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Neuropeptide Y Receptor Binding Sites in Rat Brain: Differential Autoradiographic Localizations with ^{125}I-Peptide YY and ^{125}I-Neuropeptide Y Imply Receptor Heterogeneity

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Neuropeptide Y (NPY) receptor binding sites have been localized in the rat brain by *in vitro* autoradiography using picomolar concentrations of both ^{125}I-NPY and ^{125}I-peptide YY (PYY) and new evidence provided for differentially localized receptor subtypes. Equilibrium binding studies using membranes indicate that rat brain contains a small population of high-affinity binding sites and a large population of moderate-affinity binding sites. ^{125}I-PYY (10 pM) is selective for high-affinity binding sites (K_D = 23 pM), whereas 10 pM ^{125}I-NPY labels both high- and moderate-affinity sites (K_D = 54 pM and 920 pM). The peptide specificity and affinity of these ligands in autoradiographic experiments match those seen in homogenates. Binding sites for ^{125}I-PYY are most concentrated in the lateral septum, stratum oriens, and radiatum of the hippocampus, amygdala, piriform cortex, entorhinal cortex, several thalamic nuclei, including the reuniens and lateral posterior nuclei, and substantia nigra, pars compacta, and pars lateralis. In the brain stem, ^{125}I-PYY sites are densest in a variety of nuclei on the floor of the fourth ventricle, including the pontine central grey, the supragenual nucleus, and the area postrema. ^{125}I-NPY binding sites are found in similar areas, but relative levels of NPY binding and PYY binding differ regionally, suggesting differences in sites labeled by the two ligands. These receptor localizations resemble the distribution of endogenous NPY in some areas, but others, such as the hypothalamus, contain NPY immunoreactivity but few binding sites.

Neuropeptide Y (NPY), a 36 amino acid peptide putative neurotransmitter, is widely distributed in the central and peripheral nervous systems. NPY was isolated initially from porcine brain and is homologous to peptide YY (PYY) and other members of the pancreatic polypeptide family (Tatemoto et al., 1982). Whereas PYY is found mainly in endocrine cells in the gastrointestinal tract, NPY is most concentrated in the nervous system and the adrenal gland (Lundberg et al., 1984; Lukinius et al., 1986; Miyachi et al., 1986). Sparse populations of PYY-immunoreactive neurons also have been identified in the brain stem, spinal cord, hypothalamus, and median eminence of the rat (Broome et al., 1985; Ekman et al., 1986).

Numerous studies have indicated that NPY acts directly on a variety of target systems (O'Donohue et al., 1985; Gray and Morley, 1986). NPY also can block or enhance transmission at a variety of neuroeffector junctions. Although these effects have been demonstrated most often at noradrenergic sympathetic synapses, NPY may also interact with acetylcholine (Stjernquist et al., 1983; Kilborn et al., 1985; Potter, 1985), substance P (Walker et al., 1988) histamine, and PGF_{2alpha} (Edvinsson et al., 1984).

Immunocytochemical localizations also support a role for NPY as a neurotransmitter. In the brain, NPY occurs in both neuronal fibers and cell bodies (Chronwall et al., 1985; Yamazoe et al., 1985; de Quidt and Emson, 1986a, b; Gray and Morley, 1986), and in several regions *in situ* hybridization reveals NPY mRNA in neuronal cells (Gehlert et al., 1987). In the peripheral nervous system, NPY is colocalized with norepinephrine in sympathetic ganglia (Gray and Morley, 1986), and physiological effects of NPY resemble those elicited by noradrenergic stimulation. Like norepinephrine, NPY constricts blood vessels (Emson and de Quidt, 1984), and NPY-promoted insulin release is antagonized by phentolamine, an alpha noradrenergic antagonist (Alwmark and Ahren, 1987). NPY distribution in the central nervous system also resembles that of norepinephrine (Hokfelt et al., 1983a, b; Everitt et al., 1984; Blessing et al., 1986; Harfstrand et al., 1987a). Colocalization of NPY with norepinephrine is exceptionally marked in the A1 and A6 groups of the brain stem. Physiological effects following NPY injection in the brain are similar to those of norepinephrine and include regulation of feeding (Stanley and Leibowitz, 1984, 1985; Clark et al., 1985; Stanley et al., 1985; Kuenzel et al., 1987), blood pressure (Fuxe et al., 1983; Harfstrand, 1986), and the neuroendocrine system (Kalra and Crowley, 1984; Kerkerian et al., 1985; McDonald et al., 1985; Harfstrand et al., 1987b). PYY exerts similar effects when given intracerebroventricularly, suggesting that both peptides can activate the same receptors (Fuxe et al., 1982; Morley et al., 1985; Broome et al., 1985; Kuenzel et al., 1987).

A single class of high-affinity binding sites for NPY has been reported in brain, based on binding of ^{125}I-NPY (Unden et al., 1984; Saria et al., 1985) and ^{125}I-N-succinimidyl 3-(4-hydroxy 5-iodophenyl) propionate-NPY (^{125}I-Bolton Hunter NPY) (Chang et al., 1985). In preliminary studies, receptors have been

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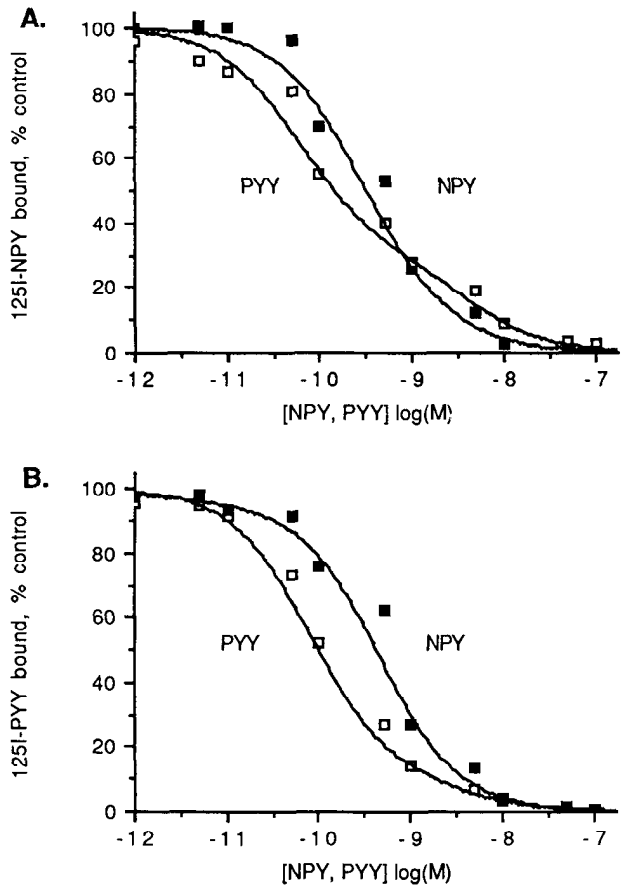

Figure 1. ^{125}I-NPY and ^{125}I-PYY binding to rat brain membranes. *A*, Ten picomoles/liter of ^{125}I-NPY was incubated with increasing concentrations of NPY (■) or PYY (□). *B*, Ten picomoles/liter ^{125}I-PYY was incubated with increasing concentrations of NPY (■) or PYY (□). Representative experiments performed in triplicate are shown ($n = 3$ except for ^{125}I-NPY vs NPY, $n = 4$ for ^{125}I-NPY vs PYY, and $n = 3$ for ^{125}I-PYY vs NPY and PYY).

localized by autoradiography with ^{3}H-NPY (Martel et al., 1986; Unnerstall et al., 1986) or ^{125}I-NPY (Nakajima et al., 1986; Harfstrand et al., 1986; Martel et al., 1987). Two binding sites for ^{125}I-PYY recently have been reported in brain (Inui et al., 1988). The properties of NPY and PYY receptors in rat brain and dorsal root ganglion (DRG) cell cultures have been characterized in detail using high-pressure liquid chromatography (HPLC)-purified monoiodinated ^{125}I-PYY and ^{125}I-NPY (Walker and Miller, 1988; Walker et al., 1988). Equilibrium binding assays in these studies demonstrate that ^{125}I-NPY and ^{125}PYY recognize a minor population of high-affinity sites and a major population of moderate-affinity sites in the brain. ^{125}I-PYY is better able to discriminate the high- and moderate-affinity sites. We now report the detailed localization of NPY receptors in rat brain using both ^{125}I-NPY and ^{125}I-PYY as ligands for *in vitro* autoradiography and provide evidence for differential distribution of the high- and moderate-affinity sites.

Materials and Methods

Materials. Unlabeled PYY and NPY were obtained from Peninsula (Belmont, CA), kainic acid and BSA from Sigma (St. Louis, MO), ^{3}H-Ultrafilm from LKB, ^{125}I from Amersham (Arlington Heights, IL), and protein assay reagent from Biorad (Richmond, CA).

Preparation of ^{125}I-PYY and ^{125}I-NPY. PYY and NPY were iodinated as described previously (Walker and Miller, 1988). Briefly, the reaction mixture contained 0.25 M phosphate buffer, 2.8 nmol peptide, 0.5 nmol ^{125}I, and 36 nmol chloramine T. The reaction was stopped after 1 min with 525 nmol sodium metabisulfite and transferred to a test tube containing 1.0 ml Sephadex QEA-A25 equilibrated with 0.08 M Trizma HCl, 0.08 M NaCl, 0.02 M HCl, and 0.02% BSA, pH 8.6.

