

**NEUROPEPTIDE Y BINDING SITES IN RAT BRAIN AND  
THEIR RELEVANCE TO THE POSSIBLE ANXIOLYTIC-LIKE  
ACTIVITY OF RECEPTOR LIGANDS**

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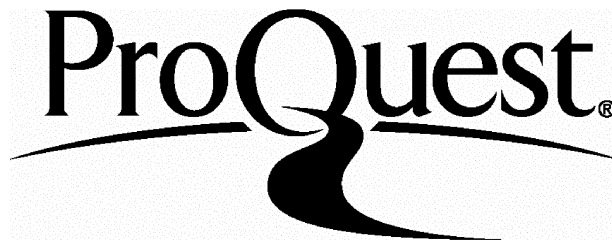
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This Thesis is for Mandy

with love.

## **ABSTRACT**

Neuropeptide Y was first discovered in 1982 and found to be a member of the pancreatic polypeptide family. It is one of the most abundant peptides found in the mammalian brain, where it often acts as a co-transmitter modulating the effects of a variety of other transmitters. Its effects are mediated by specific neuropeptide Y receptors of which at present there are thought to be at least three subtypes.

Possible sites of action of neuropeptide Y with respect to its putative anxiolytic effects were investigated using neuropeptide Y receptor autoradiography to visualise its receptor binding sites in the rat brain. Areas rich in receptor binding sites include the hippocampus, claustrum, amygdala, septum, stria terminalis and cerebral cortex. Receptor subtypes were also visualised showing the Y<sub>1</sub> receptor subtype to be prominent in the cortex and claustrum whereas the Y<sub>2</sub> receptor subtype was found in the hippocampus, septum, amygdala and stria terminalis.

Neuronal activity following central administration of neuropeptide Y was also investigated using the expression of Fos-like immunoreactivity as a marker of this activity. Areas expressing Fos-like immunoreactivity were the entorhinal cortex, amygdalopiriform transitional area, nucleus accumbens, ventral hippocampus, paraventricular thalamic nucleus and the dorsal endopiriform nucleus. Regions showing activity and receptor binding sites were considered strong candidates for further investigation.

Evidence is presented for an anxiolytic-like action of neuropeptide Y in rats as detected in the elevated x-maze and Vogel's conflict models of anxiety. The neuropeptide Y receptor subtypes responsible for this action were investigated using various selective neuropeptide Y receptor subtype agonists. The anxiolytic-like effects of neuropeptide Y might be mediated by the Y<sub>1</sub> receptor subtype.

Finally, evidence is presented showing the hippocampus to be a site of action of neuropeptide Y in producing anxiolytic-like effects.

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## Abbreviations

ADP	Adenosine diphosphate
cAMP	Cyclic adenosine monophosphate
CDP	Chlordiazepoxide
CNS	Central nervous system
CSF	Cerebrospinal fluid
ECT	Electroconvulsive treatment
FLI	Fos-like immunoreactivity
GABA	$\gamma$ -Amino butyric acid
GTP	Guanosine triphosphate
i.c.v.	Intracerebroventricular
IEG	Immediate-early gene
i.m.	intramuscular
i.p.	intraperitoneal
IP <sub>3</sub>	Inositol triphosphate
LC	Locus coeruleus
LH	Luteinizing hormone
NA	Noradrenaline
NEN	New England Nuclear
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
p.o.	per os (oral administration)
PP	Pancreatic polypeptide
PP56	D-myo-inositol 1,2,6-triphosphate
PVN	Hypothalamic paraventricular nucleus
PY	Nonmammalian (fish) pancreatic peptide Y
PYY	Peptide YY
SCN	Suprachiasmatic nucleus
SH	Spontaneously hypertensive rats
VIP	Vasoactive intestinal peptide
WKY	Wistar-Kyoto rats

## **Publication**

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Localization of neuronal activity mediated by neuropeptide Y in rat brain : the  
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**CHAPTER 1**  
**INTRODUCTION**

## 1.1 Discovery of neuropeptide Y

The story of neuropeptide Y (NPY) began with the isolation of insulin from the chicken pancreas (Kimmel et al., 1975). During this process (avian) pancreatic polypeptide (PP) was discovered as a by product. PP was subsequently isolated from many other species. Soon after, antibodies raised against avian PP were developed and PP-like immunoreactivity detected in the central nervous system (CNS) of various species (Loren et al., 1979). As all attempts to extract PP from mammalian brain failed, it was soon concluded that the PP-like immunoreactivity present in the brain was due to the existence of a PP-like peptide with a related, but different structure. The missing peptide was later isolated by Tatemoto and Mutt (1978) using a novel chemical assay for detecting C-terminal amide structures. A C-terminal  $\alpha$ -amide group is a characteristic feature of many biologically active peptide hormones, and so the chances were quite high that such a technique might reveal undiscovered peptides. Using this technique they had already isolated peptide YY (PYY) from porcine intestine, and it was at first thought that PYY might also occur in the brain (Tatemoto and Mutt, 1980). They soon found upon isolation from the brain of a peptide amide that was thought to be PYY, and despite it having distinct structural and biological similarities to both PYY and PP, that it was a previously uncharacterised peptide. They named this peptide neuropeptide Y (NPY) (Tatemoto et al., 1982).

## 1.2 Structure

NPY is a member of the pancreatic polypeptide family. This group is so called because PP was the first member to be discovered. Other members of the group include PYY and the non-mammalian (fish) pancreatic peptide Y (PY) (Figure 1.1).

NPY shows a high degree of conservation between species, with the peptide sequences being known for several mammals (human, pig, cow, sheep, rat, rabbit, and guinea pig), chickens, goldfish, and the ray *Torpedo marmorata* (Figure 1.1). On comparison of the sequences of these species it is clear that NPY is one of the most highly conserved neuroendocrine peptides (Wahlestedt and Reis, 1993). This high degree of conservation of NPY sequence between species may be suggestive of an important functional role.

The sequences of porcine NPY and PYY exhibit a 69% sequence homology and both of these peptides activate most NPY-responsive receptors with similar potencies. The sequence of PP however, shows a larger variation between the various species, and also from NPY and PYY.

NPY itself is a 36 amino acid peptide, 5 of which are tyrosine residues. Two of these tyrosine residues are located one each at the N and C-termini, hence the name neuropeptide Y (Y being the single letter amino acid code for tyrosine). A distinct tertiary structure has been proposed for the PP family of

### NPY Amino Acid Sequences

NPY human	YPSKPDNPGEDAPAEDMARYYSALRHYNLITRQRY		
NPY rat	YPSKPDNPGEDAPAEDMARYYSALRHYNLITRQRY		
NPY pig	YPSKPDNPGEDAPAED <u>L</u> ARYYSALRHYNLITRQRY	97%	
NPY sheep	YPSKPDNPGD <u>D</u> APAED <u>L</u> ARYYSALRHYNLITRQRY	94%	
NPY goldfish	YPT <u>K</u> PDNPGEG <u>A</u> PAE <u>E</u> L <u>A</u> KYYYSALRHYNLITRQRY	86%	
NPY torpedo	TPSKPDNPGEG <u>A</u> PAED <u>L</u> A <u>K</u> YYYSALRHYNLITRQRY	92%	
NPY Lampetra	<u>F</u> <u>P</u> <u>N</u> <u>K</u> <u>P</u> <u>D</u> <u>S</u> <u>P</u> <u>G</u> <u>E</u> <u>D</u> <u>A</u> <u>P</u> <u>A</u> <u>E</u> <u>D</u> <u>L</u> <u>A</u> <u>R</u> <u>Y</u> <u>L</u> <u>S</u> <u>A</u> <u>V</u> <u>R</u> <u>H</u> <u>Y</u> <u>N</u> <u>L</u> <u>I</u> <u>T</u> <u>R</u> <u>Q</u> <u>R</u> <u>Y</u>	83%	

### PYY Amino Acid Sequences

PYY rat	YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY		(67%)
PYY pig	YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY		(69%)
PYY human	YPI <u>K</u> PEAPGEDASPEEL <u>N</u> RYYSALRHYNLNLVTRQRY	94%	(67%)

### PP Amino Acid Sequences

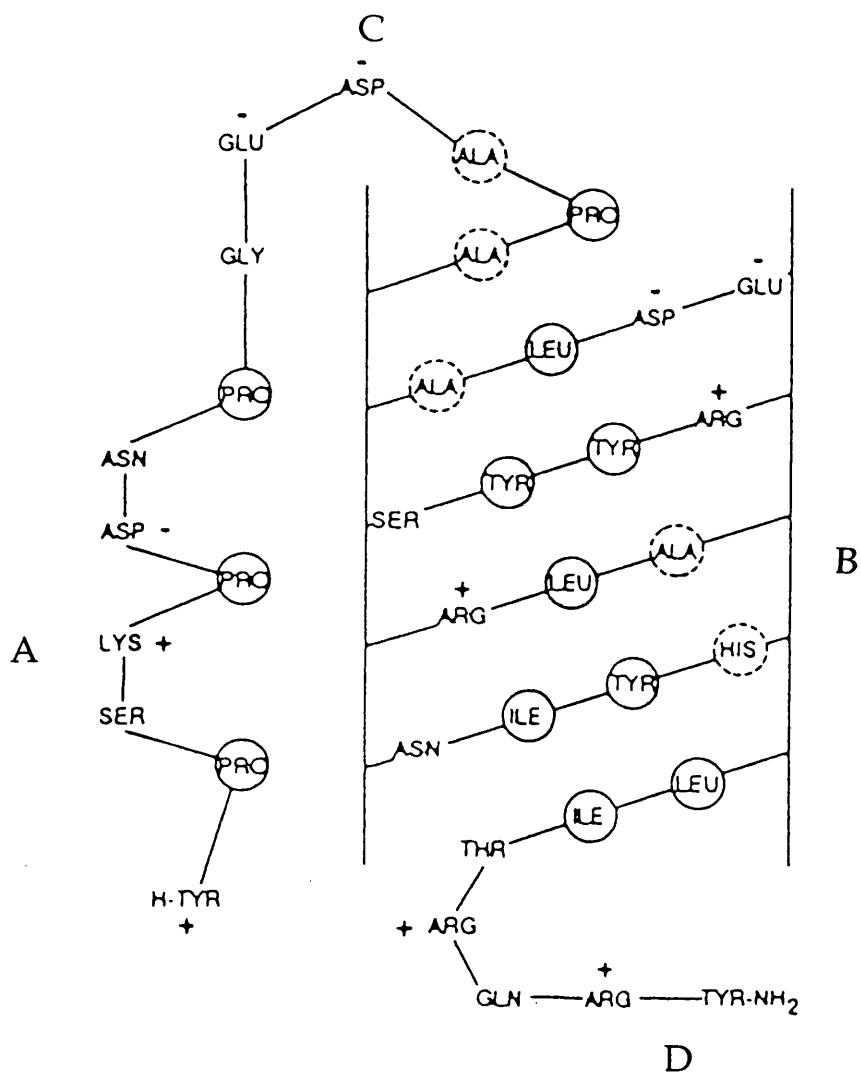
PP pig	APLEPVYPGD <u>D</u> ATPEQMAQYAAELRRYINMLTRPRY		(50%)
PP human	APLEPVYPGD <u>N</u> ATPEQMAQYAA <u>D</u> LRRYINMLTRPRY	94%	(50%)
PP rat	APLEP <u>M</u> YPGD <u>A</u> THE <u>Q</u> RA <u>Q</u> Y <u>E</u> T <u>Q</u> LRRYIN <u>T</u> LTRPRY	78%	(47%)

*Figure 1.1* A comparison of several species differences in the amino acid sequences of NPY, PYY and PP (examples taken from Wahlestedt and Reis, 1993). Any variations from the top sequence are shown as being double underlined. The % figure following several of the peptide sequences shows the percentage sequence homology compared to the top peptide of the group. The percentage figure in the brackets following the sequences shows the percentage sequence homology compared to NPY in the corresponding species.

peptides (Glover et al., 1983; 1985). This is based on the high resolution X-ray analysis of crystalline avian PP, and is referred to as the PP fold. The PP fold is a globular structure comprising a left-handed polyproline II-like helix (residues 1-8) which is closely packed against an amphiphilic  $\alpha$ -helix (residues 13-32) through hydrophobic interactions. These two antiparallel helices are linked by a  $\beta$ -turn (residues 9-12). This confers a hairpin-like structure to the molecule where the tyrosine residue in position 1 and amino acid residues 30-36 are located in close proximity to one another. The C-terminal region (residues 33-36) has no regular structure, and extends away from the molecule. Computer prediction suggests that because sequence homology between NPY and avian PP conserves the residues that are most important to maintain the integrity of the PP fold, NPY also maintains a compact folded conformation characterised by intramolecular hydrophobic interactions (Allen et al., 1987), (Figure 1.2).

### 1.3 Structure-activity relationships

A large number of structure-activity relationship studies have been undertaken with regards to NPY, and the majority of these support the model for NPY forming a hairpin-like structure, with N- and C-terminals in close proximity. Deletion of the tyrosine residue in position 1 of the molecule markedly decreases its affinity for its receptor binding sites (Cadieux et al., 1990; Martel et al., 1990; Quirion et al., 1990). It is suggested that this tyrosine



*Fig. 1.2* The tertiary structure of neuropeptide Y as predicted by computer using a model obtained from X-ray analysis of crystalline avian pancreatic polypeptide (Allen et al.,1987). Important areas are A a left-handed polyproline II-like helix (residues 1-8), B an amphiphilic  $\alpha$ -helix (residues 13-32), here represented on a helical net diagram, which is closely packed against A by hydrophobic interactions, C a  $\beta$ -turn (residues 9-12) which links the two antiparallel helices and D the C-terminal region (residues 33-36) which adopt no regular structure. Charged residues are marked and hydrophobic residues are circled.



may help in stabilising the hairpin structure of NPY (MacKerrel et al., 1989), and/or directly participates in the interaction with the receptors. The importance of the N-terminal end of the polyproline helix has been further highlighted by the fact that the potency of analogues comprised of the most essential C-terminal residues is enhanced by the addition of one or more residues from the N-terminal end, attached via a flexible linking structure (Beck-Sickinger et al., 1990). Fuhlendorff et al. (1990b) have also shown that both the N- and C-terminal portions are important for adequate binding affinity to receptors. They suggest that a folded conformation in which the N- and C-terminals are in close proximity is important for receptor recognition and activation. In studies where certain residues from the polyproline region were replaced, such as to increase interactions between the N- and C-terminal  $\alpha$ -helix and to stabilise the hairpin-like structure of NPY, an increase in the affinity of these analogues was obtained (Minakata and Iwashita, 1990). Bouvier and Taylor (1992) introduced a lactam bridge between a lysine and aspartic acid residue which were then substituted into positions 18 and 22 of NPY or an analogue where the polyproline portion has been replaced by a spacer molecule. Both the substituted residues were part of the proposed  $\alpha$ -helical section of the molecule and so by linking them together, this should stabilise the helix. Using this technique they were able to demonstrate that the proline rich sequence 1-9 stabilises the  $\alpha$ -helical structure in the C-terminal half of the molecule. These results suggest that the PP fold is important for receptor binding, and that stabilisation of the amphiphilic  $\alpha$ -helix may promote receptor activation.

There has been some suggestion that the biological effects of NPY may not be totally due to receptor interactions. McLean et al. (1990) suggest that at low micromolar concentrations of peptide, some of the effects may be due to the amphiphilic  $\alpha$ -helix of NPY altering the membrane bilipid layer structure.

#### 1.4 Distribution of neuropeptide Y

##### 1.4.1 Peripherally

NPY is widely distributed throughout the peripheral nervous system. A large component of this is in the sympathetic nervous system where it coexists with noradrenaline. Within the sympathetic nervous system large numbers of NPY immunoreactive neurones, and high concentrations of NPY have been shown in the superior cervical, stellate and coeliac ganglia. NPY containing sympathetic neurones have been shown which innervate vascular smooth muscle, the heart, the pineal gland, the eye, the middle ear, thyroid gland, respiratory tract, gut, biliary tract, juxtaglomerular apparatus and urogenital tract. The adrenal medulla also contains numerous NPY containing cells (see O'Donohue et al., 1985).

NPY is not only found in the peripheral sympathetic nervous system. It has also been shown in non-sympathetic neurones in the myenteric plexus and the submucosal ganglia.

### 1.4.2 Centrally

NPY has been identified in abundance within the CNS of both rat and man (Allen et al., 1983; Adrian et al., 1983). It is one of the most abundant neuropeptides yet discovered. It has been shown to be widely distributed throughout the CNS within discrete areas. In the rat, the highest levels of NPY are found in the hypothalamic areas, the nucleus accumbens, periaqueductal gray matter and the septum. Moderate/high levels are found in the cortical regions including the hippocampus, in the thalamus and in the basal ganglia. The pons and the cerebellum show very low levels, or even an absence of NPY immunoreactivity.

A similar distribution has been demonstrated for NPY in the human brain with the highest levels in the nucleus accumbens, the hypothalamus, the basal ganglia and the amygdala. Moderate levels are seen in certain cortical areas such as the hippocampus, in the septum and in the periaqueductal gray matter. The thalamus, substantia nigra, globus pallidus, pons and cerebellum show the lowest levels of NPY.

Both the rat and the human spinal cord have been shown to contain moderate levels of NPY (Gibson et al, 1984; Allen et al, 1984).

### 1.4.3 Co-localization of neuropeptide Y

#### 1.4.3a Co-localization in the peripheral nervous system

NPY has been found to coexist with other transmitters in the peripheral nervous system. It is found to be closely associated with catecholamines in the adrenal medulla and in the autonomic nervous system (Emson and Dequidt, 1984). Many of the endocrine cells of the medulla have been found to exhibit the coexistence of NPY and catecholamines (Allen et al., 1983; Varndell et al., 1984). Enkephalin has also been shown to be present in many of these cells (Varndell et al., 1984).

Sympathetic ganglia also contain high concentrations of NPY (Allen et al., 1983). In humans the NPY-immunoreactive cells of the sympathetic ganglia also contain dopamine- $\beta$ -hydroxylase and tyrosine hydroxylase (Lundberg et al., 1983). Also the NPY concentrations in these areas are often depleted after 6-hydroxydopamine treatment in rats (O'Donahue et al., 1985). This suggests that NPY coexists with noradrenaline in these cells.

#### 1.4.3b Co-localization in the central nervous system

As in the peripheral nervous system, the CNS also exhibits many instances of coexistence of NPY with other transmitters and neuropeptides (Everitt et

al.,1984; Hökfelt et al.,1983a; 1983b; Hunt et al.,1981; Jacobowitz and Olschowka,1982; Lundberg et al.,1980). NPY has been found to coexist with catecholamines in some central neurones. Coexistence with noradrenaline occurs in the A1 cell group of the ventral lateral medulla, the A2 group of the dorsal medulla oblongata and the A4 and A6 (locus coeruleus) group of the pons. Coexistence also occurs with adrenaline in the C1 and C2 cell groups in the ventral and dorsal medulla.

A number of amino acid and neuropeptide transmitters have also been found to coexist with NPY in the CNS. In the cerebral cortex, NPY is co-localized with GABA and with somatostatin (Vincent et al.,1982; Chronwall et al.,1984). Coexistence with somatostatin also occurs in the striatum, hippocampus, lateral septum, nucleus accumbens and olfactory tubercle, and a few neurones of the periventricular nucleus of the hypothalamus (Vincent et al.,1982; Chronwall et al.,1984; Hendry et al.,1984). In the noradrenergic A1/A2, the adrenergic C1/C2 cell groups and also the hypothalamic arcuate nucleus, FMRFamide coexists with NPY and the catecholamines (Hökfelt et al.,1983b; Chronwall et al.,1985). Finally, NPY may coexist with enkephalin in the ventral region of the spinal cord (Hunt et al.,1981) and with vasoactive intestinal polypeptide (VIP) in the dorsal motor nucleus of the vagal nerve (Dai et al.,1986).

## 1.5 Neuropeptide Y receptors

NPY has been shown to be involved in many physiological processes in both the peripheral and central nervous systems (see section 1.6). It seems that it exerts its effects via specific receptors, as generally these effects are not blocked by known antagonists for other neurotransmitter receptor sites. The only other known endogenous ligand that acts upon the same receptors is PYY, which itself shows considerable sequence homology with NPY (see section 1.2).

### 1.5.1 Receptor distribution in the central nervous system

A large number of studies have reported on the distribution of NPY receptor binding sites in the rat brain (Ohkubo et al.,1990; Martel et al., 1986; Lynch et al., 1989; Walker and Miller, 1988; Dumont et al.,1990). Martel et al. (1986) used [<sup>125</sup>I] Bolton Hunter labelled NPY ([<sup>125</sup>I]-BH NPY) in an autoradiographic study of the rat brain in order to map the distribution of NPY receptors. They reported high densities of NPY receptor binding sites in the olfactory bulb, superficial layers of the cortex, ventral hippocampus, lateral septum, various thalamic nuclei and the area postrema. Low to moderate levels were seen in the striatum, paraventricular nucleus of the hypothalamus, amygdala and the dorsal horn of the spinal cord. Low densities were observed in the globus pallidus, superior colliculus, the central gray and the cerebellum. All white matter areas showed an absence of specific receptor binding sites.

The distribution of NPY receptors can be seen to bear some resemblance to the distribution of NPY-like immunoreactivity. In some areas however, there seems to be a mismatch between the areas with high affinity receptors for NPY and those containing NPY itself (Agnati et al., 1989). There is a high degree of such a mismatch within the thalamic and hypothalamic regions of the brain. In most thalamic midline nuclei and lateral thalamic nuclei there are high amounts of high affinity NPY binding sites, but very few NPY immunoreactive nerve terminal networks. In the hypothalamus however, the opposite seems to be the case. There are high amounts of NPY immunoreactive nerve terminals, but very few high affinity NPY receptor binding sites. The existence of mismatches such as these can be explained by the idea of volume transmission, the presence of low affinity binding sites and the existence of NPY receptor subtypes. The theory put forward by Agnati et al. (1989) is that NPY neuronal systems may not only operate via classical synaptic (or wiring) transmission alone, but also via the mechanism of volume transmission. Volume transmission might be seen in areas with relatively low levels of low affinity NPY binding sites linked to the synapse and high amounts of high affinity binding sites in the surrounding areas. In this situation, NPY may induce its effects by diffusing in the extracellular fluid to surrounding high affinity binding sites. The classical synaptic transmission may occur in areas where a low level of high affinity binding sites and a high level of low affinity binding sites linked to the synapse exists.

## 1.5.2 Receptor subtypes

### 1.5.2a Sympathetic neuroeffector junction

The NPY receptor seems to exist as several subtypes. The existence of two subtypes was first proposed by Wahlestedt et al. (1986). They studied the effects of NPY, PYY, desamido-NPY and several C-terminal fragments of NPY and PYY on smooth muscle preparations in vivo. The C-terminal fragments used were NPY (19-36), NPY (24-36), PYY (13-36), PYY (24-36) and PYY (27-36). They found that NPY and PYY had three distinct effects on the sympathetic neuroeffector junction in this preparation (see Figure 1.3). NPY and PYY were shown to: a) have a direct post-junctional effect, leading in this case to the constriction of the guinea-pig iliac vein; b) potentiate the postjunctional vasoconstrictor effects of noradrenaline and histamine in the rabbit femoral artery and vein respectively; and c) have a prejunctional effect by suppressing the stimulated release of noradrenaline from sympathetic nerve terminals in the rat vas deferens. In all of these actions NPY and PYY were approximately equipotent, but the fragments varied in their effects. In the guinea-pig iliac vein, the preparation showing a direct postjunctional effect of NPY, neither desamido-NPY nor the fragments had any effect. Neither did they show any effect in potentiating the effects of noradrenaline and histamine in the rabbit femoral artery and brain preparations. In the rat vas deferens preparation however, in which NPY and PYY suppressed the release of noradrenaline prejunctionally, the C-terminal fragment PYY (13-36) did show



an effect. The latter effect was not emulated by the remaining shorter fragments nor by desamido-NPY. These results suggested that there was a difference between the pre- and postjunctional receptors. The C-terminal amide was necessary for both sites to be activated. Whilst the postjunctional effects required the complete sequences of NPY and PYY, the C-terminal fragment NPY (13-36) was sufficient for the prejunctional effects to be seen. On the basis of this and subsequent studies, Wahlestedt et al. (1987a) proposed the existence of two NPY receptor subtypes. The postjunctional receptor which required the complete sequence of NPY or PYY for activation was termed the  $Y_1$  subtype, and the prejunctional receptor, requiring only the 13-36 C-terminal fragment was termed the  $Y_2$  subtype. Later studies suggested that although the  $Y_1$  receptor was the predominant postjunctional subtype on vascular smooth muscle,  $Y_2$  receptors could also exist postjunctionally in this tissue (Wahlestedt et al., 1990b).

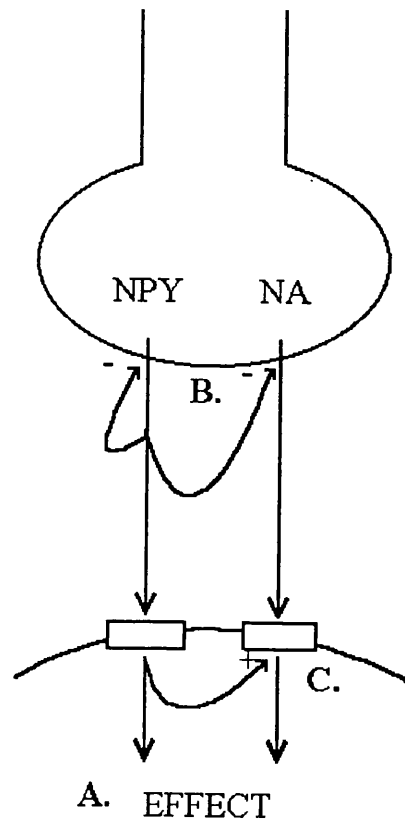


Fig. 1.3 Simplified schematic diagram of the three types of synaptic actions and interactions of neuropeptide Y (NPY) and noradrenaline (NA). Model based on studies at various neuroeffector junctions (Wahlestedt et al,1986). **A** direct postsynaptic actions of the two transmitters. **B** Presynaptic negative feedback control of itself and NA release. **C** Postsynaptic enhancement of NA effects.

### 1.5.2b Brain receptor heterogeneity

The brain also displays subtypes of NPY receptor, as shown by the work of Lynch et al. (1989). They used a combination of receptor autoradiography and membrane binding studies to compare [<sup>125</sup>I]-BH NPY (10pM) and [<sup>125</sup>I]-PYY (10pM) binding to NPY receptors in the rat brain. In the membrane binding study they found that [<sup>125</sup>I]-BH NPY and [<sup>125</sup>I]-PYY binding was inhibited by both NPY and PYY. [<sup>125</sup>I]-BH NPY (10pM) binding to membranes was displaced by unlabelled NPY with a Hill slope of 0.81, indicating some heterogeneity in the binding sites. NPY could only discriminate this weakly however and the data fit a one site model with an IC<sub>50</sub> of 0.37nM (r<sup>2</sup> = 0.96). Using the data supplied by the authors, the Cheng-Prusoff equation gives a Ki value of 0.36nM. When PYY was used to displace [<sup>125</sup>I]-BH NPY binding a Hill slope of 0.59 was obtained and the data fitted a two site model with IC<sub>50</sub> values of 0.048 and 5.3 nM (r<sup>2</sup> = 0.96). The Ki values calculated as above are 0.04 and 5.24nM respectively. Fitting of the latter data to a one site model was very poor (r<sup>2</sup> = 0.65) and gave a Ki value (calculated from the supplied data) of 0.13nM. This suggests that NPY may occupy both high and moderately high affinity binding sites that are more clearly distinguished by PYY than NPY.

When [<sup>125</sup>I]-PYY (10pM) was used as the radioligand, NPY displaced binding with a Hill slope of 0.94. The data fit a one site model with an IC<sub>50</sub> of 0.5nM (r<sup>2</sup> = 0.96). The Ki value calculated from this data is 0.35nM. PYY displaced the [<sup>125</sup>I]-PYY binding to give a Hill slope of 0.84, which again fits a one site model

(IC<sub>50</sub> of 0.1nM, r<sup>2</sup> = 0.95). From published data, this gives a Ki of 0.09nM. Taken altogether, these results suggest that whereas 10pM [<sup>125</sup>I]-BH NPY may label both high and moderately high affinity binding sites, 10pM [<sup>125</sup>I]-PYY selectively labels high affinity sites.

Lynch et al. (1989) then examined the regional distribution of both [<sup>125</sup>I]-BH NPY and [<sup>125</sup>I]-PYY receptor binding sites in the rat brain using receptor autoradiography. They found that the distribution of binding sites for both of these radioligands was very similar, although the level of [<sup>125</sup>I]-PYY binding was greater than that for [<sup>125</sup>I]-BH NPY in all regions. They found that the ratio of [<sup>125</sup>I]-BH NPY to [<sup>125</sup>I]-PYY binding was not consistent throughout all the brain regions, suggesting differences in the sites labelled by these two radioligands.

### 1.5.2c Y<sub>1</sub> receptors

As already mentioned (see section 1.5.2a), the Y<sub>1</sub> receptor was first proposed as a postjunctional receptor at the sympathetic neuroeffector junction in vascular smooth muscle preparations (Wahlestedt et al., 1986; Wahlestedt et al., 1987a). The receptor at this site was later shown to be of the Y<sub>1</sub> receptor type as the binding of radiolabelled PYY was displaced by both NPY and the Y<sub>1</sub> receptor agonist [Pro<sup>34</sup>] NPY (Grundemar et al., 1992). This Y<sub>1</sub> receptor site causes vasoconstriction in some vessels via two mechanisms. It has a direct

postjunctional effect and it can also potentiate the vasoconstrictor effects of noradrenaline (Wahlestedt et al., 1986).

There is also much evidence to suggest the presence of NPY Y<sub>1</sub> receptors in the brain. Feeding behaviour in rats can be stimulated by the central administration of NPY, an effect which is also seen with the Y<sub>1</sub> agonist [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY, but much less so with the Y<sub>2</sub> agonist NPY (13-36) (Flood and Morley, 1989). NPY-induced reduction in spontaneous locomotor activity and anxiety also seem to involve the Y<sub>1</sub> receptor, although this has not been shown by the direct use of a Y<sub>1</sub> receptor agonist ( Heilig et al., 1988b; Heilig et al., 1989).

(i) Structural requirements of Y<sub>1</sub> receptor ligands

A number of structure-activity relationships have been studied in order to distinguish the Y<sub>1</sub> receptor from the other subtypes in terms of which part of the NPY molecule is required for Y<sub>1</sub> receptor activation. Several studies have demonstrated that, following deletion of the first N-terminal Tyr<sup>1</sup> residue, the resultant compound NPY (2-36) showed a decrease in activity. Rioux et al, studying the effects of NPY as a coronary vasoconstrictor, showed a 12-fold reduction in potency following N-terminal Tyr<sup>1</sup> deletion (Rioux et al., 1986). Furthermore, subsequent shortening of the molecule by deleting N-terminal residues Tyr<sup>1</sup> to Glu<sup>15</sup> or Tyr<sup>1</sup> to Ala<sup>18</sup> caused major losses of potency.

Similar effects have also been demonstrated at vascular NPY receptors (Grundemar et al., 1992) and at an expressed human NPY Y<sub>1</sub>-like receptor (Larhammer et al., 1992). Boublik et al. introduced D-amino acid substitutions into different positions of the NPY molecule and found that substitution of any of the first five residues caused a marked reduction in potency (Boublik et al., 1990). Finally, the importance of the intact N-terminus of NPY for full Y<sub>1</sub> receptor activation is demonstrated by a loss in affinity of the Y<sub>1</sub> receptor agonist [Pro<sup>34</sup>] NPY to the Y<sub>1</sub> receptor following the substitution of a Lys<sup>4</sup> in place of the Glu<sup>4</sup> residue (Schwartz et al., 1990).

The importance of the C-terminus has also been studied. Fuhlendorff et al. (1990a) made substitutions of two residues in the C-terminal end of the molecule. They effectively combined the C-terminal hexapeptide of PP with the PP-fold of NPY to create the novel compound [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY. This compound was found to be a specific high-affinity agonist for Y<sub>1</sub> receptors (Fuhlendorff et al., 1990a). So the exact sequence of residues in the C-terminal portion of NPY seems to be of less importance than for the N-terminal region.

The importance of both N- and C-terminals for Y<sub>1</sub> receptor activation indicates that there may be some structural requirements of the central section of the molecule in order to ensure the correct configuration of both terminals upon presentation to the receptor site. The central portion of the NPY molecule is proposed to form a hairpin-like structure (discussed in section 1.2) which may be of importance for Y<sub>1</sub> receptor activation. Substitution of a proline residue

into the NPY molecule to make [Pro<sup>20</sup>] NPY breaks the hairpin loop structure, with a loss of Y<sub>1</sub> receptor affinity (Fuhlendorff et al., 1990b). Experiments where the central turn region and various amounts of the helical region were removed and replaced by a spacer molecule to retain the N- and C-terminals in a similar spacial orientation demonstrated the resultant analogues to be active at Y<sub>1</sub> receptors (Krstenansky et al., 1989).

(ii) Y<sub>1</sub> receptor clones

In 1990, Eva et al reported on the molecular cloning of a novel rat guanine nucleotide-binding protein-coupled receptor, FC5 (Eva et al.,1990). Its primary structure resembled that of the superfamily of neuropeptide receptors. However, it was not until 1992, after seeing similarities between expression patterns of the receptor-encoding mRNA and localization of NPY Y<sub>1</sub> receptor subtype in rat brain, that they were able to show the receptor to be of the Y<sub>1</sub> subtype (Krause et al., 1992). Two groups have also reported the cloning of human Y<sub>1</sub> receptors (Herzog et al.,1992; Larhammar et al.,1992). These receptors like the rat receptor, are members of the G protein-coupled receptor superfamily. The cloned receptor has 384 amino acids and, like other members of this superfamily, has seven putative transmembrane domains (see Fig. 1.4).

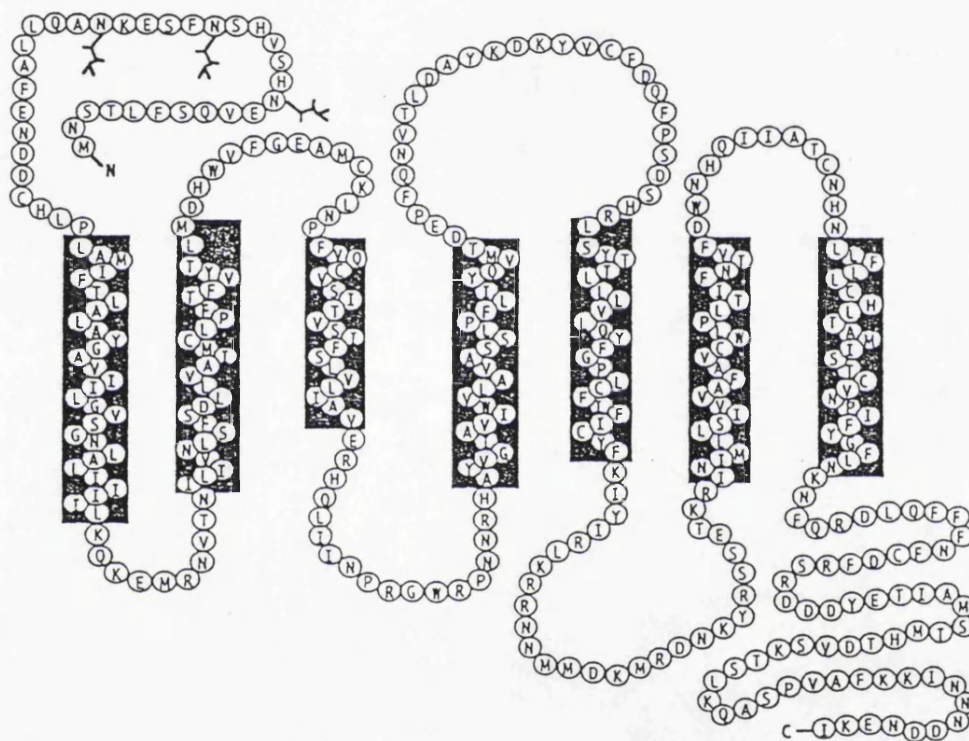


Fig. 1.4 Amino acid sequence and proposed structure of the human NPY Y1 receptor (Larhammar et al,1992) showing the seven membrane spanning domains (shaded areas) and possible N-linked glycosylation.



### 1.5.2d Y<sub>2</sub> receptors

The NPY Y<sub>2</sub> receptor subtype was first proposed as being the presynaptic NPY receptor at the sympathetic neuroeffector junction (Wahlestedt et al.,1986; Wahlestedt et al.,1987a). It is thought to have a modulatory presynaptic role, attenuating the release of transmitters. As well as being present on noradrenergic neurones, Y<sub>2</sub> receptors have also been reported in cholinergic motor neurones in the rat uterine cervix where they inhibit the cholinergic motor response (Stjernquist and Owman,1990). Grundemar et al. (1990) have also demonstrated the presence of NPY Y<sub>2</sub> receptors in non-adrenergic, non-cholinergic nerve fibres. They found NPY and NPY (13-36) to suppress capsaicin-sensitive sensory nerve-mediated contraction in the guinea-pig airway. They suggest that the fibres involved may be of the C-fibre type.

#### (i) Structural requirements for Y<sub>2</sub> receptor ligands

The requirements for Y<sub>2</sub> receptor activation are a lot less stringent with respect to the N-terminal region of its ligands compared to the Y<sub>1</sub> receptor subtype requirements. Thus C-terminal fragments of NPY can exhibit high affinity for the Y<sub>2</sub> receptor (Wahlestedt et al., 1986). Using the suppression of electrically-evoked contractions of the rat vas deferens as a measure of ligand activity, it was found that the shortest C-terminal fragment length required for Y<sub>2</sub> receptor activation was the 12 residues from NPY (25-36) (Grundemar and

Håkanson, 1990).

An intact C-terminal region of NPY is required for  $Y_2$  receptor activation. When a proline residue is substituted at residue 34, and/or leucine at residue 31, there is a loss of affinity for the  $Y_2$  receptor subtype (Fuhlendorff et al., 1990a). Minakata et al. (1989) studied the importance of the C-terminus by systematic substitutions of amino acids. They suggest that His<sup>26</sup> is important for  $Y_2$  receptor recognition. The C-terminal amine group is essential for receptor activation as desamide NPY is inactive at the  $Y_2$  receptor (Wahlestedt et al., 1986).

The hairpin loop structure does not seem essential for  $Y_2$  receptor recognition. NPY analogues with the first 4 N-terminal residues joined to the last 12 C-terminal residues, thus deleting the hairpin loop, were almost as potent at  $Y_2$  receptors as NPY itself (Beck-Sickinger et al., 1992). Upon substitution of a proline residue at position 20 in the NPY amino acid sequence, an effect that breaks the hairpin loop, Fuhlendorff et al found that the resultant analogue [Pro<sup>20</sup>] NPY was still active at the  $Y_2$  receptor (Fuhlendorff et al., 1990b). Substitution of PP (1-30) (the PP-fold) for NPY (1-30) showed a high affinity for the  $Y_2$  receptor subtype.

(ii) Y<sub>2</sub> receptor structure?

