cell lines indicating that they are distinct molecular entities (Sheikh *et al.*, 1989a,b). The two receptor subtypes also have differing molecular weights in affinity cross linking studies (Sheikh and Williams, 1990). The study of the Y-1 subtype has been facilitated by the discovery of [Leu^{31}-Pro^{34}] NPY which is highly selective for the Y-1 subtype of the receptor (Fuhlendorff *et al.*, 1990). The localization of these subtypes indicates that distinct receptor populations may mediate the known effects of NPY on the brain. [Leu^{31}-Pro^{34}] NPY and NPY were equipotent in increasing blood pressure indicating that the Y-1 subtype mediates the peripheral vasoconstriction (Fuhlendorff *et al.*, 1990; Potter *et al.*, 1991). Central administration of NPY has been reported to produce a vasodepressor response (Barraco *et al.*, 1990; Harland *et al.*, 1988; Scott *et al.*, 1989; Tseng *et al.*, 1989) while the localization of NPY receptors in cardiovascular regulatory centers such as the subfornical organ (Y-1 and Y-2), hypothalamus (Y-2) and in the brainstem cardiovascular region, the nucleus of the solitary tract (Y-2), appears to be mostly Y-2. This indicates that the regulation of blood pressure by NPY may be mediated by different receptor subtypes in the periphery when compared to the CNS. The Y-2 receptor present in the hypothalamus may be the location in the brain where NPY may exert endocrine effects (Aguirre *et al.*, 1991). NPY could potentially exert its cognitive effects (Flood *et al.*, 1987) via receptor populations in both the cerebral cortex and hippocampus which were primarily Y-1 and Y-2 respectively.

At the present time, NPY receptor subtypes cannot be adequately differentiated on the basis of second messenger generation. The second messenger identified for the Y-1 receptor appears to be somewhat heterogeneous at this point. The Y-1 receptor in a human erythroleukemia cell line is responsible for a mobilization of intracellular calcium via an activation of phosphoinositide hydrolysis (Daniels *et al.*, 1989). On the other hand, Y-1 receptors found in a human neuroepithelioma cell line (SK-N-MC) also mediate a mobilization of intracellular calcium; however, NPY does not appear to alter inositol phosphate metabolism (Aakerlund *et al.*, 1990; Lobaugh and

Blackshear, 1990). Rather, in this cell line, the Y-1 receptor is responsible for an inhibition of adenylate cyclase by NPY (Aakerlund *et al.*, 1990; Lobaugh and Blackshear, 1990; Wahlestedt *et al.*, 1990). In the rat brain, NPY has been demonstrated to increase both the concentrations of inositol phosphates (Hinson *et al.*, 1988; Widdowson and Halaris, 1990) as well as inhibit the activity of adenylate cyclase (Westlind-Danielsson *et al.*, 1987, 1988; Widdowson and Halaris, 1990). The best studied Y-2 receptor in the rat brain is found in the CA3 region of the hippocampus. Application of NPY and the long C-terminal fragments in this region produces an inhibition of neurotransmitter release (Colmers *et al.*, 1991) which is distinct from the ability of NPY to inhibit adenylate cyclase (Klapstein *et al.*, 1990). In studies using rat dorsal root ganglion cells (Bleakman *et al.*, 1991; Perney and Miller, 1989; Walker *et al.*, 1988), NPY produced an increase in the synthesis of inositol phosphates with an inhibition of intracellular calcium presumably through an interaction with a Y-2 receptor. The reduction in intracellular calcium appears to be directly related to protein kinase C activation since down regulation of protein kinase C by long term treatment with phorbol esters resulted in a reduction of the NPY effect on calcium (Ewald *et al.*, 1988). Interestingly, NPY produces an inhibition of adenylate cyclase in the brainstem (Härfstrand *et al.*, 1987a), a region where primarily Y-2 receptors were localized. In addition to the generally recognized Y-1 and Y-2 receptor subtypes, it has become apparent that additional subtypes of NPY receptors may exist (Michel, 1991). Clearly, more selective pharmacological tools will be useful in elucidating the functional coupling and physiological role of NPY receptor subtypes.

The distribution of the two NPY receptor subtypes sites overlapped in many brain regions. Few areas, such as the lateral septum (Y-2) and the cerebral cortex (Y-1), appeared to contain a single receptor subtype. It is unlikely that these receptor populations will be differentiated by the presence of the neurotransmitter since NPY is quite abundant in the brain while a few PYY immunoreactive neurons have been reported in select brain regions (Broome *et al.*, 1985; Ekman *et al.*, 1986). High levels of NPY immunoreactivity can also be found in the cerebral cortex where a moderate level of Y-1 binding is found indicating a region of the brain where the transmitter–receptor localization matches. In the brainstem, a high level of NPY immunoreactivity is found in the nucleus of the solitary tract where a relatively high level of the Y-2 receptor can be found. On the other hand, there is also a mismatch between some regions of the brain which contain high levels of NPY immunoreactivity and those which contain high densities in the receptor. High levels of NPY cell bodies and terminals can be found in the hypothalamus while only low levels of the Y-2 receptor can be detected. Low levels of NPY were found in the thalamus while high levels of the receptor were found in this region where both subtypes of the receptor were located, often in distinct thalamic nuclei. Therefore, the receptor mismatch cannot be explained by the localization of one receptor subtype in regions containing NPY immunoreactivity. The receptor–transmitter discrepancy has been previously noted with NPY (Martel *et al.*, 1988) as well as other neurotransmitters (Herkenham, 1987; Kuhar, 1985) and the reasons for this are presently unclear. Interestingly, PYY immunoreactivity is found in brain regions such as the brainstem and hypothalamus (Broome *et al.*, 1985; Ekman *et al.*, 1986), regions where the Y-2 receptor is predominant indicating that PYY containing neurons may be in proximity to Y-2 receptor populations. Further study will be necessary to determine which peptide is the endogenous ligand for each of the receptor subtypes.

In conclusion, the distribution of Y-1 and Y-2 receptors has been examined in the rat brain. The receptors exhibit a distinct distribution in a few brain regions, but, in general, the subtypes can be co-localized to a number of brain regions.

Note added in proof

Recently, a third NPY receptor, the Y-3 receptor, has been identified (Rimland *et al.*, *Molec. Pharmacol.* **40**, 869–875, 1991; Wahlestedt *et al.*, *Life Sci.* **50**, PL-7–PL-12, 1992). Since this receptor has very low affinity for PYY and ^{125}I-labeled PYY, it is unlikely that any appreciable binding to this site occurred in our study.

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