The iodinated peptide was extracted in batch rinses, which were pooled and purified on an Altex C_{18} ultrasphere ion-pairing column (5-μm particle size, 0.46 × 25 cm). The mobile phase consisted of acetonitrile and an aqueous buffer: 0.1 M phosphoric acid, 0.02 M triethylamine, 0.05 M $NaClO_4$, all brought to pH 3.0 with NaOH. Two major monoiodinated peaks eluted after the uniodinated peptide at 34% (vol/vol) acetonitrile and 30% acetonitrile for ^{125}I-NPY and ^{125}I-PYY, respectively. Peak fractions were pooled and concentrated under vacuum, then desalted on a Bio-gel P-2 column (75 × 2.5 cm) equilibrated with 0.01 M Tris, 0.1% BSA, and 0.02% sodium azide, pH 7.4. The peak fractions were pooled and concentrated under vacuum, then stored at −20°C. For both PYY and NPY, the HPLC peak that offered maximal synthetic yield and specific binding to rat brain synaptosomes was selected as the choice peak and was used in all future experiments. Trypsin digestion experiments indicated that ^{125}I-NPY was monoiodinated on the N-terminal tyrosine and ^{125}I-PYY was monoiodinated on the C-terminal tyrosine (Walker and Miller, 1988).

Membrane-binding studies. Binding studies were performed as described elsewhere (Walker and Miller, 1988). Briefly, the entire brain minus the cerebellum and brain stem was dissected and homogenized with a Teflon pestle for 10–12 strokes in ice-cold 0.32 M sucrose. The

Table 1. Competition binding to rat brain membranes with 10 pM radioligand

Radioligand competitor	^{125}I-NPY NPY	^{125}I-NPY PYY	^{125}I-PPY NPY	^{125}I-PPY PYY
Hill slope	0.81 ± 0.07	0.59 ± 0.01	0.94 ± 0.04	0.84 ± 0.05
One-site model				
IC_{50} (nM)	0.37 ± 0.052	0.15 ± 0.04	0.50 ± 0.12	0.10 ± 0.02
r^2	0.96 ± 0.01	0.65 ± 0.10	0.95 ± 0.01	0.96 ± 0.01
Two-site model				
$IC_{50(1)}$ (nM)	—	0.048 ± 0.0068		
$IC_{50(2)}$ (nM)		5.3 ± 1.8		
r^2		0.96 ± 0.01		

Competition binding data were analyzed by the following equation, where $B_{(c)}$ refers to the specific binding at competitor concentration (C), $B_{(0)}$ refers to the specific binding in the absence of competitor, and IC_{50} is the competitor concentration producing half-maximal inhibiton: $B_{(c)}/B_{(0)} = 1 - [C]/(IC_{50} + [C])$. The one-site and two-site models correspond to 1 or 2 additive and independent terms, respectively; the IC_{50}s in the two-site model are desiganted $IC_{50(1)}$ and $IC_{50(2)}$. The coefficient of variation (r^2) is the proportion of the total variation in $B_{(c)}/B_{(0)}$ that can be explained by the regression model. Parameters are listed for the simplest model required to obtain a coefficient of variation ≥0.95. All values are presented as mean ± SEM. ($n \geq 3$).

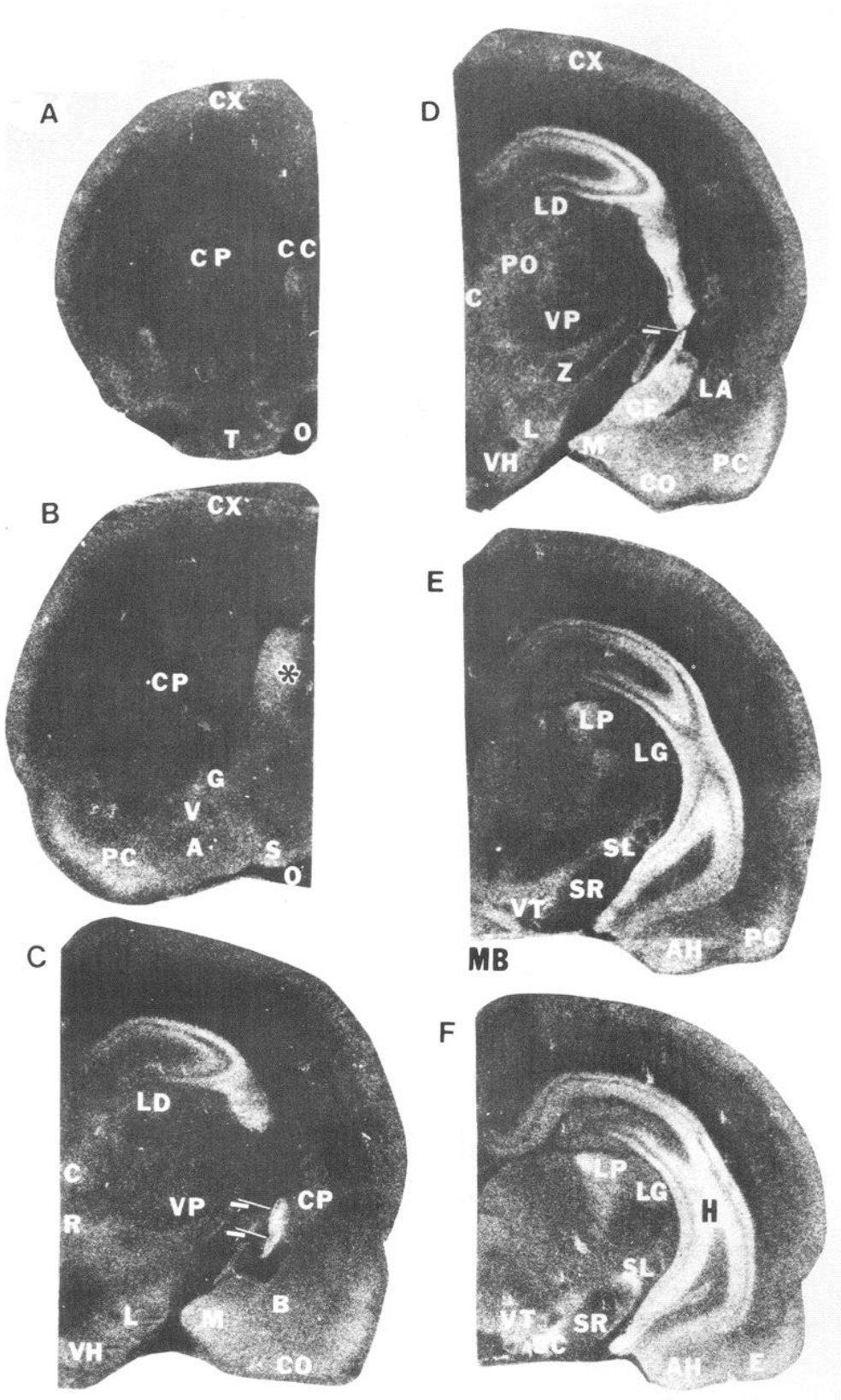

Figure 2. ^{125}I-PYY binding in the rat forebrain. Eight-microliter sections of rat brain were incubated in 5 pM ^{125}I-PYY. Negligible levels of autoradiographic grains were observed in slices incubated with 200 nM unlabeled PYY along with the radiolabel (not shown). High levels of binding are found in the lateral septum (*), the piriform cortex (*PC*), the stria terminalis (*arrows* in *C, D*), several nuclei of the amygdala (*M, B, CO, CC*), mamillary body (*MB*), the stratum oriens and radiatum around CA3 of the hippocampus (*H*), the pars compacta (*SC*) and lateralis (*SL*) of the substantia nigra (*SR*), and the lateral posterior nucleus of the thalamus (*LP*). Moderate levels are found in the cerebral cortex (*CX*), and the caudate-putamen (*CP*). Low levels are found in the globus pallidus (*G*), and the corpus callosum (*CC*). Abbreviations: *A,* anterior amygdala; *AH,* amygdalohippocampal area; *B,* bed nucleus stria terminalis; *C,* centromedian thalamic nucleus; *CC,* corpus callosum; *CE,* central amygdaloid nucleus; *CO,* cortical amygdala; *CP,* caudate putamen; *CX,* cerebral cortex; *E,* entorhinal cortex; *G,* globus pallidus; *L,* lateral hypothalamus (in *C,D*); *LA,* lateral amygdala; *LD,* lateral dorsal thalamic nucleus; *LG,* lateral geniculate; *LP,* lateral posterior nucleus of thalamus; *M,* medial amygdala; *MB,* mamillary body; *O,* optic chiasm; *PC,* piriform cortex; *PO,* posterior thalamic nucleus; *R,* reuniens thalamic nucleus; *S,* supraoptic nucleus, hypothalamus; *SC,* substantia nigra, pars compacta; *SL,* substantia nigra, pars lateralis; *SR,* substantia nigra, pars reticulata; *T,* olfactory tubercle; *V,* ventral pallidum; *VH,* ventromedial hypothalamic nucleus; *VP,* ventral posterior thalamic nucleus; *VT,* ventral tegmental areas; *Z,* zona incerta; *, lateral septum; *arrow,* stria terminalis.