Unlike the NPY Y<sub>1</sub> receptor subtype, the Y<sub>2</sub> subtype has not as yet been cloned. However to try and characterize the structure of this receptor, Sheikh and Williams covalently cross-linked [<sup>125</sup>I-Tyr<sup>36</sup>]PYY to this receptor (Sheikh and Williams, 1990). They affinity labelled Y<sub>2</sub> receptors in rat hippocampus and rabbit kidney membranes using different cross-linking reagents. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and autoradiography analysis showed a major labeled protein band of M<sub>r</sub> = 50,000. This was unaffected by reducing agents and thus the Y<sub>2</sub> receptor subtype is a structurally distinct glycoprotein which is not disulphide-linked to other subunits. A M<sub>r</sub> = 50,000 protein band has also been reported following cross-linking [<sup>125</sup>I]-PPY to porcine hippocampal receptors (Inui et al., 1989).

1.5.2e Y<sub>3</sub> receptors

In most model systems PYY mimicks the effects of NPY with a potency similar to or slightly greater than NPY. This is also the case in model systems used for defining Y<sub>1</sub> and Y<sub>2</sub>-like receptors. Recently it has been observed that PYY in some cases is unable to mimic the actions of NPY, or even has the opposite effect. This has led to the proposal for the existence of a third receptor subtype termed the Y<sub>3</sub> receptor. These receptors are suggested to exist in both the brain and the periphery.

Autoradiographic analysis of [<sup>125</sup>I]-BH NPY binding in the rat brain stem has shown that although the binding is displaceable by NPY, PYY does not displace NPY from this region (Nakajima et al., 1986). Härfstrand et al. (1989) demonstrated that unlike NPY, which shows interactions with  $\alpha_2$ -adrenoceptors in the nucleus of the tractus solitarius (NTS), PYY shows no such interactions. Upon injection of NPY and its related peptides into the NTS, Grundemar et al. (1991a;1991b) found a discrepancy in the characteristics of the receptors mediating the cardiovascular effects in this region. Whilst NPY, the Y<sub>1</sub> receptor agonist [Pro<sup>34</sup>] NPY, and the Y<sub>2</sub> receptor agonist NPY (13-36) all produced a similar dose dependent decrease in arterial blood pressure in anaesthetised rat, PYY was inactive despite being both a Y<sub>1</sub> and Y<sub>2</sub> receptor agonist. The NTS therefore seems to contain an NPY receptor subtype, distinct from the Y<sub>1</sub> and Y<sub>2</sub> classification, and termed the Y<sub>3</sub>-like receptor.

In the periphery, Higuchi et al. (1988) found that PYY was of a very much lower potency than NPY in inhibiting nicotine-stimulated noradrenaline and adrenaline release in bovine adrenal chromaffin cells. These findings were confirmed by Wahlestedt et al. (1992) who found that [<sup>125</sup>I]-BH NPY binding to bovine adrenal chromaffin cells was displaced by NPY, [Pro<sup>34</sup>] NPY and NPY (13-36), but [<sup>125</sup>I]-PYY showed no specific binding in these cells. Similar binding characteristics are seen in the rat intestine and cardiac ventricular membranes. [<sup>125</sup>I]-BH NPY binding to both these tissues was displaceable by NPY, but PYY was far less potent in its ability to displace the bound radioligand (Nguyen et al., 1990; Balasubramaniam et al., 1990).

Tepperman and Whittle (1991) assessed the effects of both NPY and PYY rat gastric mucosal blood flow and found that although NPY decreased the blood flow, PYY did so in an inconsistent manner. Finally, opposite effects of NPY and PYY were seen in a study of their effects on plasma immunoreactive atrial natriuretic factor (IR-ANF) in rats (Baranowska et al., 1987). NPY injection increased IR-ANF, whereas PYY decreased it.

The evidence is therefore very strong for the existence of another NPY receptor subtype, the  $Y_3$  NPY receptor, which is insensitive to the effects of PYY. There may also be further subtypes which are at present indistinguishable using the presently available agonists and antagonists.

### 1.5.3 Signal transduction mechanisms for neuropeptide Y

Several studies have shown a link between the NPY receptor and a G-protein and so NPY is proposed to belong to the G protein-coupled receptor family. NPY inhibits adenylate cyclase activity in rat atrial cells and atrial membranes. This inhibitory action is blocked by prior treatment with pertussis toxin (Kassis et al., 1987). Pertussis toxin is known to specifically ADP-ribosylate the  $\alpha$ -subunits of two GTP-binding proteins,  $G_i$  and  $G_o$ . In this way it uncouples the receptor from the G-protein, blocking the effects on the adenylate cyclase (Ui, 1984). Feth et al. (1991) demonstrated that, in the presence of GTP $\gamma$ S, [ $^{125}$ I]-NPY binding to permeabilized SK-N-MC cells (a  $Y_1$ -

like receptor system) was inhibited in a dose dependent manner. This inhibition was abolished by pretreatment with pertussis toxin (Feth et al.,1991). GTP $\gamma$ S is a stable form of GTP which binds to the G-protein and converts it to the low affinity state, therefore decreasing the amount of ligand bound. Finally the uncoupling effect of pertussis toxin on NPY receptors in sensory neurones is reversed by the perfusion of GTP and the purified  $\alpha$ -subunit of G<sub>o</sub> (Ewald et al.,1988). These results demonstrate that the NPY signal transduction is via G-proteins, probably G<sub>i</sub> or G<sub>o</sub>.

All three of the presently defined receptor subtypes have been demonstrated to inhibit adenylate cyclase activity upon activation. Krause et al. (1992), using a recombinantly expressed NPY Y<sub>1</sub> receptor, showed that Y<sub>1</sub> receptor agonists were able to inhibit adenylate cyclase. NPY has also been shown to inhibit adenylate cyclase in SK-N-BE2 cell, a Y<sub>2</sub>-like model system (Wahlestedt et al.,1990b). Balasubramaniam and Sheriff (1990) showed that the NPY fragment NPY (18-36) blocked the inhibitory effects of NPY on adenylate cyclase activity in rat cardiac ventricular membranes, a Y<sub>3</sub>-like receptor system. It would appear, therefore, that inhibition of adenylate cyclase is a universal signalling mechanism associated with NPY receptor activation.

Finally, it seems that in many systems NPY receptor activation alters the concentration of free intracellular Ca<sup>2+</sup>, and that this occurs via several mechanisms. NPY may cause a Ca<sup>2+</sup> influx through L-type channels, as the dihydropyridine-type Ca<sup>2+</sup> entry blockers, nifedipine and felodipine, attenuate

the splenic vasoconstrictor response to NPY in vivo (Lundberg et al.,1988). NPY may also inhibit  $Ca^{2+}$  influx through N-type channels, a possible mechanism for pre-synaptic inhibition of transmitter release. Hirning et al. (1990) showed that  $Ca^{2+}$  currents via N-type channels were inhibited by NPY. NPY also inhibited N-type  $Ca^{2+}$  currents in acutely dissociated rat vagal afferent (nodose) neurones (Wiley et al.,1990). The latter effect was blocked by pretreatment with pertussis toxin, suggesting the involvement of a G-protein. The mobilization of  $Ca^{2+}$  from intracellular stores has also been demonstrated following NPY administration. This can occur following phospholipase C activation as demonstrated by Perney et al. (1989) who found that NPY can stimulate the synthesis of inositol triphosphate ( $IP_3$ ) and increase cytosolic free calcium in cultured rat sensory neurones. The mobilization of  $Ca^{2+}$  can also occur independently of  $IP_3$ . Mihara et al. (1989) found that although NPY caused transient rises in intracellular free  $Ca^{2+}$ , it was unable to significantly increase  $IP_3$  generation in smooth muscle cells cultured from porcine aorta.

It is apparent from the study of second messenger systems associated with NPY receptors that it is not possible to distinguish between the receptor subtypes on this basis. It may be that the second messenger systems involved with NPY receptor subtypes are not subtype specific, but rather are dependent on the cell-type. This is clearly shown in a study of a cloned NPY  $Y_1$  receptor subtype (Herzog et al.,1992). These authors expressed the  $Y_1$  receptor clone in two types of cell. When expressed in human embryonic kidney (293) cells, the receptor coupled to a pertussis toxin-sensitive G protein that

mediates the inhibition of cAMP accumulation. In Chinese hamster ovary cells however, the expressed receptor is not coupled to the inhibition of adenylate cyclase, but instead causes the elevation of intracellular  $\text{Ca}^{2+}$ .

### 1.5.3a Fos: a possible third messenger for neuropeptide Y receptor signalling?

The signal transduction mechanisms discussed above all represent relatively short-lived biochemical events causing mainly rapid responses in neurones. However, stimulation also elicits slower, long term responses, that are linked to alterations in gene expression (for reviews see Morgan and Curran, 1989; Sheng and Greenberg, 1990). This type of response is likely to play a role in the development and adaptive plasticity of the nervous system. There are two classes of gene that are responsive to synaptic stimulation and membrane electrical activity. These are genes whose transcription is rapidly and transiently stimulated, the immediate-early genes (IEGs), and those whose expression is induced much more slowly, the late response genes. It has been proposed that the IEG expression results in proteins that regulate the expression of late response genes, which in turn have specific effector functions (Morgan and Curran, 1988).

One of the first IEGs to be identified was the *c-fos* proto-oncogene (Greenberg and Ziff, 1984). Following an extracellular stimulus, the second messenger systems of the cell are activated. Some of these lead to an induction of the *c-fos*



IEG, and another IEG, *c-jun* (Curran, 1988).  $Ca^{2+}$  is thought to be an important second messenger in regulating the expression of these IEGs (Greenberg and Ziff, 1986; Morgan and Curran, 1986; 1988). The resultant protein products, Fos and Jun, form a heterodimeric protein complex in the nucleus that binds to the DNA regulatory element, the AP-1 binding site (Curran and Franza, 1988; Rauscher et al, 1988). In this way, it can regulate nearby promoters, thereby either stimulating or repressing transcription of the late response genes, depending upon which member of the Jun family it complexes with (Chiu et al., 1989; Schutte et al., 1989).

As already mentioned (see above, section 1.5.3), NPY receptor activation alters the concentration of free intracellular  $Ca^{2+}$ . In so doing it could possibly induce *c-fos* expression, and stimulate these 'third messenger' systems. Earnest et al. (1992), have studied Fos expression in the suprachiasmatic nucleus (SCN) of rodents as a result of constant retinal light stimulation, in order to study its rhythmic variations during the circadian cycle. Neuropeptide Y has been shown to affect the circadian rhythm when injected into the SCN of hamsters (Albers et al., 1984). Earnest et al. (1992) found that in discrete areas of the SCN, the density of Fos-immunoreactivity was doubled during the subjective night compared to the subjective day. Furthermore, the Fos-immunoreactivity was confined to an area of SCN that was coextensive with NPY-immunopositive fibres. It therefore seems likely that NPY may have long term effects in the central nervous system via induction of IEG expression.

#### 1.5.4 Neuropeptide Y receptor agonists

All NPY receptor agonists thus far are peptides based on the NPY sequence of amino acids (see Figure 1.5.4). The three currently defined receptor subtypes all share NPY as a common agonist. However PYY, despite being approximately equipotent with NPY at the Y<sub>1</sub> and Y<sub>2</sub> receptor subtypes, does not seem to recognise the Y<sub>3</sub> receptor (see section 1.5.2e for references). C-terminal fragments of NPY retain agonist activity at the Y<sub>2</sub> receptor, but fail to activate the Y<sub>1</sub> receptor subtype. The reverse is seen with the substituted analogues [Pro<sup>34</sup>] NPY and [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY. These analogues show agonist activity at the Y<sub>1</sub> receptor whilst failing to show any activity at the Y<sub>2</sub> receptor (Fuhlendorff et al.,1990a).

#### 1.5.5 Neuropeptide Y receptor antagonists?

Research into NPY and its receptor subtypes has been largely hampered by the absence so far of any high affinity and specific antagonists. These would be important for the absolute pharmacological definition of receptor subtypes, as well as to assist in determining the physiological roles of NPY. Several groups have reported antagonistic effects of compounds, but as yet these have had little influence on NPY research.

NPY (pig)                    YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY  
 PYY (pig)                    YPAKPEAPGEDASPEELSRYYASLRHYLNLVTRQRY  
 NPY (13-36)                - - - - - PAEDLARYYSALRHYINLITRQRY  
 NPY (18-36)                - - - - - ARYYSALRHYINLITRQRY  
 [Pro<sup>34</sup>] NPY                YPSKPDNPGEDAPAEDLARYYSALRHYINLITRPRY  
 [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY        YPSKPDNPGEDAPAEDLARYYSALRHYINLLTRPRY

**Fig 1.5a**

Receptor Subtype	Agonist order of potency
Y <sub>1</sub>	PYY ≥ NPY ≥ [Pro <sup>34</sup> ] NPY > NPY (13-36)
Y <sub>2</sub>	PYY > NPY > NPY (13-36) > [Pro <sup>34</sup> ] NPY
Y <sub>3</sub>	NPY ≥ [Pro <sup>34</sup> ] NPY ≥ NPY (13-36) > PYY

**Fig 1.5b**

**Fig 1.5** NPY receptor agonists. **a** Amino acid sequences of various NPY receptor agonists. Variations from the parent molecule (NPY) are shown as underlined. **b** The general order of potency of several NPY receptor agonists at specific receptor subtypes. The assay systems in which the agonist potencies were determined were: Y<sub>1</sub> - cloned Y<sub>1</sub> receptor (Herzog et al., 1992), human SK-N-MC neuroblastoma cells (Fuhlendorff et al., 1990a; Wahlestedt et al., 1992); Y<sub>2</sub> - human SK-N-BE(2) neuroblastoma cells (Wahlestedt et al., 1992), rat vas deferens (Wahlestedt et al., 1987a); Y<sub>3</sub> - rat gastric mucosa (Tepperman and Wittle, 1991), adrenal chromaffin cells (Wahlestedt et al., 1992; Higuchi et al., 1988), and nucleus of the tractus solitarius (Grundemar et al., 1991a; 1991b).

### 1.5.5a He90481

In 1990, Michel and Motulsky showed a competitive antagonist effect of the H<sub>2</sub> receptor agonist He90481. NPY is able to increase the free intracellular Ca<sup>2+</sup> concentrations in HEL cells, a Y<sub>1</sub>-like model system. He90481 was able to shift the dose-response curve for NPY to the right, leaving the same maximal NPY-stimulated Ca<sup>2+</sup> increase. Linear Schild plots were obtained with a slope not significantly different from unity. A pA<sub>2</sub> value of 4.43 (affinity = 37.5 μM) was obtained from these data. Two factors however limit the usefulness of this compound as an antagonist. Firstly, it has a low affinity for NPY receptors, and secondly, it is not specific for NPY receptors. It also competes for α<sub>2</sub>-adrenoceptors and is an agonist at H<sub>2</sub> histamine receptors in guinea-pig heart with a potency about 1000 times greater than for NPY receptors.

### 1.5.5b PP56

Edvinsson et al. (1990) demonstrated that preincubation of guinea-pig basilar artery with D-myo-inositol 1,2,6-triphosphate (PP56), significantly attenuated NPY-induced contractions. Significant attenuation of these NPY-induced contractions was seen at 0.1 μM PP56. Schild plot analysis revealed a straight line with a slope that differed from unity. The antagonism thus produced was not competitive nor reversible. The effect of this compound on endothelin 1, histamine, bradykinin, noradrenaline, 5-hydroxytryptamine and prostaglandin

$F_{2\alpha}$  receptors was also evaluated, but it did not significantly attenuate the contractions produced by any of these compounds. Tests were conducted on the effects of PP56 on the NPY potentiation of NA-induced vasoconstriction in rabbit femoral artery (Adamsson and Edvinsson, 1991). PP56 was shown to inhibit this potentiating effect of NPY at a much lower concentration than for the effect on the direct NPY induced vasoconstriction. This may reflect the involvement of different receptor subtypes. The antagonistic effects of PP56 have also been demonstrated in other systems. PP56 was found to antagonise NPY induced inhibition of relaxation induced by acetylcholine, substance P and VIP in guinea-pig cerebral, coronary and uterine arteries (Edvinsson et al., 1992). In vivo, using the pithed rat, PP56 significantly inhibited the pressor response to NPY at a dose which did not itself influence the pressor actions of preganglionic nerve stimulation or bolus injections of phenylephrine or vasopressin (Sun et al., 1991). In an attempt to study the pre- and postsynaptic antagonistic effects of PP56 in vivo, Potter et al. (1992) used an anaesthetised dog experiment where both pre- and postsynaptic actions of exogenous NPY or NPY released from the cardiac sympathetic nerve can be recorded in the same preparation. PP56 was seen to antagonise NPY in both the presynaptic effect, as measured by the inhibitory effect of NPY on cardiac vagal action, and postsynaptic effect, as measured by recording arterial blood pressure. Centrally administered PP56, given as a pretreatment before NPY administration, has been shown to cause a significant decrease in NPY-induced hyperphagia, however, a similar effect was also seen with galanin (Heilig et al., 1991). In the same study PP56 had no effect on NPY induced hypoactivity. It also failed to

displace any [<sup>125</sup>I]-BH NPY bound to membranes or to cells displaying either Y<sub>1</sub> or Y<sub>2</sub> receptor subtypes alone. This latter finding suggests that PP56 is not a competitive receptor antagonist.

PP56 is an isomer of inositol 1,4,5-trisphosphate (IP<sub>3</sub>). Inositol phosphates occur naturally in the body within membrane phospholipids. They are utilised by cells as second messengers involved in the intracellular release of stored calcium and also to stimulate calcium entry. IP<sub>3</sub> is considered responsible for this very specific action, but it differs from PP56. Therefore it is not known whether PP56 or a degradation product of PP56 interacts with the turnover or influences the second messenger role of IP<sub>3</sub>. It is possible that PP56 may exert its NPY antagonistic effects on some part of the second messenger system, as NPY has been shown to increase IP<sub>3</sub> accumulation in certain cell systems (Hägglad and Fredholm, 1987).

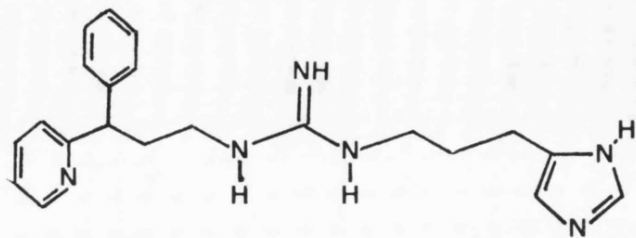
#### 1.5.5c Neuropeptide Y (18-36)

The NPY C-terminal fragment NPY (18-36) is a Y<sub>2</sub> receptor agonist, but it has also been shown to inhibit [<sup>125</sup>I]-BH NPY binding in rat cardiac ventricular membranes (Balasubramaniam and Sheriff, 1990). It was also found to inhibit NPY stimulated adenylate cyclase activity in the same tissue, which is a PYY insensitive, or Y<sub>3</sub>-like receptor system. As already mentioned in section 1.5.4, NPY (18-36) is a partial agonist at Y<sub>1</sub>-like NPY receptors in HEL cells for the

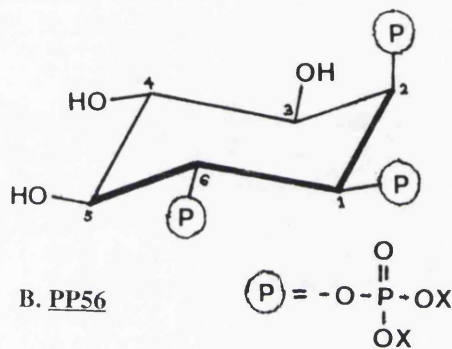
inhibition of cyclic AMP accumulation and for intracellular  $\text{Ca}^{2+}$  mobilisation. It is also a full agonist at  $\text{Y}_2$ -like NPY receptors in various tissues (Michel et al., 1990).

#### 1.5.5d Benextramine

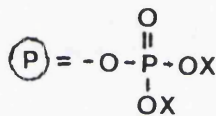
Benextramine is an irreversible  $\alpha$ -adrenoceptor antagonist, but it has also been reported to inhibit the NPY-induced increase in diastolic pressure in pithed rats and NPY binding to rat brain membranes (Doughty et al., 1990). These authors subsequently examined analogues of benextramine to look for structure activity relationships and found that the benzene moiety (see Figure 1.6) was important for its NPY antagonist activity. They noticed some similarities between the non-peptide benextramine molecule and the C-terminal region of NPY (Doughty et al., 1993). Both have an extended, polycationic structure containing an aromatic ring. This aromatic ring is a methoxybenzyl moiety in benextramine and a phenolic moiety of Tyr<sup>36</sup> in the case of NPY. The distance between the phenyl and the positively charged inner N-atom of benextramine has the same number of atoms as that between the phenolic moiety of Tyr<sup>36</sup> and the positively charged guanidinium group of Arg<sup>35</sup> in NPY, indicating the possibility of common hydrophobic and ionic interactions at the NPY receptor.



A. He 90481

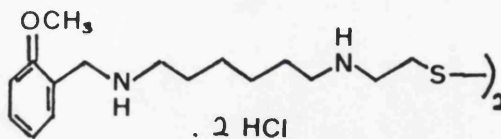


B. PP56



C. NPY (18-36)

18  
Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-  
28 36  
Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH<sub>2</sub>



D. Benextramine

(*N,N*-bis-[6-[(2-methoxybenzyl)amino]hex-1-yl]cystamine

E. PYX-1 & PYX-2

PYX-1 : Ac-[3-(2,6-dichlorobenzyl)-Tyr<sup>27</sup>,D-Thr<sup>32</sup>] NPY (27-36)

PYX-2 : Ac-[3-(2,6-dichlorobenzyl)-Tyr<sup>27,36</sup>,D-Thr<sup>32</sup>] NPY (27-36)

Fig. 1.6 NPY receptor antagonists.



The usefulness of benextramine as an antagonist is dampened by the high concentrations needed to inhibit NPY binding to rat brain membranes. An  $IC_{50}$  of  $100\mu\text{M}$  was required, a level considerably higher than that required in its interactions with  $\alpha$ -adrenoceptor binding sites ( $2\mu\text{M}$ ). This NPY antagonist may therefore have nonspecific effects in addition to those involving the NPY system when used in vivo.

#### 1.5.5e PYX-1 and PYX-2

In 1990, Tatemoto introduced two peptides which he claimed had NPY antagonistic properties (Tatemoto, 1990). They are based on the C-terminal structure of NPY itself from residue 27 to 36. They are Ac-[3-(2,6-dichlorobenzyl)-Tyr<sup>27</sup>, D-Thr<sup>32</sup>]NPY (27-36) and Ac-[3-(2,6-dichlorobenzyl)-Tyr<sup>27,36</sup>, D-Thr<sup>32</sup>]NPY (27-36), PYX-1 and PYX-2, respectively (Figure 1.6). Their antagonistic properties were tested against NPY-stimulated  $\text{Ca}^{2+}$  mobilization in human erythroleukemia (HEL) cells, which represent a selective  $Y_1$ -like receptor model system. PYX-2 displayed a higher affinity than PYX-1, PYX-2 being active in the 10-100nM range as opposed to the 100-1000nM range for PYX-1. Their selectivity for the NPY receptor subtypes has not as yet been shown.

### 1.5.5f Antisense oligodeoxynucleotides

Due to the lack of any useful NPY receptor antagonist for studying the role of specific NPY receptor subtypes in CNS physiology, Wahlestedt et al. (1993) used a different approach to antagonise the effects of NPY at the  $Y_1$  receptor subtype. They constructed an antisense oligodeoxynucleotide corresponding to the  $NH_2$ -terminus of the rat  $Y_1$  receptor. When this was added to rat cortical neurones they found a selective decrease in  $Y_1$  receptor density and an attenuation of the decrease in adenosine 3',5'-monophosphate (cAMP) seen after  $Y_1$  receptor activation. Upon repeated injection of this oligodeoxynucleotide into the lateral cerebral ventricle of rats a similar selective decrease in cortical  $Y_1$  receptor density was seen. Animals treated in this way displayed anxiety-like behaviour suggesting that NPY may have an important behavioural role in mammalian physiology.

## 1.6 Central nervous system physiology of neuropeptide Y

### 1.6.1 Feeding behaviour

In 1984, Clark et al. found that i.c.v. injection of NPY produced an increase in feeding behaviour in rats. The exact site of action of NPY in the brain was then unknown, but later work by Stanley and Leibowitz (1984) showed that it may exert its effects in the hypothalamic paraventricular nucleus (PVN) as direct

injection into this site produced dose-dependent increases in both feeding and drinking behaviour (Stanley and Leibowitz, 1984). In this study they found that the effects of NPY on drinking behaviour also took place in the absence of food, thus suggesting that it was a primary effect, and not an indirect effect due to increased eating. However, in some studies drinking is only effected secondary to eating or even not at all (Morley et al.,1987; Parrott et al.,1986). Of the three basic macronutrients, it seems that carbohydrate intake is stimulated preferentially by NPY (Stanley et al.,1985; Morley et al.,1987). The physiological role of NPY in feeding behaviour is highlighted by the finding that hypothalamic PVN NPY concentrations increase with time after starvation, and then return quickly to normal following food ingestion (Sahu et al., 1988).

#### 1.6.2 Sedation

The i.c.v. injection of NPY (0.4-4.0nmol/5 $\mu$ l saline given over 1min) into rats has been shown to reduce open field and home cage activity in a dose dependent manner (Heilig and Murison,1987a). Since Fuxe et al. (1983) have reported NPY to produce both EEG changes and behavioural signs of sedation, with increased synchronized and reduced desynchronized activity, it is possible that the decreases in locomotor activity are a result of a sedative effect of NPY. This theory was further supported by the finding that NPY (2nmol/5 $\mu$ l saline given over 1min) protects against the development of stress-induced gastric erosions in the rat, an effect which is often associated with sedatives (Heilig

and Murison,1987b).

### 1.6.3 Endocrine

NPY plays an important role in central endocrine regulation. Firstly NPY has an effect on the secretion of luteinizing hormone (LH) from the pituitary. This effect is different depending on the hormonal state of the animal. In normal male rats for example, i.c.v. injection of NPY (10µg/3µl saline) stimulated LH release (Allen et al.,1985). In contrast to this, i.c.v. NPY (0.02-5.0µg/2µl saline) administration in ovariectomized rats caused a reduction in plasma LH relative to pretreatment levels (McDonald et al.,1985). The pretreatment levels were however higher than for normal rats. In the latter study a decrease in the plasma concentration of growth hormone (GH) was also seen following central NPY treatment.

Secondly, NPY has been shown to have an activating effect on the pituitary-adrenocortical axis (Wahlestedt et al.,1987b). Injection of NPY (50pmol/0.5µl saline given over 30s) into the area of the hypothalamic PVN produced increases in circulating adrenocorticotrophic hormone (ACTH) and corticosterone levels. This effect of NPY may be mediated by corticotropin-releasing factor (CRF) as an increase in CRF-immunoreactivity has been shown in the hypothalamus following NPY treatment (Haas and George,1987). This latter finding is of interest as both NPY and CRF are highly concentrated in the

hypothalamus, and NPY neurones may project to the PVN, the major site of CRF synthesis (Bai et al.,1985).

There is also evidence to suggest that NPY may play a role in modulating the central hormonal control of plasma volume and electrolyte control. Willoughby and Blessing (1987) demonstrated that NPY could cause the secretion of vasopressin when injected into the supraoptic nucleus of rats.

Finally, NPY has been found to inhibit thyroid-stimulating hormone (TSH) secretion (Härfstrand et al.,1987) and to increase plasma insulin levels (Moltz and McDonald,1985) following i.c.v. administration.

#### 1.6.4 Circadian rhythms

A large number of physiological and behavioural mechanisms follow daily patterns of activity which are known as circadian rhythms. In higher animals, the circadian pacemaker follows an approximate 24 hour cycle. This is normally synchronized with the external 24 hour light-dark cycle which we all encounter daily. The suprachiasmatic nucleus (SCN) of the hypothalamus has been shown to be a putative circadian pacemaker as its destruction eliminates the circadian cycle of a large number of functions (Rusak and Zucker,1979 ; Takahashi and Zatz,1982). Since NPY immunoreactivity localization studies have shown a dense innervation of the SCN (Allen et al.,1983), it seemed that

NPY may play a role in the control of circadian rhythms. Albers et al.(1984) demonstrated that injection of PP (0.2µg/0.2µl saline), and also NPY, into the SCN of hamsters caused a shift in the animals' circadian rhythms of locomotor activity. When the peptides were administered within a 10 hour interval prior to the onset of wheelrunning (a measure of locomotor activity), they caused an advancement of the phase of activity. When given between 6 and 14 hours after the onset of wheelrunning, the peptides produced phase delays in the rhythms of activity.

#### 1.6.5 Memory

High densities of NPY receptor binding sites are found in the hippocampus and amygdaloid hippocampal areas of the brain (Martel et al.,1986) as are NPY-like-immunoreactive perikarya and fibre terminals (Allen et al.,1983). As this region is involved in learning and memory processes, Flood et al. (1987) examined the effects of NPY on memory retention in mice. They found that i.c.v. administration of NPY given after training of the mice led to an improvement in memory retention. They also demonstrated the ability of NPY to reverse the amnesia induced by the cholinergic muscarinic antagonist scopolamine or by the protein synthesis inhibitor anisomycin.

### 1.6.6 Central control of blood pressure

The central administration of NPY in order to study its effects on blood pressure has produced inconsistent results. Vallejo and Lightman (1986) administered NPY (470pmol/10 $\mu$ l i.c.v given over 2min) to anaesthetized rats and found increases in both blood pressure and heart rate. Härfstrand (1986), however, using a higher dose range than in the previous study (750-1250pmol as opposed to 0.9-470pmol) found that NPY (dissolved in 30 $\mu$ l mock CSF and given over 3min) lowered both blood pressure and heart rate.

Microinjection of NPY into the posterior hypothalamus produced dose-related increases in blood pressure (Martin et al.,1988). Varying effects are seen upon the microinjection of the peptide into the nucleus of the solitary tract. In this region, a biphasic response is produced with low doses producing hypertension and higher doses giving hypotension (Carter et al.,1985).

Maccarrone and Jarrot (1985) have measured the regional brain concentrations of NPY immunoreactivity in age matched Wistar-Kyoto (WKY) and spontaneously hypertensive (SH) rats. They found lower levels in the cortex, cervical and thoracic spinal cord, the midbrain and medulla oblongata-pons of the SH rats. In subsequent studies they also found elevated levels of NPY in selected hypothalamic nuclei of SH rats (Maccarrone and Jarrot,1986).

Finally, in normal rats NPY has been shown to increase the number of  $\alpha_2$ -

adrenoceptor binding sites in medulla oblongata membranes in vitro, and to increase their affinity in vivo (Agnati et al.,1983). In the spontaneously hypertensive rats however, this receptor interaction seems to be abolished as NPY is unable to up regulate the number of  $\alpha_2$ -adrenergic binding sites (Agnati et al.,1983).

#### 1.6.7 Central nervous system pathophysiology

There is evidence to suggest the involvement of NPY in several pathophysiological disorders of the CNS. It may be involved in schizophrenia as NPY concentrations in the temporal cortex were found to be reduced in schizophrenic patients (Widerlöv et al.,1988a). There also seems to be an involvement in Alzheimer's-type dementia. A reduction in cortical and hippocampal NPY-immunoreactivity has been shown in Alzheimer's patients (Beal et al.,1986a; Beal et al.,1987). Furthermore those regions with the most severe loss of NPY-immunoreactive neurones and axons, mainly the CA1 region of the hippocampus and the entorhinal cortex, are also those areas most affected by the Alzheimer's disease process (Chan-Palay et al.,1986).

A link between NPY and Huntington's disease has also been demonstrated. In post mortem Huntington's disease brains, an increase in both somatostatin and NPY-like immunoreactivity has been found in the caudate nucleus and the putamen compared to control brains (Dawbarn et al.,1985). It was also



observed that upon immunohistochemical staining of the caudate and putamen of control and Huntington's disease brains, the number of NPY-positive neurones was increased compared to controls. Also in this region NPY was shown to induce increases in dopamine turnover (Beal et al.,1986b). Dopamine is known to aggravate chorea in Huntington's disease (Martin,1984), and so it may be that NPY is involved in this process.

#### 1.6.7a Depression and anxiety

There is a large amount of evidence suggesting the possibility of NPY being involved in both depression and anxiety. The anatomical distribution of NPY in the CNS shows high amounts in several limbic areas such as the nucleus accumbens, septum, amygdala as well as the cerebral cortex and the hypothalamus, all regions believed to be involved with emotions. NPY has been shown to coexist with noradrenaline in several brain areas (Everitt et al.,1984) which, together with the fact that noradrenergic pathways of the brain are suggested to regulate affective and anxiety states (Siever and Davies,1985; Redmond and Huang,1979), points to a possible role for NPY in depression and anxiety. NPY has also been demonstrated to have effects on functions such as circadian rhythms (Albers et al.,1984), feeding behaviour (Clark et al.,1984; Stanley and Leibowitz,1984) and on the hypothalamic pituitary-adrenocortical axis (Wahlestedt et al.,1987b) which are often affected in major depressive disorders.

NPY levels in the CSF have been shown to be decreased in patients with major depression (Widerlöv et al.,1988b), although in previous studies increased levels had been found (Ekman et al.,1984). Other studies have shown no change in the CSF concentrations of NPY in depressive patients (Berrettini et al.,1987) which has led to the hypothesis that it may not be decreased levels of NPY, but an alteration in the processing or metabolism of NPY that occurs in depressed patients.

Chronic antidepressant treatment in rats has been found to increase NPY-like-immunoreactivity in the rat brain (Heilig et al.,1988a). Heilig et al. (1988a) administered both imipramine and zimeldine over a three week period and found increases in the frontal cortex of 40 and 60%, respectively. Imipramine increased hypothalamic NPY-like-immunoreactivity by 65% whereas zimeldine had no effect in this region. Another treatment for depression is electroconvulsive treatment (ECT). Several studies have found that repeated ECT increased the concentrations of NPY in several areas of the rat brain, whereas a single treatment of ECT was without effect (Stenfors et al.,1989; Wahlestedt et al.,1990a). The effect on peptide concentrations was highly specific for NPY as no effect on substance P (SP), neurokinin A (NKA), neurotensin (NT), vasoactive intestinal polypeptide (VIP) or galanin (GAL) concentrations were found following treatment (Stenfors et al.,1989). It is possible therefore that imipramine, zimeldine and ECT are producing their antidepressant effects via interactions with NPY systems of the brain.

A useful model for evaluating antidepressant activity of compounds is olfactory bulbectomy-induced muricide (mouse killing behaviour) (Ueki, 1982). Using this model of depression, Katoaka et al (1987) were unable to show a direct effect of NPY (5 and 10µg/µl) when injected into the medial amygdala, an area involved in the regulation of muricide, in suppressing muricide. They were, however, able to demonstrate an 80% suppression of muricide when NPY (10µg/µl) was administered into the medial amygdala in combination with the maximal-ineffective dose of noradrenaline. This study provides direct evidence that NPY may be involved in depression, although probably not primarily, through its interactions with the noradrenergic system.

Anxiety is often seen as part of the depressive syndrome, and so human studies of depression may also be providing results relevant to anxiety itself. In a study of CSF NPY concentrations in depressed patients, Widerlöv et al (1988b) found a negative correlation between NPY concentrations and anxiety level scores. Tricyclic antidepressants are also used as a treatment for anxiety (Modigh,1987). These findings, plus the fact that NPY has been shown to have sedative effects in rats (Heilig and Murison,1987a; Fuxe et al.,1983) prompted Heilig et al. (1989) to study the effects of NPY in several animal models of anxiety.

Heilig et al. (1989) studied the effects of i.c.v. NPY (0.2 - 5.0 nmol/5µl, 5µl/min) and the NPY Y<sub>2</sub> receptor agonist NPY (13-36) (0.4 - 2.0 nmol/5µl, 5µl/min) in the elevated x-maze, and in the Vogel's drinking conflict test. They

found that both 1 and 5 nmol NPY showed anxiolytic-like effects in the x-maze, with 0.2 and 1 nmol NPY showing anxiolytic-like activity in the Vogel conflict test. In the x-maze test, 5 nmol NPY showed possible sedative activity by reducing the total number of entries into either open or closed arms (see sections 2.4.5a and 5.1 for explanation of terms). NPY (13-36) showed no significant anxiolytic-like effect in either model. Since the NPY  $Y_2$  receptor agonist showed no anxiolytic-like activity at the doses used, they suggest that the anxiolytic effects of NPY might be mediated by the  $Y_1$  receptor. Inspection of their graphical data shows that, although not significant, NPY (13-36) does show a small, dose-dependent tendency towards an increase of % time spent in the open arms. It is possible, therefore, that higher doses than those tested by Heilig et al. (1989) might induce significant anxiolytic-like effects. Considering that NPY (13-36) is less potent than NPY in most model systems (see figure 1.5), it would have been wise to test this selective agonist over at least the same dose range as that used for NPY.

Heilig et al. (1989) also suggest that NPY might exert its anxiolytic-like activity via an interaction with  $\alpha_2$ -adrenoceptors. (For review of possible  $\alpha_2$ -adrenoceptor interactions see section 6.1). The authors try to test this hypothesis by attempting to block the effects of NPY using a subthreshold dose of the  $\alpha_2$ -selective antagonist, idazoxan, which at suprathreshold levels would itself show anxiogenic activity. Unfortunately they were unable to find a dose of idazoxan which would block the anxiolytic-like effects of NPY without having a behavioural effect itself. In the Vogel conflict model,

idazoxan (2mg/kg, i.p.) seemingly blocked the effects of 1nmol NPY without having an effect in itself, suggesting to the authors an interaction between NPY and  $\alpha_2$ -adrenoceptors. However, the dose of idazoxan used in this study as 'subthreshold', may have been unable to show any anxiogenic effects itself because the behavioural responses of the saline control animals were already very suppressed. Thus it is possible that 2mg/kg was not a subthreshold dose of idazoxan and therefore it blocked the anxiolytic-like effects of NPY by inducing anxiogenesis.

This study has therefore raised several questions which remain unanswered. Firstly, is the NPY  $Y_1$  receptor subtype responsible for the anxiolytic-like activity of NPY, and secondly, is this effect mediated by an interaction between the NPY receptor and  $\alpha_2$ -adrenoceptors?

#### 1.6.7b Anxiety and the hippocampus: the behavioural inhibition system

In his book, 'The neuropsychology of anxiety', Gray (1982) describes the possible interaction between several brain regions that mediate behavioural responses to the environment. One of these integrated brain pathways is the 'behavioural inhibition system', which mediates behaviour responses to novelty and conditioned stimuli for punishment and non-reward, resulting in passive avoidance and extinction. The conditioned stimuli affecting this system, ie punishment and non-reward, are the equivalent of the emotions 'fear' and

'conditioned frustration'. These emotions, as well as those generated by novel stimuli, are part of the general emotion, 'anxiety'. The 'behavioural inhibition system' has the functions of inhibiting all ongoing behaviour, and performing the maximum possible analysis of current environmental stimuli, especially novel ones.

Based on a review of animal studies, Gray (1982) suggested that the brain regions comprising the 'behavioural inhibition system' were the septal and hippocampal areas, otherwise known as the septo-hippocampal system. Lesions of the septal area, or of the hippocampus, caused behaviour similar to that seen after giving anxiolytic drugs. Stimulation of the septal areas has the opposite effect. He proposes that the septo-hippocampal system, along with several other areas, acts as a comparator, predicting the expected state of the animals' world in the next instant of time (about 0.1 sec), and comparing this with the actual state of the world (as supplied to the septo-hippocampal system by the sensory analysis systems via the entorhinal cortex). If the actual event matches the predicted event, the system continues with the next prediction, and so on. If however, the actual event does not match the predicted one, or if the predicted one is aversive, then the system takes control of behaviour causing behavioural inhibition, increased arousal, and increased attention (Gray, 1982; 1988).