Table 2. Specificity of ^{125}I-PYY and ^{125}I-NPY binding to rat brain tissue sections

Ligand	Region	IC_{50} (pM) PYY	IC_{50} (pM) NPY
^{125}I-PYY	Lateral septum	100	600
	Hippocampus	50	600
	Piriform cortex	75	800
^{125}I-NPY	Lateral septum	–	50
	Hippocampus	150	200
	Thalamus	800	–

Sections of rat brain were incubated with approximately 10 pM ^{125}I-NPY or ^{125}I-PYY with varying concentrations in labeled NPY or PYY as described in Materials and Methods. Concentrations of unlabeled peptide varied from 0.1 pM to 500 nM. Autoradiograms generated were quantified in each region, and data are shown as concentrations inhibiting 50% of total binding in the absence of unlabeled peptide. Nonspecific binding was determined as described in Materials and Methods.

Table 3. Autoradiographic distribution of ^{125}I-PYY and ^{125}I-NPY binding sites

Region	^{125}I-PYY bound (fmol/mg protein)	^{125}I-NPY bound (fmol/mg protein)	Ratio
Telencephalon			
Cortex			
Retrosplenial	3.4 ± 0.3 (21)	0.95 ± 0.07	3.6
Parietal	5.6 ± 0.2 (20)	1.49 ± 0.06	3.8
Frontal	5.3 ± 0.2 (20)	1.26 ± 0.08	4.2
Piriform	11.2 ± 0.3 (47)	1.60 ± 0.11	7.0
Temporal	6.6 ± 0.5 (9)	1.80 ± 0.15	3.7
Occipital	5.1 ± 0.3 (5)	1.25 ± 0.14	4.1
Entorhinal	9.1 ± 0.5 (15)	1.68 ± 0.13	5.4
Hippocampus			
CA3–4	15.5 ± 0.4 (35)	2.42 ± 0.07	6.4
CA1–2	10.9 ± 0.7 (4)	1.35 ± 0.08	4.7
Dentate gyrus	4.8 ± 0.3 (4)	1.05 ± 0.08	4.6
Amygdalohippocampus	10.2 ± 0.7 (9)	1.86 ± 0.14	5.4
Globus pallidus	3.5 ± 0.2 (10)	0.60 ± 0.05	5.8
Caudate putamen	4.2 ± 0.2 (32)	0.80 ± 0.07	5.2
Claustrum/endopiriform	8.7 ± 0.4 (10)	1.92 ± 0.18	4.5
Medial septum	3.6 ± 0.1 (3)	0.46 ± 0.18	7.8
Lateral septum	16.2 ± 1.2 (23)	1.35 ± 0.07	12.0
Diagonal band	5.4 ± 0.2 (6)	0.54 ± 0.13	10.0
Nucleus accumbens	5.2 ± 0.2 (5)	0.63 ± 0.10	8.3
Ventral pallidum	8.5 ± 0.6 (21)	0.89 ± 0.06	9.6
Bed nucleus of the stria terminalis			
Anterior	5.7 ± 0.2 (8)		
Ventral	5.6 ± 0.2 (3)		
Dorsal	5.6 ± 0.2 (3)	1.14 ± 0.10	4.9
Medial	10.3 ± 0.5 (9)	1.00 ± 0.16	10.3
Amygdala			
Lateral	6.8 ± 0.5 (11)	1.06 ± 0.21	6.4
Posterior cortical	12.1 ± 0.5 (25)	1.45 ± 0.10	8.3
Anterior cortical	8.5 ± 0.2 (3)	1.52 ± 0.14	5.6
Anterior	6.5 ± 0.2 (8)	1.40 ± 0.14	4.6
Medial	11.3 ± 1.5 (8)	1.23 ± 0.16	9.2
Central	9.3 ± 2.0 (6)	1.42 ± 0.20	6.5
Basal	8.5 ± 0.5 (6)	1.26 ± 0.19	6.7
Anterior olfactory nucleus	13.3 ± 0.9 (5)	2.29 ± 0.04	5.8
Olfactory tubercle	7.7 ± 0.4 (14)	1.23 ± 0.07	6.3
Islands of Calleja	10.2 ± 0.6 (5)	2.55 ± 0.18	4.0
White matter			
Stria terminalis	16.1 ± 0.6 (15)	2.51 ± 0.13	6.4
Corpus callosum	1.6 ± 0.2 (7)	0.40 ± 0.05	4.0
Internal capsule	2.7 ± 0.5 (4)	0.05 ± 0.05	–
Optic tract	2.0 ± 0.3 (4)	0.10 ± 0.07	–
Lateral olfactory tract	1.6 ± 0.2 (3)		
Anterior commissure	4.3 ± 0.9 (3)		
Diencephalon			
Median preoptic	9.1 ± 0.4 (9)	0.57 ± 0.11	16.0
Lateral preoptic	7.5 ± 0.4 (9)	0.74 ± 0.15	10.1
Hypothalamus			
Medial mamillary	7.2 ± 0.4 (9)	1.74 ± 0.31	4.1
Lateral mamillary	9.0 ± 0.7 (14)	0.85 ± 0.15	10.5
Supraoptic	7.9 ± 0.6 (15)	1.26 ± 0.23	6.2
Suprachiasmatic	3.9 ± 0.2 (8)	0.83 ± 0.13	4.7
Periventricular	4.6 ± 0.8 (4)		
Anterior	4.8 ± 1.8 (4)		
Lateral	6.6 ± 0.4 (12)	0.91 ± 0.11	7.3
Arcuate	7.0 ± 0.6 (6)	0.83 ± 0.15	8.4
Posterior	7.3 ± 0.2 (4)	0.69 ± 0.09	10.5
Dorsal/dorsalmedial	5.2 ± 1.2 (4)	0.45 ± 0.16	11.5
Thalamus			
Centromedian	8.1 ± 0.4 (13)	1.75 ± 0.28	4.6
Medial dorsal	7.2 ± 0.4 (16)	1.82 ± 0.12	4.0
Lateral dorsal	4.7 ± 0.3 (12)	2.09 ± 0.10	2.2
Ventral lateral	5.4 ± 1.4 (8)	1.71 ± 0.05	3.2
Ventral medial	5.1 ± 1.5 (6)	0.92 ± 0.10	5.5
Rhomboid	8.3 ± 0.9 (6)	1.93 ± 0.19	4.3
Reuniens	8.2 ± 1.3 (6)	2.38 ± 0.41	3.4
Medial geniculate	5.1 ± 0.4 (26)	0.86 ± 0.15	5.9
Dorsal lateral geniculate	5.5 ± 0.3 (13)	1.12 ± 0.15	4.9
Ventral lateral geniculate	4.2 ± 0.2 (3)	0.28 ± 0.02	15.0
Lateral posterior	12.1 ± 0.6 (25)	1.82 ± 0.11	6.6
Posterior	6.4 ± 0.4 (12)	1.54 ± 0.21	4.3
Lateral habenula	6.4 ± 0.5 (9)	0.83 ± 0.02	7.7
Medial habenula	6.1 ± 0.6 (10)	0.71 ± 0.12	8.6
Zona incerta	6.0 ± 0.4 (14)	0.51 ± 0.09	11.8
Mesencephalon			
Interpeduncular nucleus	4.2 ± 0.9 (9)	0.89 ± 0.20	4.7
Substantia nigra			
Pars compacta	9.1 ± 0.5 (28)	1.01 ± 0.09	9.0
Pars reticularis	2.9 ± 0.2 (25)	0.45 ± 0.14	6.4
Pars lateralis	12.2 ± 0.4 (38)	1.28 ± 0.23	9.5
Ventral tegumental area	8.3 ± 0.3 (31)	0.77 ± 0.08	10.7
Interfascicular nucleus	8.6 ± 1.0 (8)		
Prerubral field	4.4 ± 0.2 (4)		
Red nucleus	2.9 ± 0.2 (10)		
Periaqueductal grey	6.8 ± 0.4 (17)		
Inferior colliculus	4.4 ± 0.2 (3)		
Superior colliculus	5.6 ± 0.4 (11)		
Anterior pretectal nucleus	2.6 ± 0.3 (6)		
Motor nucleus 3	7.7 ± 0.7 (6)		