## 1.7 Aims of this study

The suggestion that NPY may be involved in the anxiety process, and specifically in the reduction of anxiety levels, makes it an exciting target for research. The psychobiology of anxiety seems to be very complicated with not one, but many neurotransmitter systems interacting to produce an overall effect. The findings that NPY is often co-localised with other neurotransmitters, many of which are already associated with anxiety disorders, increase the interest in the involvement of NPY, as does its ability to modulate the effects of other transmitters.

In the present study the distribution of NPY receptors has been determined and the areas of the brain activated by central NPY administration assessed to characterise the possible sites of action of the peptide. This was firstly achieved by using autoradiography to map the distribution of receptor binding sites, and then by using the expression of the *c-fos* immediate early gene as a marker of neuronal activity following central NPY administration.

Finally, the effects of NPY and several analogues were tested in three different behavioural models of anxiety. The effects of drugs administered both *i.c.v.* and directly into the specific brain regions suggested by the autoradiography and *c-fos* work were examined to determine the site of action of NPY and the receptor subtype involved.

## **CHAPTER 2**

## **METHODS**



## Part I : RECEPTOR AUTORADIOGRAPHY

### 2.1. Neuropeptide Y receptor autoradiography

Several groups of workers have reported on the localization of NPY receptors in the rat brain (Martel et al., 1986; Ohkubo et al., 1990), but all used vastly different parameters in their assays. In order to carry out autoradiographic studies as part of the present study, it was necessary to vary the parameters in order to obtain the optimum experimental conditions.

#### 2.1.1 Preparation of rat brains for autoradiography

Male Wistar rats (250-310g) were killed by decapitation. The brains were quickly removed and frozen by immersion in isopentane cooled to about -30°C in liquid nitrogen. 20µm thick sections were then cut on a cryostat at -20°C, thaw mounted onto gelatin-coated slides, and stored at -20°C until use.

#### 2.1.2 Optimising conditions

The conditions described by Martel et al. (1986) were used as a starting point for the assay, and the following protocol was devised.

Sections were pre-incubated for 60 min at room temperature in Krebs-Ringers buffer (NaCl (118mM),  $\text{KH}_2\text{PO}_4$  (1.18mM), KCl (4.7mM),  $\text{NaHCO}_3$  (25mM),  $\text{MgSO}_4$  (4.7mM),  $\text{CaCl}_2$  (2.5mM) and glucose (5.55mM)) at pH 7.4 in order to wash out any endogenous ligands from the section, and then allowed to dry. They were then incubated for 120 min with 100 $\mu\text{l}$ /section of the same buffer solution containing 0.1% bovine serum albumin, 0.05% bacitracin and 0.1nM [ $^{125}\text{I}$ ] Bolton Hunter NPY ([ $^{125}\text{I}$ ]-BH NPY -2200Ci/mmol, NEN). Specifically bound [ $^{125}\text{I}$ ]-BH NPY was determined by the difference in binding seen in the absence and presence of 1 $\mu\text{M}$  NPY in the incubation solution. At the end of the incubation period, excess buffer was removed and the sections washed by sequential transfer through 4 rinses (4 min each) of buffer, and finally dipped in distilled water to remove ions. Incubated sections were then dried under a stream of cold air.

For the purposes of optimising conditions, the brain sections were wiped off the slide using damp filter papers, and placed in vials. The amount of radioactivity bound to the section was then measured by a gamma radiation counter.

The parameters varied to achieve optimum conditions were wash time, wash temperature, displacer concentration, incubation time and pre-incubation time. Only one variable was changed in each experiment, and the newly found optimum condition of that variable was included in the subsequent experiment.

### 2.1.3 Optimum conditions

The optimum conditions as determined by the described experiments were then used for mapping out the NPY receptor localization throughout the brain.

Pre-incubation was reduced to 30 min in Krebs-Ringer buffer with GTP (200 $\mu$ M) followed by a 5 min wash in Krebs-Ringer buffer alone. The incubation time was reduced to 60 min with 1 $\mu$ M NPY as the displacer for the non-specific sections. The sections were washed for a 3x5 min period in Krebs-Ringer buffer at room temperature before drying under a stream of cold air (see Figures 3.1.1-3.1.6).

### 2.1.4 Visualising receptor binding sites

After drying, the sections were juxtaposed against radiation sensitive film (Ultrafilm, Amersham), along with a [<sup>125</sup>I] microscale standard (Amersham).

Following a 3 day exposure period, the film was developed using Kodak D-19 at room temperature for 4 min, and after a 1 min wash was fixed for 5 min. The resultant films were then analysed quantitatively for receptor density using a Quantimet 970 image analyser (Cambridge Instruments).

## Part II : Receptor subtype autoradiography

### 2.2 Ligands used for receptor subtype autoradiography

For the purpose of this study, the parameters for the assay were similar to those used in the previous study (Chapter 2, Part I). However, the [<sup>125</sup>I]-BH NPY was replaced by [<sup>125</sup>I]-PYY (2200 Ci/mmol; NEN). The same concentration of labelled PYY was used as it mimicks the effects of NPY in most model systems with a similar potency to NPY. Walker and Miller (1988) used rat brain membranes to determine the K<sub>d</sub> values for both [<sup>125</sup>I]-NPY and [<sup>125</sup>I]-PYY. For each ligand they found both high and moderately high affinity binding sites. At the high affinity binding sites, the K<sub>d</sub> values for [<sup>125</sup>I]-NPY and [<sup>125</sup>I]-PYY were 54.0 ± 32.6pM and 23.5 ± 6.5pM respectively. At the moderately high affinity binding sites, the K<sub>d</sub> values were 0.92 ± 0.34pM and 1.9 ± 0.66pM respectively. It is, however, difficult to draw any definitive conclusions from this data. This is because all the K<sub>d</sub> values have a large standard error of the mean and the sample sizes are small (n=3). PYY binds to Y<sub>1</sub> and Y<sub>2</sub> receptor subtypes, but not to Y<sub>3</sub> receptors (Nakajima et al.,1986; Harfstrand et al.,1989; Grundemar et al.,1991a;1991b). The K<sub>i</sub> values for the displacement of [<sup>125</sup>I]-BH NPY and [<sup>125</sup>I]-PYY by unlabelled NPY are approximately 0.36nM and 0.35nM respectively (calculated from data supplied by Lynch et al. (1989) using the Cheng-Prusoff equation). Thus 1μM NPY was also used in this study to determine the non-specific binding of [<sup>125</sup>I]-PYY. This also allowed the

displacement to be achieved using a different ligand to that used for the label, a far better method of obtaining non-specific binding.

$Y_1$  and  $Y_2$  receptor binding sites were highlighted by displacement with  $1\mu\text{M}$  NPY (13-36) and [ $\text{Leu}^{31},\text{Pro}^{34}$ ] NPY, respectively. NPY (13-36) is a  $Y_2$  receptor ligand (Wahlestedt et al.,1986;1987a), and so it will displace labelled ligand from  $Y_2$  receptors leaving label bound to  $Y_1$  receptors. [ $\text{Leu}^{31},\text{Pro}^{34}$ ] NPY does the reverse, displacing ligand from the  $Y_1$  receptor subtype and thus leaving label bound to  $Y_2$  receptors. The remainder of the protocol was the same as for Chapter 2, Part I.

**Part III: Localization of neuronal activity mediated by NPY in rat brain: the use of Fos-like immunoreactivity as an anatomical marker of peptide receptor activation.**

2.3.1 Method of anaesthesia

Because we were investigating the effects of NPY in the brain, and it is unable to cross the blood brain barrier, it was necessary to administer the drug directly into the brain by injecting into the right lateral ventricle. This method of drug administration required the implantation of cannulae into rat brains using a surgical technique. Anaesthesia was brought about by the i.p. administration of chloral hydrate (400mg/kg in distilled H<sub>2</sub>O vehicle).

2.3.2 Surgery

Male Wistar rats (Harlan Olac) in the weight range of 250-310g were anaesthetised and their heads shaved. The animals were then placed in a Kopf stereotaxic frame with the tooth bar adjusted for the flat skull position. An incision was made in the skin of the head, and the tissue surrounding the skull was scraped away using a scalpel. The stereotaxic position for the placement of a guide cannula was determined in relation to the relevant skull

Brain Region Injected	Coordinates P / L / V	Injection Depth	Volume Injected	Pretreatment Time
<u>Fos studies:</u>				
Intracerebro-ventricular (i.c.v.)	0.8/-1.5/2.6	3.6mm	1µl	3 h #
<u>Behavioural studies:</u>				
Intracerebro-ventricular (i.c.v.)	0.8/-1.5/2.6	3.6mm	1µl	1 h # <sup>1</sup>
Ventral Hippocampus	5.8/-5.4/5.5	6.5mm	0.5µl	30min # <sup>1</sup>

**Table 2.1 The stereotaxic coordinates used for each brain area studied, the injection dynamics involved and the pretreatment times allowed.** Stereotaxic coordinates were based on the Paxinos and Watson rat brain atlas (1986). All coordinates relate to the position relative to the skull reference point bregma. P: posterior to bregma, L: lateral to bregma, V: ventral to the dura. # and #<sup>1</sup> for an explanation of these pretreatment times refer to sections 2.3.3 and 2.4.3 respectively.

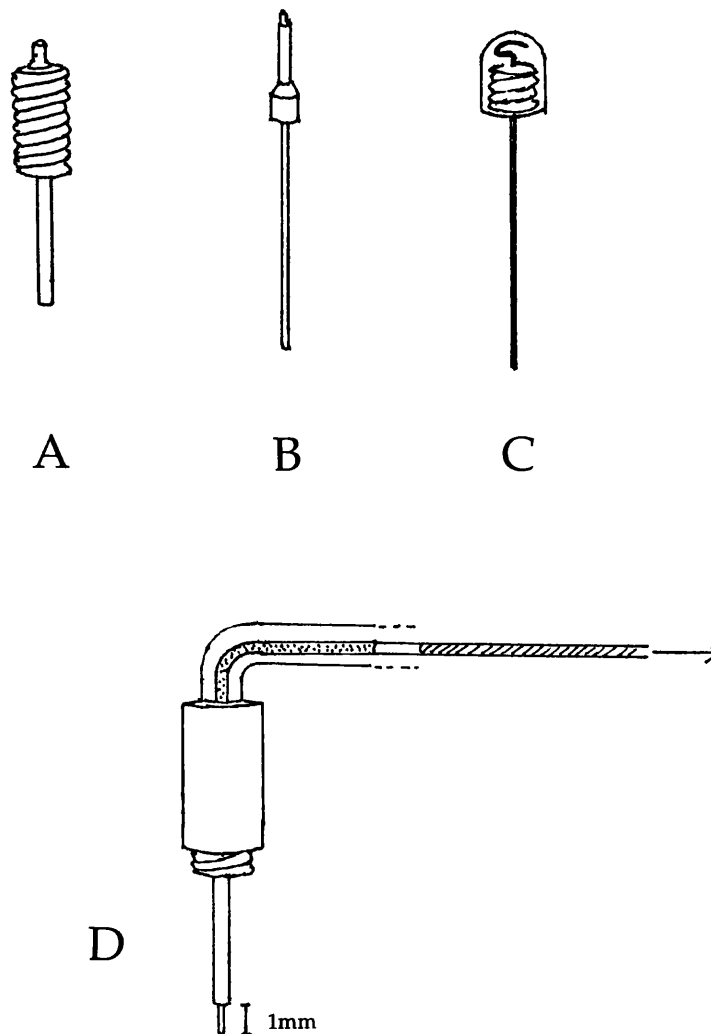
bregma and a dot of ink used to mark this position on the skull surface (Table 2.1). Three holes were drilled with a dental drill for the attachment of screws to the skull surface. Another hole was drilled at the position marked for the guide cannula. A chronic guide cannula (22 gauge:Plastics One Inc.) was then attached to the arm of the stereotaxic frame and lowered slowly to the correct depth in the brain. The guide cannula was cemented in place using dental acrylic (Sevriton:De Trey), which was allowed to dry before the surrounding skin was secured with stitches. The animals were then removed from the frame and placed under a lamp to keep them warm whilst they recovered. The guide cannula was closed to prevent infection by inserting a dummy cannula (Plastics One Inc.). When the animals were fully conscious they were transferred to individual, single size cages and allowed a free supply of food and water.



During the week following surgery, animals were handled daily to reduce the stress associated with the handling procedure.

### 2.3.3 Intracerebral drug administration

Approximately 7 days following surgery, animals were injected with physiological saline (1µl), with or without 1nmol NPY (Sigma). This was administered via an internal cannula (28 gauge:Plastics One Inc.) inserted into the guide cannula. The internal cannula was 1mm longer than the guide





**Figure 2.1** The various components of the injection system. **A** Guide cannula. **B** Injection cannula. **C** Dummy cannula. **D** Diagram showing the operating of the injection system. The internal injection cannula is inserted into and extends 1mm beyond the tip of the chronically implanted guide cannula. This is connected to the syringe via a connecting tube.  represents solution containing drug,  represents sterile H<sub>2</sub>O. The two solutions are separated by a small air bubble which also aids in visualising the injection process. When not injecting, the guide cannula is closed by inserting the dummy cannula.

cannula (Figure 2.1). The internal cannula was attached to a 50µl Hamilton syringe via a 40cm length of connecting tubing (Plastics One Inc.). The syringe and connecting tube were filled with saline and a small air bubble was then introduced into the internal cannula by drawing air in with the syringe. This was to detect whether the injection was successfully entering the brain. The correct volume of solution to be injected was drawn up into the internal cannula, and inserted into the guide cannula. The injection was then administered under the control of a microinjection pump (CMA/100, Carnegie Medicin), programmed to give the dose over a 5 min period. Animals were unrestrained and in their home cages during the injection process, and during the subsequent 3 h of pretreatment time. The 3 h pretreatment time was selected for two reasons. Firstly, Fos-like immunoreactivity (FLI) reaches a maximum in rat brain sections approximately 2 h following stimulation (conditions optimised in the laboratory of R.A. Leslie, Oxford), and secondly, Heilig et al. (1989) suggest that the anxiolytic-like effects of NPY are maximal 1 h after pretreatment. Thus, combining these two factors suggests that, in looking for FLI resulting from the anxiolytic-like effects of NPY, a 3 h pretreatment time would be optimal.

#### 2.3.4 Preparation of rat brains for Fos immunohistochemistry

Three hours later the rats were re-anaesthetised with sodium pentobarbitone (Euthetal:Rhôme Merrieux) and perfused transcardially, firstly with 100ml

saline, followed by 100ml 4% paraformaldehyde in 0.1M sodium phosphate buffer . The brains were then removed and post-fixed in the same fixative for at least another 24h at 4°C.

100µm brain sections were cut on a vibratome and collected into an 'immunobuffer'(pH7.4), containing NaCl (0.1M), KCl (5mM), Na<sub>2</sub>HPO<sub>4</sub> (8mM), NaH<sub>2</sub>PO<sub>4</sub> (15mM), Tris-HCl (10mM), Triton-X 100 (0.3%) and merthiolate (0.04%). All chemicals were purchased from BDH except merthiolate which was purchased from Sigma. Fos-like immunoreactivity (FLI) was detected in these brain sections using immunohistochemistry.

### 2.3.5 Fos immunohistochemistry

All solutions were made up in 'immunobuffer'(pH 7.4) unless otherwise stated. All incubations and washes took place at room temperature (20-22°C). Sections were pre-incubated in 1% H<sub>2</sub>O<sub>2</sub> for 30 min, washed 3 times in buffer and left for a further 30 min in 2% normal rabbit serum (Vector labs). Sections were then incubated for 3 days in a polyclonal primary antiserum (dilution 1:2000), raised in sheep against residues 2-16 of the N-terminal region of the Fos molecule (Cambridge Research Biochemicals). After a further 3 washes the sections were incubated in biotinylated rabbit anti-sheep secondary antiserum (1:200, Vector labs) for 24h. Sections were then washed a further three times and incubated in avidin-biotin complex (Vector labs) for 2h. After a final 3

washes in 50mM Tris buffer (pH 7.4) the sections were incubated in diaminobenzidine (0.05% in 0.1M phosphate buffer pH 7.4) for 10 min. H<sub>2</sub>O<sub>2</sub> (0.1%) was then added to the diaminobenzidine and once a colour change had occurred (approximately 10min), the sections were washed three times in 50mM Tris-buffer (pH 7.4), mounted on microscope slides and allowed to dry. Once dry they were dehydrated in absolute alcohol and defatted in HistoClear (10min each). They were then mounted with Styrolite mounting medium and covered with a coverslip ready for investigation.

#### 2.3.6 Quantification of results

Quantification of cells showing FLI was performed by counting the number of immunoreactive cells seen in a light microscope (Leitz) equipped with a circular grid patterned ocular and a 10X bright field objective. Any cell containing an amount of brown immunoreactive staining was counted if it was on or near the plane of focus. The size of the circular brain section area surveyed was approximately 0.03mm<sup>2</sup>. One field was viewed per section, with one section being viewed per animal. Differences in FLI levels between peptide treated and control brains were analyzed using the Mann-Whitney Test. Results were considered significant at  $p < 0.05$ .

## Part IV: Behavioural Studies

### 2.4.1 Anaesthesia and surgery

Male Wistar rats in the weight range of 250-310g were anaesthetised using medetomidine (0.03ml/100g,i.m.; Domital, FKB animal health), followed by fentanyl (0.6ml/100g,i.p.; Sublimaze, Janssen Pharmaceuticals, Belgium). Surgery was performed as in section 2.3.2. After surgery was completed and the animal removed from the frame, the anaesthesia was reversed using a mixture of atipamezole (0.02ml/100g; Antisedan, FKB animal health) and nalbuphine (0.02ml/100g; Dupont) i.m.. This method of anaesthesia was considered of much greater benefit to both the animal and experimenter. Within five min of the reversal being administered the animals were awake and walking around. Animals were then transferred to individual, single size cages and allowed free access to food and water. Animals were additionally given a mixture of soft food pellets (pellets soaked in water) and sliced fresh fruit to aid recovery.

During the week following surgery, animals were handled, and weighed daily. Only animals which had regained their initial body weight by the time of the first experiment were used.

#### 2.4.2 Protocol for animal re-use

Animals were re-used for three different models testing for anxiety. The animals were first tested in the elevated x-maze, 6-8 days after surgery. On days 11-13 after surgery the animals were tested in the social interaction model. Finally, on days 22-24 the animals were tested in the Vogel apparatus.

#### 2.4.3 Method of intracerebral injection

The method for administering the intracerebral microinjections was as for section 2.3.3. For volumes of injections used see Table 2.1. The volume of injection was administered over a 1 min period, the injection cannula being left in place for a further 1 min to ensure that the drug had diffused away. A 1 h pretreatment time was allowed for drugs administered i.c.v.. This was in line with the suggested time course of action for NPY (Heilig et al., 1989). Microinjection into the hippocampus was given 0.5 h prior to behavioural testing to take into account that the effects of the drug should develop much quicker when given directly into a discrete brain region.

#### 2.4.4 Drug usage

All drugs used were peptides specific for all or different subtypes of NPY

receptors. All peptides were dissolved in a sterile distilled water before being made up to the correct volume with sterile saline. This was necessary to prevent the peptides from denaturing due to the formation of salts with saline. The solutions were then frozen in aliquots until used. NPY, PYY, NPY (13-36) and [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY were obtained from Bachem UK.

#### 2.4.5 Elevated x-maze

##### 2.4.5a Apparatus

The x-maze (also known as the +-maze) consisted of two open, and two enclosed arms (with an open roof), arranged so that the two open arms were opposite each other (Fig.2.2). The arms were 45cm long and 15 cm wide, the walls of the closed arms being 15cm high. Two lines were marked on the floor of each arm, one at the entrance to the arm and end at the midpoint of each arm. The maze was elevated to a height of 70 cm (see Fig 2.2). A video camera was placed in the same room as the maze so that the animals' activity on the maze could be seen by the observer in an adjacent room.

##### 2.4.5b Procedure

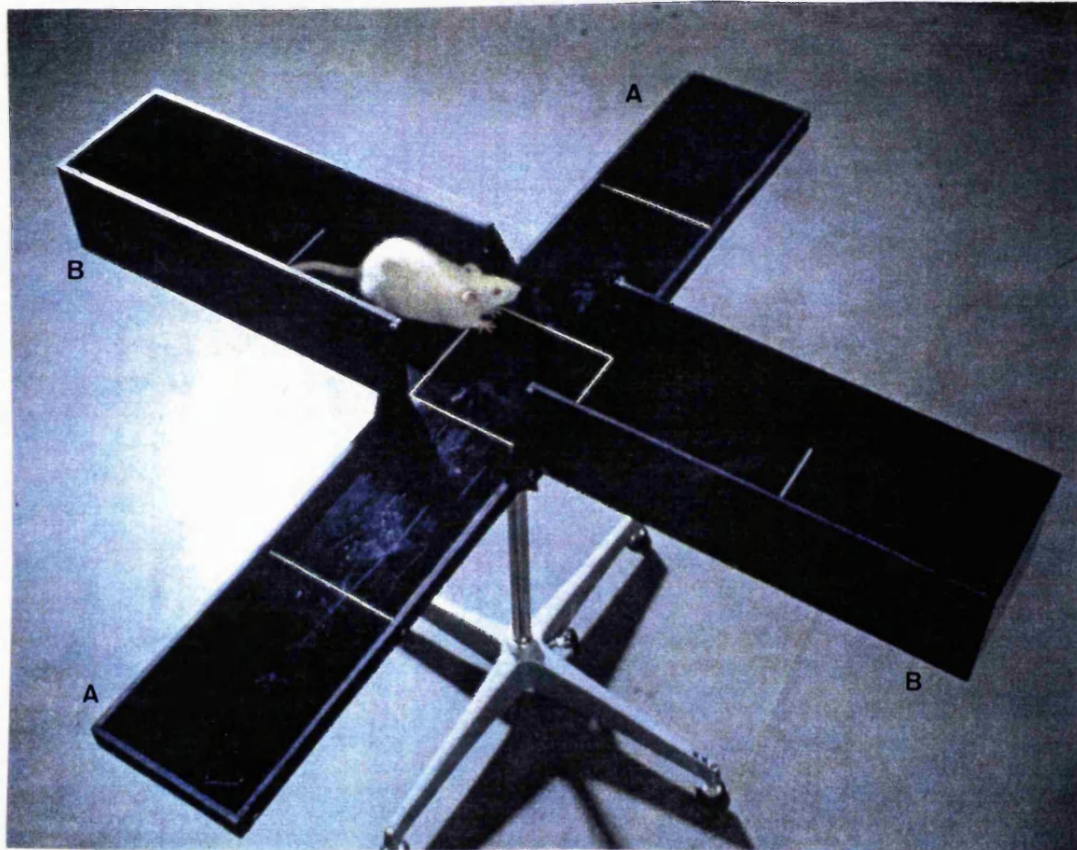
Following pretreatment with the appropriate agent (for pretreatment times and

volumes see Table 2.1), animals were placed on the centre of the maze facing a closed arm. The number of entries into either open or closed arms was measured as was the time spent in each of these arms. Entry into an arm was taken as having all four feet crossing the line at the entrance to the arm. As well as these measurements, the number of entries and time spent in the end of the open arms, taken as all four feet crossing the line at the midway point of the open arms, was measured. Finally, the overall activity of the animals was measured as the total number of line crossings to give some indication of a drug's sedative or stimulant effects. Animals were tested over a 5min period after which time they were returned to their home cages and the maze cleaned to remove any scent that may be on the maze, and which in turn might influence other animals.

#### 2.4.5c Validation of x-maze

In order to validate the test, the effects of the anxiolytic compound, chlordiazepoxide hydrochloride (CDP; 2.5mg/kg p.o. dissolved in water), was tested in the model. Oral administration was used for validation as the oral doses that are required to exhibit an effect were already established in this laboratory. Also, the pretreatment times were identical to those to be used for i.c.v. administration of NPY. Ideally, however, the model should have been validated by i.c.v. administration of CDP.





**Figure 2.2** Photograph showing the layout of an elevated x-maze. Open arms and closed arms are marked **A** and **B** respectively, each arm being 45cm in length and 15cm in width. The walls are clearly seen around the closed arms of the maze and are 15cm in height. The entire maze is elevated 70cm above the ground.

## 2.4.6 Social interaction test

### 2.4.6a General test procedure

It is normal practice to house all animals to be used in the social interaction test individually for at least 3 days prior to the test. The rats used in this experiment had been housed individually since surgery, a period of 11-13 days. The animals were housed in dimly lit conditions.

The animals were weighed and allocated into weight-matched pairs ( $\pm 20\text{g}$ ). Rats were not allocated to a test pair if they had been housed adjacent to one another. Each animal of a test pair received the same drug treatment at the specified pretreatment time.

The test was started by placing each member of a pair of rats in opposite corners of the test arena and then leaving them for 15 min whilst recording their behaviour on videotape, and scoring their social interactions. The total time spent in social interaction was scored for each rat by measuring the social behaviours outlined in Table 2.2. Locomotor activity was measured by line crossings of a matrix drawn on the floor of the test arena.

---

Name of interaction	Description of interaction
<b>1: Participation of single rat (A) required only.</b>	
ALLOGROOM	A grooms B
CLIMB OVER/ CRAWL UNDER	A crawls under or climbs over B which displays no response to this behaviour
FOLLOW	A follows B in close attendance
SNIFFING	A investigates by sniffing in any region of B other than the anogenital or skull cap regions of B
<b>2: Participation of both rats required.</b>	
BOXING	Both A and B stand on their hind legs facing each other with their forelimbs extended.
WRESTLING	Both rats involved in a variety of behaviours including crawling under/over, tumbling and various posturing.

---

**Table 2.2** The types of behaviour classed as social interaction for the purposes of the social interaction model of anxiety. A and B refers to each of a pair of animals in the test arena.

#### 2.4.6b Validation of test procedure

In order to validate this model for the detection of anxiolytic-like activity, social interaction was scored in animals, previously implanted with an intraventricular cannula, 1 h following administration of CDP (2.5mg/kg p.o., dissolved in water). Behaviour was scored in exactly the same way as described in paragraph 2.4.6a. For comment on route of administration see section 2.4.5c.

#### 2.4.7 Vogel's drinking conflict test

##### 2.4.7a Apparatus

The apparatus and procedure used for this model were based on previous studies by Vogel et al. (1971). The test chamber was a metal box, with a floor consisting of metal bars. A drinking spout protruded from one wall, and the chamber was illuminated by a small bulb in the roof. The complete apparatus was enclosed in a sound attenuated chamber.

##### 2.4.7b General test procedure

Two days before the Vogel test, animals were water deprived for 18 h

overnight in order to allow the animals to be familiarised to the test chamber on the day before the actual test. The day before the test, the animals were placed individually in the test chambers and allowed to drink, unpunished. After having drunk for a continuous three sec, the animals were allowed to drink for a further 3 min before being placed back into their home cages. They were then allowed to drink freely in their home cages for about 4 h before being water deprived again overnight. On the day of the test, animals were pretreated with the various treatments at the specified time before testing. To test for anxiolytic effects, animals were placed in the test chambers and allowed to drink continually for 3 sec, after which time an electric shock (2.5mA, 0.2s) was applied between the water spout and the cage floor. Thereafter the animals were shocked every further 5 sec of cumulative drinking time for a 3 min period. The total number of shocks accepted during the test was recorded automatically.

#### 2.4.7c Validation of test procedure

In order to validate this test for its use as a model of anxiety, animals, previously implanted with an intraventricular cannula were tested 1 h following administration of CDP (2.5mg/kg p.o., dissolved in water). For discussion on route of administration see section 2.4.5c.

#### 2.4.7d Effects on thirst

This test relies on the animals thirst to drive them into a conflict between drinking and a noxious electrical stimulus. It is important therefore to test for any effect of NPY on thirst in this apparatus. This was studied as for the complete Vogels' test, except that the stimulators were switched off, and the time spent drinking during a 10 min period was measured.

#### 2.4.7e Effects on shock threshold

In order to test for any effects of NPY on the sensitivity of the animals to the electrical stimulus, the animals were treated as before, but instead of being tested on in the normal Vogel model, an alternative protocol was devised to find the shock threshold of each animal. The animals were placed in the Vogel apparatus and the shockers were set to manual control. Whilst drinking, the electrical stimulus was applied through the water spout in increments (commencing 0.2mA; 0.2s pulse duration; 0.1mA step, 10s between subsequent stimuli) and the shock threshold for several stages of response determined. The stages used were the stimulus required to stop the animal drinking, to cause a head movement away from the spout, or to cause a body movement away from the spout. To confirm each shock threshold, the stimulus strength was brought down below the threshold in a step-wise manner, and then increased again.

#### 2.4.8 Validation of cannula placement

In order to check that an animal had received the intracerebral injection at the correct site within the brain, animals were injected as for the experiments, but with diluted china ink. They were then sacrificed (after a time period corresponding to the pretreatment for that area) and their brain removed. For animals who had received i.c.v. injections, the brains were then cut with a razor to check for the spread of dye throughout the ventricular system. The brains of animals that were injected elsewhere were cut into a smaller thickness segment surrounding the cannula wound, and placed in a 4% paraformaldehyde solution. This was left to fix for at least 48 h at 5°C. Brains were then frozen by immersion in isopentane at -30°C and cut at 20µm sections. These were thaw mounted on gelatin coated slides and the sections stained with cresyl violet. The placement of the cannula could then be seen by the injection site of the dye when viewed under a microscope.

#### 2.4.9 Statistical analysis of data

When data which were to be compared consisted of only the control and a treatment group, a Student's t-test was used. When data for multiple treatments were compared, standard parametric ANOVA was performed. When ANOVA showed the data to be significant, multiple comparisons of the treatment groups versus control group were carried out using Dunnet's test.

**CHAPTER 3**  
**RESULTS PART I & II**  
**NPY RECEPTOR AUTORADIOGRAPHY**



### 3.1 Part I: Neuropeptide Y receptor autoradiography

When looking at the role of an endogenous ligand on CNS function, it is important to have some knowledge of where the ligand may be having its effects in the brain. One way of obtaining some idea of this is by mapping out the regions of the brain which contain receptor binding sites for the ligand in question. The technique used for this purpose is known as receptor autoradiography, and is widely used with all sorts of ligands. The overall picture of binding sites is obtained by incubating brain sections with radioligand and then, after washing, placing the sections against a film that is sensitive to the radiation given off by the ligand which is bound to the receptor binding sites. Non-specific binding to the section is determined by displacing the radioligand from the binding sites with a 'cold' (unlabelled) ligand for the same receptors. In this way, it is possible to obtain an accurate picture of exactly where the receptors for the ligand are situated in the brain, and therefore where it may be having its effects.

#### 3.1.1 Optimising conditions

All the optimisation experiments show the results of single experiments conducted in triplicates.

The concentration of [<sup>125</sup>I]-BH NPY used for these studies (0.1nM) was taken

from previous studies by Martel et al. (1986) and Aicher et al. (1991).

(i) Final wash time.

Experiments where the duration of the final wash was varied showed that at room temperature the highest specific binding was obtained after the 8 and 12 min wash duration (Figure 3.1.1a). The greatest percentage specific binding was obtained after the 12 min and this was considered the optimum wash time at room temperature (Figure 3.1.1b). When the wash was performed at 0°C (the incubation being at room temperature) the level of specific binding remained fairly constant over 60 min (Figure 3.1.2a). This suggests that the dissociation of the specific binding is temperature dependent, a feature of receptor binding. However when compared to the total binding, the resultant percentage specific binding increased throughout the experiment and had not peaked even after 60 min.(Figure 3.1.2b) although continuation past 60 min would probably have shown that the dissociation of the bound ligand at 0°C was a slowed down version of the dissociation curve at room temperature. This is probably due to the dissociation of the non-specific binding not being temperature dependent. Since the percentage specific binding would determine the clarity of an autoradiograph, the 0°C wash temperature at 60 min gives the same results as 12 min at room temperature, and on comparing the autoradiographs of sections washed in this way (not shown), the longer wash period at 0°C caused damage to these sections and thus the optimum conditions for washing were

taken as 12 min at room temperature.

(ii) Displacing concentration

The effect of varying the concentration of NPY used to displace the specifically bound [<sup>125</sup>I]-BH NPY from the sections (Figure 3.1.3) showed that the optimal specific binding was obtained with a displacing concentration of 1μM NPY. Incubation time experiments showed that the optimal incubation time to obtain the greatest specific binding levels was 60 min (Figure 3.1.4).

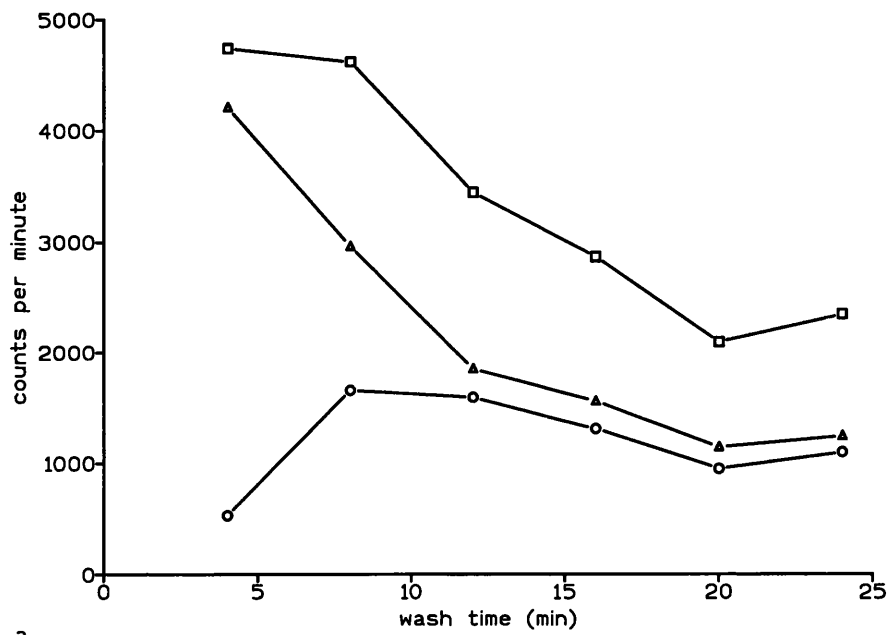
(iii) Effects of GTP

Finally the effects of GTP(200μM) in both the pre-incubation medium and incubation medium were studied. GTP added to the pre-incubation wash caused an increase in total and specific but a net decrease in non-specific binding (Figure 3.1.5). This may be due to a G-protein mechanism, where binding of GTP might cause the receptor to enter a low affinity state. This might in turn cause the receptors to release any bound endogenous ligand, and thus leaving more receptor binding sites unoccupied and therefore 'available' to bind [<sup>125</sup>I]-BH NPY at the later incubation stage.

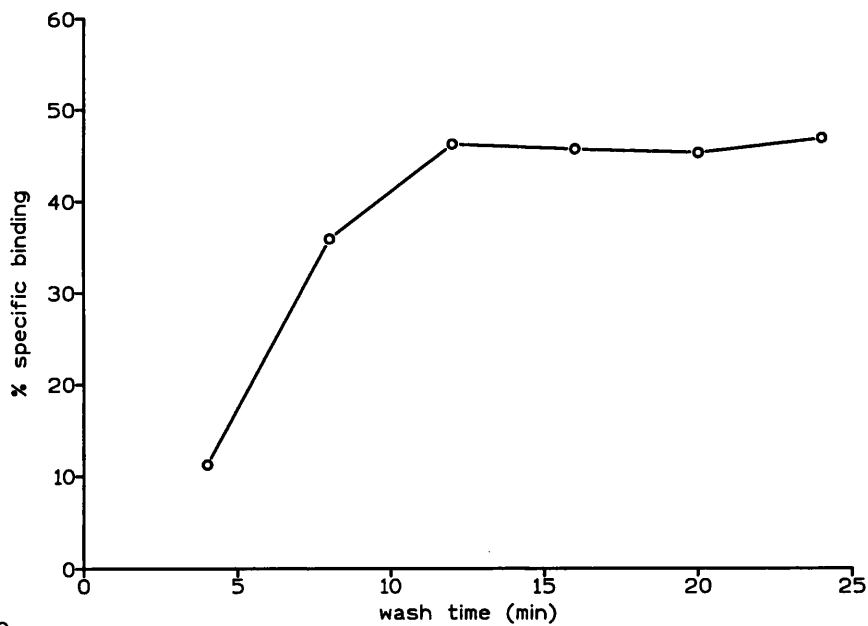
GTP added to the incubation medium had the opposite effects with much less

specific binding resulting (Figure 3.1.6). Again, if a G-protein binds the GTP, it may be changed to its low affinity state and therefore will bind much less [<sup>125</sup>I]-BH NPY.

Thus using these experiments the optimal conditions were obtained as being a pre-incubation of 30min in Krebs-Ringers buffer containing GTP(200μM) and a 5min wash in Krebs-Ringers buffer. These sections were then dipped into distilled water to remove any ions. A 60 min incubation time was then used followed by 3x4min washes in Krebs-Ringer buffer at room temperature.

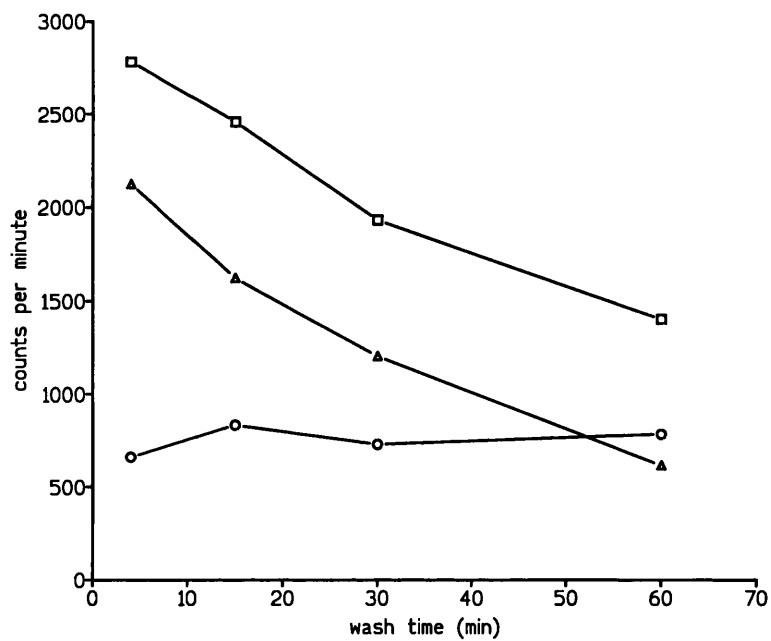


a

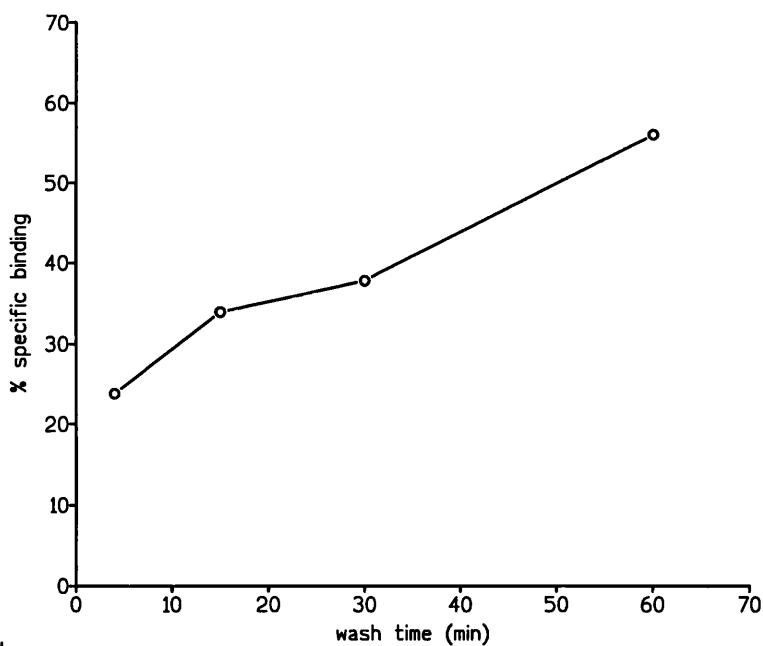


b

**Fig.3.1.1 a,b.** The effects of wash time at room temperature on the binding of [ $^{125}$ I]-BH NPY (0.1nM) to rat brain sections. Sections were transferred through the appropriate number of 4min washes in Krebs-Ringers buffer to obtain the total wash times shown. **a** Mean radioactivity counts obtained from whole rat brain sections (representative set of data. n=1, measured in triplicate). Total binding (□) represents binding in the absence of a displacing agent. Non-specific binding (Δ) represents binding in the presence of NPY (1μM) to displace [ $^{125}$ I]-BH NPY from specific binding sites (○). **b** The percentage specific binding showing specifically bound radioactivity as a percentage of the total radioactivity bound.

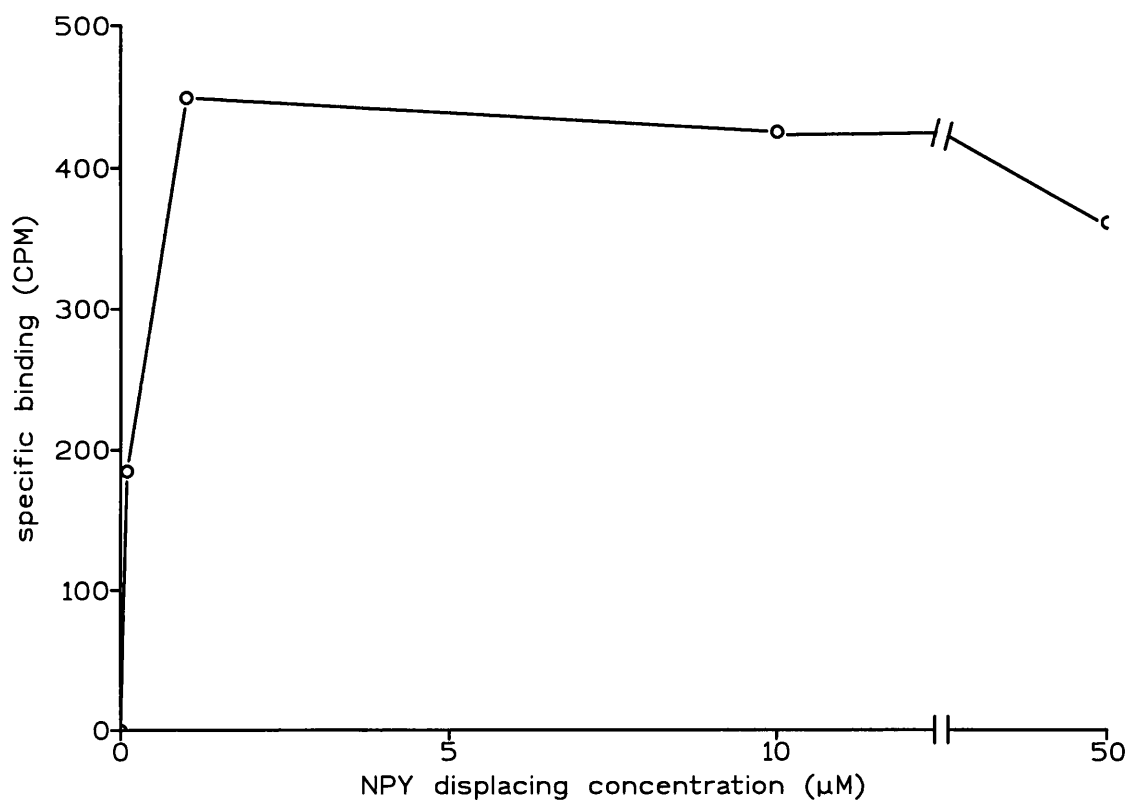


a

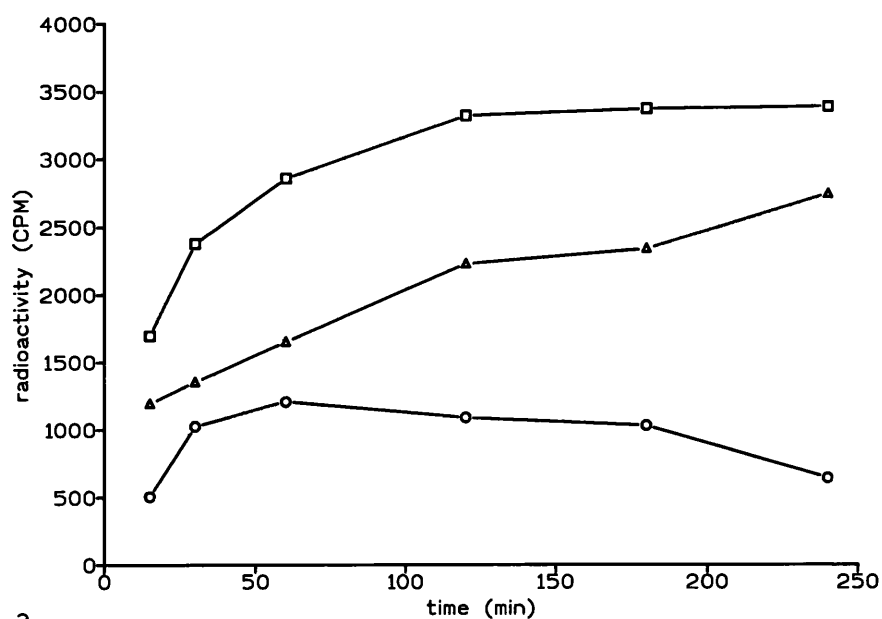


b

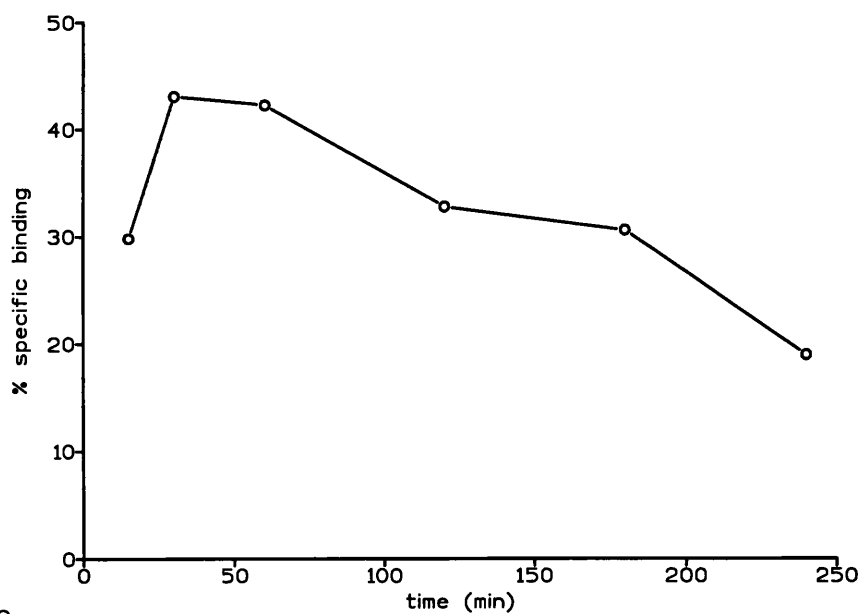
**Fig.3.1.2 a,b.** The effects of wash time at 0°C on the binding of [<sup>125</sup>I]-BH NPY (0.1nM) to rat brain sections. Sections were transferred through the appropriate number of 4min washes in Krebs-Ringers buffer to obtain the total wash times shown. **a** Mean radioactivity counts obtained from whole rat brain sections (representative set of data. n=1, measured in triplicate). Total binding (□) represents binding in the absence of a displacing agent. Non-specific binding (Δ) represents binding in the presence of NPY (1μM) to displace [<sup>125</sup>I]-BH NPY from specific binding sites (○). **b** The percentage specific binding showing specifically bound radioactivity as a percentage of the total radioactivity bound.



**Fig.3.1.3** The effects of varying the concentration of NPY used to displace specifically bound [<sup>125</sup>I]-BH NPY (0.1nM) from rat brain sections. Values represent the mean radioactivity counts obtained from whole brain sections incubated with the displacing agent (0.1-50μM) (representative set of data. n=1, measured in triplicate).



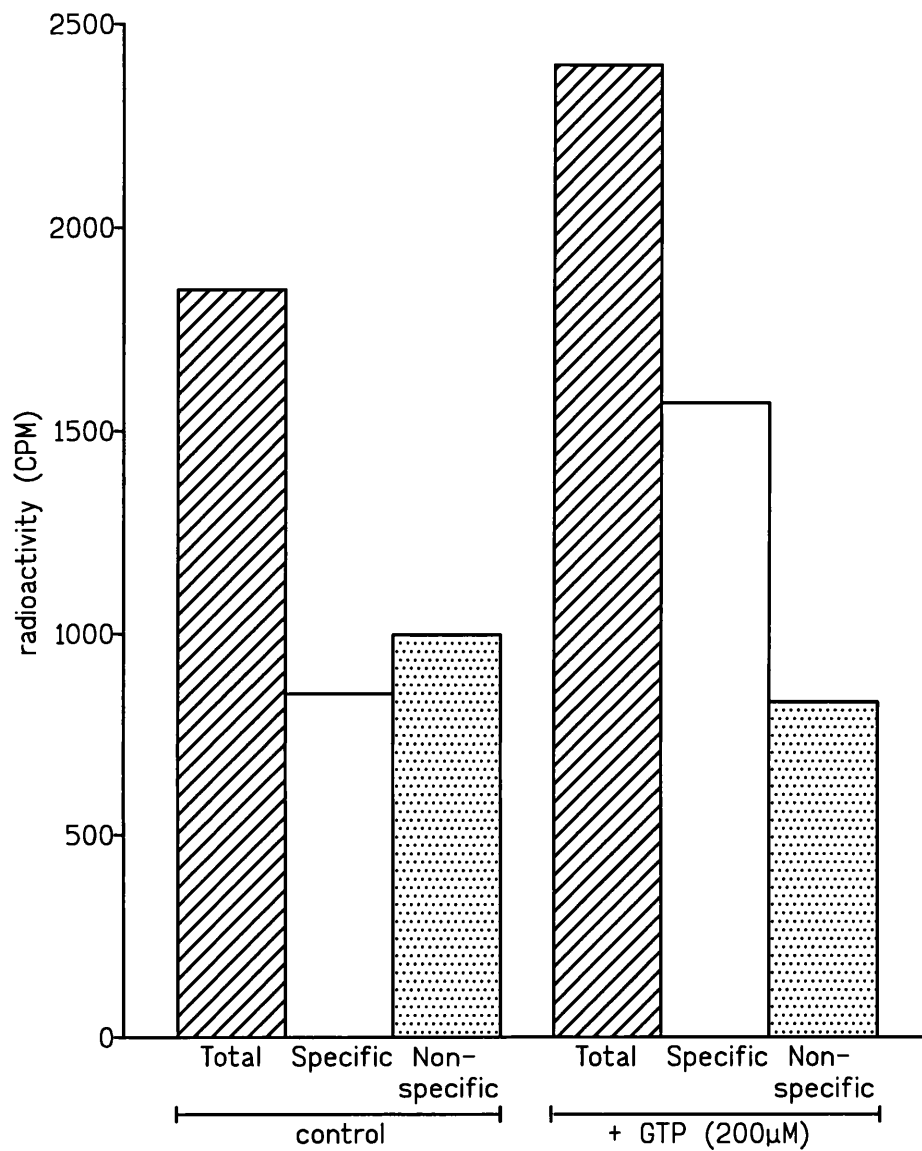
a



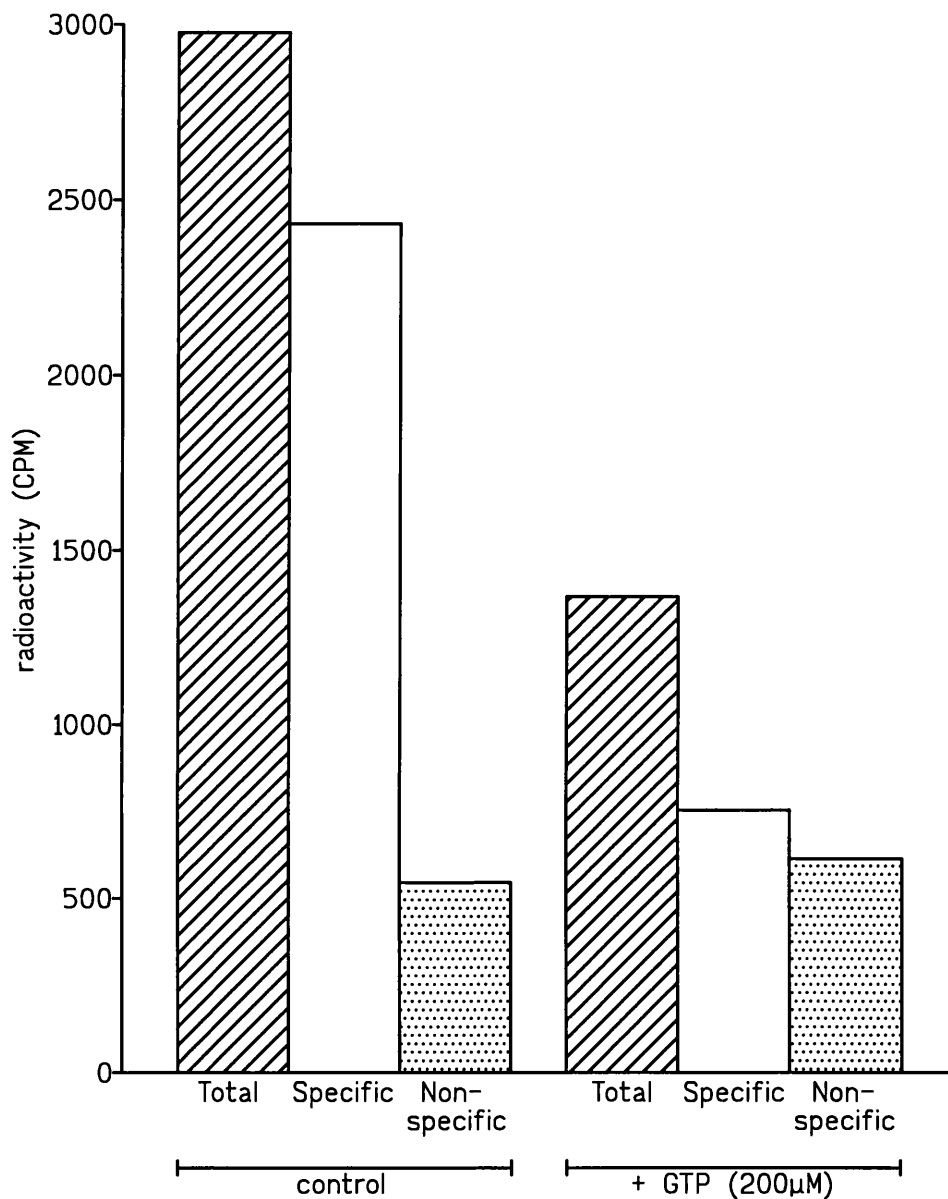
b

**Fig.3.1.4** The effects of incubation time on the binding of [<sup>125</sup>I]-BH NPY (0.1nM) to rat brain sections. **a** Total binding (□) was determined in the absence of a displacing agent. Non-specific binding (Δ) was determined in the presence of NPY (1μM) to displace the radioactivity bound to specific (○) NPY receptor binding sites. **b** The percentage specific binding showing specifically bound radioactivity as a percentage of the total bound radioactivity. Each value is the mean radioactivity counts obtained from triplicate brain sections (n=1).





**Fig.3.1.5** The effect of GTP (200µM) in the pre-incubation wash on the binding of [<sup>125</sup>I]-BH NPY (0.1nM). GTP was added to the same Krebs-Ringers buffer solution as used to pre-incubate the control sections. Values are the mean radioactivity counts obtained from whole rat brain sections (representative set of data. n=1, measured in triplicate). Non-specific binding was determined in the presence of NPY (1µM).



**Fig.3.1.6** The effect of GTP (200 μM) in the incubation medium on the binding of [<sup>125</sup>I]-BH NPY (0.1 nM). GTP was added to the same Krebs-Ringers buffer solution as used to pre-incubate the control sections. Values are the mean radioactivity counts obtained from whole rat brain sections (representative set of data. n=1, measured in triplicate). Non-specific binding was determined in the presence of NPY (1 μM).

### 3.1.2 Neuropeptide Y receptor distribution

The distribution of [<sup>125</sup>I]-BH NPY binding sites, as determined from analysis of serial brain sections by autoradiography, are shown in Figures 3.1.7-3.1.8. The receptor binding site densities in several brain regions are quantified in Figures 3.1.9-3.1.12. Very high densities of NPY binding sites were present in the ventral hippocampus, mainly localised in the stratum oriens and the stratum radiatum (Figure 3.1.7). Other regions which showed very high densities of NPY binding sites are the stria terminalis, claustrum, posteromedial cortical amygdaloid nucleus, the lateral anterior olfactory nucleus (Figure 3.1.7) and the dorsal and intermediate lateral septal nuclei (Figure 3.1.8). High densities of NPY binding sites were found in the dorsal hippocampus, again localised mainly in the oriens layer and the stratum radiatum, in the amygdalohippocampal area (Figure 3.1.7), the substantia nigra pars compacta, the dorsal anterior olfactory nucleus and the ventral lateral septal nucleus (Figure 3.1.8). Moderate levels of NPY binding were seen in the dentate gyrus, the dorsal and ventral medial geniculate nucleus, the anterior cortical amygdaloid nucleus (Figure 3.1.7), the olfactory tubercle, the laterodorsal and lateral posterior thalamic nuclei and, the posterior thalamic nucleus, the zonal layer of the superior colliculus, the ventral anterior olfactory nucleus, the septohypothalamic nucleus (Figure 3.1.8) and layers I and II of several cortical areas (Oc1B and Oc1M) (Figures 3.1.7 & 3.1.8). Low levels of NPY binding sites were found in the subiculum, the dorsal lateral geniculate nucleus, the caudate putamen, the entorhinal and piriform cortex (Figure 3.1.7),

the nucleus accumbens, the central amygdaloid nucleus, the lateral olfactory tract (Figure 3.1.8), several thalamic nuclei including the anteroventral, anterodorsal and laterodorsal thalamic nuclei, the substantia nigra pars reticularis, the optic tract, the dorsal cortex of the inferior colliculus and layers I and II of several cortical regions (frontal cortex areas 1 and 2, and the occipital cortex area 2MM) (Figure 3.1.7). Very low densities were found in the optic tract, reticular and ventrolateral thalamic nuclei, globus pallidus, the cerebellum, inner layers of the cerebrocortex, external cortex and central nucleus of the inferior colliculus and the pontine nucleus.

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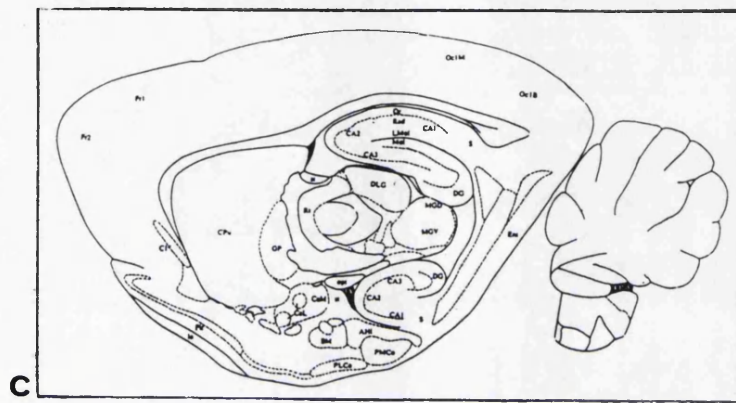
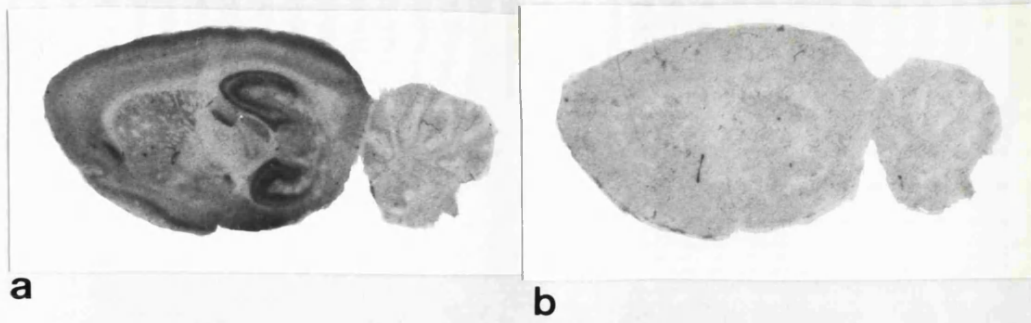
aca	anterior commissure	LSD	lateral septal nu, dorsal
AcbC	accumbens nu, core	LSI	lateral septal nu, intermediate
AcbSh	accumbens nu, shell	LSV	lateral septal nu, ventral
ACo	anterior cortical amygdaloid nu	LV	lateral ventricle
AHiPM	amygdalohip area, posteromed	Me	medial amygdaloid nu
AOB	accessory olfactory bulb	MGD	medial geniculate nu, dorsal
AOD	anterior olfactory nu, dorsal	MGV	medial geniculate nu, ventral
AOE	anterior olfactory nu, external	Mol	molecular layer dentate gyrus
AOL	anterior olfactory nu, lateral	Oc1B	occipital cortex, area 1, binocular
AOP	anterior olfactory nu, posterior	Oc1M	occipital cortex, area 1, monocular
AOV	anterior olfactory nu, ventral	Oc2MM	occipital cortex, area 2, mediomedial
AV	anteroventral thalamic nu	Op	optic nerve layer sup col
BM	basomedial amygdaloid nu	opt	optic tract
CA1-3	field CA1-3 of Ammon's horn	Or	oriens layer hippocampus
CeL	central amygdaloid nu, lateral	OT	nu optic tract
CeM	central amygdaloid nu, medial	PF	parafascicular thalamic nu
CIC	central nu inferior colliculus	PIL	posterior intralaminar thalamic nu
Cl	claustrum	Pir	piriform cortex
CL	centrolateral thalamic nu	PLCo	posterolateral cortical amygdaloid nu
cp	cerebral peduncle, basal	PMCo	posteromedial cortical amygdaloid nu
CPu	caudate putamen	PN	pontine nuclei
DCIC	dorsal cortex inf colliculus	Po	posterior thalamic nuclear group
DG	dentate gyrus	Rad	stratum radiatum hippocampus
DLG	dorsal lateral geniculate nu	Rt	reticular thalamic nu
ECIC	external cortex inf colliculus	S	subiculum
ent	entorhinal cortex	SHy	septohypothalamic nu
Fr1	frontal cortex, area 1	SNC	substantia nigra, compact
Fr2	frontal cortex, area 2	SNL	substantia nigra, lateral
GP	globus pallidus	SNR	substantia nigra, reticular
Gu	gustatory thalamic nu	SO	supraoptic nu
HDB	nu horizontal limb diagonal band	st	stria terminalis
InG	intermediate gray layer sup col	SuG	superficial gray layer sup col
InWh	intermediate white layer sup col	TT	tenia tecta
LC	locus coeruleus	Tu	olfactory tubercle
LD	laterodorsal thalamic nu	VL	ventrolateral thalamic nu
LH	lateral hypothalamic area	VM	ventromedial thalamic nu
LMol	lacunosum moleculare layer hip	VP	ventral pallidum
lo	lateral olfactory tract	VPL	ventral posterolateral thalamic nu
LOT	nu lateral olfactory tract	VPM	ventral posteromedial thalamic nu
LP	lateral posterior thalamic nu	ZI	zona incerta
LPO	lateral preoptic area	Zo	zonal layer superior colliculus

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**Table 3.1 Abbreviations used for brain regions in all Chapter 3 figures**

**Legend for figure 3.1.7(i),(ii) & 3.1.8(i),(ii). Autoradiographs illustrating NPY receptor distribution in the rat brain.** Photographs show a parasagittal brain section 3.9mm (fig.3.1.7(i)), 2.9mm (fig.3.1.7(ii)), 1.9mm (fig.3.1.8(i)) and 1.4mm (fig.3.1.8(ii)) lateral to bregma. The dark areas indicate bound [<sup>125</sup>I]-BH NPY in the absence (a) or presence (b) of 1μM NPY displacing ligand. c Schematic diagram of the above sections showing the position of several brain regions (adapted from *The rat brain in stereotaxic coordinates* (1986), second edition Paxinos and Watson). Abbreviations for brain regions are found in Table 3.1.

(i)



(ii)

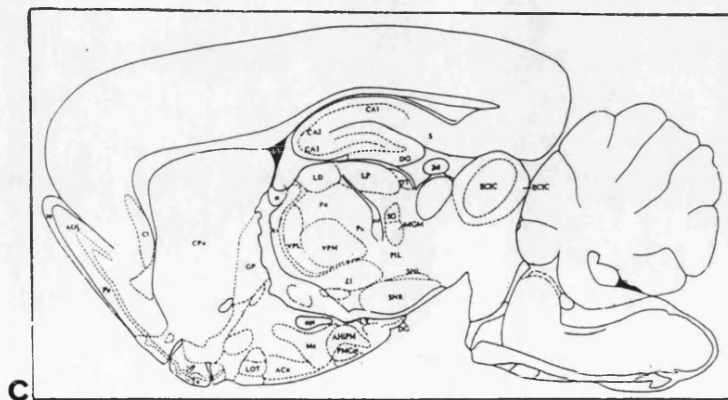
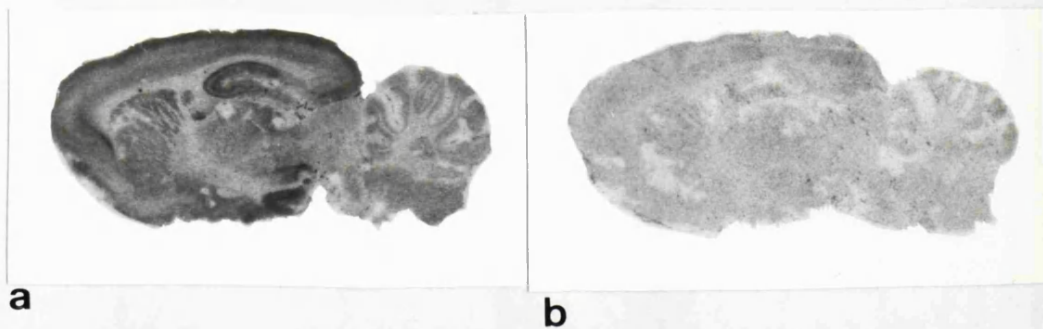
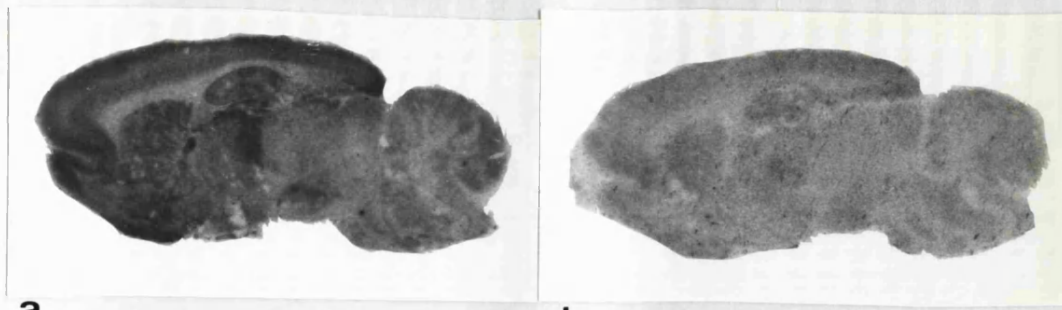


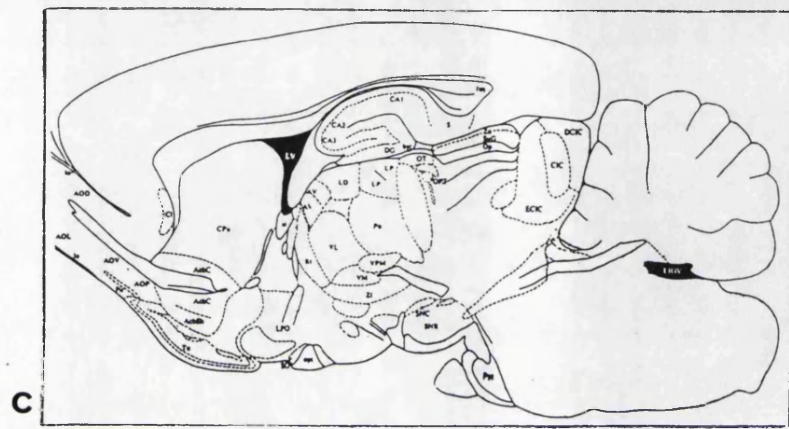
Figure 3.1.7(i) & (ii). (Legend p109)

(i)



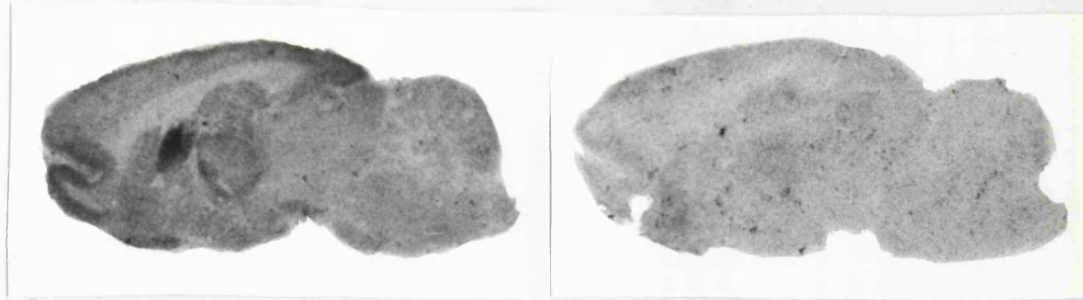
a

b



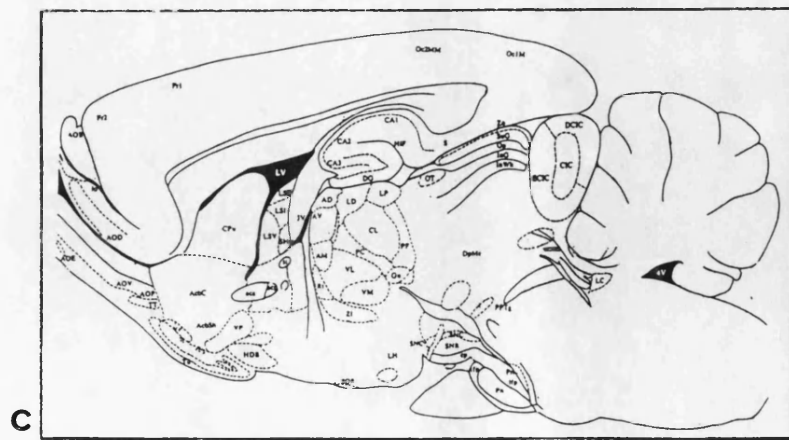
c

(ii)



a

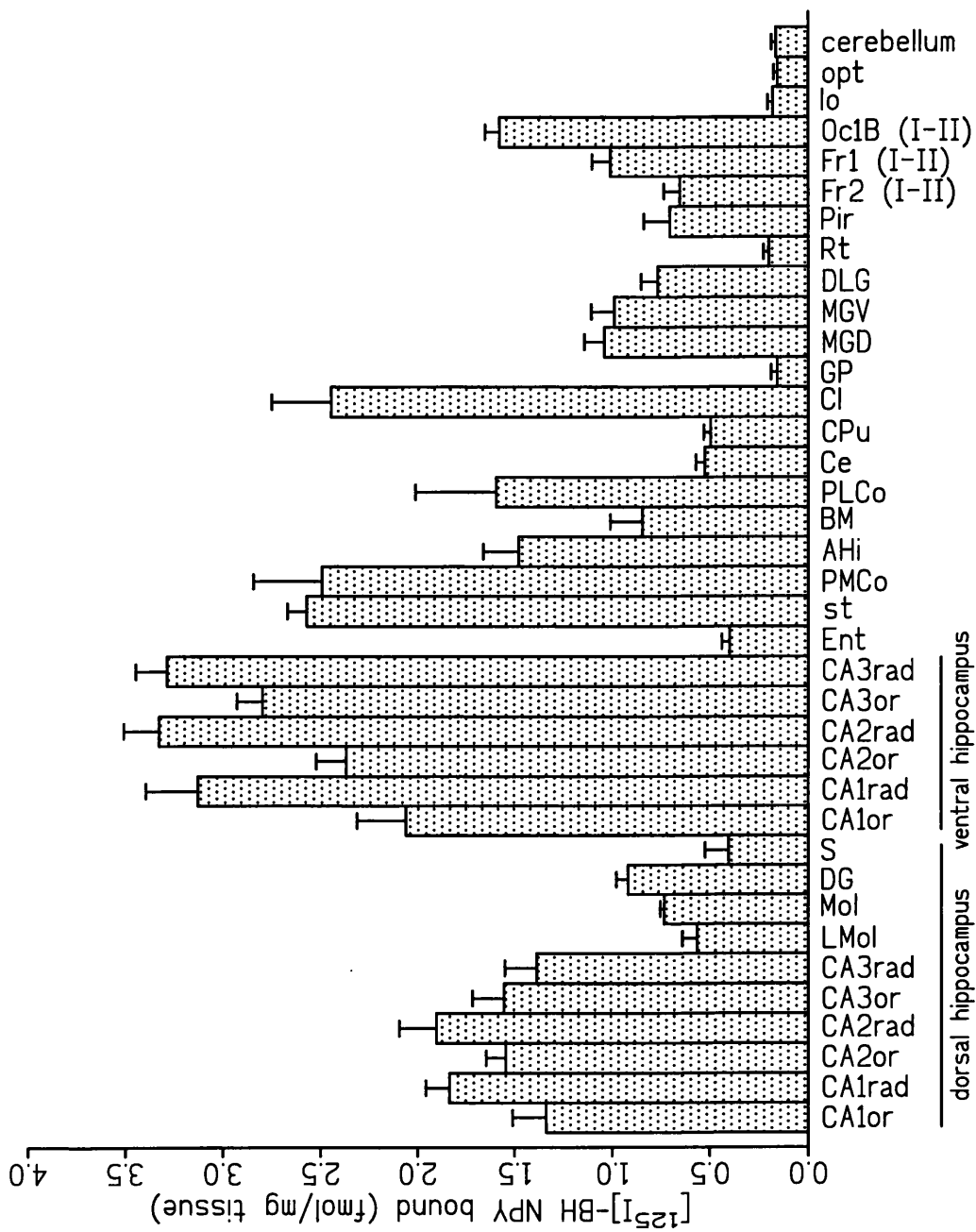
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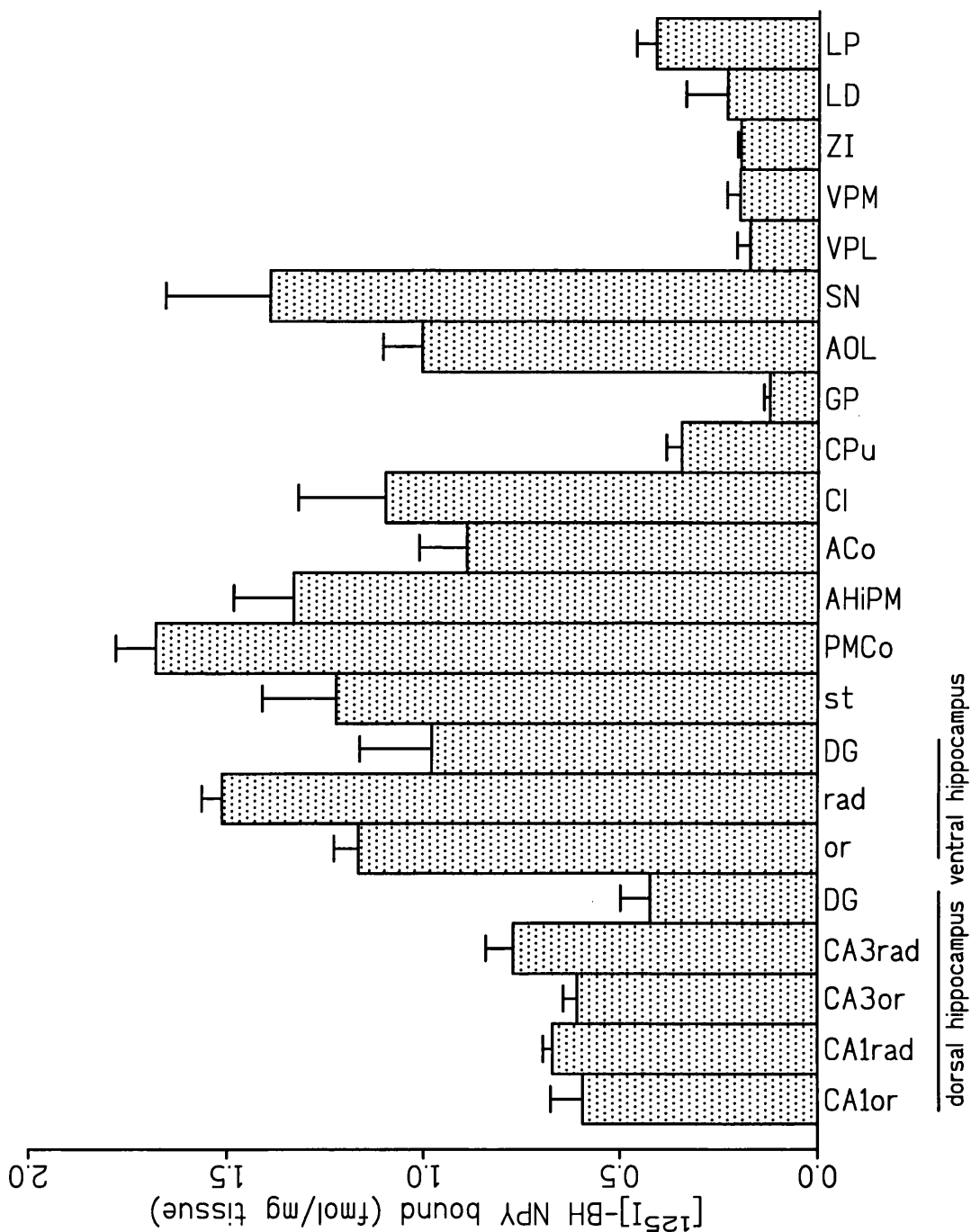
c

Figure 3.1.8(i) & (ii). (Legend p109)