Table 3. Continued

Region	^{125}I-PYY bound (fmol/mg protein)	^{125}I-NPY bound (fmol/mg protein)	Ratio
Metencephalon and mylencephalon			
Pontine nucleus	3.3 ± 0.3 (8)		
Pontine reticular formation	5.8 ± 0.6 (3)		
Motor nucleus 7	7.05 ± 0.4 (19)		
Motor nucleus 6	4.4 ± 0.3 (6)		
Motor nucleus 5	4.8 ± 0.1 (4)		
Mesencephalic nucleus 5	6.6 ± 0.2 (3)		
Sensory nucleus 5	5.4 ± 0.7 (4)		
Spinal nucleus 5	2.4 ± 0.2 (6)		
Substantia gelatinosa 5	3.9 ± 0.2 (3)		
Superior olive	6.0 ± 0.2 (16)		
Dorsal parabrachial	7.2 ± 0.3 (9)		
Ventral parabrachial	6.7 ± 0.3 (6)		
Central grey pons	10.0 ± 0.5 (9)		
Dorsal tegument nucleus	4.9 ± 0.6 (11)		
Sphenoid	14.0 ± 1.5 (6)		
Supragen.	9.0 ± 0.2 (5)		
Preopositus hypoglossal	6.1 ± 0.4 (18)		
Vestibular nucleus			
Medial	4.0 ± 0.1 (5)		
Lateral	3.1 ± 0.2 (3)		
Spinal	3.4 ± 0.2 (5)		
Cochlear nucleus			
Dorsal	4.4 ± 0.5 (5)		
Ventral	3.0 ± 0.5 (5)		
Raphe magnus	5.6 ± 0.4 (6)		
Raphe pallidus	6.6 ± 0.4 (5)		
Raphe obscurus	4.2 ± 0.7 (3)		
Raphe pontine	7.4 ± 0.5 (4)		
Medial reticulum			
Gigantocellular	4.7 ± 0.3 (13)		
Intermediate	4.4 ± 0.2 (6)		
Lateral	3.6 ± 0.2 (3)		
Inferior olive	5.2 ± 0.4 (17)		
Hypoglossal	3.1 ± 0.2 (4)		
Cuneate/gracilis nucleus	3.2 ± 0.4 (4)		
Nucleus ambiguus	4.6 ± 0.3 (12)		
Nucleus tractus solitarius	5.7 ± 0.4 (7)		
Dorsal motor vagus	3.6 ± 0.7 (4)		
Area postrema	15.7 ± 1.3 (4)		
Cerebellum			
Granule cell layer	4.2 ± 0.4		
Molecular layer	2.4 ± 0.4		

^{125}I-PYY and ^{125}I-NPY autoradiography was performed at a concentration of 10 pM as described. Nonspecific binding was determined in the presence of 500 nM unlabeled ligand. Autoradiograms were quantitated on a Loats image analysis system, and ODs were converted to fmol/mg protein using ^{125}I standards in brain paste. Anatomical areas were defined using toluidine blue-stained sections and compared to the neuroanatomical atlas of Paxinos and Watson (1986).

homogenate was spun at 800 × *g* for 10 min, and the supernatant was reserved. The pellet was rehomogenized and spun at 800 × *g* for 10 min. The supernatants were combined and spun at 17,000 × *g* for 20 min. The resulting crude mitochondrial pellet was resuspended in ice-cold buffer A [137 mM sodium chloride, 5.4 mM potassium chloride, 0.44 mM potassium phosphate (monobasic), 1.26 mM calcium chloride, 0.81 mM magnesium sulfate, 0.5% BSA, 0.1% bacitracin, 20 mM HEPES, pH 7.4]. For these experiments, buffer A was supplemented with 1 mM dithiothreitol, 100 mg/liter streptomycin sulfate, 1 mg/liter aprotinin, 10 mg/liter soybean trypsin inhibitor, and 1 μM captopril. The protein concentration in the homogenate was approximately 0.4 mg/ml as determined using Biorad protein reagent (Bradford, 1976).

For the binding experiments, plastic microfuge tubes were prepared with the binding buffer, iodinated ligand, and competing ligand so that the total volume was 500 μl. Nonspecific binding was measured by including 100 nM unlabeled NPY in incubations with ^{125}I-NPY and 100 nM PYY in incubations with ^{125}I-PYY. Binding was initiated by adding 500 μl of membranes, and tubes were incubated for 1 hr at 22°C. The binding was stopped by centrifugation at 8700 × *g* for 15 sec. Pellets were rinsed and dried, transferred to 12 × 75 mm tubes, and counted in a gamma counter.

Competition curves were analyzed with a modified version of a non-linear least squares computer curve fitting program (Yamakoa et al., 1981). Specific binding was fit to an adsorption isotherm with one or more additive and independent sites.

Autoradiographic procedure. Rats were perfused with 0.32 M sucrose as described previously (Lynch et al., 1986a, b). The brains were removed, embedded in brain paste, and kept frozen until 8 μm cryostat sections were cut. The sections were thaw mounted onto gelatin/chrome alum subbed slides and kept at −20°C until autoradiography was performed.

For autoradiography, sections were preincubated in buffer A for 10 min at 22°C. The sections were then transferred to buffer A containing 40,000 dpm/ml ^{125}I-PYY or ^{125}I-NPY (10 pM) plus any inhibitors used. Sections were incubated in this solution for 1 hr at 22°C. After two 5-min rinses in buffer A, sections were dried rapidly under a stream of cool dry air, desiccated overnight, and then exposed to LKB Ultrafilm for 3–6 weeks. Autoradiograms were quantitated using a Loats Associates image analysis system (Westminster, MD) and grain densities converted to fmol-bound ligand/mg protein using standards prepared from brain paste and sodium iodide-125. Anatomic localizations, including nuclear subdivisions, were derived by inspection of adjacent toluidine blue-stained sections using the atlas of Paxinos and Watson (1986). Nonspecific binding was measured by including 500 nM unlabeled NPY in the incubations with ^{125}I-NPY and 500 nM unlabeled PYY in the incubations with ^{125}I-PYY.

Stability of ^{125}I-PYY and ^{125}I-NPY. To confirm that the ligands were not degraded under our incubation conditions, samples of media before and after incubation with sections were chromatographed on a Brownlee RP-300 HPLC column attached to a Waters HPLC system. Each sample (200–500 μl) was applied to the column (equilibrated with 0.1% trifluoroacetic acid) and eluted with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid. The column was run at 2.0 ml/min, and 2 ml fractions were collected and counted in a gamma counter. For both ^{125}I-PYY and ^{125}I-NPY, the total ^{125}I eluted as single peak whose mobility was identical before and after incubation (data not shown).

Lesioning studies. To determine the cellular localization of NPY receptors in the hippocampus, rats received kainic acid lesions of the hippocampal CA3–4 pyramidal cells (McGinty et al., 1983). The integrity of hippocampal afferents was confirmed using cholinesterase staining (Lynch et al., 1986a).

Results

Specificity of ^{125}I-NPY and ^{125}I-PYY binding

^{125}I-NPY and ^{125}I-PYY binding are inhibited by both PYY and NPY. Binding of 10 pM ^{125}I-NPY to membranes is inhibited by NPY with a Hill slope of 0.81, indicating some heterogeneity in the binding sites (Table 1, Fig. 1). However, NPY discriminates this heterogeneity weakly, so that a one-site model fits the data with an IC_{50} of 0.37 nM (Table 1; $r^2 = 0.96$). When PYY is the competitor, the competition curve is shallower (Hill slope = 0.59), and the data are poorly fitted by a one-site model ($r^2 = 0.65$). A two-site model fits the data with IC_{50} of 48 and 5300 pM ($r^2 = 0.96$). Thus, 10 pM ^{125}I-NPY appears to occupy high- and moderate-affinity binding sites that can be distinguished more clearly by PYY than NPY.

When ^{125}I-PYY is the radioligand and NPY is the competitor, the competition curve gives a Hill slope of 0.94. This indicates