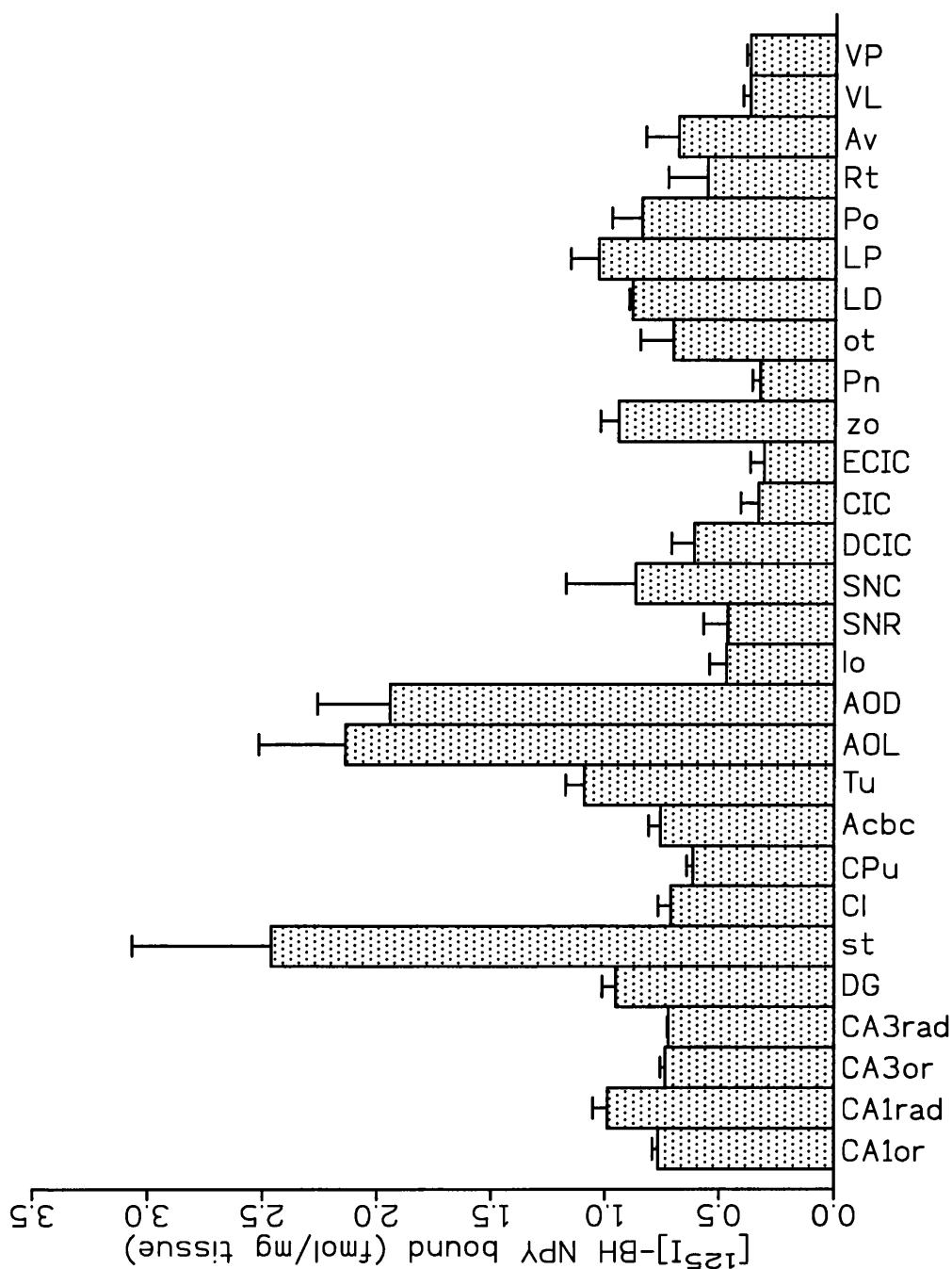




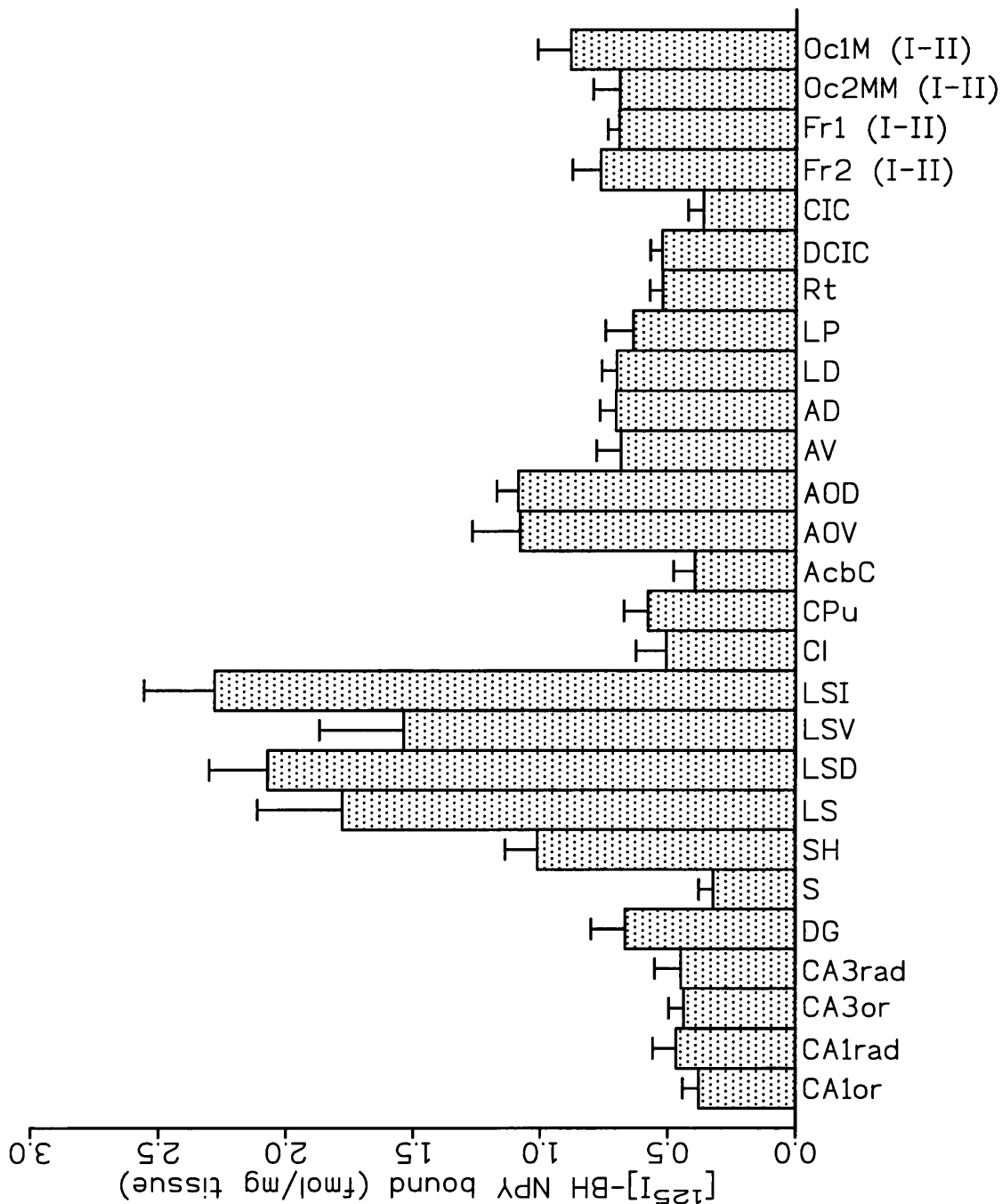
**Figure 3.1.9. NPY receptor distribution in a parasagittal section 3.9mm lateral to bregma.** Values are the mean  $\pm$  s.e.m. receptor density (fmol/mg tissue) calculated from the specific binding of [ $^{125}$ I]-BH NPY (0.1nM) to the section. Specific binding was determined as the difference between the amount of [ $^{125}$ I]-BH NPY bound to the section in the absence and presence of 1 $\mu$ M NPY. For abbreviations see Table 3.1.



**Figure 3.1.10. NPY receptor distribution in a parasagittal section 2.9mm lateral to bregma.** Values are the mean  $\pm$  s.e.m. receptor density (fmol/mg tissue) calculated from the specific binding of [<sup>125</sup>I]-BH NPY (0.1nM) to the section. Specific binding was determined as the difference between the amount of [<sup>125</sup>I]-BH NPY bound to the section in the absence and presence of 1 $\mu$ M NPY. For abbreviations see Table 3.1



**Figure 3.1.11. NPY receptor distribution in a parasagittal section 1.9mm lateral to bregma.** Values are the mean  $\pm$  s.e.m. receptor density (fmol/mg tissue) calculated from the specific binding of [<sup>125</sup>I]-BH NPY (0.1nM) to the section. Specific binding was determined as the difference between the amount of [<sup>125</sup>I]-BH NPY bound to the section in the absence and presence of 1 $\mu$ M NPY. For abbreviations see Table 3.2.



**Figure 3.1.12. NPY receptor distribution in a parasagittal section 1.4mm lateral to bregma.** Values are the mean  $\pm$  s.e.m. receptor density (fmol/mg tissue) calculated from the specific binding of [<sup>125</sup>I]-BH NPY (0.1nM) to the section. Specific binding was determined as the difference between the amount of [<sup>125</sup>I]-BH NPY bound to the section in the absence and presence of 1 $\mu$ M NPY. For abbreviations see Table 3.1.

## 3.2 Part II: receptor subtype autoradiography

Having obtained an overall map of the general distribution of NPY receptor binding sites throughout the rat brain, it was considered desirable to try and differentiate between any specific receptor subtypes that might exist in the rat brain. This was achieved by using the basic assay optimised in Chapter 2 part I, but by using a different selection of ligands to probe for the various subtypes. The concentrations of displacing ligands used were based on those used by Dumont et al. (1990).

### 3.2.1 Neuropeptide Y receptor subtype distribution

The effects of the specific agonists used to displace [<sup>125</sup>I]-PYY binding from rat brain sections are illustrated for Y<sub>1</sub> and Y<sub>2</sub> receptor subtypes in Figure 3.2.1. The quantification of the binding site densities are shown in Table 3.2. Using a cloned human NPY-Y<sub>1</sub> receptor, [<sup>125</sup>I]-PYY binding has been shown to be maximally displaced with 1µM [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY (Herzog et al.,1992). In the human neuroblastoma cell line SK-N-MC, which display Y<sub>1</sub> receptor subtypes, 0.1µM [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY only displaced approximately 85% of [<sup>125</sup>I]-BH NPY binding and had not appeared to have reached a maximum (Fuhlendorff et al.,1990a). Thus to maximally inhibit PYY binding to Y<sub>1</sub> receptor sites, the concentration of [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY required should be 1µM. In the SMS-KAN human neuroblastoma cell lines, which have Y<sub>2</sub> receptor subtypes, labelled

[<sup>125</sup>I]-BH NPY binding was only displaced by about 85% with 0.1nM NPY (13-36), which again had not reached a maximum, suggesting that at least 1μM NPY might be required to maximally displace binding. With the Y<sub>2</sub> receptor agonist NPY (13-36) present, only the Y<sub>1</sub> receptors should be labelled by the [<sup>125</sup>I]-PYY. With the Y<sub>1</sub> receptor agonist [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY present, only Y<sub>2</sub> receptors should be labelled. In extrapolating binding data from cloned human Y<sub>1</sub> receptors (Herzog et al., 1992) to binding in rat brain sections, I am assuming that the Y<sub>1</sub> receptors have the same affinity for the ligands used in both systems. The same assumption is made for the receptors expressed in human neuroblastoma cells (Fuhlendorf et al., 1990a) and rat brain sections. However, it is possible that they may be different. Therefore, it would have been preferable to study the displacement of [<sup>125</sup>I]-PYY by NPY (13-36) and [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY in rat brain sections prior to carrying out further binding studies in this thesis.

Under the conditions used in this study, the main brain regions containing the Y<sub>1</sub> receptor subtype are the outer layers (I and II) of the cerebrocortical regions. Here they exist at a much greater density than the Y<sub>2</sub> receptors (see Figure 3.2.1). Other regions where the Y<sub>1</sub> receptor subtype exists in a greater density than the Y<sub>2</sub> receptors are the claustrum, the anteroventral thalamic nucleus and the dorsal cortex of the inferior colliculus. Regions where they exist in approximately equal density are the caudate putamen and the entorhinal cortex. However, in all other brain regions examined, the Y<sub>1</sub> receptor densities were less than those of the Y<sub>2</sub> receptors. The highest densities of Y<sub>2</sub> receptor

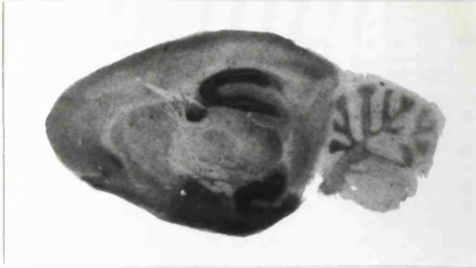
binding sites were found in the ventral hippocampus, the lateral septum, the stria terminalis and the posteromedial cortical amygdaloid nucleus. High densities were also found in the dorsal hippocampus and the remaining areas of the amygdala. Moderate levels of binding to the  $Y_2$  site were demonstrated in the anterior olfactory nucleus and the piriform cortex. Moderate/low levels were observed in the cerebellum and the nucleus accumbens. Very low levels were found in the caudate putamen, the entorhinal cortex and the cerebrocortical areas. Quantitative analysis of binding to  $Y_1$  and  $Y_2$  receptor binding sites is presented in Table 3.2.

**Legend for figure 3.2.1 Autoradiographic distribution of NPY receptor subtypes in the rat brain.** Photographs show [<sup>125</sup>I]-PYY binding to parasagittal rat brain sections 3.9mm (i) and 1.9mm (ii) lateral to bregma. **a** Total [<sup>125</sup>I]-PYY binding, **b** binding in the presence of 1μM Y<sub>2</sub> receptor agonist NPY (13-36), **c** binding in the presence of 1μM Y<sub>1</sub> receptor agonist [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY and **d** non-specific binding in the presence of 1μM NPY. For schematic diagrams illustrating several regions from these sections see Figures 3.1.7 and 3.1.8. For abbreviations of brain regions see Table 3.1.

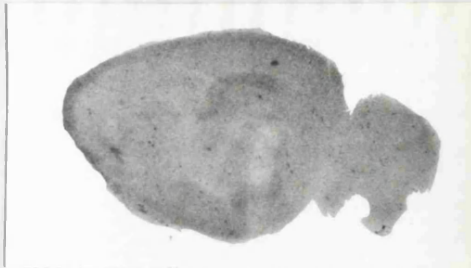


(i)

a) Total



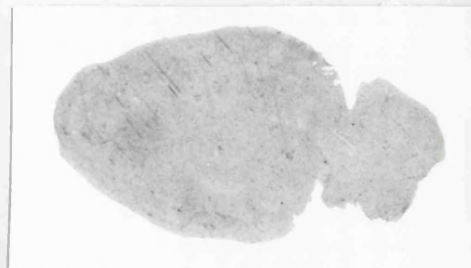
b) + 1 $\mu$ M NPY (13-36)



c) + 1 $\mu$ M [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY



d) Non-specific (+ 1 $\mu$ M NPY)

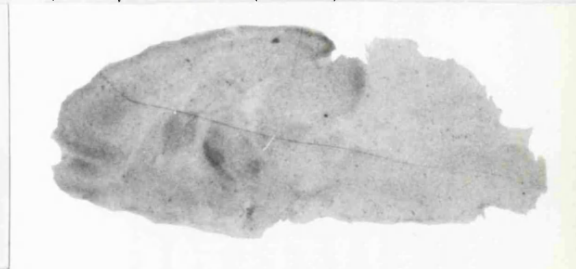


(ii)

a) Total



b) + 1 $\mu$ M NPY (13-36)



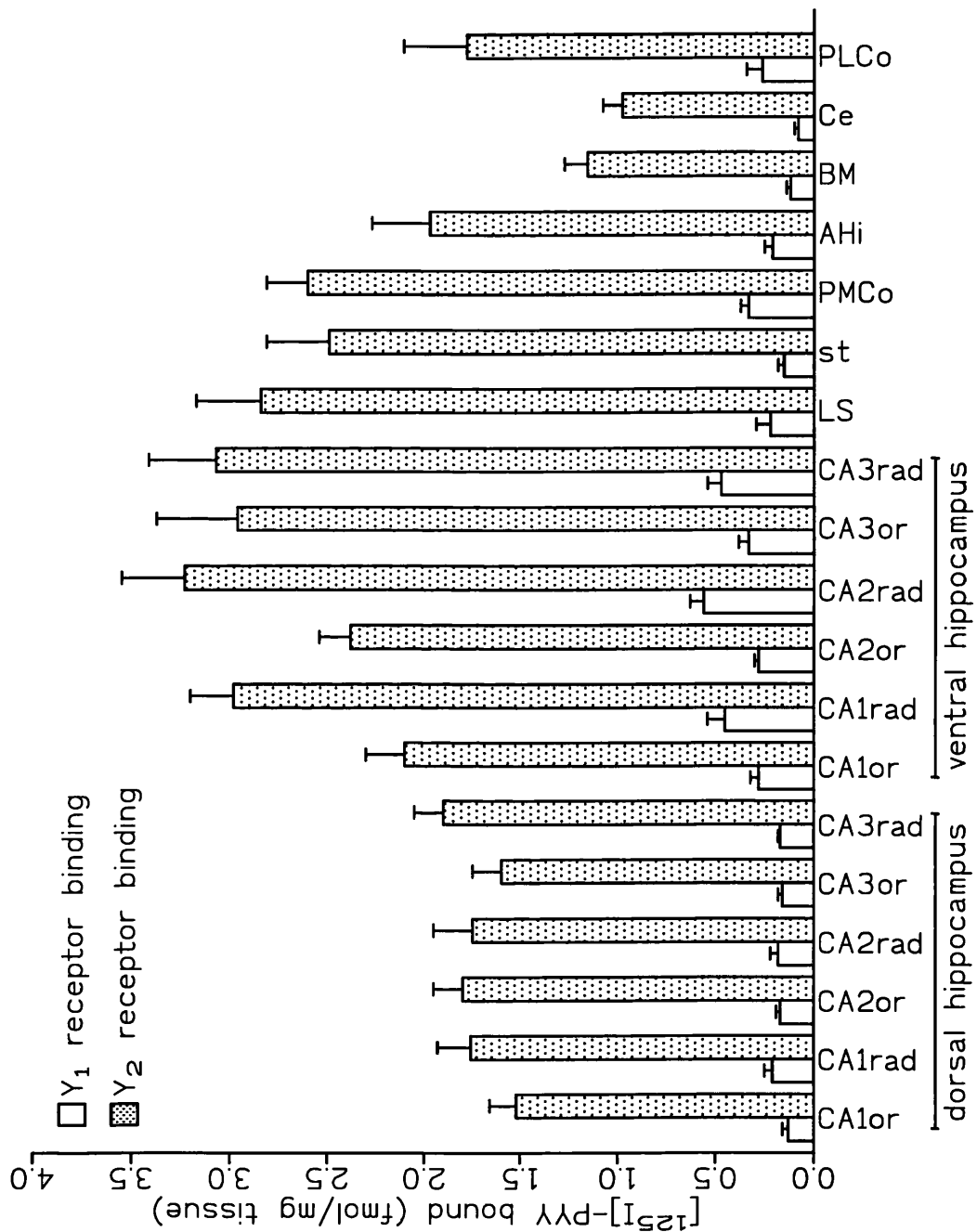
c) + 1 $\mu$ M [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY



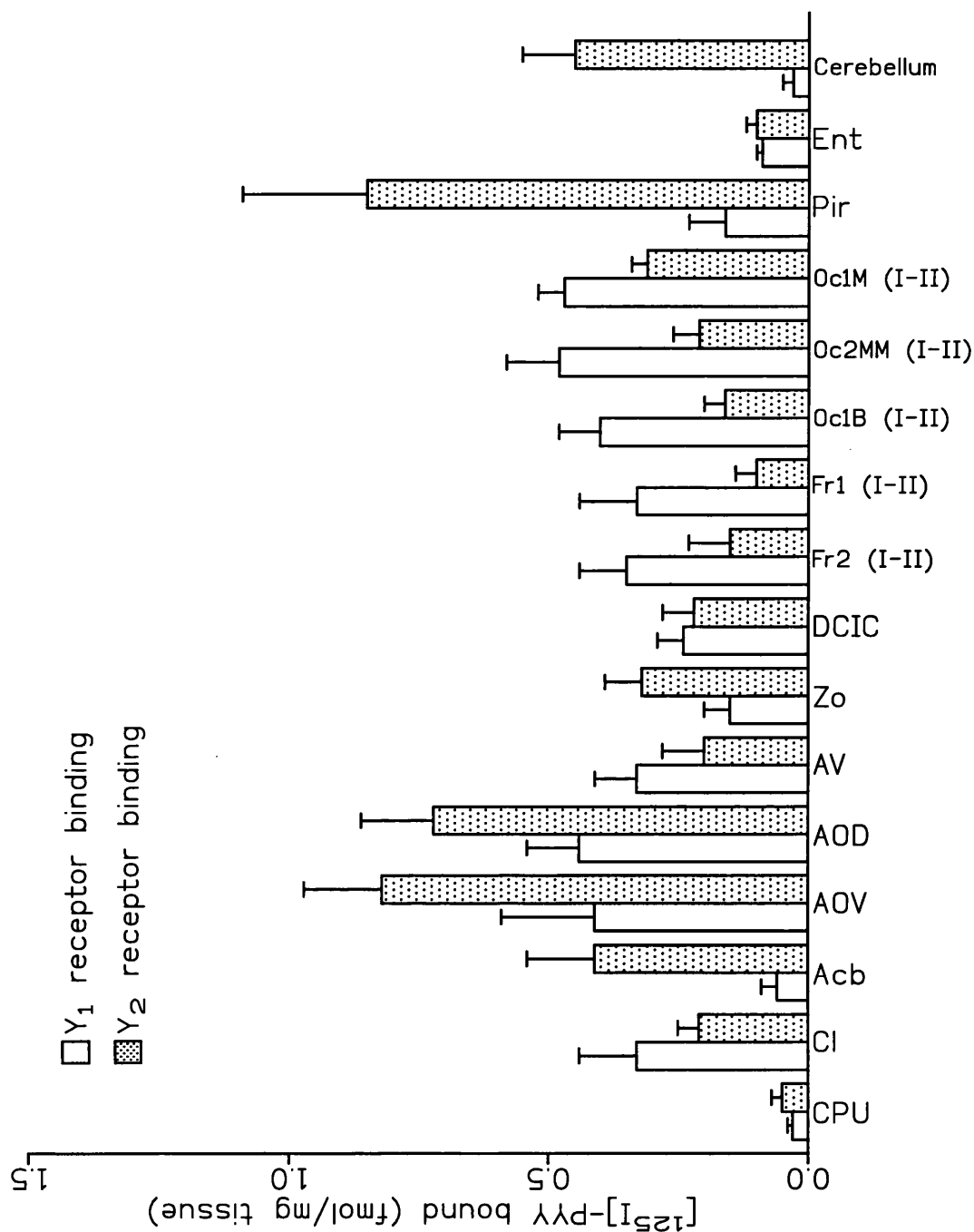
d) Non-specific (+ 1 $\mu$ M NPY)



Figure 3.2.1. (legend p119)



**Figure 3.2.2** Graph showing the densities of NPY receptor subtypes in the rat brain. Y<sub>1</sub> and Y<sub>2</sub> receptor densities were determined from the differences in [<sup>125</sup>I]-PYY bound to sections in the presence and absence of the Y<sub>1</sub> receptor agonist [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY and the Y<sub>2</sub> receptor agonist NPY (13-36) respectively. For abbreviations of brain regions see Table 3.1.



**Figure 3.2.3** Graph showing the densities of NPY receptor subtypes in the rat brain. Y<sub>1</sub> and Y<sub>2</sub> receptor densities were determined from the differences in [<sup>125</sup>I]-PYY bound to sections in the presence and absence of the Y<sub>1</sub> receptor agonist [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY and the Y<sub>2</sub> receptor agonist NPY (13-36) respectively. For abbreviations of brain regions see Table 3.1.

**Table 3.2 Quantitative autoradiographic distribution of [<sup>125</sup>I]-PYY binding sites in rat brain.**

Brain region	<u>[<sup>125</sup>I]-PYY binding (fmol/mg tissue)</u>			Percentage of specific binding	
	Specific binding	Y <sub>1</sub> binding	Y <sub>2</sub> binding	%Y <sub>1</sub>	%Y <sub>2</sub>
<b>Dorsal Hippocampus</b>					
Oriens layer					
CA1	3.24 ± 0.42	0.13 ± 0.03	1.52 ± 0.14	4.0	47
CA2	3.79 ± 0.63	0.17 ± 0.02	1.80 ± 0.15	4.5	48
CA3	3.40 ± 0.46	0.16 ± 0.02	1.60 ± 0.15	4.7	47
Stratum radiatum					
CA1	3.79 ± 0.50	0.21 ± 0.04	1.76 ± 0.17	5.5	46
CA2	3.75 ± 0.51	0.18 ± 0.04	1.75 ± 0.02	4.7	47
CA3	3.69 ± 0.40	0.17 ± 0.01	1.90 ± 0.15	4.7	52
<b>Ventral hippocampus</b>					
Oriens layer					
CA1	4.05 ± 0.64	0.28 ± 0.04	2.10 ± 0.20	6.9	52
CA2	4.38 ± 0.60	0.28 ± 0.02	2.38 ± 0.16	6.3	54
CA3	4.51 ± 0.49	0.33 ± 0.05	2.96 ± 0.41	7.4	66
Stratum radiatum					
CA1	4.89 ± 0.61	0.45 ± 0.09	2.98 ± 0.22	9.2	61
CA2	4.93 ± 0.61	0.56 ± 0.07	3.23 ± 0.32	11	66
CA3	4.76 ± 0.69	0.47 ± 0.07	3.07 ± 0.34	9.9	64
Lateral septum	4.71 ± 0.75	0.22 ± 0.07	2.84 ± 0.33	4.6	60
Stria terminalis	4.11 ± 0.65	0.15 ± 0.03	2.49 ± 0.32	3.6	61
<b>Amygdaloid complex</b>					
PMCo	5.18 ± 0.54	0.33 ± 0.04	2.60 ± 0.21	6.4	50
AHi	3.37 ± 0.50	0.21 ± 0.04	1.97 ± 0.30	6.4	59
Bm	2.60 ± 0.44	0.12 ± 0.02	1.16 ± 0.12	4.7	44
Ce	1.89 ± 0.34	0.08 ± 0.02	0.98 ± 0.10	4.2	52
PLCo	3.85 ± 0.69	0.26 ± 0.08	1.78 ± 0.32	6.9	46
<b>Basal ganglia</b>					
CPu	0.29 ± 0.06	0.03 ± 0.01	0.05 ± 0.02	11	19
Cl	1.33 ± 0.20	0.33 ± 0.11	0.21 ± 0.04	25	16
Acb	0.85 ± 0.22	0.06 ± 0.03	0.41 ± 0.13	7.5	48

continued over

**Table 3.2 (continued)**

Brain region	<u>[<sup>125</sup>I]-PYY binding (fmol/mg tissue)</u>			Percentage of specific binding	
	Specific binding	Y <sub>1</sub> binding	Y <sub>2</sub> binding	Y <sub>1</sub>	Y <sub>2</sub>
<b>Cortical areas</b>					
Frontal cortex					
Area 1 layers I-II	0.58 ± 0.11	0.33 ± 0.11	0.10 ± 0.04	57	17
Area 2 layers I-II	0.68 ± 0.10	0.35 ± 0.09	0.15 ± 0.08	51	23
Occipital cortex					
Area 1B layers I-II	1.10 ± 0.23	0.40 ± 0.08	0.16 ± 0.04	36	14
Area 1M layers I-II	0.47 ± 0.05	0.31 ± 0.03	0.11 ± 0.02	65	23
Area 2MM layers I-II	0.48 ± 0.10	0.21 ± 0.05	0.09 ± 0.03	44	18
Piriform cortex	2.37 ± 0.68	0.16 ± 0.07	0.85 ± 0.24	6.9	36
Entorhinal cortex	0.58 ± 0.09	0.09 ± 0.01	0.10 ± 0.02	16	18
<b>Olfactory areas</b>					
Anterior olfactory nucleus					
dorsal	2.26 ± 0.40	0.44 ± 0.10	0.72 ± 0.14	19	32
ventral	1.58 ± 0.42	0.41 ± 0.18	0.82 ± 0.15	26	52
AV	1.11 ± 0.10	0.33 ± 0.08	0.20 ± 0.08	30	18
Zo	1.07 ± 0.20	0.15 ± 0.05	0.32 ± 0.07	14	30
DCIC	0.90 ± 0.10	0.24 ± 0.05	0.22 ± 0.06	26	24
Cerebellum, granular layer	0.85 ± 0.11	0.03 ± 0.02	0.45 ± 0.10	3.7	52

Values represent the mean ± SEM of four to five determinations (except for when marked with a # where n=3) using a concentration of 0.1nM [<sup>125</sup>I]-PYY. Nonspecific labelling in the presence of 1µM NPY was subtracted from all readings. The values for binding at the Y<sub>1</sub> site represent label bound in the presence of the Y<sub>2</sub> receptor agonist NPY (13-36) (1µM). Values for binding at the Y<sub>2</sub> site represent label bound in the presence of the Y<sub>1</sub> receptor agonist [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY (1µM). For explanation of abbreviations see Table 3.1

### 3.3 Discussion

The general distribution of both [<sup>125</sup>I]-PYY and [<sup>125</sup>I]-BH NPY binding sites in this study seem to be similar although there are regional differences in relative densities between the two ligands (see Figure 3.2.4). The results of this study are in general agreement with those of other studies (Martel et al.,1986; Ohkubo et al.,1990). The main differences between the distribution of [<sup>125</sup>I]-BH NPY and [<sup>125</sup>I]-PYY binding sites in this study were in the cerebellum. The density of [<sup>125</sup>I]-PYY binding in the cerebellum was approximately five times greater than that for [<sup>125</sup>I]-BH NPY binding in a separate experiment despite similar levels of binding in the other regions. Several groups (Lynch et al.,1989; Ohkubo et al.,1990) have suggested that the differences in regional levels of PYY and NPY binding could be due to receptor heterogeneity.

As with other studies of total NPY receptor distribution, there are similarities between the NPY receptor binding sites and the distribution of NPY-like immunoreactivity shown in other studies (see Gray and Morley,1986). Areas showing high density of [<sup>125</sup>I]-BH NPY binding sites and high levels of NPY-like immunoreactivity include the nucleus accumbens, septum, amygdala, olfactory nucleus, the cerebral cortex, caudate putamen, claustrum and the hippocampus.

The presence of both NPY immunoreactivity and binding sites in several brain areas suggests some physiological roles. The very high and moderate densities

of NPY receptors in the hippocampus and cerebral cortex, respectively, are suggestive of a role in higher cognitive functions. Also the high densities of NPY receptors and NPY immunoreactivity in the septum and hippocampus suggest a role for NPY in the limbic system and hence emotions.

Based on the above conditions optimised in other laboratories, the present study demonstrates different distributions of the  $Y_1$  and  $Y_2$  NPY receptor subtypes in the rat brain. The results generally confirm the findings of Dumont et al. (1990). However, the latter study only reported on a limited number of brain regions, i.e. general cortical areas, the anterior olfactory nucleus, the hippocampal formation, and certain un-named 'brain stem regions'. In the present study, a more detailed report of brain regions showing the two [ $^{125}$ I]-PYY-labelled receptor subtypes is presented, to enable the subsequent studies of this thesis. From the autoradiographs of  $Y_1$  receptor binding, the  $Y_1$  sites were generally distributed at very low densities, but were especially concentrated in the superficial layers of cortical areas and the claustrum. The  $Y_2$  receptors however were especially concentrated in hippocampal, septal, olfactory, amygdaloid and cerebellar areas. The  $Y_2$  receptor seems to be the dominant subtype in the brain as overall densities are much higher than those for the  $Y_1$  receptor. Many areas exhibit both receptor subtypes, although the  $Y_1$  receptor tends to be present at lower densities. However, it is important to note that the total  $Y_1$  and  $Y_2$  binding taken together does not reach that of the specific PYY binding. This could have several explanations, the first of which is that the concentration of displacing ligands used are not optimally displacing

[<sup>125</sup>I]-PYY binding from their selective receptor subtypes. The concentrations of the ligands used should, based on previous studies in other laboratories (see section 3.2.1) be sufficient to displace [<sup>125</sup>I]-PYY from their receptor subtypes. However, it is possible that the receptor ligands used are not as specific for their receptors as previously thought. Herzog et al (1992) found that at a concentration of 1μM, the Y<sub>2</sub> 'specific' agonist PYY (13-36) displaced 20% [<sup>125</sup>I]-PYY binding from cloned human NPY Y<sub>1</sub> receptors. Similarly, Fuhlendorff et al. (1990a) found NPY (13-36) to displace about 20% of [<sup>125</sup>I]-BH NPY binding to the Y<sub>1</sub> expressing cells, SK-N-MC. They also showed the Y<sub>1</sub> 'specific' ligand [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY, at a concentration which displaced about 85% binding from Y<sub>1</sub> sites (0.1μM), to displace about 30% of [<sup>125</sup>I]-PYY binding to the Y<sub>2</sub> expressing cells, SMS-KAN. Thus it seems that at the concentrations required to maximally inhibit [<sup>125</sup>I]-PYY binding from their 'specific' receptors, these ligands displace binding at the 'other' receptor subtypes. Therefore, we can only refer to these ligands as being 'relatively specific' at the concentrations required to maximally displace [<sup>125</sup>I]-PYY from its binding sites. Despite this, in the area 1M of the occipital cortex, the addition of the binding to the two receptor subtypes accounts for 88% of the total specific PYY binding in that area. Finally there is also the possibility of the existence of further NPY receptor subtypes which are, as yet, undiscovered. It would seem however that further ligands are required before it is possible to quantify accurately NPY receptor subtype distribution in the rat brain.



### 3.4 Conclusion

The distribution of [<sup>125</sup>I]-BH NPY and [<sup>125</sup>I]-PYY binding sites were found to be similar, both also resembling the distribution of NPY immunoreactivity in the rat brain. Several brain areas of importance for emotional and cognitive functions were found to have moderate/high receptor densities. These included the cortex, hippocampus, septum and amygdala. Y<sub>1</sub> and Y<sub>2</sub> receptor subtypes were identified and found to show differential distributions throughout the rat brain. The Y<sub>1</sub> receptor was prominent in the cortex and the claustrum, whereas the Y<sub>2</sub> receptor was more widely distributed, but found mainly in the hippocampus, septum and amygdaloid areas. These areas are possible sites of action for NPY with regards to the modulation of anxiety.

CHAPTER 4  
RESULTS PART III  
NPY-INDUCED FOS-LIKE  
IMMUNOREACTIVITY

Part III: Localization of neuronal activity mediated by NPY in rat brain: the use of Fos-like immunoreactivity as an anatomical marker of peptide receptor activation.

4.1 Introduction

When studying the effects of a centrally active substance, a regional marker of neuronal activation would be useful in order to visualize where the compound exerts its effects. It has recently been suggested that the generation of Fos protein in nerve cells provides such a marker, so that when cells are activated an increase in Fos protein is observed (Hunt et al., 1987). The Fos protein is a nuclear phosphoprotein that binds to DNA. It is thought to function as a nuclear "third messenger" molecule that couples transient extracellular signals to long term changes in cell function by regulating specific gene expression. Fos is the protein produced upon the expression of the *c-fos* immediate-early gene (IEG), also known as a nuclear proto-oncogene, the mammalian homologue of the *v-fos* oncogene found in two murine osteogenic sarcoma viruses (for review see Doucet et al., 1990). Histochemical studies in the rat CNS suggest that the expression of the *c-fos* IEG, measured by Fos protein immunocytochemistry, is a marker of neuronal activity (Sagar et al., 1988). In this respect it may be possible to map functional neuronal pathways.

The objective of the present study is to determine the functional pathways

involved in the actions of NPY, specifically related to the possible anxiolytic effects, and to see if there is any correlation with the autoradiographic locations of the NPY receptor binding sites.

## 4.2 Results

### 4.2.1 Effect of central neuropeptide Y administration

Initial studies were carried out to determine the dose of NPY to use in the subsequent studies. Brain slices from rats treated with either 1 and 5nmol NPY were visually compared and no obvious differences in FLI were apparent between the two doses. FLI was visualised as dark brown dots showing the immunopositive cells against a light brown non-specific background.

Both NPY-treated and control brains showed regions containing FLI (see Table 4.1a). There were significant increases in FLI in NPY treated animals when compared to saline treated controls in the CA1 region of the ventral hippocampus, the dorsal endopiriform cortex, the amygdalopiriform transition area, the entorhinal cortex, the nucleus accumbens, and the posterior paraventricular thalamic nucleus (see Figures 4.1 and 4.2). An animal which had a cannula surgically implanted, but received no injection, showed similar levels of FLI as the saline control animals (see Table 4.1a).

Several areas which are known to contain NPY receptor binding sites in autoradiographic studies failed to exhibit a significant increase in FLI following i.c.v. NPY administration. The CA1 region of the dorsal hippocampus was one such area as was the CA2 region of the hippocampus and occipital cortex (Table 4.1b).

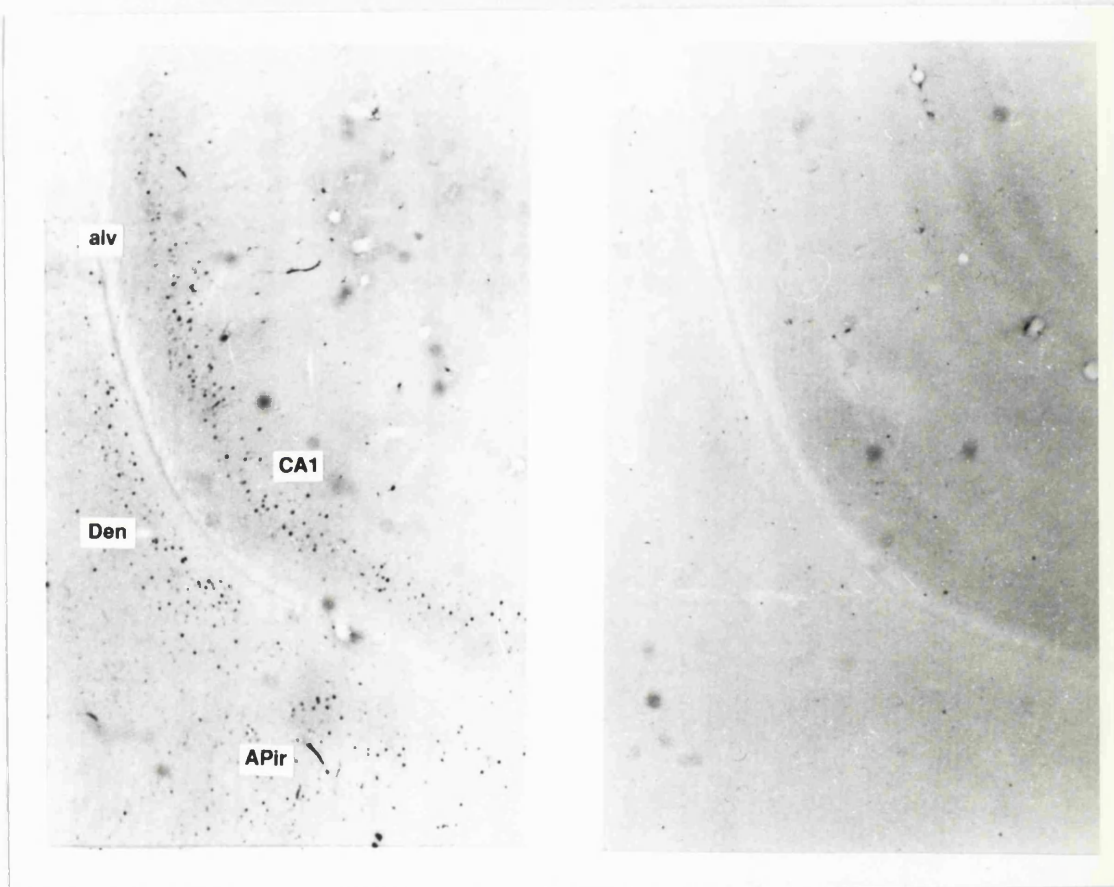
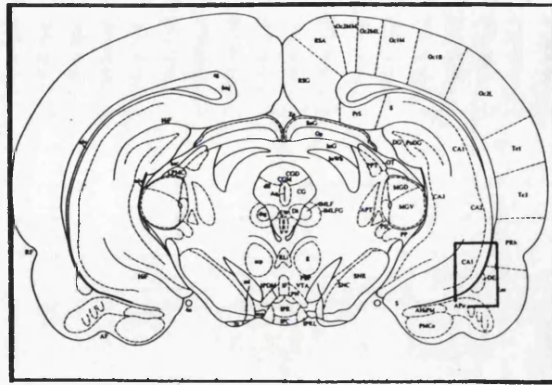
Brain region	NPY(1nmol/1µl)	Saline (1µl)	Sham injected <sup>#</sup>
entorhinal cortex	6.80 ± 2.69 (5)	0.40 ± 0.24 (5)	1
amygdalopiriform transition area	8.80 ± 3.22 (5)	0.80 ± 0.49 (5)	4
nucleus accumbens	19.00 ± 2.55 (4)	6.75 ± 2.28 (4)	14
ventral hippocampus CA1 area	14.00 ± 2.55 (5)	5.20 ± 1.46 (5)	9
paraventricular thalamic nucleus	21.40 ± 3.50 (5)	10.00 ± 1.34 (5)	16
dorsal endopiriform nucleus	10.60 ± 1.66 (5)	5.40 ± 0.51 (5)	6

**Table 4.1a.**

Brain region	NPY(1nmol/1µl)	Saline (1µl)	Sham injected <sup>#</sup>
hippocampus CA2	2.40 ± 1.17 (5)	1.60 ± 0.75 (5)	2
occipital cortex	12.20 ± 4.21 (5)	4.80 ± 1.66 (5)	5

**Table 4.1b.**

**Table 4.1a & 4.1b.** FLI in several brain regions 3 hours following i.c.v. administration of saline either alone or containing NPY (1nmol). Values are the mean ± standard error of the mean number of cell bodies exhibiting FLI in an area approx. 0.03mm<sup>2</sup>. Figures in brackets are the number of animals in the group. **4.1a.** Regions showing significant increases in FLI after treatment with NPY. All regions compared to saline controls using Mann-Whitney U test and considered significant at p<0.05. **4.1b.** Regions which despite having NPY receptor binding sites show no significant increases in FLI. # The sham injected animal (n=1) had identical surgery and procedure (i.e. the injection cannula was inserted), but was not injected. Values represent the number of cell bodies exhibiting FLI in an area approx. 0.03mm<sup>2</sup> for a single animal.

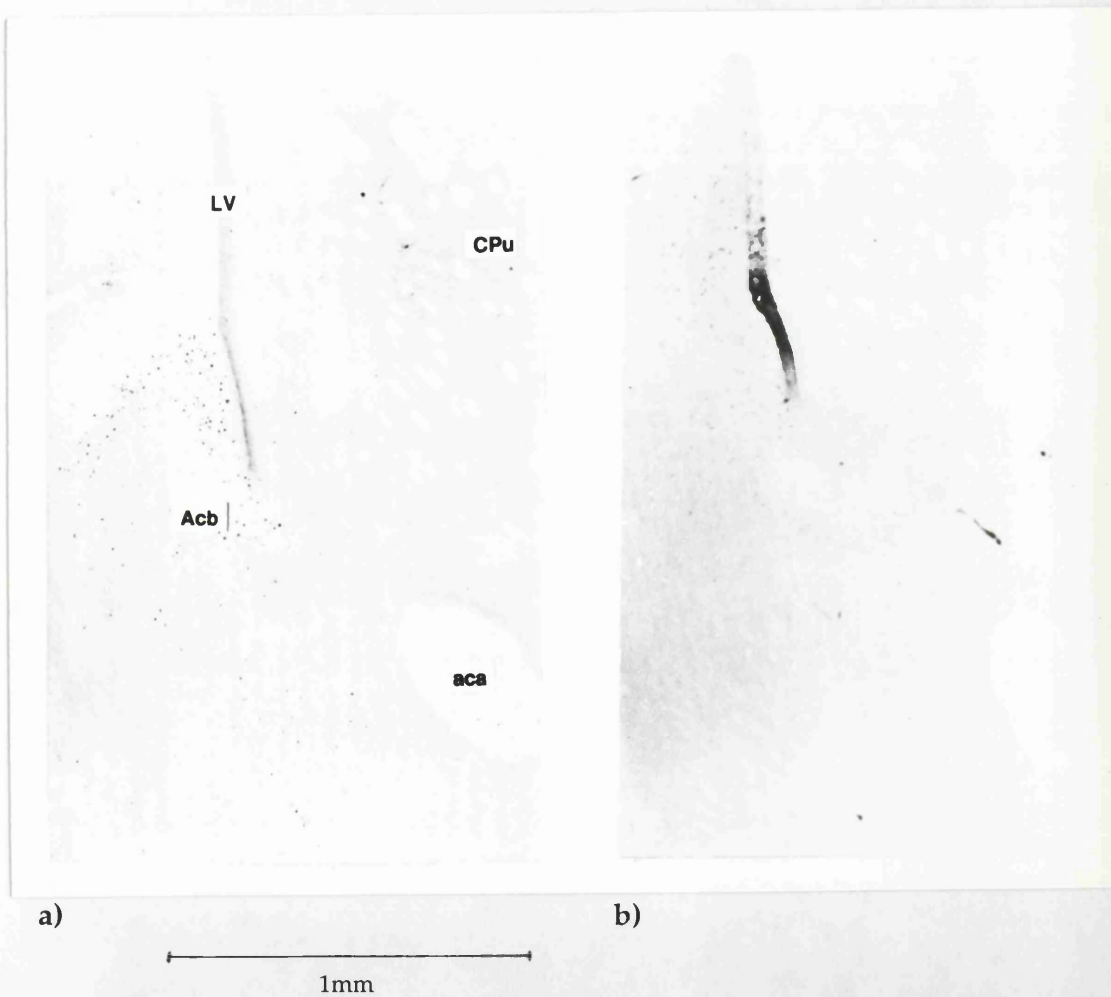
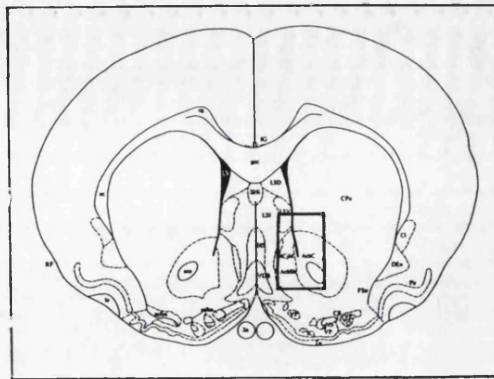


a)

b)

1mm

**Figure 4.1a,b.** Photographs showing Fos-like immunoreactivity (FLI) in the region of the rat brain highlighted above (coronal section 5.8mm caudal to bregma). FLI can be seen as a darkening of the cells. The number of cells exhibiting FLI are significantly increased in the CA1 region of the ventral hippocampus (CA1), the dorsal endopiriform nucleus (Den) and the amygdalopiriform transition area (APir) of the NPY treated (a) compared to the saline treated (b) animals. Also shown is the alveus hippocampus (alv).



**Figure 4.2a,b.** Photographs showing Fos-like immunoreactivity (FLI) in the region of the rat brain highlighted above (coronal section 1mm rostral to bregma). FLI can be seen as a darkening of the cells. The number of cells exhibiting FLI are significantly increased in the accumbens nucleus (Acb) of the NPY treated (a) compared to the saline treated (b) animals. Also shown are the caudate putamen (CPu), anterior commissure anterior (aca), and a lateral ventricle (LV).



### 4.3 Discussion

The purpose of this study was to attempt to visualise those regions of the brain that may be responsible for the reported anxiolytic-like effects of NPY. Heilig et al. (1989) demonstrated NPY (1nmol) to produce such an effect, but at a higher dose (5nmol) also produced sedation. The dose of the peptide used in this study was therefore 1nmol to show those areas of neurones activated in response to any possible anxiolytic-like effects, and to minimise any activation due to the sedative or any other effects. A simple visual comparison of brain slices taken from rats treated with either 1 or 5nmol NPY showed no obvious differences in FLI between the two doses, adding further weight to the choice of dose. There is a possibility that when administering a sedative dose of NPY, the sedative effect on neuronal activity might decrease *c-fos* expression and thus might hide any effects we are trying to see due to anxiety.

The use of FLI to map active neurones is an extremely sensitive technique. It is important that the conditions are carefully controlled as *c-fos* can be activated by many stimuli. Such stimuli include stress, to which these animals are prone due to the nature of the techniques employed in this study, and mechanical stimuli such as an intracerebroventricular (i.c.v.) injection procedure which can evoke the synthesis of large amounts of FLI in rat brains (Ceccatelli et al., 1989). The surgery involved in this study, and the presence of a chronically implanted cannula, might therefore be expected to stimulate the brain, and hence increase *c-fos* expression. The sham animal implanted with

a cannula, but which received no injection on the day of the experiment, showed FLI of a similar magnitude to that of control animals. This suggests that surgery, handling and cannula implantation can influence FLI due to their inherent excitatory nature with respect to central neuronal activity. As well as the possibility of false positives, false negatives should also be considered. The work of Hunt et al. (1987) has shown that sometimes FLI fails to adequately map functional pathways.

There appears to be a fairly good agreement between those brain regions showing significant increases in FLI and those containing NPY receptors or NPY-immunoreactivity. The nucleus accumbens, endopiriform nucleus, hippocampus, paraventricular thalamic nucleus and most amygdaloid areas have all been shown to possess NPY immunoreactivity (for review see Gray and Morley, 1986). The presence of NPY receptor binding sites has been demonstrated in the CA1 region of the ventral hippocampus, entorhinal cortex and the amygdalopiriform transition area (see section 3). It is important to note however that many regions shown to contain NPY or its receptors failed to show any increase in FLI. The CA2 region of the hippocampus and the superficial layers of the occipital cortex both show NPY receptor binding but no significant increases in FLI were evident in either case. The presence of both FLI and NPY receptors in the hippocampus are suggestive of a role in higher cognitive function and in the limbic system. This makes the hippocampus an interesting site of study with respect to the behavioural models of anxiety.

A limitation of this study is that it only shows those cells activated and not those inhibited by NPY. NPY is thought to have an anxiolytic action in rats if given i.c.v. at the dose employed in this study. It is possible therefore, that the anxiolytic effects of NPY could be due to an inhibition of neuronal activity, which may not be apparent using the present technique. The neuronal pathways relating to the present observations may include those associated with anxiolysis, but not exclusively. NPY is believed to be involved in numerous central activities. These include the control of circadian rhythm, the control of brain electrical activity, central control of blood pressure, thermoregulation, sedation, locomotion, stimulation of feeding, insulin secretion, melatonin biosynthesis, inhibition of libido and LH secretion and enhancement of memory. Thus, an area showing increases in FLI when challenged with NPY could be involved in any one of these processes. It is possible that the regions shown to be activated could be other neurones in a functional pathway or GABA interneurones. It is also possible that the cells exhibiting FLI are not neuronal, but are glial cells. Experiments to stain the immunopositive cells with a neurone specific antibody and stain were unsuccessful. The sections were much too thick to see where the neurone specific stain was bound. The possibility thus remains that we may be seeing glial cell activity and not viewing functional neuronal pathways. Thinner sections (50µm or less) would be needed for this verification.

As already pointed out, Fos is a nuclear protein. This limits the staining of the protein to activated cell bodies. Other techniques could be used to try to obtain

similar information about functional pathways, each with their advantages and disadvantages. 2-Deoxyglucose autoradiography shows glucose utilization throughout the cell, and thus also shows alterations in functional activity in the terminals. These two techniques could ideally be used side by side or even on the same brain using alternate sections. This would create a much more complete picture of cellular activity. Other possible techniques that could be used are electrophysiology and microdialysis of transmitter release. These latter two techniques would not be useful in this study as they only give information about a localised brain region and not, as in the case of this study, the entire brain. They could be used however as a more detailed study of regions shown to be activated in the *c-fos* or 2-deoxyglucose studies.

In conclusion, it would seem that FLI appears to provide a useful marker of NPY-induced cellular activity in mammalian brain. Further studies, concerning the time course of the response and the background levels of FLI produced due to the procedures used, may prove this technique to be useful for studying those areas of the brain which are important in the function of NPY. Increased FLI was found in several brain areas, including the ventral hippocampus, following NPY administration. As this area has also been shown to display a high density of NPY receptor binding sites (see section 3.2), and is part of the limbic system, it warrants further investigation as a possible site of action for the anxiolytic-like effects of NPY.

**CHAPTER 5**  
**RESULTS PART IV**  
**BEHAVIOURAL STUDIES**

## 5. Behavioural studies of anxiety

It is important when investigating the role of a ligand in CNS physiology to try and link any biochemical and immunohistochemical findings to the in vivo situation. No matter how much in vitro work is done, until this work is applied to the whole animal situation it will be unclear as to the physiological relevance of any findings. In this study, the role of NPY in the modulation of anxiety has been investigated in vivo by examining its effects in behavioural models of anxiety.

Behavioural models of anxiety are an important aspect of research in this field, and many different models exist. The problem with using these models is that in the whole animal situation, with the type of tests used, it is impossible to be certain that any one test is showing direct effects on anxiety, or if the activity seen is an indirect result of the effect of the ligand on some other system, eg. sight or smell. For this reason it is important to use several models of anxiety, and to compare the results between them. In this particular study we have used three such models, the elevated x-maze, social interaction and Vogel's drinking conflict tests. All three of these models are very different in their approach, and so represent a comprehensive study into the effects of NPY on anxiety.

## 5.1 Elevated x-maze

The elevated x-maze model of anxiety is derived from procedures used by Montgomery (1955). He showed that exposure to an elevated open maze alley evoked an approach-avoidance conflict that was stronger than that evoked by exposure to an enclosed maze alley. Rats placed in a living cage and allowed access to an enclosed alley explored a lot more than those allowed access to an elevated open alley and furthermore, the latter animals showed more retreats away from the alley to the back of their living cage. He then used a Y-maze in which he was able to vary the ratio of open to enclosed arms, and found that animals preferred the enclosed arms in all cases. He interpreted the animals' responses as an approach-avoidance conflict, in which the animals' exposure to a novel stimulus evokes both an exploratory drive and a fear drive. Thus, elevated open maze alleys seem to evoke fear to a greater extent, and therefore cause more avoidance behaviour.

The Y-maze concept was extended by Handley and Mithani (1984) who added an extra arm to the maze to produce what they called the elevated +-maze. This comprised two open and two enclosed arms, and they described the extent to which drugs effect the ratio of open to closed arm activity as a measure of anxiety. This behavioural model was further validated by Pellow et al. (1985) using clinically effective anxiolytics and anxiogenic agents. They were able to show that neither antidepressants nor major tranquillisers had any specific effects in this model, demonstrating the selectivity of this model for

anxiolytic/anxiogenic agents.

#### 5.1.1 Validation of test procedure

Animals treated with chlordiazepoxide (2.5mg/kg,p.o. dissolved in water) showed significant increases in the % time spent in the open arms, % entries into the open arms, time spent in the end of the open arms, and the number of entries into the end of the open arms (Figure 5.1.1) compared to vehicle treated control animals.

#### 5.1.2 Effects of i.c.v. neuropeptide Y administration

Animals tested on the elevated x-maze 1 h after treatment with NPY showed a decreased preference for the closed arms of the maze. This was indicated by dose dependent increases in the % time spent in the open arms, % entries into open arms and time spent in the end of the open arms (Figure 5.1.2). The increases in % time spent in the open arm and the time spent in the end of the open arm were significant at both the 1 and 5nmol levels of treatment, whereas the increase in % entries into the open arms only reached significance at the 5nmol level. A small increase in number of entries into the end of the open arms was insignificant at either dose.



A dose-dependent decrease in both the total number of entries and the number of line crossings was observed. This only reached significance however after 5nmol NPY (Figure 5.1.3).

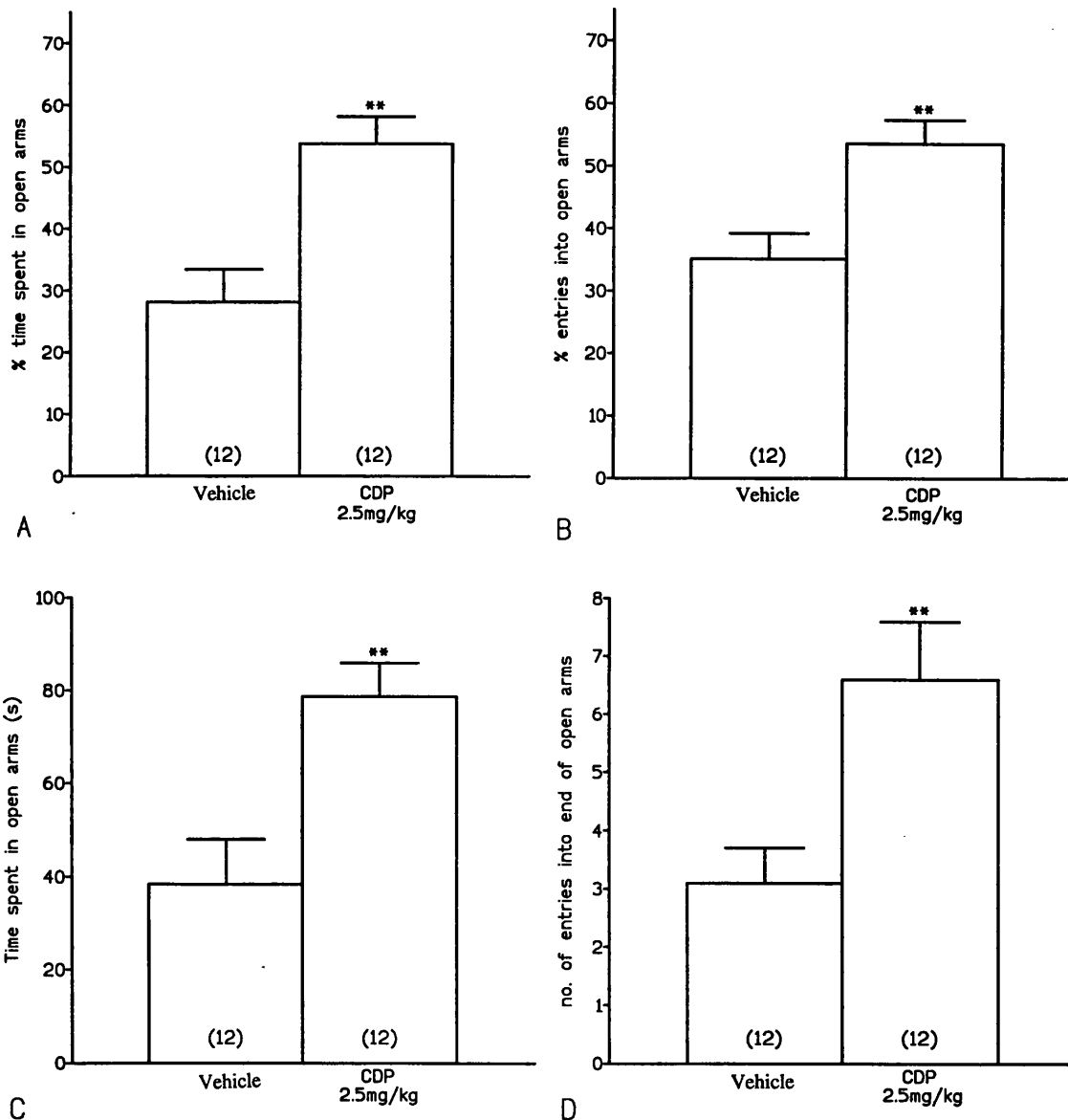
### 5.1.3 Effects of various neuropeptide Y agonists

Animals treated with NPY (5nmol) showed significant increases in the time spent in the open arms (Figure 5.1.4). No significant differences were seen in any other parameter measured. There did appear to be a tendency towards an increase with both [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY and PYY (44% and 60% increase in % time spent in the end of the open arms respectively), although neither was significant.

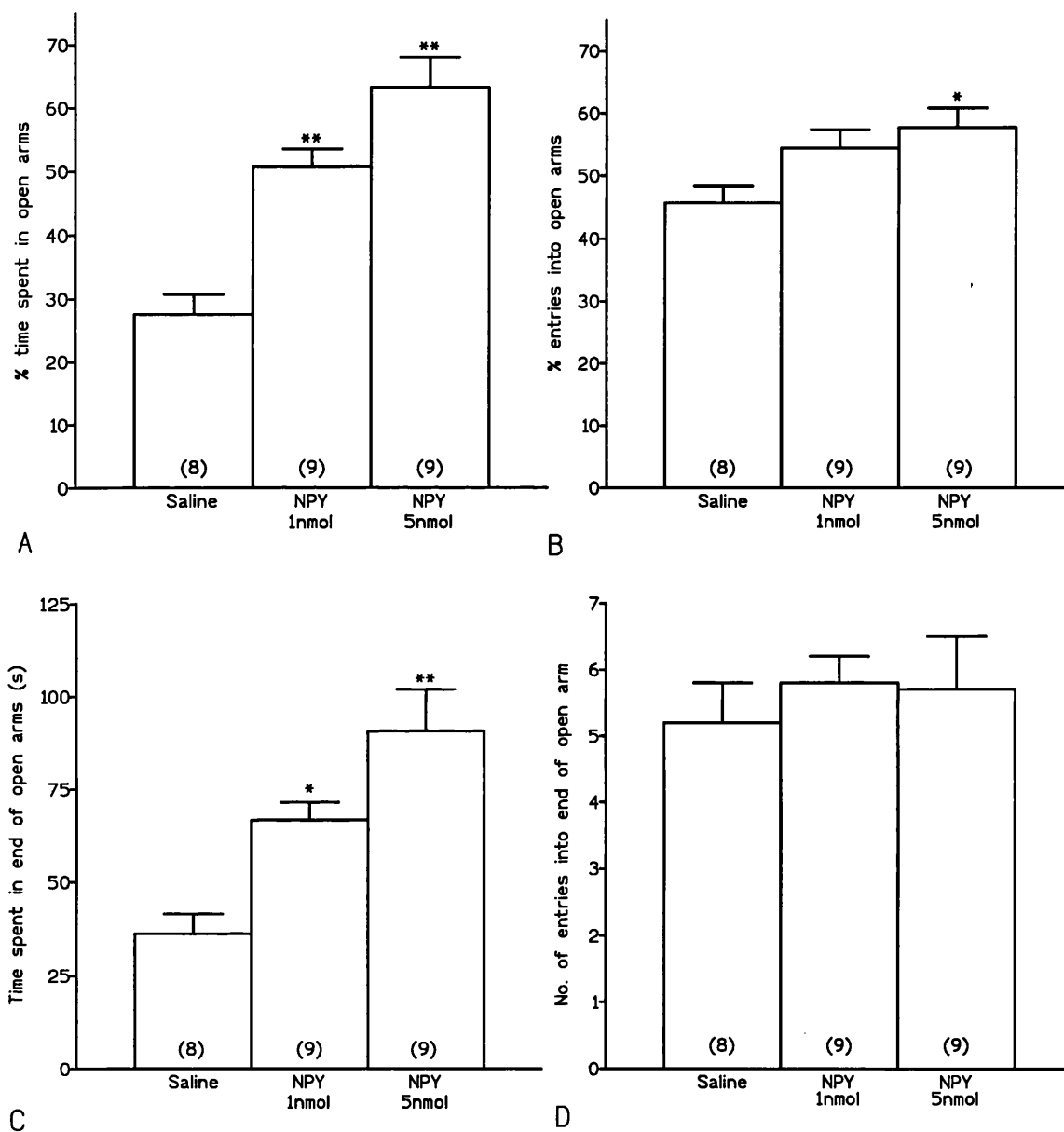
There were no significant effects with any of the agonists on either the total number of entries or the number of line crossings (Figure 5.1.6).

### 5.1.4 Effects of neuropeptide Y in the ventral hippocampus

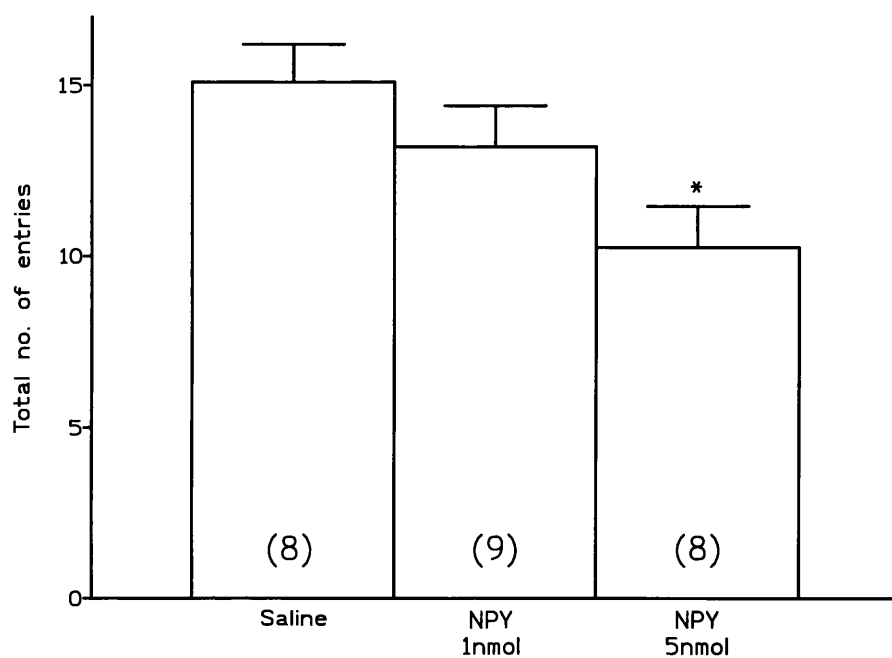
At the lower of the two doses tested, NPY (0.5nmol) showed a trend toward a decreased preference for the closed arms of the maze. The only significant increase however was for the % time spent in the open arms. At the higher dose, NPY (1nmol) caused significant increases in all four of the parameters



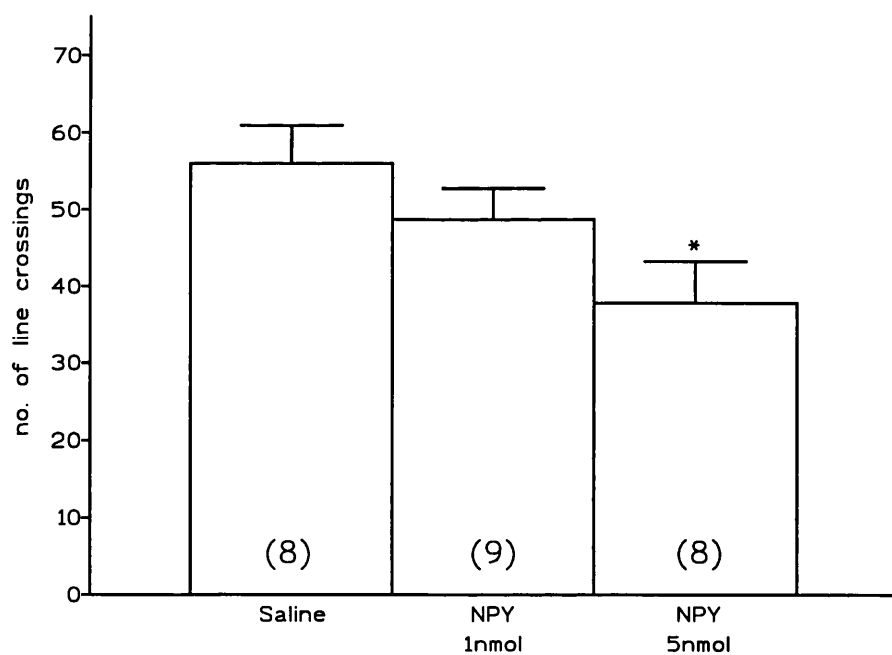
**Fig. 5.1.1 A,B,C,D.** Behaviour during a 5min elevated x-maze test 1 h after p.o. treatment with either water alone or containing CDP (2.5mg/kg). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Percentage time spent in open arms of the maze. **B** Percentage entries into the open arm of the maze. **C** The time spent in the end of the open arms. **D** Number of entries into end of open arms. \* & \*\* indicate differences versus water treated controls at 5% & 1% significance levels (Students t-test).



**Fig. 5.1.2 A,B,C,D.** Behaviour during a 5min elevated x-maze test 1 h after i.c.v injection of 1 $\mu$ l saline either alone or containing NPY (1.0 & 5.0 nmol). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Percentage time spent in open arms of the maze.  $F(23,2)=22.99$ ,  $P<0.0001$ . \*\* indicates a difference versus the saline treated control at a significance level of 1% on Dunnett's test. **B** Percentage entries into the open arm of the maze.  $F(23,2)=4.41$ ,  $P<0.05$ . \* indicates a difference versus saline control at a significance level of 5% on Dunnett's test. **C** The time spent in the end of the open arms.  $F(23,2)=11.98$ ,  $P<0.01$ . \* & \*\* indicate differences versus the saline controls at significance level of 5% & 1% respectively. **D** Number of entries into end of open arms was not significant,  $F(23,2)=0.025$ .

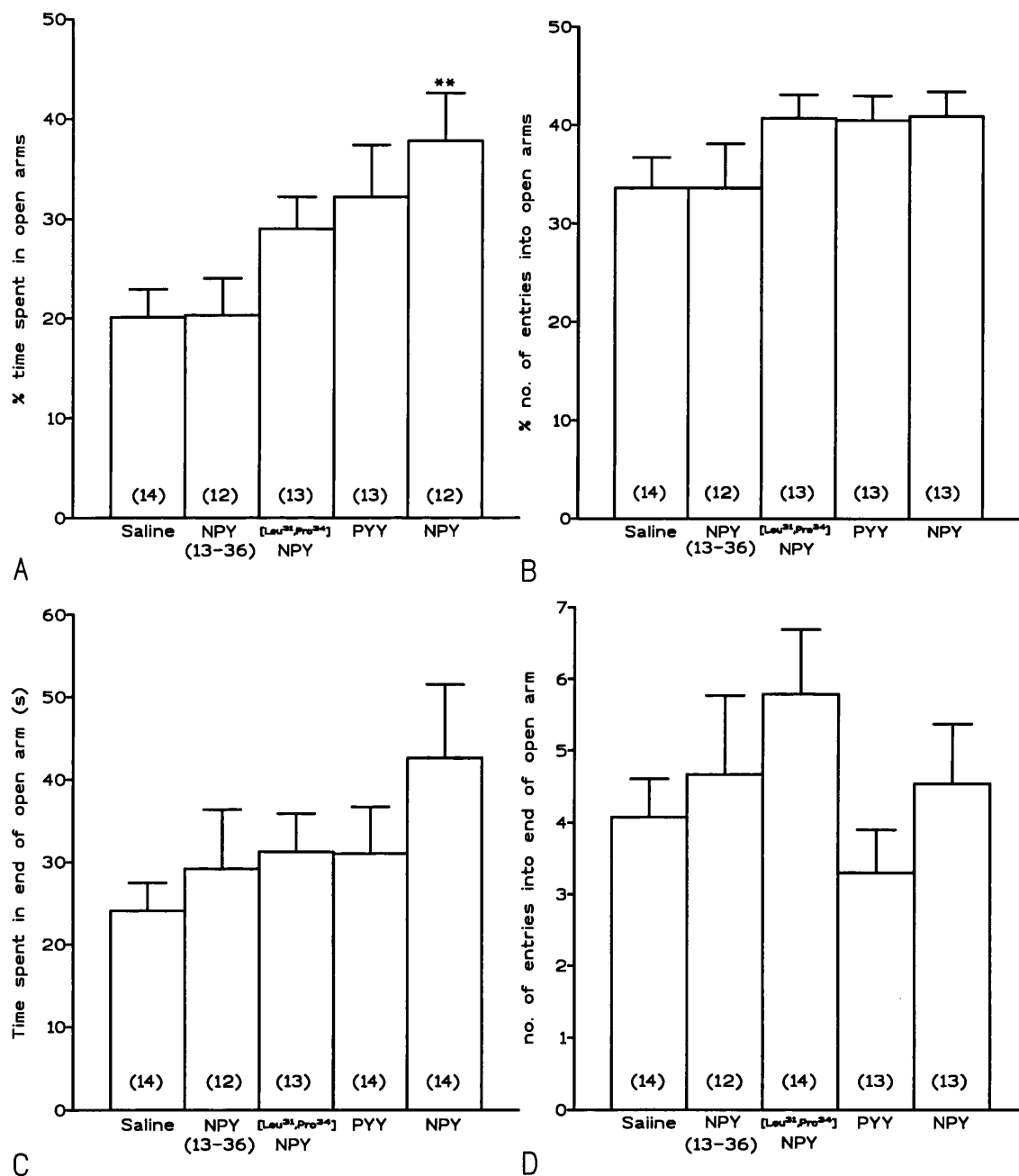


A

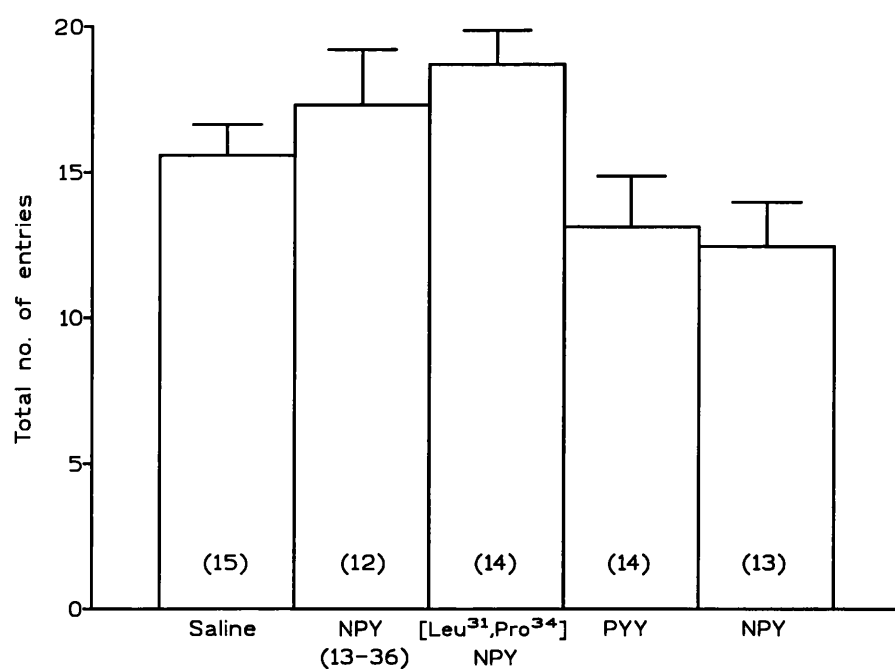


B

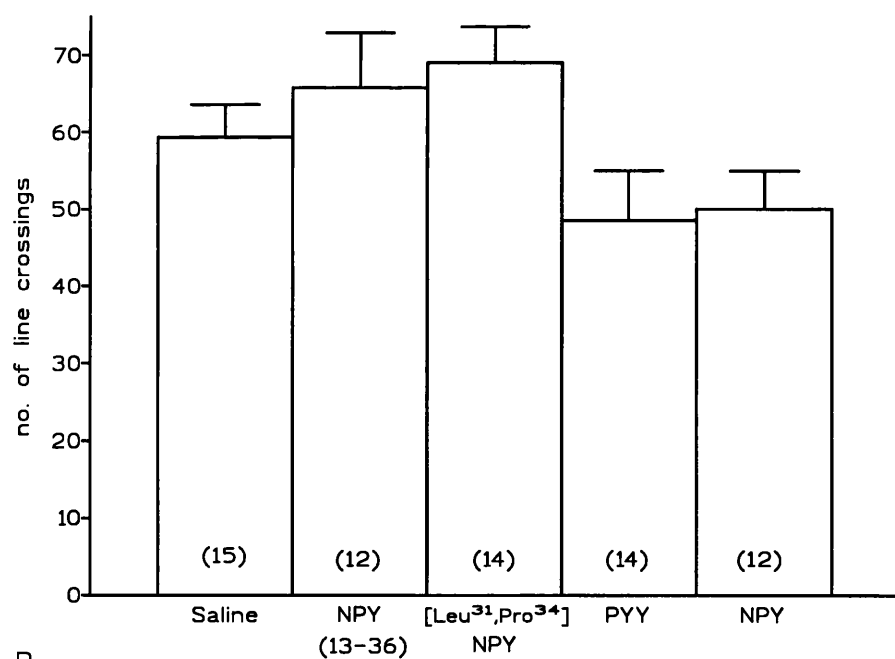
**Fig. 5.1.3 A,B.** Behaviour during a 5min elevated x-maze test 1 h after i.c.v. administration of 1 $\mu$ l saline either alone or containing NPY (1.0 & 5.0nmol). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Total number of entries into any arms of the maze.  $F(2,2)=4.287$ ,  $P<0.05$ . \* indicates a difference versus the saline treated control at a significance level of 5% on Dunnet's test. **B** Number of line crossings made.  $F(2,2)=3.92$ ,  $P<0.05$ . \* indicates a difference versus saline control at a significance level of 5% on Dunnet's test.



**Fig. 5.1.4 A,B,C,D.** Behaviour during a 5min elevated x-maze test 1 h after i.c.v. injection of 1µl saline either alone or containing NPY (13-36), [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY, PYY or NPY (5.0 nmol). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Percentage time spent in open arms of the maze.  $F(59,4)=3.63$ ,  $P<0.05$ . \*\* indicates a difference versus the saline treated control at a significance level of 1% on Dunnet's test. **B** Percentage entries into the open arm of the maze was not significant,  $F(60,4)=1.62$ . **C** The time spent in the end of the open arms was not significant,  $F(62,4)=1.30$ . **D** Number of entries into end of open arms was not significant,  $F(61,4)=0.31$ .