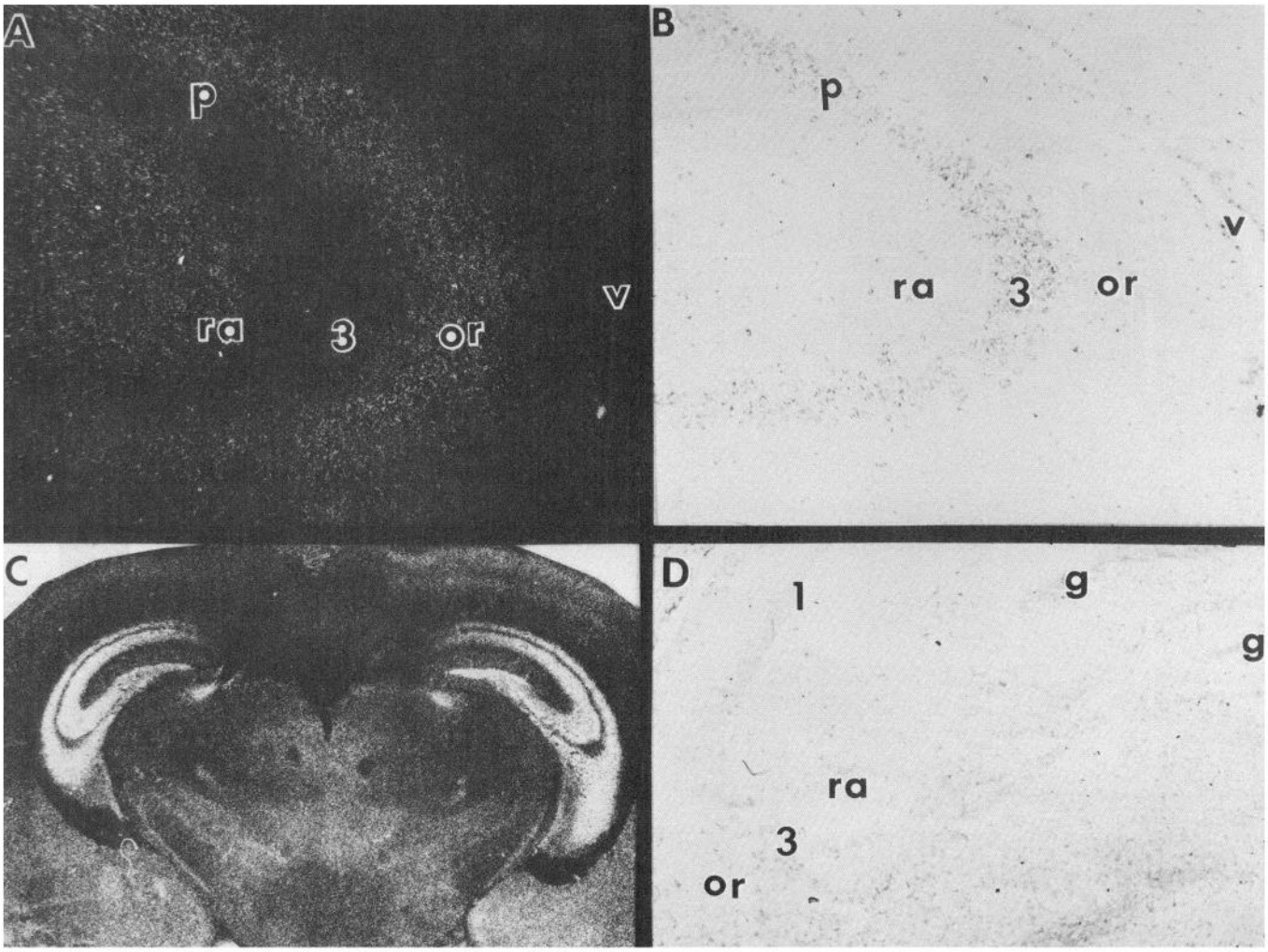

Figure 3. ^{125}I-PYY binding sites in the hippocampus. In the hippocampus, ^{125}I-PYY binding sites are concentrated in the stratum oriens (*or*) and radiatum (*ra*) of CA3 (*3*), with lower levels around CA1, 2 (*p*). v, Ventricle. (Autoradiographic image in *A*, tissue staining in *B*.) Lesioning of the hippocampal CA3 pyramidal cells with quinolinic acid (*D*) does not destroy the high levels of sites in the stratum oriens and radiatum (*C*).

some ability of NPY to discriminate the heterogeneity in binding sites, but the data are still fit by a one-site model with an IC_{50} of 0.50 nM ($r^2 = 0.96$). Thus, unlabeled NPY competes similarly for sites labeled by ^{125}I-NPY or ^{125}I-PYY. Such is not observed with PYY as the competitor. When ^{125}I-PYY is the radioligand, the competition curve produced by PYY has a Hill slope of 0.84. The data are fit well by a one-site model with $IC_{50} = 0.10$ nM ($r^2 = 0.95$). The competition curve produced by unlabeled PYY is dependent on the radioligand. When 10 pM ^{125}I-NPY is the radioligand, the curve is shallow; when 10 pM ^{125}I-PYY is the radioligand, the curve is relatively steep. This indicates that 10 pM ^{125}I-NPY labels both high- and moderate-affinity binding sites, whereas 10 pM ^{125}I-PYY selectivity labels high-affinity sites.

Binding of ^{125}I-PYY and ^{125}I-NPY to rat brain tissue sections is specific and saturable. Specific to nonspecific ratios are approximately 10:1 for most brain regions with ^{125}I-PYY and approximately 3:1 with ^{125}I-NPY, consistent with the greater hydrophobicity of ^{125}I-NPY (Walker and Miller, 1988). PYY and NPY both potently inhibit binding of ^{125}I-PYY and ^{125}I-NPY (Table 2). PYY has a lower IC_{50} for inhibiting ^{125}I-PYY than ^{125}I-NPY binding, whereas NPY has a similar IC_{50} for the 2 ligands. Inhibition curves with PYY and NPY for both ligands are shallow (data not shown), consistent with the relationships determined in the membrane binding assays. The binding affinities match those seen in homogenates, confirming that the same sites are labeled in our autoradiographic experiments.

Localization of ^{125}I-PYY binding sites

In the forebrain, PYY binding sites are enriched in several regions (Table 3, Fig. 2). Highest levels are found in the lateral septum and the hippocampus, where binding is mainly in the stratum oriens and radiatum around CA3 (Fig. 3). Binding is unaltered in animals with unilateral lesions of the hippocampal pyramidal cells of CA3, suggesting that the receptors here are presynaptic (Fig. 3). It also is conceivable that binding sites reside, at least in part, on elements, such as glia, that are unaffected by neuronal lesions. Few binding sites are found in the dentate gyrus, whereas moderate amounts are present in CA1–2. Binding is relatively high in the amygdala, particularly the cortical nuclei, but low in the bed nuclei of the stria terminalis. The cerebral cortex displays heterogeneous binding, with highest levels in the entorhinal and piriform regions. Cortical binding is highest in layer 2 and in layers 5–6 (Fig. 4).

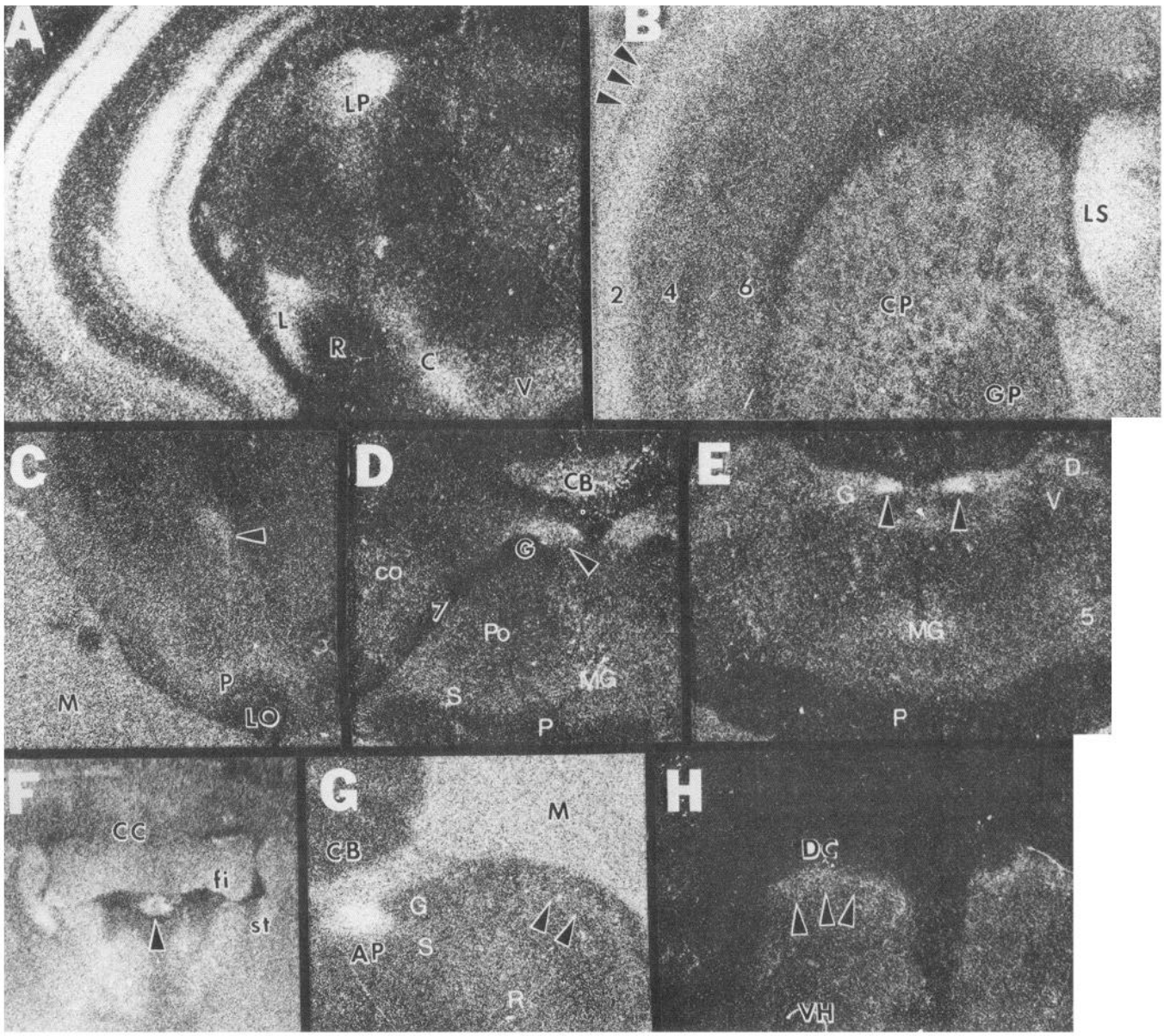

Figure 4. Localization of ^{125}I-PYY and ^{125}I-NPY binding in rat brain. Eight-micrometer sections were incubated with ^{125}I-PYY (*A–E, G–H*) or ^{125}I-NPY (*F*) as described. Enlargements are printed directly from ^{3}H-Ultrafilm. Among the regions with the highest levels of binding are the lateral posterior nucleus of the thalamus (*LP*) and the pars lateralis (*L*) and compacta (*C*) of the substantia nigra. Very low levels are found in the pars reticularis (*R*). In the cortex, binding is highest in layer 2, with lower levels in layers 4 and 6. The *arrows* in *B* show the border between the cortical surface and the embedding medium. The caudate (*CP*) is more intensely labeled than the globus pallidus (*GP*). *LS,* Lateral septum. The claustrum (*arrow* in *C*) and piriform cortex (*P* also are discretely labeled, whereas the lateral olfactory tract is not (*LO*). *M,* brain paste embedding medium. The subfornical organ also is labeled by ^{125}I-NPY (*arrow* in *F*) *fi,* fimbria; *st,* stria terminalis. In the brain stem, binding is highest in the prepositus hypoglossal nucleus (*arrow* in *D*), the sphenoid nucleus (*arrow* in *E*), and the area postrema (*AP*); *CB,* cerebellum. The substantia gelatinosa is labeled in both the medulla (*arrows* in *G*) and the spinal cord (*arrows* in *H*); *DC,* dorsal column; *VH,* ventral horn. Abbreviations: *A*: *LP,* lateral posterior thalamus; *L,* pars lateralis substantia nigra; *R,* pars reticularis substantia nigra; *C,* pars compacta substantia nigra; *V,* ventral tegmental area. *B*: ▶, surface of cerebral cortex; *2,* layer 2 of cerebral cortex; *4,* layer 4 of cerebral cortex; *6,* layer 6 of cerebral cortex; *CP,* caudate putamen; *LS,* lateral septum; *GP,* globus pallidus. *C*: ◀, claustrum; *P,* piriform cortex; *LO,* lateral olfactory tract; *M,* brain paste medium. *D*: *CB,* cerebellum, granule cell layer; ◀, prepositus hypoglossal nucleus; *G,* genu, facial nerve; *7,* facial nerve; *C,* cochlear nuclei; *S,* superior olivary nucleus; *PO,* pontine reticular formation; *P,* pyramidal tract; *MG,* raphe magnus. *E*: *G,* pontine central grey; ▲, sphenoid nucleus; *D,* dorsal parabrachial nucleus; *V,* ventral parabrachial nucleus; *MG,* raphe magnus; *P,* pyramidal tract; *5,* trigeminal sensory nucleus. *F*: *cc,* corpus callosum; ▲, subfornical organ; *fi,* fimbria; *st,* stria terminalis. *G*: *CB,* cerebellum; *AP,* area postrema; *G,* gracilis nucleus; *S,* nucleus tractus solitarius; *R,* reticular formation of medulla; ▲, substantia gelatinosa of trigeminal spinal tract. *H*: *DC,* dorsal column; ▲, substantial gelatinosa; *VH,* ventral horn.

In the diencephalon, ^{125}I-PYY binding sites are concentrated in some thalamic nuclei, particularly the lateral posterior nucleus (Fig. 3). Levels are also high in the centromedian, medial dorsal, reuniens, and rhomboid nuclei. Within the medial geniculate nucleus, binding densities vary, with more binding caudally and laterally in the nucleus. Hypothalamic binding is relatively low, with lateral areas consistently higher than medial areas.

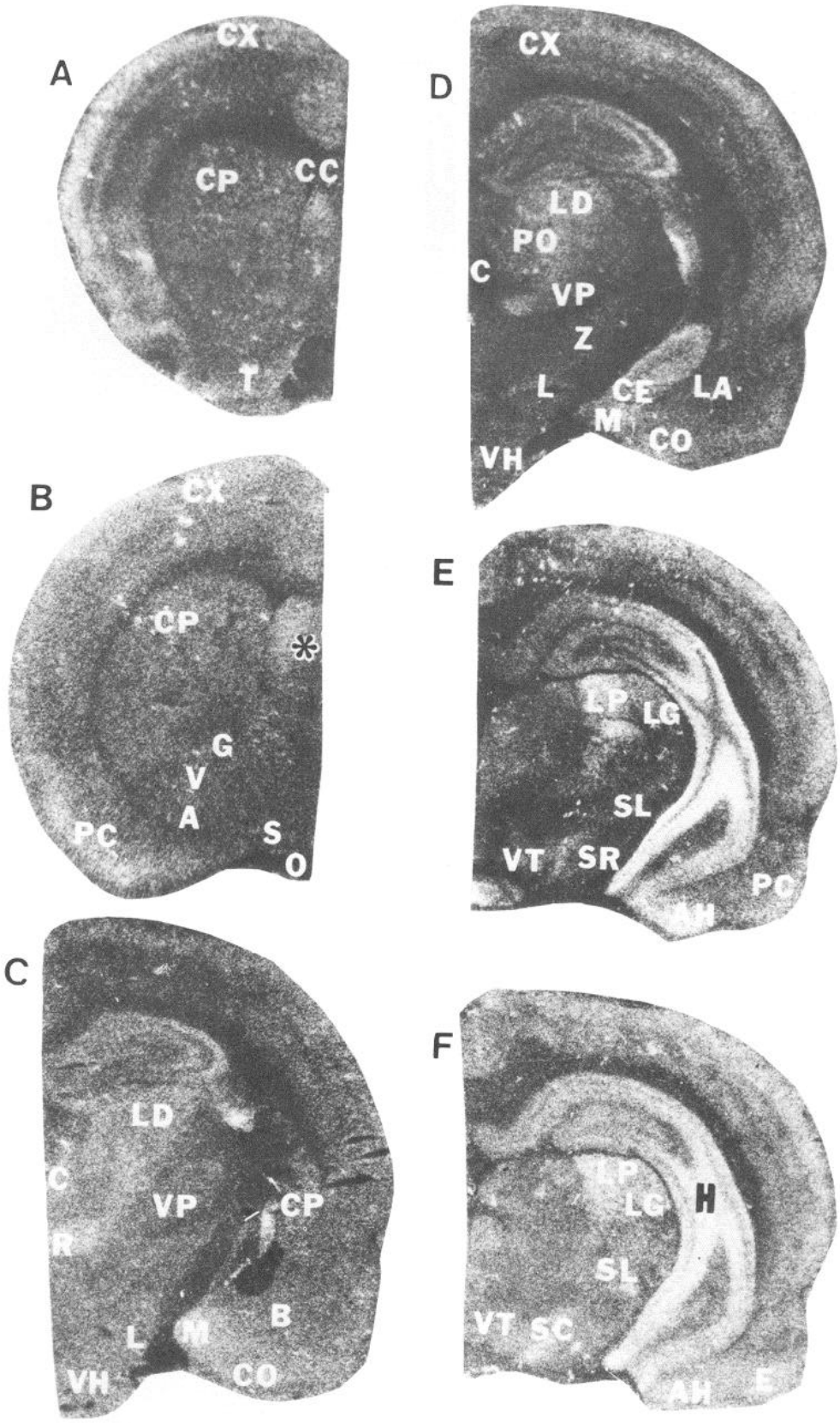

Figure 5. ^{125}I-NPY binding in rat forebrain. Eight-micrometer sections of rat forebrain were incubated with 5 pM ^{125}I-NPY. Sections in which 200 nM unlabeled NPY was included contained negligible autoradiographic grains (not shown). Binding is high in the stria terminalis (*arrows* in *C*) and the stratum oriens and radiatum of CA3 of the hippocampus (*H*). Moderate levels of binding are found in the lateral septum (*), caudate putamen (*CP*), piriform cortex (*PC* in *E*), frontal cerebral cortex (*CX*), and certain nuclei of the amygdala (*B, M, CO*), and substantia nigra [particularly the pars compacta (*SC*) and lateralis (*SL*)]. Low levels are found in the corpus callosum (*CC*) and globus pallidus (*G*). Abbreviations are the same as in the legend for Figure 2.

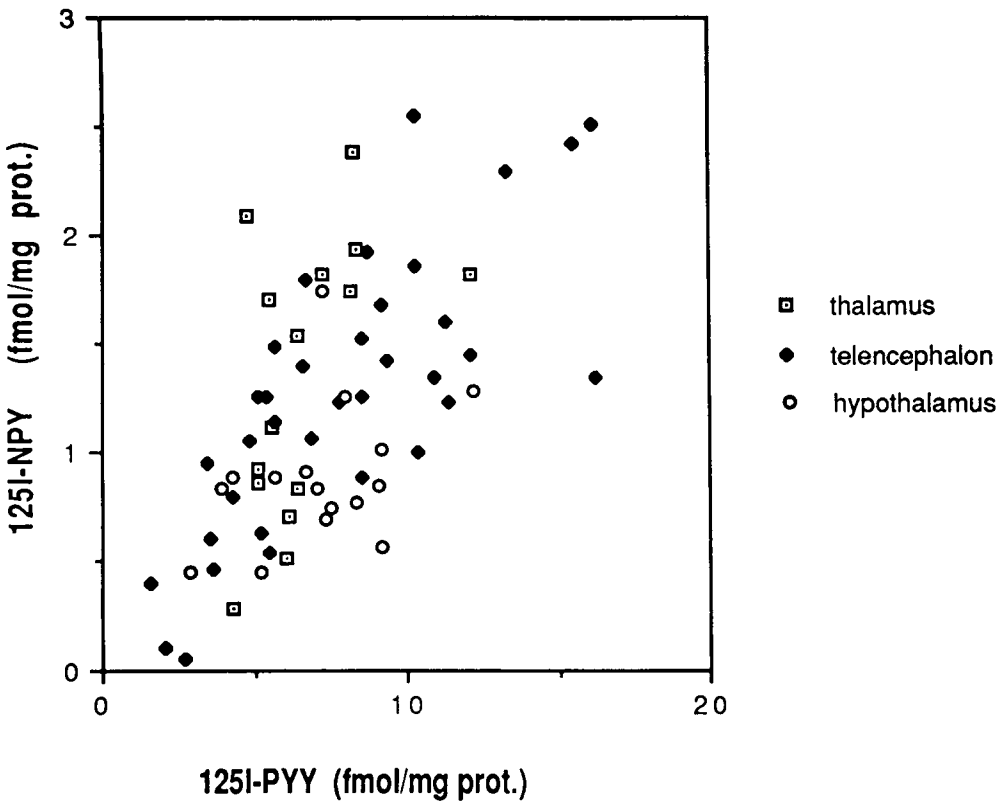

Figure 6. Comparison of ^{125}I-PYY and ^{125}I-NPY binding. Data from Table 2 were plotted, with ^{125}I-PYY binding on the abscissa and ^{125}I-NPY binding on the ordinate. Points are from the telencephalon (◆), thalamus (⊡), or hypothalamus (○).