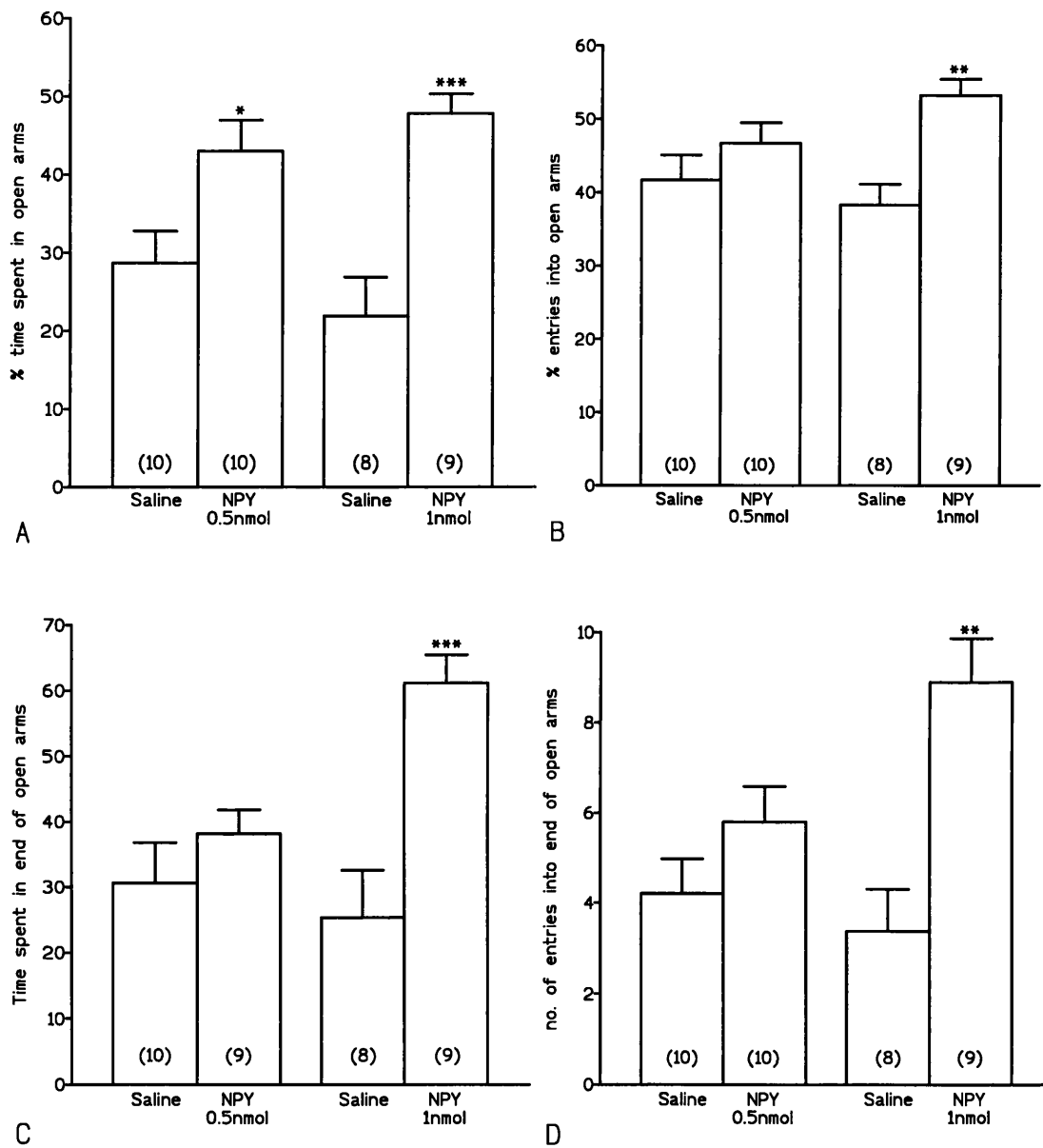


A



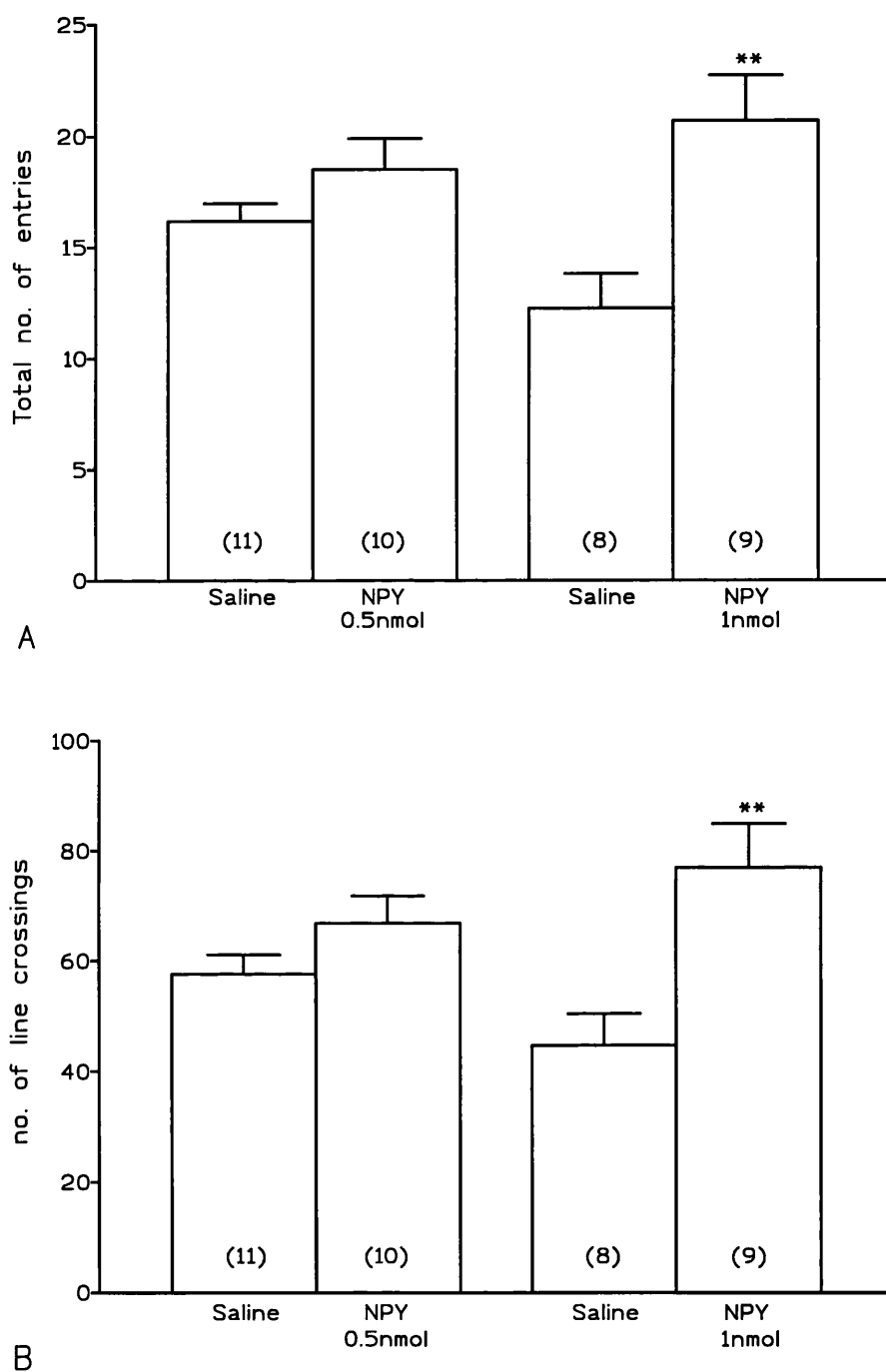
B

**Fig. 5.1.5 A,B.** Behaviour during a 5min elevated x-maze test 1 h after i.c.v. administration of 1µl saline either alone or containing NPY (13-36), [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY, PYY & NPY (5.0 nmol). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Total number of entries into any arms of the maze.  $F(6,3,4)=3.22$ ,  $P<0.05$ . No significance obtained on comparing the groups to the saline treated controls on Dunnet's test. **B** Number of line crossings made.  $F(6,2,4)=2.743$ ,  $P<0.05$ . No significance obtained on comparing the groups to the saline treated controls on Dunnet's test.



**Fig. 5.1.6 A,B,C,D.** Behaviour during a 5min elevated x-maze test 0.5 h after injection of 0.5µl saline either alone or containing NPY (0.5 & 1.0nmol) into the ventral hippocampus. Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Percentage time spent in open arms of the maze. **B** Percentage entries into the open arm of the maze. **C** The time spent in the end of the open arms. **D** Number of entries into end of open arms.

\*, \*\* & \*\*\* indicate differences versus the corresponding saline controls at 5%, 1% & 0.1% significance levels (Students t-test).



**Fig. 5.1.7 A,B.** Behaviour during a 5min elevated x-maze test 0.5 h after administration of 0.5 $\mu$ l saline either alone or containing NPY (0.5 & 1.0nmol) into the ventral hippocampus. Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Total number of entries into arms of the maze. **B** Number of line crossings made.

\*\* indicates a difference versus the corresponding saline treated control at the 1% significance level (Student's t-test).



measuring anxiety-like behaviour (Figure 5.1.6).

NPY (1nmol) also produced significant increases in both the total number of entries and the number of line crossings, whereas NPY (0.5nmol) had no effect (Figure 5.1.7).

## 5.2 Social interaction test

The social interaction test (File and Hyde, 1978; 1979; File, 1980) is based on the environmental suppression (unfamiliarity of the test arena and rat partner, and high illumination) of normal social behaviours displayed by rats when paired together. Anxiolytic drugs should release the suppressed behaviour and therefore increase the time spent in social interaction between rats.

### 5.2.1 Validation of test procedure

Chlordiazepoxide (5mg/kg,p.o. dissolved in water) increased time spent in social interaction for both the 5 and 15min test by 110% and 108% respectively (Figure 5.2.1a). No significant changes in locomotor activity, as measured by the number of line crossings made, were seen (Figure 5.2.1b).

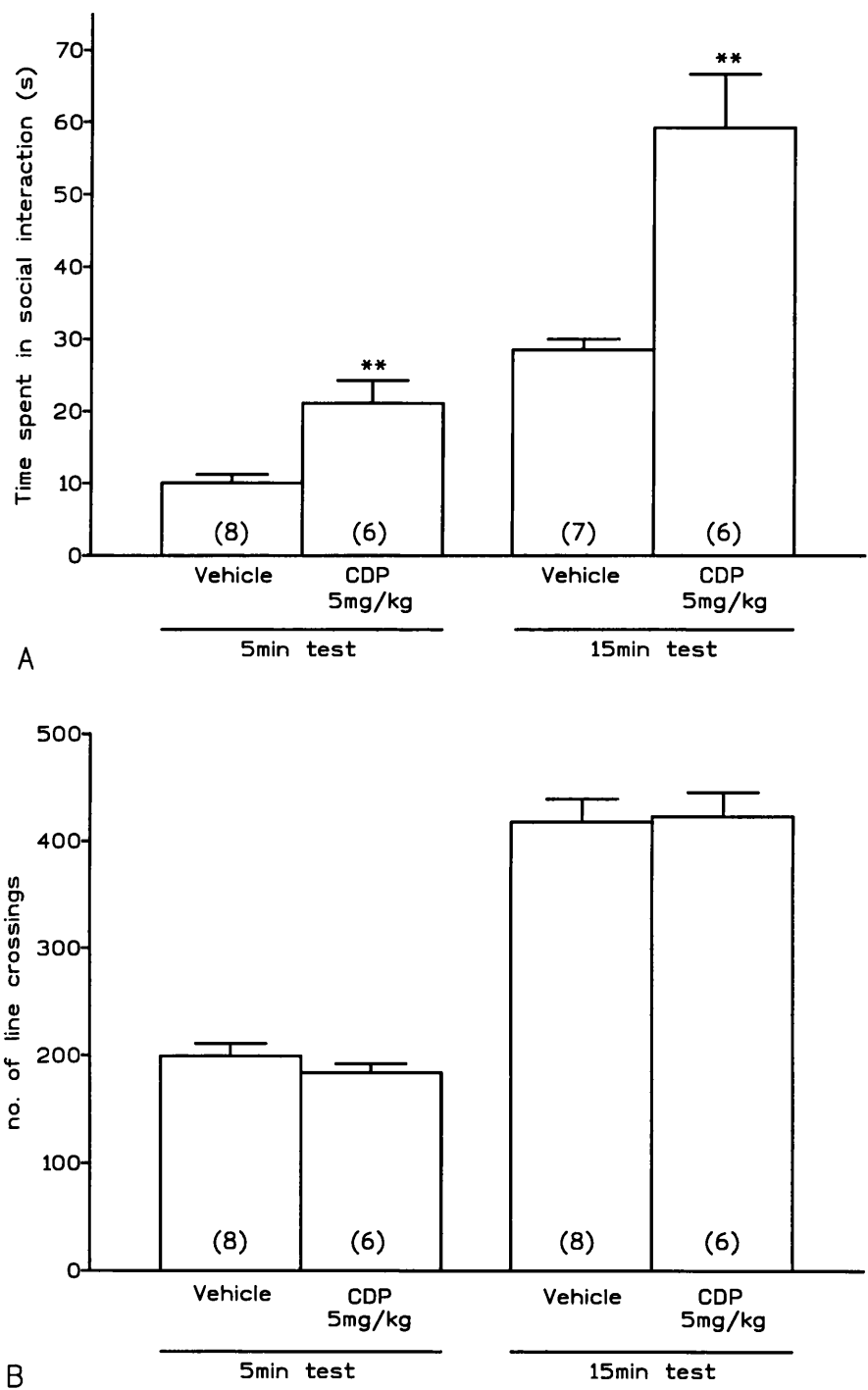
### 5.2.2 Effect of i.c.v. neuropeptide Y administration

The i.c.v. administration of 1nmol or 5nmol NPY to animals 1 h prior to testing caused small, but insignificant increases in social interaction of 45% and 41% respectively over the first 5min of the test period. During the full 15min test a similar result was seen with 50% and 27% increases, respectively (Figure 5.2.2a).

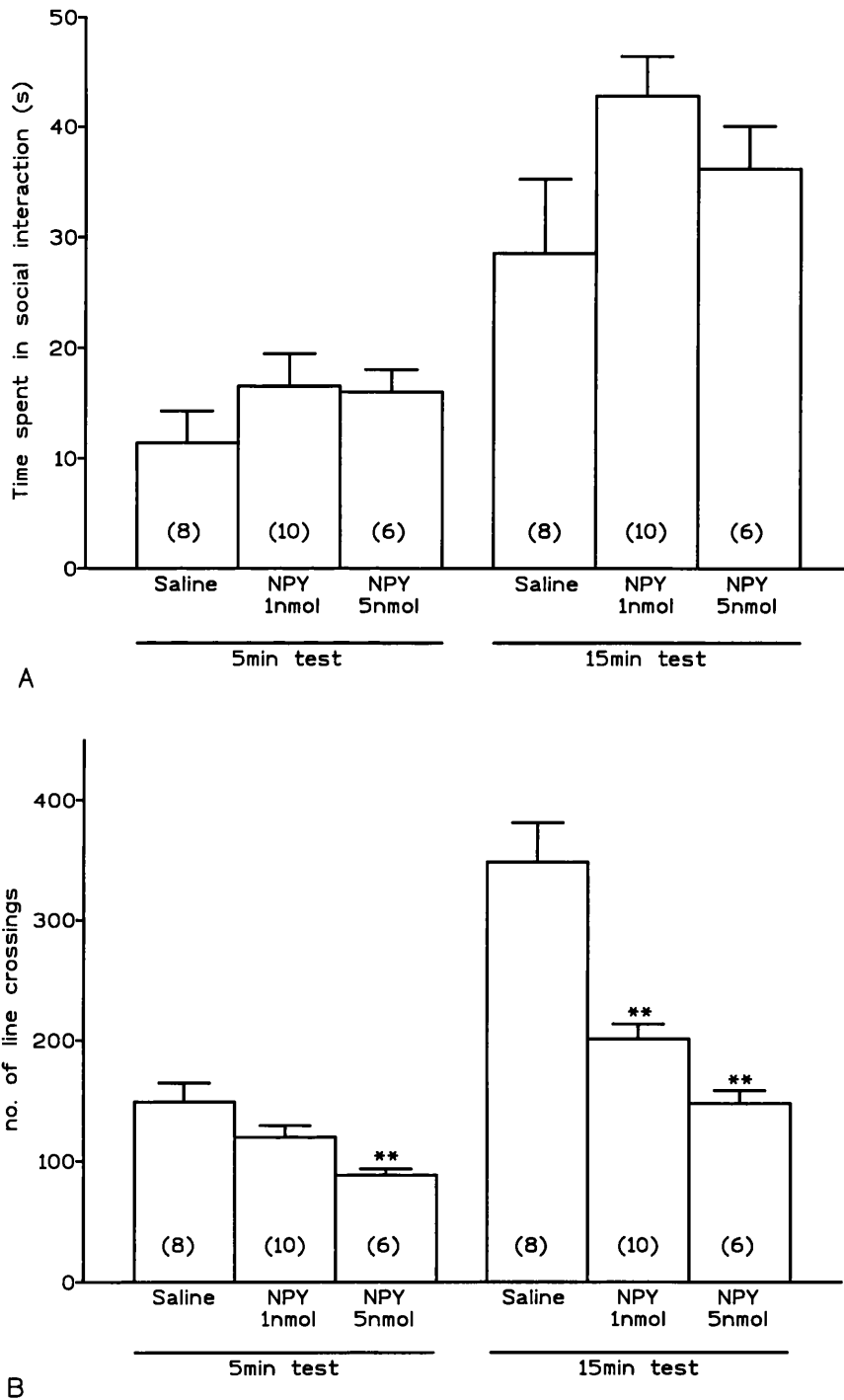
A large decrease in locomotor activity was also seen during these tests. NPY (1nmol) had no significant effect on the number of line crossings over the first 5min of the test period, whereas over 15min a significant decrease of 41% was seen. NPY (5nmol) showed significant decreases of 42% and 58% over the first 5min and 15min tests respectively (Figure 5.2.2b).

### 5.2.3 Effects of various neuropeptide Y agonists

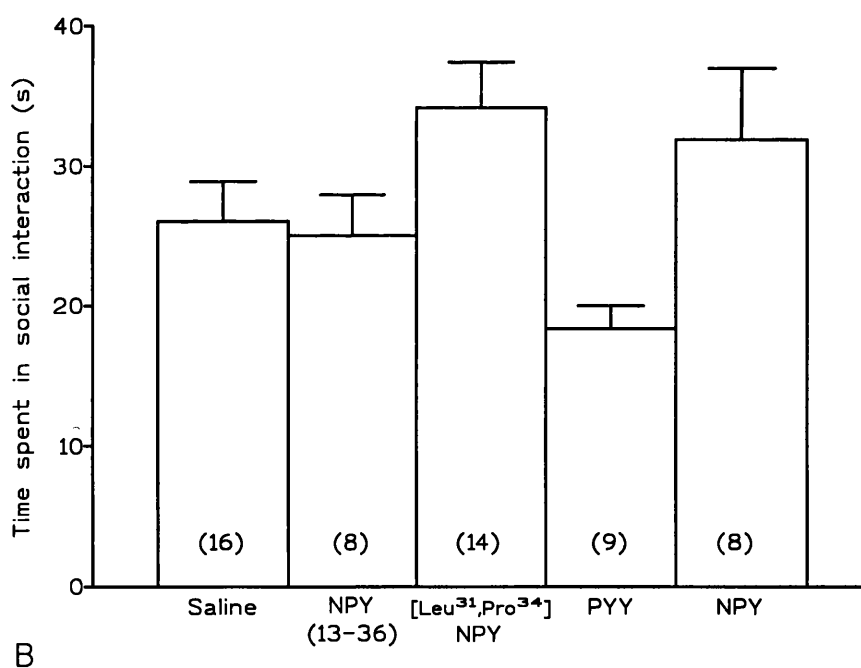
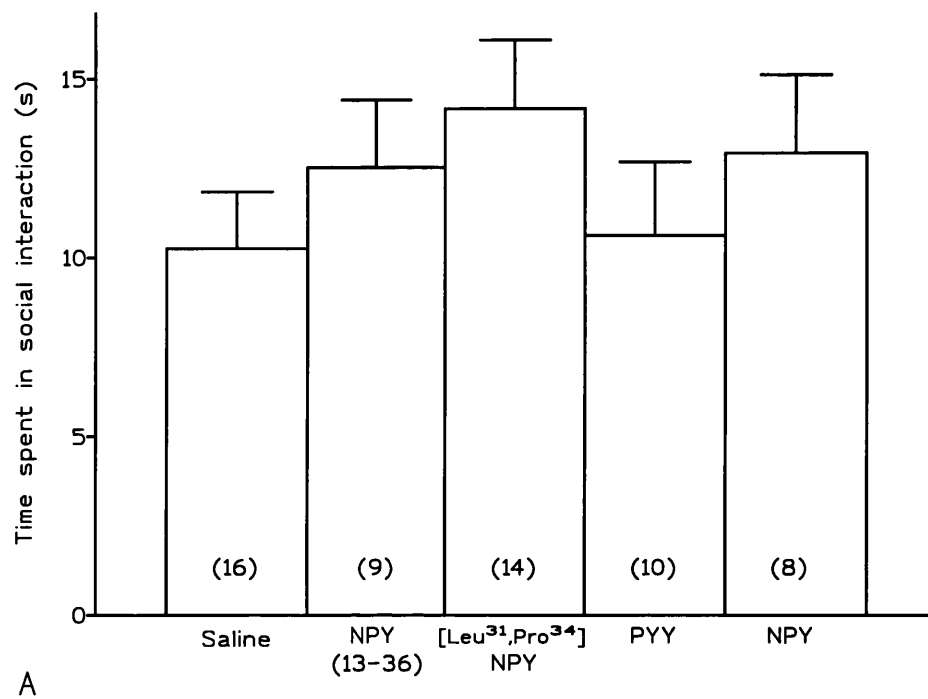
There were no significant increases in the time spent in social interaction during either the 5 or 15min test periods following i.c.v. administration of various NPY agonists (Figure 5.2.3). However, both NPY and [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY showed consistent but insignificant increases in social interaction over both time periods.



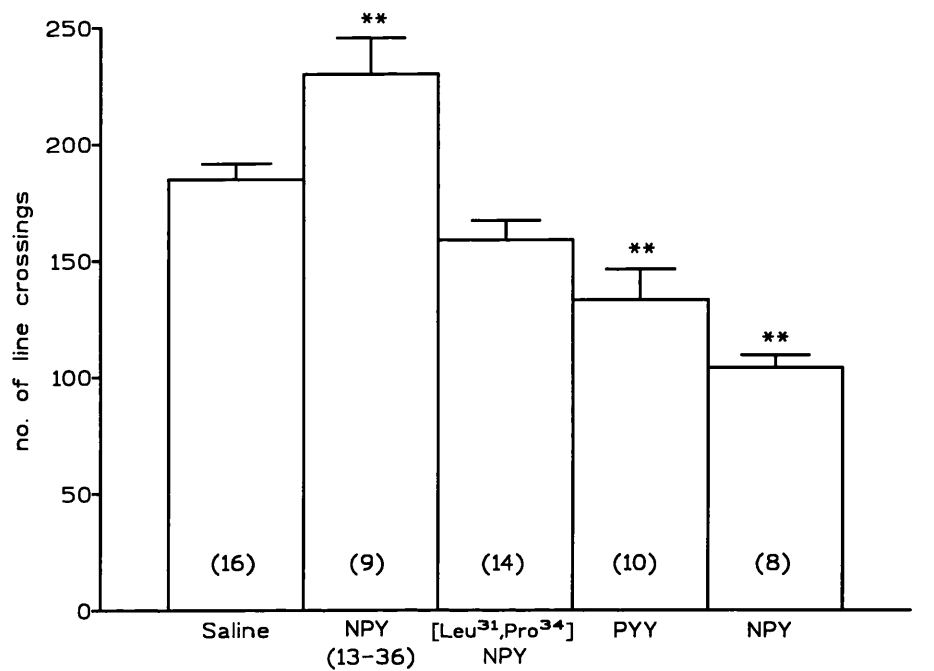
**Fig. 5.2.1 A,B.** Behaviour during a 5 and 15 min social interaction test 1 h after oral administration of water either alone or containing CDP (5mg/kg). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Time spent in social interaction. **B** Number of line crossings. \*\* indicates differences versus saline treated controls at the 1% significance level (Students t-test).



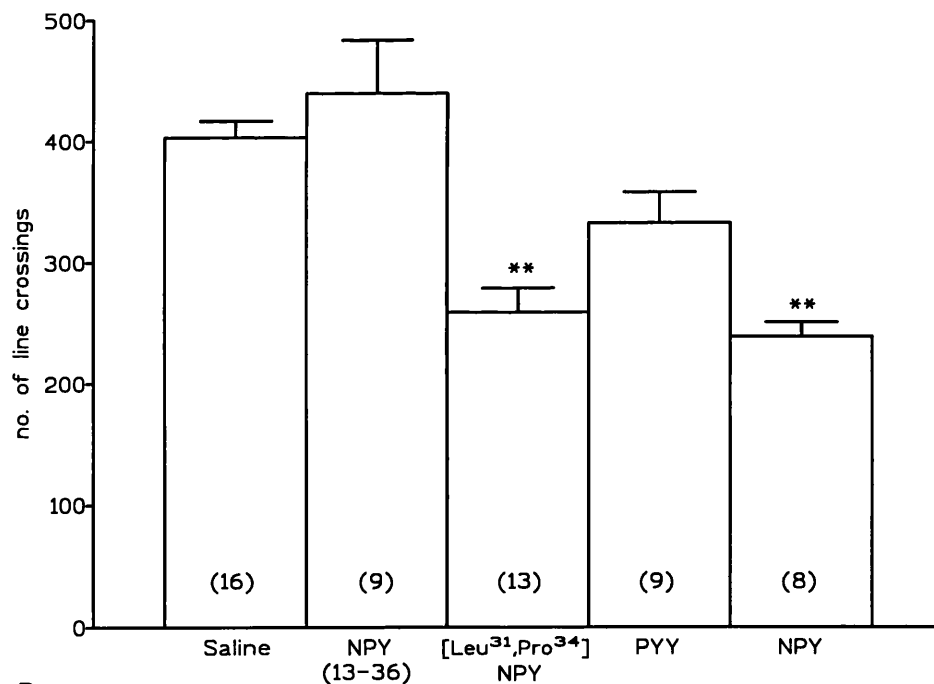
**Fig. 5.2.2 A,B.** Behaviour during a 5 and 15 min social interaction test 1 h after i.c.v administration of 1 $\mu$ l saline either alone or containing NPY (1.0 & 5.0nmol). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Time spent in social interaction was not significant,  $F(2,12)=1.03$  &  $2.28$  for the 5 and 15min tests respectively. **B** Number of line crossings.  $F(2,12)=6.02$  &  $22.36$  for the 5 and 15min tests respectively,  $P<0.01$  &  $0.0001$ . \*\* indicates differences versus saline treated controls at the 1% significance level (Dunnet's test).



**Fig. 5.2.3 A,B.** Behaviour during a 5 and 15 min social interaction test 1 h after i.c.v. administration of 1µl saline either alone or containing NPY (13-36), [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY, PYY & NPY (5.0 nmol). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Time spent in social interaction during the 5min test was not significant,  $F(5,4)=0.85$ . **B** Time spent in social interaction during the 15min test.  $F(5,4)=3.375$ ,  $P<0.05$ . No significance was obtained on comparing individual means versus saline controls (Dunnet's test).

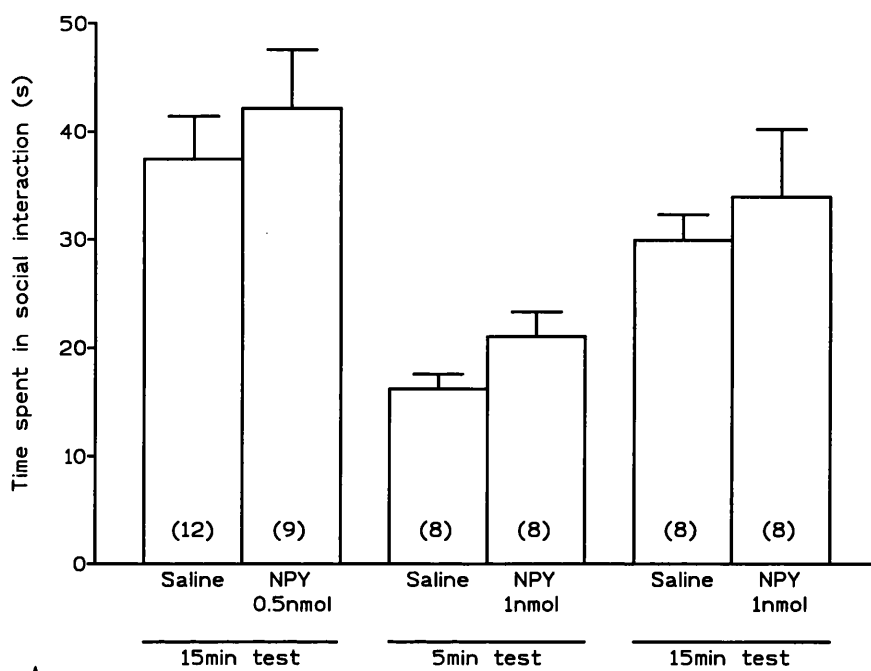


A

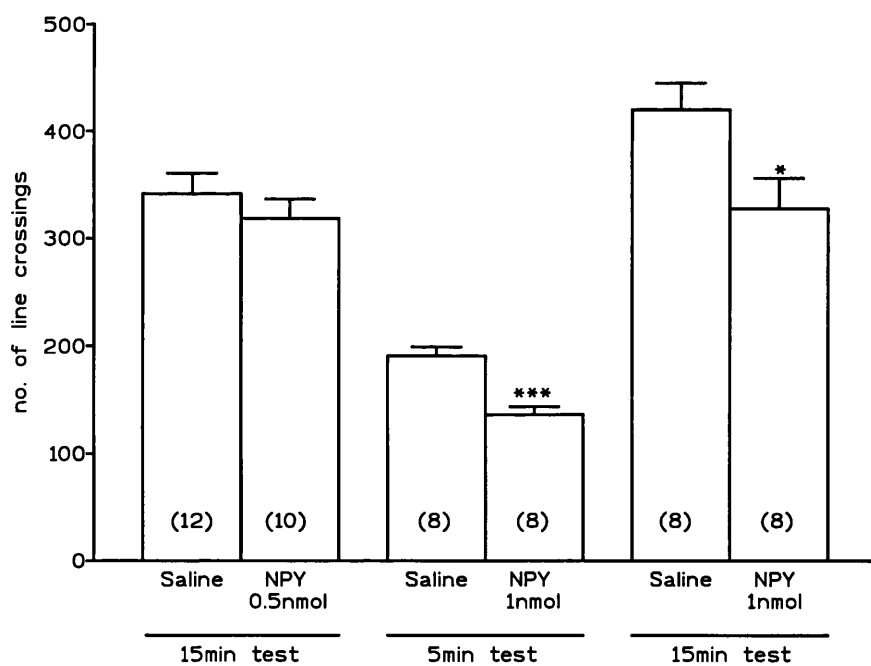


B

**Fig. 5.2.4 A,B.** Behaviour during a 5 and 15 min social interaction test 1 h after i.c.v. administration of 1 $\mu$ l saline either alone or containing NPY (13-36), [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY, PYY & NPY (5.0 nmol). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Total number of line crossings made during the 5min test,  $F(52,4)=18.96$ ,  $P<0.0001$ . \*\* indicates a difference versus saline controls at a significance level 1% on Dunnet's test. **B** Total number of line crossings made during the 15min test.  $F(50,4)=13.42$ ,  $P<0.0001$ . \*\* indicates a difference versus saline controls at a significance level of 1% (Dunnet's test).



A



B

**Fig. 5.2.5 A,B.** Behaviour during a 5 and 15 min social interaction test 0.5 h after administration of 0.5µl saline either alone or containing NPY (0.5 & 1.0nmol) into the ventral hippocampus. Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group. **A** Time spent in social interaction. **B** Number of line crossings.

\* & \*\*\* indicate differences versus the corresponding saline treated controls at the 5% & 0.1% significance levels respectively (Students t-test).

The locomotor activity was significantly decreased by PYY (28%) and NPY (44%) over the first 5min of the test, and by both [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY (36%) and NPY (41%) over 15 min. NPY (13-36) was found to increase locomotor activity during the 5min test (Figure 5.2.4).

#### 5.2.4 Effect of microinjection of neuropeptide Y in the ventral hippocampus

The time spent in social interaction following microinjections of NPY (0.5 and 1nmol) into the ventral hippocampus showed a tendency to increase over both test periods. Locomotor activity was decreased with NPY (1nmol) at both 5 and 15min (Figure 5.2.5).

#### 5.3 Vogel's drinking conflict test

The Vogel test (Vogel et al., 1971) is based on the conflict between reward and punishment. The test uses water deprived rats which are punished for drinking. The aversive stimulus in this test is an electrical stimulus accompanying the acceptance of a water reward. The animal quickly associates the two conflicting stimuli and adjusts its behaviour accordingly. Thus their water intake will be low, even after a considerable period of water deprivation. Anxiolytic agents will decrease the conflict which exists in this situation and the animal will drink more, and thus receive more electric shocks than a



control animal. As this model also involves the behaviour of drinking and shock perception, it is important that the agent used is tested for effects on these parameters, and these effects considered when interpreting the effects of this test.

### 5.3.1 Validation of test procedure

The anxiolytic compound chlordiazepoxide caused a dose dependent increase in the number of shocks accepted by the animals, which reached significance at 5mg/kg (p.o. dissolved in water). This demonstrates the validity of the Vogel's test for the detection of anxiolytic-like effects (Figure 5.3.1).

### 5.3.2 Effects of i.c.v. neuropeptide Y administration

Animals receiving i.c.v. administration of NPY (1 and 5nmol) accepted more shocks than control animals (Figure 5.3.2). The increases in the number of shocks accepted were very large being 240% and 308% for 1 and 5nmol NPY, respectively.

These results were shown to be independent of any possible effects of NPY on thirst as no significant difference was seen during a 10min free drinking test (Figure 5.3.3). There was also no effect of NPY in a perceived shock threshold

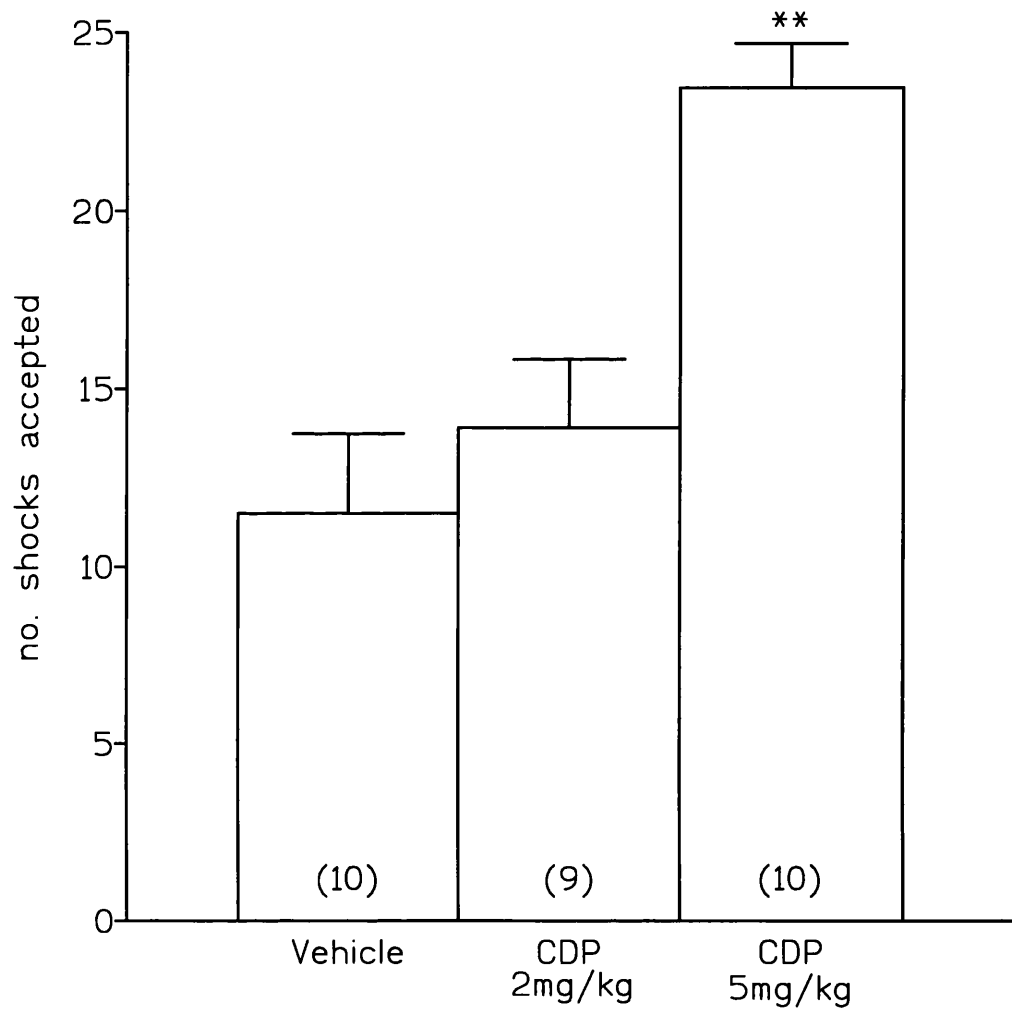
experiment (see Figure 5.3.6).

### 5.3.3 Effect of various neuropeptide Y agonists

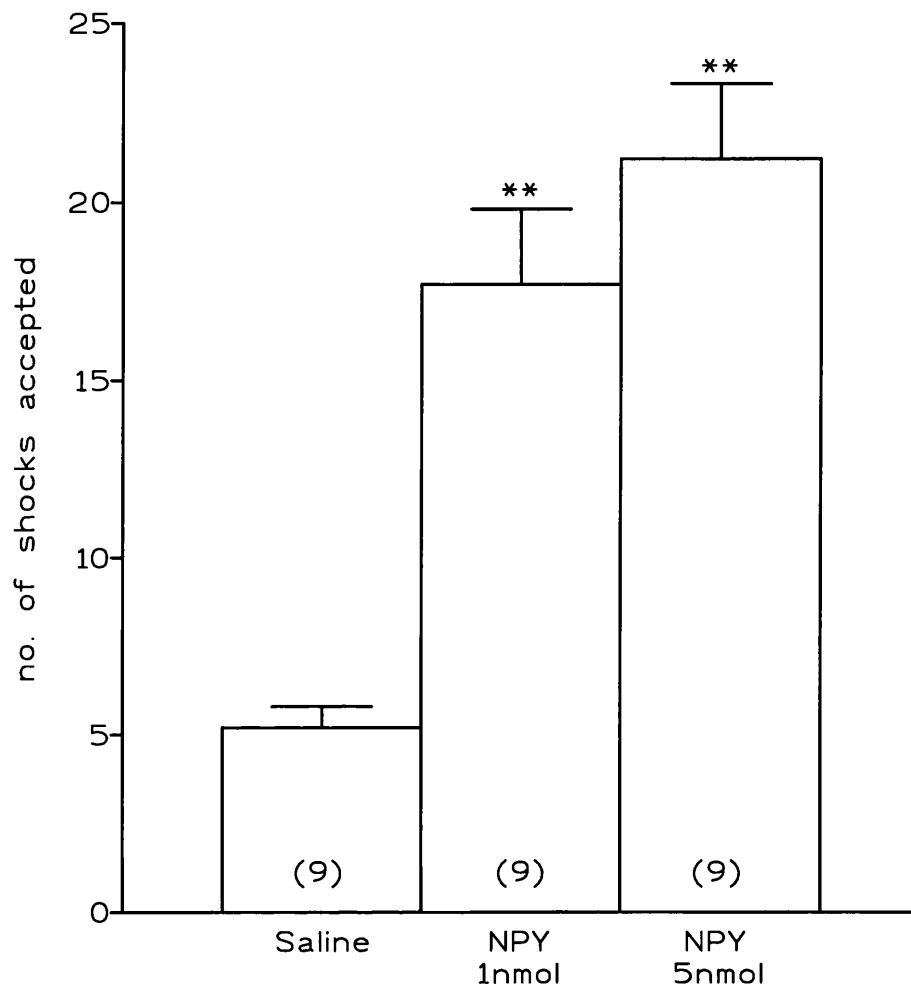
There were large, significant increases in the number of shocks accepted following i.c.v. administration of [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY (132%), PYY (249%) and NPY (211%). NPY (13-36) showed a small, but insignificant increase (Figure 5.3.4). None of the agonists had any effects in a 10min free drinking experiment (Figure 5.3.5) or a perceived shock threshold experiment (Figure 5.3.6) indicating the increase in number of shocks accepted to be a genuine effect of anxiolysis and not an effect on thirst or pain perception.