In the midbrain, ^{125}I-PYY binding sites are enriched in areas associated with dopaminergic cell bodies, such as the pars compacta and lateralis of the substantia nigra and the ventral tegmental area. Levels of ^{125}I-PYY binding are low in the rest of the midbrain.

^{125}I-PYY binding in the hindbrain is low except for a few nuclei, mostly at the floor of the 4th ventricle, including the pontine central grey, sphenoid and supragenual nuclei, and the area postrema (Fig. 4).

Localization of ^{125}I-NPY binding

The distribution of ^{125}I-NPY binding resembles that of ^{125}I-PYY (Fig. 5). The claustrum/endopiriform nucleus and islands of Calleja have especially high levels. The lateral septum and hippocampus also have high levels, and the layering of binding in the hippocampus and cerebral cortex is the same as for ^{125}I-PYY. Hippocampal ^{125}I-NPY binding sites are highest in the stratum oriens and radiatum, and cortical binding is greatest in layer 2. Unilateral lesions of hippocampal pyramidal cells (described in legend to Fig. 3), which do not alter ^{125}I-PYY binding, also do not affect ^{125}I-NPY binding (data not shown). ^{125}I-NPY sites are also concentrated in the anterior olfactory nucleus, mamillary body, the nuclei reuniens and rhomboid of the thalamus, and the stria terminalis, but only modest amounts occur in the thalamic lateral posterior nucleus and substantia nigra pars lateralis and compacta.

Comparison of ^{125}I-NPY and ^{125}I-PYY binding

Although the distributions of ^{125}I-PYY and ^{125}I-NPY are qualitatively similar, the amount of ^{125}I-PYY bound is greater than the amount of ^{125}I-NPY bound in all regions. This may reflect a greater specific activity of ^{125}I-PYY or a greater free concentration of ^{125}I-PYY due to its hydrophilicity (Walker and Miller, 1988). However, the ratio of ^{125}I-PYY to ^{125}I-NPY binding is not the same in all brain regions (Table 3). In the telencephalon, the lateral septum and diagonal band bind relatively more ^{125}I-PYY than ^{125}I-NPY. The piriform cortex, the entorhinal cortex, and the hippocampal CA3–4 region all have high ratios of ^{125}I-PYY bound to ^{125}I-NPY bound. In the diencephalon, thalamic regions generally have low ratios, and hypothalamic regions have high ratios.

If the amount of ^{125}I-PYY bound in different brain regions is plotted against the amount of ^{125}I-NPY bound, the graph should be a line if the 2 ligands bind to a single identical site. However, the actual graph (Fig. 6) has substantial scatter about such a line, reflecting clear differences between the levels of ^{125}I-PYY and ^{125}I-NPY binding.

Differences between ^{125}I-NPY and ^{125}I-PYY localizations are well illustrated in the mamillary bodies and the thalamus. Whereas ^{125}I-PYY binding is highest in the lateral portion of the mamillary body, ^{125}I-NPY binding is highest in the medial portion (Fig. 7). Some thalamic nuclei, particularly the reuniens nucleus, have high levels of ^{125}I-NPY sites but lower levels of ^{125}I-PYY sites (Fig. 8).

Discussion

Experiments on peripheral neuroeffector junctions have led to the proposal that NPY receptors can be classified as Y_1 and Y_2 subtypes (Wahlestedt and Hakanson, 1986; Wahlestedt et al., 1986). The proposed Y_1 subtype is located postsynaptically and mediates postjunctional effects, such as smooth muscle contraction and potentiation of neural transmission. The proposed Y_2 subtype is found presynaptically and mediates the inhibition of neurotransmitter release, probably resulting from the ability of NPY to inhibit voltage-sensitive calcium channels (Colmers et al., 1987; Walker et al., 1988).

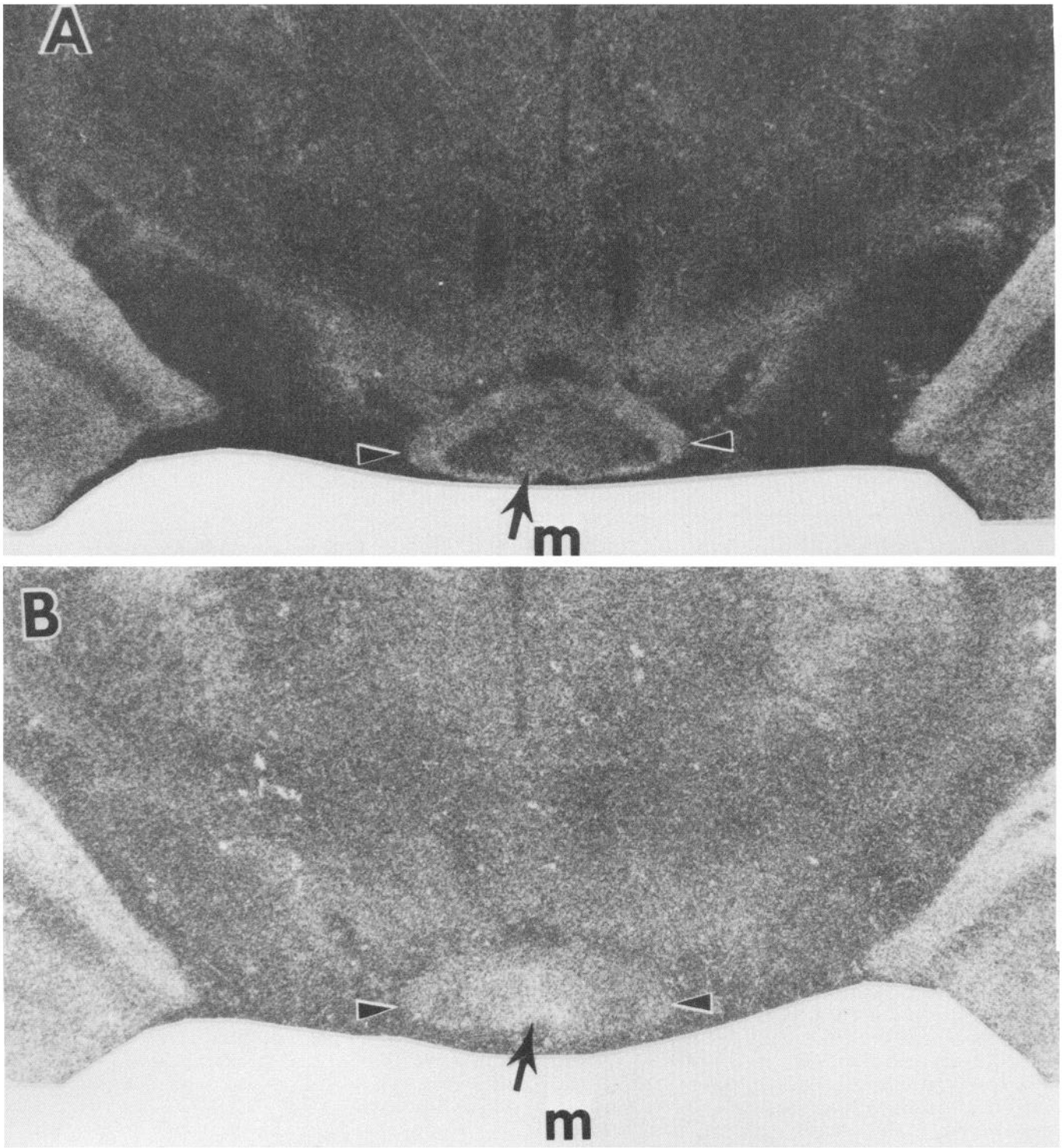

Figure 7. Localization of ^{125}I-NPY and ^{125}I-PYY binding sites in the mamillary body. Adjacent sections through the rat mamillary body were incubated in ^{125}I-PYY (*A*) or ^{125}I-NPY (*B*). Although the general pattern is similar, the PYY binding sites are found in higher amounts laterally (*arrow*), whereas NPY binding sites are greater medially. *m,* Midline of brain.