### 5.3.4 Effect of neuropeptide Y in the ventral hippocampus

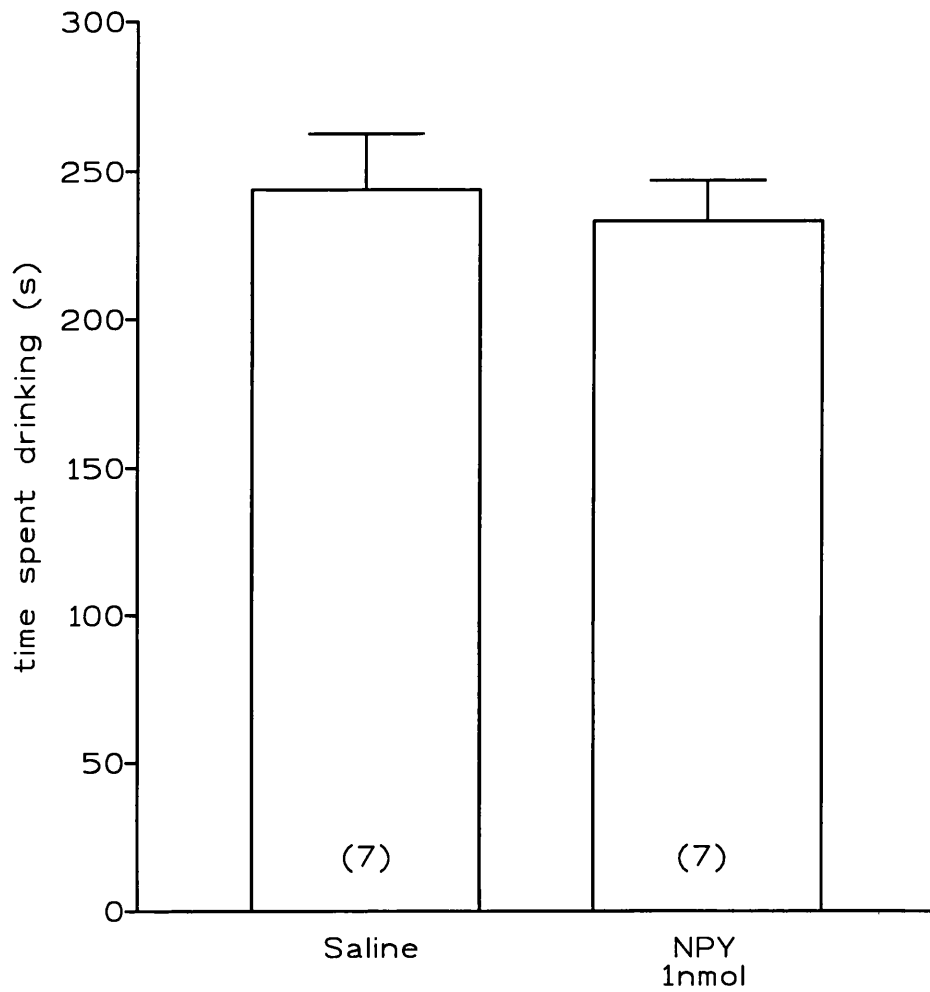
NPY (0.5 and 1nmol) increased the number of shocks accepted 0.5 h after microinjection into the hippocampus by 105% and 56% respectively (Figure 5.3.7).



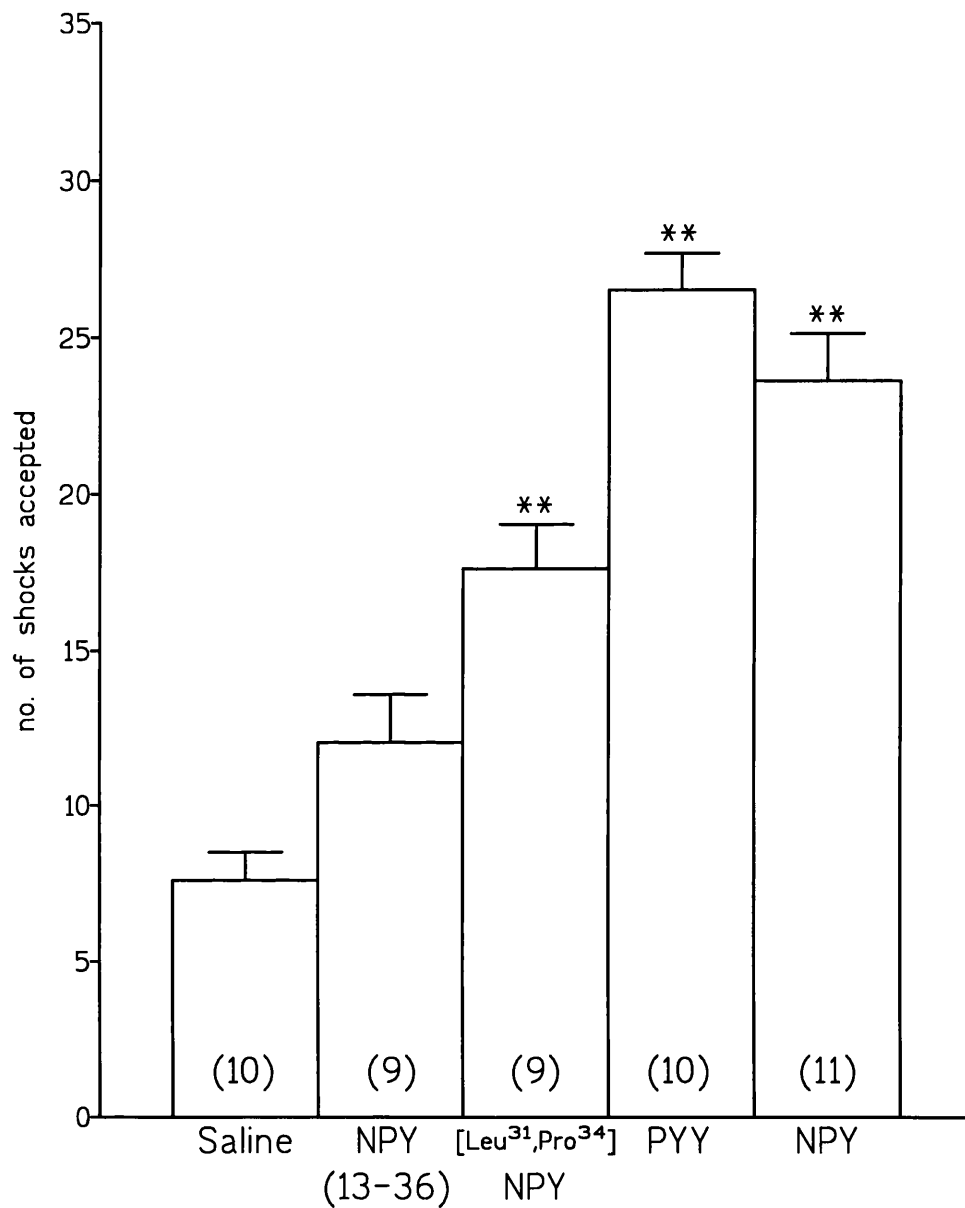
**Fig. 5.3.1.** The total number of shocks accepted during a Vogel's drinking conflict test 1 h after oral administration of water either alone or containing CDP (2 & 5mg/kg).  $F(2,2)=12.067$ ,  $P<0.001$ . \*\*\* indicates differences versus saline treated controls at the 0.1% significance level (Dunnet's test). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group.



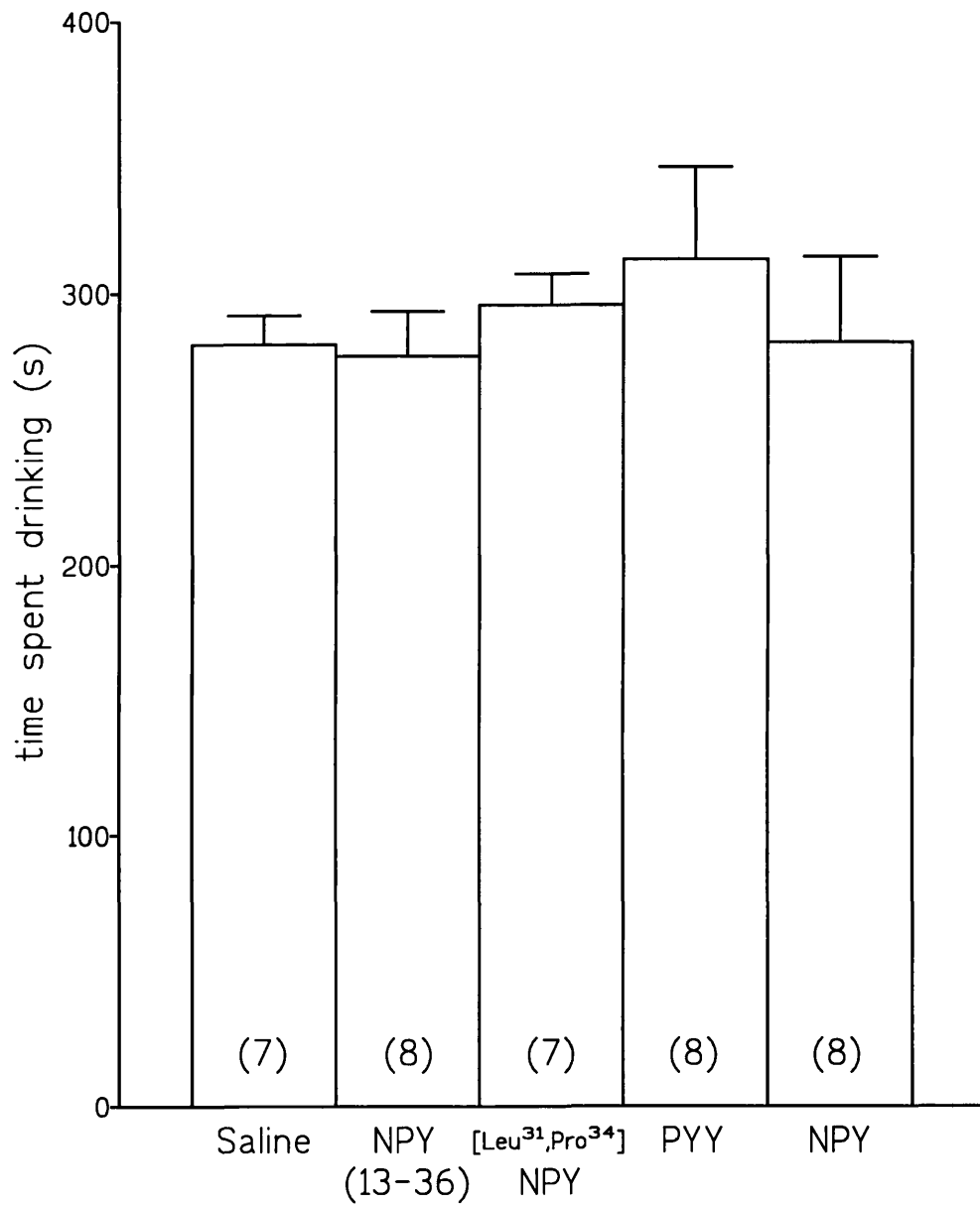
**Fig. 5.3.2.** The total number of shocks accepted during a Vogel's drinking conflict test 1 h after i.c.v. administration of saline either alone or containing NPY (1 & 5nmol).  $F(2,4,2)=23.121$ ,  $P<0.0001$ . \*\* indicates differences versus saline treated controls at the 1% significance level (Dunnet's test). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group.



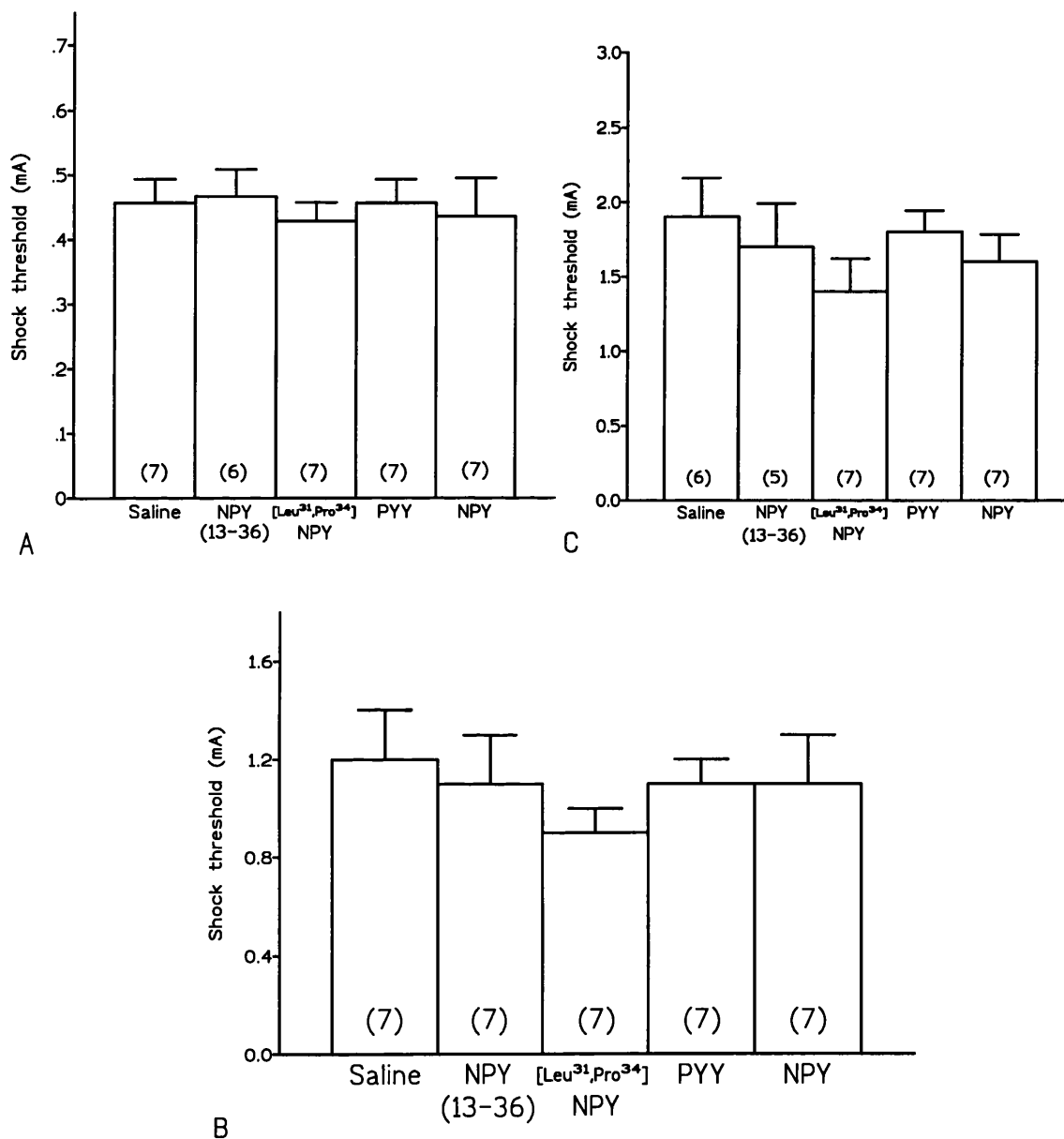
**Fig. 5.3.3.** The total time spent in unpunished drinking in the Vogel apparatus 1 h after i.c.v. administration of saline either alone or containing NPY (1nmol). No significant difference was found between the peptide and saline control groups (Students t-test). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group.



**Fig. 5.3.4.** The total number of shocks accepted during a Vogel's drinking conflict test 1 h after i.c.v. administration of saline either alone or containing NPY (13-36), [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY, PYY & NPY (5nmol).  $F(4,4)=35.74$ ,  $P<0.0001$ . \*\* indicates differences verses saline treated controls at the 1% significance level (Dunnet's test). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group.



**Fig. 5.3.5.** The total time spent in unpunished drinking in the Vogel apparatus 1 h after i.c.v. administration of saline either alone or containing NPY (13-36), [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY, PYY & NPY (5nmol) was not significant,  $F(3,4)=0.378$ . Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group.



**Fig. 5.3.6.** The shock thresholds obtained for several levels of animal response 1 h after i.c.v. administration of saline either alone or containing NPY (13-36), [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY, PYY & NPY (5nmol). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group **A** Shock threshold for causing the animal to pause in drinking was not significant,  $F(29,4)=0.246$ . **B** Shock threshold for causing the animal to sharply withdraw its head away from the drinking spout was not significant,  $F(30,4)=0.429$ . **C** Shock threshold for causing the animal to move its body away from the drinking spout was not significant,  $F(27,4)=0.913$ .



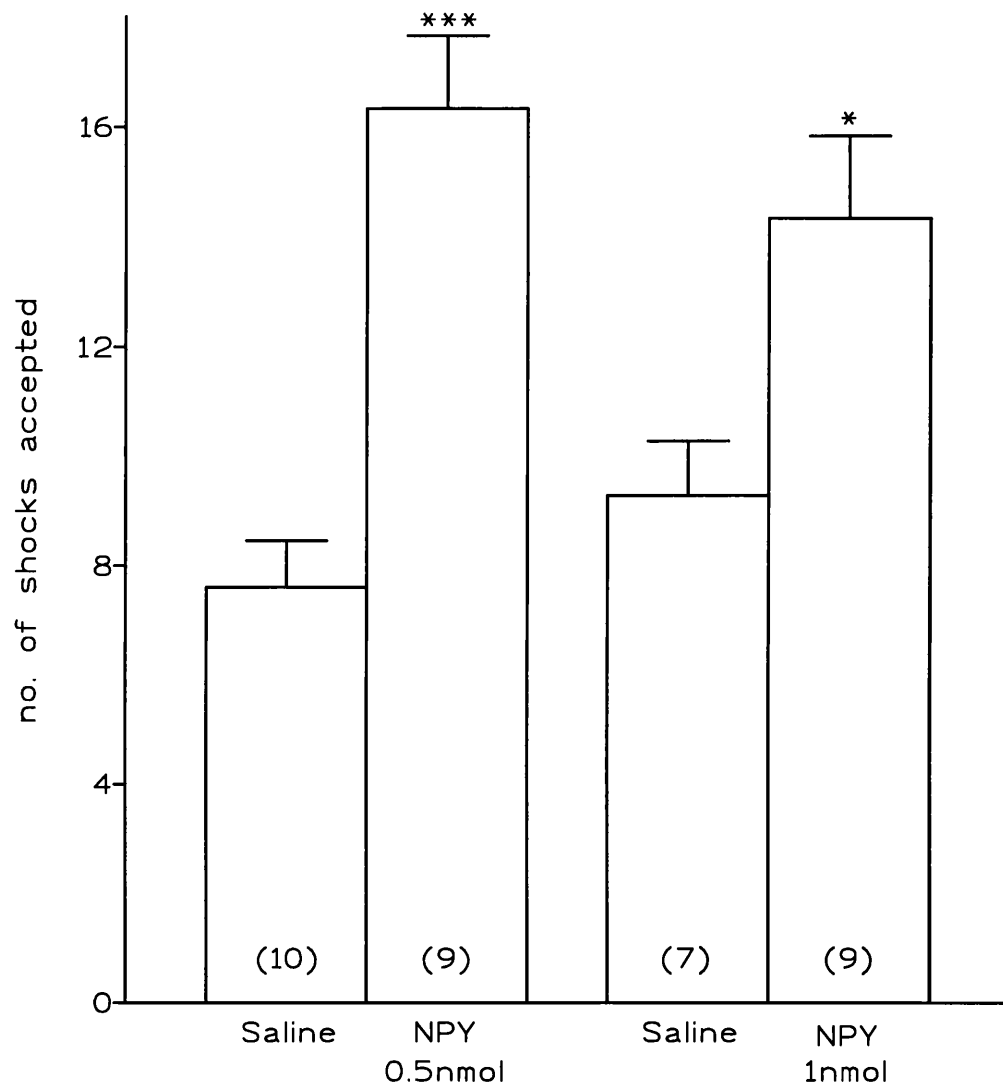


Fig. 5.3.7. The total number of shocks accepted during a Vogel's drinking conflict test 0.5h after administration of 0.5µl saline either alone or containing NPY (0.5 & 1.0nmol) into the ventral hippocampus. \* & \*\*\* indicates differences versus corresponding saline treated controls at the 5% & 0.1% significance levels respectively (Students t-test). Error bars represent standard error of the mean. Figures in brackets denote the number of animals in the group.

## 5.4 Discussion

### 5.4.1 Effects of i.c.v. neuropeptide Y administration in anxiolytic tests

It is clear from the initial experiments that NPY has a strong anxiolytic-like effect in both the x-maze and the Vogel's drinking conflict test at both 1 and 5nmol dose levels. In these models, an effect that mimicks that of an anxiolytic compound is termed an anxiolytic-like effect. This is due to the possibility that the drug might be acting on some other system which in turn produces an effect in the models. The results of several tests, each with different stimuli, is therefore desirable to ensure a 'real' anxiolytic effect is being seen. The fact that in this study NPY showed anxiolytic-like effects in two different models is strong evidence for an "anxiolytic" effect of NPY.

In the x-maze, NPY (5nmol) produced a sedative effect as shown by the decrease in overall activity (total entries and line crossings). These findings are in agreement with Heilig et al. (1989) who also administered the same doses of NPY to produce anxiolytic-like effects in the elevated x-maze and Vogel's conflict models. They also found a sedative effect of NPY at 5nmol, as observed in the present study. Previous work by Heilig and Murison (1987a) on open field and home cage activity had also found NPY to suppress activity which they interpreted to be due to sedation.

It is possible that the sedative effects of NPY may be masking any anxiolytic-

like effects in the social interaction test. This test failed to detect any anxiolytic-like activity following NPY treatment. Taken in isolation this would suggest that NPY was not anxiolytic, but after examining the potency of its effects in the other models this does not seem to be the case. Social interaction was actually increased by 50% during the 15min test following 1nmol NPY. After 5nmol NPY, however, this increase was reduced to just 27%. The sedative effects of NPY in this model are manifested more than in the x-maze, with a 58% decrease in locomotor activity after 5nmol NPY. If animals are not normally moving around the test arena then social interaction is less likely. This theory is re-enforced by observation of the animals in the test arena. After an initial walk around the perimeter of the test arena NPY treated animals tended to lie down and just occasionally get up and walk around. This suggests that the social interaction test may be unsuitable for the detection of any anxiolytic-like actions of NPY at the doses used in this study. However, it may be possible that the doses of NPY used in this model were too high. If NPY was administered at a lower dose than would cause significant sedative effect, an anxiolytic-like effect might become apparent.

#### 5.4.2 Effects of neuropeptide Y receptor agonist administration in anxiolytic tests

Having ascertained that NPY shows anxiolytic-like activity in two of the behavioural models used, it remained to try and elucidate the receptor

subtypes responsible for this effect. This was examined using several NPY analogues and fragments which are agonists at specific NPY receptor subtypes.  $Y_1$  receptor activity was detected using the  $Y_1$  receptor agonist [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY and  $Y_2$  receptor activity using the C-terminal fragment NPY (13-36). PYY was used to determine if the  $Y_3$  receptor was involved as it is ineffective at this receptor subtype. To allow for these compounds being less potent than NPY, they were administered at 5nmol dose levels (5 times greater than NPY needed to have an effect). NPY (5nmol) was also included in the tests as a positive control to ensure that the tests were detecting an effect. It is possible that problems might occur as a result of using this dose level to study the ligands' effects. As already shown (see section 5.4.1), 5nmol NPY produces significant sedative-like effects, which, in the case of the social interaction test, probably resulted in the test's failure to detect any anxiolytic-like effects. Thus by using this dose for all the ligands, we risk the possible confusion of the data due to a sedative effect. It would therefore, have been of benefit to include a lower dose of each ligand. The results of the x-maze were disappointing with the positive control (NPY) failing to cause any significant increases in any of the parameters except % time spent in the open arms. It is not surprising, therefore, that none of the other agonists caused significant changes in any of the parameters. Despite this, there was still a trend which emerged on examining the data (Figure 5.1.4a,b). There were trends toward increases in % time spent in the open arms for [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY (44%), PYY (60%) and NPY (88%), and in % entries into open arms for [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY (21%), PYY (20%) and NPY (22%). The  $Y_2$  receptor agonist NPY (13-36) showed no changes at all

in either of these parameters.

Despite the failure of the social interaction test to detect any effects in the previous experiment it was used again as a specific receptor agonist might display anxiolysis without sedation. Although no significant changes in social interaction were observed, increases were apparent for [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY (38% and 31%) and NPY (26% and 22%) over both the first 5min and the full 15 min test. Interestingly the Y<sub>2</sub> receptor agonist NPY (13-36) caused an increase in locomotor activity during the social interaction test whereas the Y<sub>1</sub> receptor agonist [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY, PYY and NPY itself caused sedative effects. Also of interest is that, whereas NPY shows significant decreases in locomotor activity over both the first 5 min and the full 15 min test, the decreases seen with PYY only reach significance over the first 5 min. Possible reasons for this are firstly that the two ligands might diffuse to, and away from, their sites of action at different rates, thus causing a time difference for their effects. Another possibility is that they might be metabolised differently in situ. If one were metabolised more quickly than the other, then it could explain these results. Also, if the metabolism of one results in active peptide fragments, then its effects might be seen to be more long lasting.

Vogel's drinking conflict test was the most interesting of the three models used in this study. [Leu<sup>31</sup>,Pro<sup>34</sup>] NPY, PYY and NPY all increased the number of shocks accepted (figure 5.3.4). NPY (13-36) also produced a small but insignificant increase in the number of shocks accepted. It is possible that if

NPY (13-36) is having a stimulant effect on the animals they might be more active in trying to drink, thus increasing the number of shocks accepted. Alternatively it might in itself be having a small anxiolytic effect either at the  $Y_2$  receptor, or possibly via a small interaction at the  $Y_1$  receptor. It is impossible to say from this study exactly which receptor subtypes are involved in the anxiolytic-like effects of NPY. This study does however strongly implicate the  $Y_1$  receptor as the principle NPY receptor subtype responsible. There is also the possibility, shown by the Vogel's test, that the  $Y_2$  receptor may have a small effect with regard to anxiolysis. Heilig et al (1989) showed that, over the dose range tested (0.2-2.0nmol), the  $Y_2$  agonist had no significant effects on the x-maze, and a small but insignificant effect in the Vogel's test at 2nmol. This accords with the results of the present study. Heilig et al. (1989) inferred from this that the anxiolytic actions of NPY were mediated via the  $Y_1$  receptor subtype. However, the dose range of NPY (13-36) used in the latter study were rather limited, not being as extensive as that used for NPY itself. In this study, therefore, a higher dose of  $Y_2$  receptor agonist was used in order to assess more fully the effects found by Heilig et al. (1989). The study by Heilig et al. (1989) also failed to address the possibility that the anxiolytic-like effects of NPY might be mediated by the NPY  $Y_3$  receptor subtype. This was presumably because at the time of this study, the evidence for a third receptor subtype was still in its infancy (see section 1.5.2e). For this reason, PYY was also used to look for any  $Y_3$  receptor effects. As PYY (5nmol) was effective in increasing the number of shocks accepted it seems unlikely that the  $Y_3$  receptor is involved as it is insensitive to PYY.

The findings of this study that the  $Y_1$  receptor is involved in mediating anxiolysis are in line with the finding that decreasing the number of  $Y_1$  receptors, by repeated injection of an antisense oligodeoxynucleotide to inhibit the receptor expression, caused an anxiety-like state in rats (Wahlestedt et al.,1993).

Data from the social interaction test demonstrated that the sedative effects of NPY are probably mediated by the  $Y_1$  receptor as well, with the  $Y_2$  receptor producing a stimulant effect.

#### 5.4.3 Ventral hippocampus

One of the aims of this study was to try and find the regions of the brain involved in the modulation of anxiety by NPY. One of the highest density of receptor binding sites found in the rat brain as a result of [ $^{125}$ I]-BH NPY autoradiography was the ventral hippocampus (CA1) (see section 3.1.1). This region also showed neuronal activity following central NPY administration as detected by FLI immunohistochemistry (see section 2.3). This made the ventral hippocampus a possible location for behavioural studies using microinjection of NPY. When given 0.5nmol NPY, a dose dependency emerged with all parameters of the x-maze showing a tendency to increase. The % time spent in the open arm showed a significant increase. 1nmol NPY caused significant increases in all the parameters including those relating to locomotor activity.

The stimulant effect on locomotor activity was very interesting as only the  $Y_2$  receptor agonist given i.c.v. caused similar effects. In the ventral hippocampus the  $Y_2$  receptor predominates over the  $Y_1$  receptor (see section 3.2) and this therefore may explain the stimulant effect of NPY when injected into this area. It is possible that the anxiolytic-like effect of NPY in this area is due to the increase in locomotor activity itself, causing the rat to make more entries into the arms. The opposite may also be possible, with the rat less 'anxious' due to the drug, the open arms no longer pose a threat, and so the animal now has unrestricted movements throughout the maze. This in itself might allow for a greater total number of entries and line crossings to be made causing an apparent increase in activity.

During the social interaction test, in contradiction to the x-maze test, microinjection of NPY into the hippocampus was found to cause a reduction in locomotor activity. This reduction was small in comparison with the increase of activity seen in the x-maze, the decrease being by 22% (15min test) compared to an increase of 71% (mean of % change in total number of entries and line crossings). It is unclear why this discrepancy occurred between the two models. Following i.c.v. NPY administration the reduction of activity seen in the social interaction test was much greater than that for the x-maze. Therefore it is possible that as the hippocampus has both  $Y_1$  and  $Y_2$  receptor subtypes, a conflict might occur with each producing opposing effects on activity. The level of sedation during the social interaction test is greater and therefore may be harder to overcome than that of the x-maze. This might be



because of the differences in stimuli present in the two tests. The x-maze could be perceived as being more 'interesting' for the rats than the social interaction test arena, with several arms to explore. This could mean that the animals are stimulated for longer before they succumb to the sedative effects of the peptide. Other possibilities are that there are different types of anxiety being measured between the two tests, that there are different motivational aspects of the tests, or even that the animals in the social interaction had previous exposure to both the x-maze, and in some cases NPY administration.

The result of the Vogel's conflict test confirm that of the x-maze that localised administration of NPY in the ventral hippocampus produces strong anxiolytic-like effects.

#### 5.4.4 Conclusion

In conclusion, this study has clearly demonstrated an anxiolytic-like effect of centrally administered NPY, an effect which seems to be mediated mainly by the  $Y_1$  receptor subtype. A sedative effect of the peptide was also demonstrated, again mediated by the  $Y_1$  receptor subtype. A stimulatory effect was seen however following  $Y_2$  receptor agonist administration. This stimulatory effect was also demonstrated in one of the models following direct microinjection of NPY into the ventral hippocampus, possibly a result of a predominantly  $Y_2$  receptor density in this area. Thus, the ventral hippocampus

may be a region which is involved in the modulation of the anxiolytic-like effects of NPY.

**CHAPTER 6**  
**GENERAL DISCUSSION**  
**AND**  
**FUTURE WORK**

## 6 General discussion

The autoradiographic analysis of NPY receptor binding sites in this study shows them to be distributed widely throughout the brain. Areas particularly rich in these receptor binding sites include the hippocampus, claustrum, amygdala, septum, stria terminalis and cerebral cortex. Further study using selective agonists for NPY receptor subtypes demonstrated that NPY Y<sub>1</sub> receptor binding sites are prominent in the cortex and claustrum, whereas the Y<sub>2</sub> receptor binding sites are prominent in the hippocampus, septum, amygdala and stria terminalis. These regions are of importance for emotional and cognitive function, and could therefore be possible sites of action for the anxiolytic-like effects of NPY.

Using the expression of FLI to map neuronal activity, several areas of the rat brain were found to be activated following i.c.v. NPY administration. These areas are the entorhinal cortex, amygdalopiriform transition area, nucleus accumbens, ventral hippocampus, paraventricular thalamic nucleus, and the dorsal endopiriform nucleus. Since NPY immunoreactivity has been identified in these areas (see Gray and Morley, 1986), and NPY receptor binding sites have been shown to be present in the ventral hippocampus, entorhinal cortex, and various amygdaloid areas (see section 3), these brain regions would appear to be promising areas for further study with respect to the physiology of NPY. Since the theme of this thesis is the possible anxiolytic-like effects of NPY receptor ligands, and one region suggested to be involved in anxiety is the

hippocampus (see section 1.6.7b), it was decided to study the effect of NPY microinjection into this site in behavioural models of anxiety.

In two behavioural models of anxiety, the x-maze and Vogel's conflict tests, i.c.v. NPY was shown to induce anxiolytic-like effects. In a third model, the social interaction test, NPY showed a small, but insignificant increase in social interaction, this activity possibly being reduced by the strong sedative-like effects shown by NPY itself in this model. Using NPY receptor subtype selective ligands, it was found that the anxiolytic-like activity of NPY might be mediated by the  $Y_1$  receptor subtype. When injected directly into the ventral hippocampus, NPY showed anxiolytic-like activity in both the x-maze and the Vogel's conflict test (see section 5.4.3). Evidence from all three results chapters in this thesis has therefore shown the hippocampus to be an area of the brain where NPY, acting on its receptors, may play an important role, and that one of these roles might be in the modulation of anxiety.

### 6.1 Possible $\alpha_2$ -adrenoceptor interaction

There seems to be an interaction between NPY and noradrenergic systems in the brain. This is not surprising as NPY and NA coexist in many neurones. An interaction was first characterised by Wahlestedt et al. (1986) on the bases of pre- and post-junctional effects. They found that at various neuroeffector junctions, three different actions or interactions could be

demonstrated (see fig 1.3). There were the separate postjunctional effects of the two transmitters, a pre-junctional negative feedback interaction, and a post-junctional potentiation of the other transmitters' effects. Agnati et al. (1983) demonstrated an interaction between NPY and  $\alpha_2$ -adrenoceptors in membranes of medulla oblongata of the rat. They found the number of  $\alpha_2$ -adrenergic binding sites to be increased following NPY pretreatment. Also in vivo, NPY was found to increase the affinity of  $\alpha_2$ -adrenoceptors (Fuxe et al.,1986).

The ability of NPY to increase the number of  $\alpha_2$ -adrenoceptor binding sites in the medulla oblongata of rats is absent in one particular strain called the spontaneously hypertensive (SH) rats (Agnati et al.,1983). This is interesting because of three other findings. Firstly, Maccarrone and Jarrott (1985) showed there to be a difference in the regional brain concentrations of NPY in SH rats compared with their controls (Wistar-Kyoto (WKY) rats). Secondly, an increase in NPY receptor binding has been found in the hippocampus and cortex of SH rats (Chang et al.,1986). Finally NPY was found to increase locomotor activity in SH rats, unlike in control WKY rats, where a decrease was seen (Heilig et al.,1989). These differences between SH and WKY rats demonstrate an interaction between NPY and  $\alpha_2$ -adrenoceptors, and suggest that any disruption to this interaction could have an effect on NPY action.

The possibility that the anxiolytic actions of NPY might be brought about by an interaction with  $\alpha_2$ -adrenoceptors was suggested by Heilig et al., (1989).

They used what they considered to be a subthreshold dose of the  $\alpha_2$ -adrenoceptor antagonist idazoxan, to block the effects of NPY in the Montgomery's x-maze test and thus they concluded that such an interaction may be responsible for this effect. Close inspection of their data however reveals two problems. Firstly they were unable to find a dose of idazoxan which blocked the effects of NPY in the x-maze without having an anxiogenic effect when used alone. Secondly, in the Vogel's conflict test where such a dose was 'found', the baseline levels of drinking were so suppressed in the control rats that any anxiogenic actions of idazoxan alone might not even have been detected. Thus the dose they used as 'ineffective' when given alone, might actually have been anxiogenic and therefore have opposed NPY's anxiolytic-like effects producing an apparent block of the action of NPY. Therefore more studies are required to determine what interactions, if any, may occur to produce the anxiolytic-like effects of NPY. One such study could be the behavioural effects of NPY on SH rats. As discussed above, there seems to be a disrupted NPY- $\alpha_2$ -adrenoceptor interaction in these rats, so any lack of NPY anxiolytic-like activity in these animals could therefore imply that in normal rats NPY anxiolysis occurs as a result of  $\alpha_2$ -adrenoceptor upregulation.

One of the principle noradrenergic nuclei of the brain is the locus coeruleus (LC). NPY has been found to lower action potentials in the LC, an effect which is much less marked in the presence of  $\alpha_2$ -adrenoceptor antagonists (Illes and Regenold,1990).  $\alpha_2$ -Adrenoceptors on the LC cell bodies have been shown to lower the firing rate of these neurones upon activation (Svensson et al.,1975).

Since there is a lot of evidence to support a LC-anxiety involvement, with decreased LC activity producing anxiolysis and increased activity producing anxiety (Redmond and Huang,1979), it is possible therefore that NPY may have effects at this site.

## 6.2 Future work

This study has provided general information about the anxiolytic-like effects of NPY, but more detailed analysis is required to elucidate these effects. The lack of any significant effect of NPY in the social interaction test could be studied at lower doses than used in this study. If lower doses were not sedative, it is possible that the 'unsedated' animal might exhibit the anxiolytic-like effects of NPY. Another important area for more work would be to study the dose-response relationships of the various NPY receptor subtype ligands in these models. This is required for absolute evidence of which receptor subtypes mediate these effects. It would also be interesting to study the effects of the receptor subtype selective ligands in the hippocampus itself.

As already mentioned (see section 6.1), the NPY receptor is suggested to interact with  $\alpha_2$ -adrenoceptors. Since  $\alpha_2$ -adrenoceptor agonists are anxiolytic-like in their effect (Handley and Mithani, 1984), it is possible that NPY may exert its anxiolytic-like effects by interacting with the  $\alpha_2$ -adrenoceptors. It



would therefore be interesting to study this interaction in more detail with respect to anxiety. A possible means of achieving this would be to study the anxiolytic-like effects of NPY in spontaneously hypertensive (SH) rats, which show a disrupted NPY- $\alpha_2$ -adrenoceptor interaction (see section 6.1).

It would be of interest to study other brain regions which could possibly be involved in the anxiolytic-like effects of NPY. The locus coeruleus (LC) is one of the principle noradrenergic nuclei of the brain, in which NPY has been found to lower action potentials (Illes and Regenold, 1990). This area should therefore be studied as a possible site for NPY's actions. Unfortunately, microinjections of NPY into the LC were attempted in the present study with promising results, but subsequent dye injection showed that the solution would have spread into the 4th ventricle and thus the effects could be from anywhere via the ventricular system. A possible alternative would be to study the behavioural effects of NPY following lesion of the LC.

Finally, this study has identified the probable subtype responsible for the main anxiolytic-like effects of NPY as the  $Y_1$  receptor. It is clear from autoradiographic studies (see section 2.2) that  $Y_1$  receptor distribution in the brain is very localised compared to  $Y_2$  receptor distribution. The main region where the  $Y_1$  receptors dominate are in the outer layers of the cortical regions. In the cortex NPY exists in interneurons (Fuxe et al., 1986), and it is possible that it interacts with NA afferents from the brain stem (eg. the LC). It would therefore be useful to study the behavioural effects of NPY either microinjected

into these areas, or i.c.v. following lesioning of these areas.

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