We have localized NPY receptors autoradiographically with both ^{125}I-PYY and ^{125}I-NPY, with the assumption that ^{125}I-PYY labels NPY receptors. Based on the differing affinities of ^{125}I-NPY and ^{125}I-PYY, we should selectively label high-affinity sites with ^{125}I-PYY and both high- and moderate-affinity sites with ^{125}I-NPY. This apparent receptor heterogeneity agrees with the biphasic Scatchard plots seen for these ligands in brain and dorsal root ganglion membrane preparations (Walker and Miller, 1988; Walker et al., 1988). Autoradiographic receptor localizations defined by ^{125}I-PYY and ^{125}I-NPY are similar but with several clear exceptions. When ^{125}I-PYY binding and ^{125}I-NPY binding are compared graphically (Fig. 7), the levels of

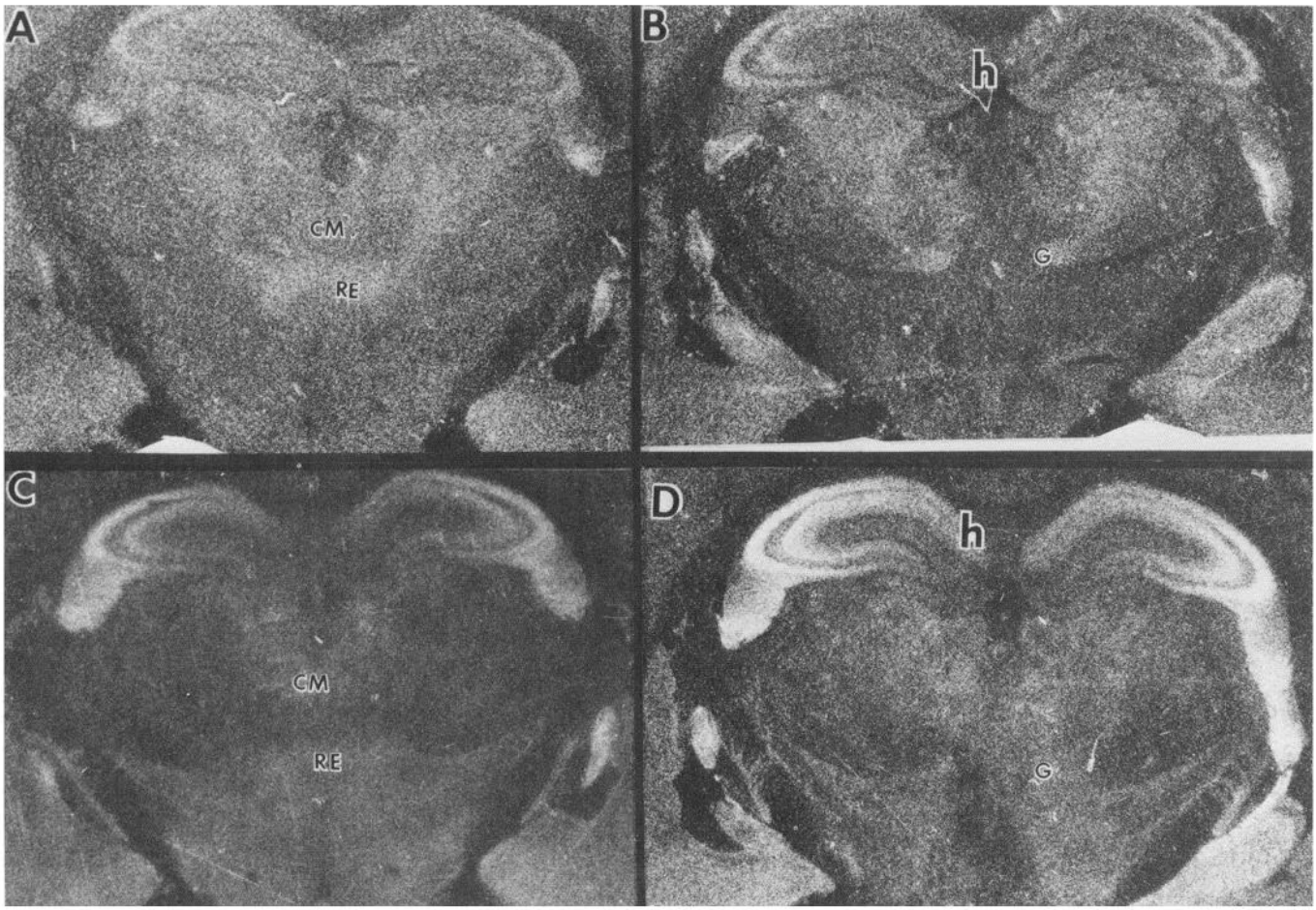

Figure 8. Localization of NPY receptors in thalamus. Adjacent sections of rat thalamus were incubated in ^{125}I-NPY (*A, B*) or ^{125}I-PYY (*C, D*). *A* and *C* are adjacent, as are *B* and *D*. NPY binding sites are found in about equal concentrations in the reuniens (*RE*), centromedian (*CM*), and gustatory (*G*) nuclei when compared to the hippocampus (*h*). In contrast, PYY binding sites are found in higher concentration in the hippocampus than in these thalamic nuclei.

binding for the 2 ligands do not directly correspond. The quantitative binding levels for the different ligands do not fit any perfect relationship. In particular, the lateral septum displays far more ^{125}I-PYY sites than ^{125}I-NPY sites, whereas the reverse is true in most thalamic regions. The pars compacta and lateralis of the substantia nigra also contain relatively more ^{125}I-PYY binding than ^{125}I-NPY binding, and the localizations of sites for the 2 ligands differ in the mamillary body. Thus in several brain areas, the 2 ligands label distinct structures.

Although the present study uses ^{125}I-NPY and ^{125}I-PYY to localize 2 populations of binding sites, the endogenous ligand for the majority of sites is most likely NPY, since relatively little authentic PYY is found in the brain (Broome et al., 1985; Ekman et al., 1986). NPY receptor localizations resemble the distribution of neuronal NPY in several brain regions (de Quidt and Emson, 1986a, b; Gray and Morley, 1986), especially the olfactory tubercle, the ventral pallidum, the stria terminalis, the thalamic rhomboid nucleus, the superficial piriform cortex, the entorhinal cortex, the amygdala (especially the medial nucleus), and the substantia gelatinosa of the medulla oblongata and spinal cord (Yamazoe et al., 1985). Localizations in the substantia gelatinosa fit with physiological evidence for NPY receptors on dorsal root ganglion cells (Walker et al., 1988).

The correspondence between NPY receptors and immunoreactivity is less clear in other brain regions. NPY levels are generally low in the hippocampus (de Quidt and Emson, 1986b; Chan-Palay et al., 1986), although NPY does have physiological effects there (Colmers et al., 1985; Brooks et al., 1987). NPY acts directly on dentate gyrus granule cells (Brooks et al., 1987) and presynaptically on CA1 neurons (Colmers et al., 1985, 1987). Receptor densities in the dentate gyrus are present but not pronounced, and lesion studies suggest the presence of presynaptic receptors in CA3.

Low levels of receptors in the hypothalamus contrast with high immunoreactivity levels. This discrepancy is most evident in the suprachiasmatic nucleus, where receptor levels are especially low despite considerable NPY immunoreactivity. In the suprachiasmatic nucleus, much of the immunoreactivity is found on neurons with cell bodies in the lateral geniculate, another area with only moderate levels of NPY receptors (Harrington et al., 1987). In the various thalamic nuclei, high receptor levels contrast with low levels of immunoreactivity.

Mismatches between transmitters and their receptors have been observed frequently, and those found here may be explained in several ways (Kuhar, 1985; Herkenham, 1987). Often, binding sites are present on the entire surface of a neuron, though functional receptors are restricted to cell bodies and dendrites where neurons receive relevant input. Alternatively, a radioli-

gand may label only a subset of receptors. If a single receptor mediates the effects of multiple frequently colocalized neurotransmitters, a receptor may be expressed in regions where only one of these transmitters is present (Schultzberg and Hokfelt, 1986). Conceivably, receptors can be activated by transmitters that diffuse large distances from their site of release.

Another possible explanation is that the levels of NPY and its receptors are differentially regulated, depending on the use of various neuronal pathways and the influence of modulating systems. For example, chronic administration of the alpha$_2$ agonist clonidine increases the specific binding of NPY in rat cerebral cortex (Goldstein et al., 1986). In the rat nucleus tractus solitarius, specific NPY binding is reduced by clonidine and increased by irreversible blockade of monoamine receptors. Chronic treatment with the phenylethanolamine-N-methyltransferase inhibitor LY 134046 increases binding of NPY in the hypothalamus (Goldstein et al., 1986). In the arcuate nucleus of the rat hypothalamus, NPY content increases following administration of the tyrosine hydroxylase inhibitor alpha-methyl-paratyrosine or the dopaminergic antagonist, haloperidol (Li and Pelletier, 1986). NPY content in the rat brain is increased by imipramine and zimelidine, which are selective blockers of norepinephrine and serotonin uptake, respectively (Heilig et al., 1988). Imipramine increases NPY content in frontal cortex and hypothalamus, whereas zimelidine changes NPY levels in frontal cortex only. Thus, discrete patterns of neuronal activity may regulate the relative ratio of NPY to NPY receptor concentrations in a particular brain region.

Physiological effects of NPY have been linked to noradrenergic effects in both the peripheral and central nervous system, suggesting that their receptor distributions might overlap. The NPY-receptor distribution we observe resembles the localization of alpha-adrenergic receptors in some but not all brain regions. Alpha$_2$ receptor distribution matches NPY receptor localization best in the substantia gelatinosa, parabrachial nucleus, reuniens nucleus of the thalamus, amygdala, lateral septum, anterior olfactory nucleus, and the stria terminalis (Unnerstall et al., 1984).

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