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Neuromodulation in the rat hippocampus by neuropeptide Y

by

Gloria Janine Klapstein

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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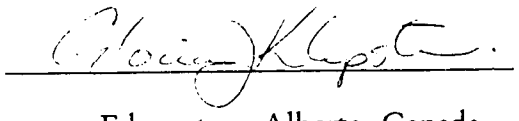

Edmonton, Alberta, Canada

June 30, 1995

There are seven windows in the head, two nostrils, two ears, two eyes and a mouth; so in the heavens there are two favorable stars, two unpropitious, two luminaries, and Mercury alone undecided and indifferent. From which and many other similar phenomena of nature such as the seven metals, etc., which it were tedious to enumerate, we gather that the number of planets is necessarily seven. . . . Moreover, the satellites are invisible to the naked eye and therefore can have no influence on the earth and therefore would be useless and therefore do not exist.

-Francesco Sizi's reply to Galileo,
on the existence of Jovian satellites.

. . . the bewilderments of the eyes are of two kinds, and arise from two causes, either from coming out of the light or from going into the light, which is true of the mind's eye, quite as much as of the bodily eye; and he who remembers this when he sees any one whose vision is perplexed and weak, will not be too ready to laugh; he will first ask whether that soul of man has come out of the brighter life, and is unable to see because unaccustomed to the dark, or having turned from darkness to the day is dazzled by excess of light. And he will count the one happy in his condition and state of being, and he will pity the other . . .

-Plato, in *The Allegory of the Cave*

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Neuromodulation in the Rat Hippocampus by Neuropeptide Y** submitted by **Gloria Janine Klapstein** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy.**

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June 9 1995
Date

I dedicate this work

to my parents

Dr. N.J. and Mrs. F.J. Kuzyk,

and to my husband

Kevin

Abstract

Neuropeptide Y and its receptors have been found in high concentrations in rat hippocampus, where they have been found to mediate presynaptic inhibition of glutamatergic transmission in area CA1. This thesis explores the pharmacology and physiology of this response, both in area CA1 and throughout the hippocampus, using the *in vitro* rat hippocampal slice preparation.

NPY, NPY analogues, and C-terminal fragments were used to construct an agonist profile of the inhibition of postsynaptic potentials in area CA1. Porcine sequence NPY (NPY), human NPY (hNPY), PYY, and NPY_{2-36} inhibited postsynaptic potentials equally in CA1, while shorter C-terminal fragments decreased in potency with decreasing length; desamido hNPY was without effect, consistent with a Y_2 receptor.

To test whether different presynaptic receptors share a mechanism of action, inhibition mediated by NPY, baclofen, or 2-chloroadenosine (2-CA) was tested in area CA1 under different conditions. The effects of all three agonists were reduced in the presence of the K^+ channel antagonist, 4-aminopyridine (4-AP, 30μM). Reduction of extracellular [Ca^{2+}] from 1.5mM to 0.75mM restored the effects of NPY and baclofen, but not that of 2-CA (except at high concentrations). Thus, presynaptic modulation can occur by more than one mechanism.

To explore the sites of NPY's actions in the hippocampus, specific afferent pathways to CA1 or CA3 pyramidal neurons or dentate granule cells, which were isolated either by selective placement of the stimulating electrode and/or by selective pharmacological antagonism. NPY inhibited all excitatory synapses tested onto pyramidal cells in CA1 and CA3, but did not inhibit either the perforant path or the

commissural inputs to dentate granule cells, nor did it directly inhibit GABAergic transmission in any of these areas.

Because it potently and selectively inhibits feedforward excitation in the hippocampus, NPY was tested in three different *in vitro* models of epilepsy. It was found that NPY could inhibit ictaform afterdischarges in the stimulus train-induced bursting (STIB) model and spontaneous, interictaform discharges in the $0Mg^{2+}$, picrotoxin, and STIB models, without altering spontaneous inhibitory events, suggesting that NPY might normally regulate excitability in the hippocampus.

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I could not have completed this work alone. Therefore, I would like to express my sincere gratitude to all those who helped through the years of hard work:

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List of Abbreviatons

2-CA	2-chloroadenosine
4-AP	4-aminopyridine
5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytryptamine, serotonin
ACSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
AD	afterdischarges
AMPA	D,L-α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid
AP	action potential
AP4	L-2-amino-4-phosphonobutyrate
APV	DL-2-amino-5-phosphonovaleric acid
ATP	adenosine triphosphate
C2-NPY	[Cys-2,8-aminooctanoic acid 5-24,D-Cys27]-NPY
CA	cornu ammonis
cAMP	cyclic adenosine 3, 5-monophosphate
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPON	C flanking peptide of neuropeptide Y
CRH	corticotropin-releasing hormone
DAGO	Tyr-D-Ala-Gly-[NMePhe]-NH$(CH_2)_2$-OH
DBH	dopamine β-hydroxylase

DRG	dorsal root ganglia
EC_{50}	effective concentration for 50% response
EDRF	endothelium-derived relaxing factor
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EPSP	excitatory postsynaptic potential
FM	frequency modulation
FMRFamide	Phe-Met-Arg-Phe-NH_2
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GI	gastrointestinal
G-protein	guanosine triphosphate-binding protein
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hNPY	human sequence NPY
HPLC	high performance (pressure) liquid chromatography
IPSP	inhibitory postsynaptic potential
LC	locus coeruleus
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LI	like immunoreactivity
LTP	long term potentiation
MCPG	(+)-α-methyl-4-carboxyphenyl-glycine
mGluR	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid

NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NBQX	6-nitro-7-sulphamoylbenzo(f)quinoxaline2,3-dione
NMDA	N-methyl-D-aspartate
NOS	nitric oxide synthase
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
PDS	paroxysmal depolarization shift
pEPSP	population excitatory postsynaptic potential
PP	pancreatic polypeptide
PNS	peripheral nervous system
PS	population spike
PY	pancreatic polypeptide Y
PYY	polypeptide YY
RIA	radioimmunoassay
RNA	ribonucleic acid
SCG	superior cervical ganglion
SCN	suprachiasmatic nucleus
SP	stratum pyramidale
SRSE	spontaneous, rhythmic, synchronous event
STIB	stimulus train induced bursting
TFA	trifluoroacetic acid
TH	tyrosine hydroxylase

CHAPTER 1

INTRODUCTION

Discovery, evolution, and characterization of NPY

Neuropeptide Y (NPY) was first purified from porcine brain (Tatemoto, et al., 1982) and sequenced (Tatemoto, 1982) in 1982 by Tatemoto et al. It belongs to a family of carboxyterminally-amidated peptides which also includes pancreatic polypeptide (PP), peptide YY (PYY) and pancreatic peptide Y (PY). The name NPY derives from the sequence of the peptide which, like PYY, contains 36 amino acids, beginning and ending with tyrosine (Y), and also from its chief locations in the central and peripheral nervous systems. The three-dimensional structure of NPY consists of a polyproline helix at the N-terminus between residues 1 and 8, and an amphiphilic α-heli-. between residues 15 and 30, connected by a β turn which folds these sections into a hairpin shape shared by all members of the NPY family of peptides and referred to commonly as the PP-fold (Schwartz et al., 1990).

NPY has been found in many different and phylogenetically distant species, ranging from human to cartilaginous fish, such as the ray *Torpedo marmorata* (Larhammar et al., 1993). The NPY in these two species differ from each other by only 3 amino acid residues (92% sequence identity). Such high sequence homogeneity suggests that, throughout the approximately 400 million years since the divergence of these species (Larhammar et al., 1993), evolutionary pressures have prevented widespread mutation of the NPY gene, leading to speculation that the actions of NPY in vertebrates are important to survival.

NPY fulfils most or all of the criteria applied to putative neurotransmitter candidates (Werman, 1966). It is *present* in synaptic vesicles in nerve terminals from which it is released (Fried et al., 1985). The presence of mRNA encoding NPY and

precursors such as preproNPY indicate that it is *synthesized* in those neurons (Higuchi et al., 1988a; Larhammar et al., 1987; Minth et al., 1984). It is *released* in a calcium-dependent manner following electrical nerve stimulation (Lundberg et al., 1989; Sheikh, et al., 1988) or upon depolarization of the cell membrane by high extracellular [K^+] (Greber et al., 1994; Sahu et al., 1988). NPY and PYY, but not PP, can be *inactivated* by endogenous peptidases, such as dipeptidyl peptidase IV, to form NPY_{3-36} and PYY_{3-36} (McDermott et al., 1992; Mentlein et al., 1993; Medeiros and Turner, 1994), which have much lower affinities for Y_1 receptors (Rioux et al., 1985). An *identity of action* is indicated by the ability of exogenous NPY and agonists to mimic stimulation of NPYergic neurons (Lacroix et al., 1994), and the ability of antagonists or specific antisera (Stanley et al., 1992) to prevent these effects. Finally, specific *receptors* have been isolated (Wahlestedt et al., 1990a; Sheikh and Williams, 1990; Nguyen et al., 1990; Mannon et al., 1991; Price and Brown, 1990; Gimpl et al., 1990), and in one case, cloned (Larhammar et al., 1992), which bind NPY and specific agonists, and which exist in tissues exhibiting NPY effects.

NPY receptor pharmacology

Diversity exists among NPY receptors, which can be distinguished pharmacologically by the relative actions of various agonists upon them. The first indication of NPY receptor heterogeneity was that C-terminal fragments of NPY or PYY could mimic some, but not all of the effects of these peptides at sympathetic neuroeffector junctions (Wahlestedt et al., 1986; Wahlestedt et al., 1987). It was concluded that NPY, PYY and the $_{13-36}$ C-terminal fragments acted at a pre-junctional

receptor to reduce the vas deferens twitch response to electrical stimulation of sympathetic afferents, but that only the full peptides could evoke contractions of the guinea pig iliac vein, by acting post-junctionally.

C-terminal fragments commonly used range from NPY_{2-36} to NPY_{26-36}. They have both decreasing affinity and biological effect at Y_2 receptors with decreasing length, and almost none at Y_1 receptors (Grundemar et al., 1993). Several cyclic, centrally-truncated analogues of NPY have also been synthesized (Krstenansky et al., 1989; McLean et al., 1990). One of these, [Cys2, 8-aminooctanoic acid5-24, D-Cys27]-NPY (C2-NPY), has been shown to be active in tissues involving Y_2 receptors, such as rat jejunum mucosa (Cox and Krstenansky, 1991) or the rat dorsal raphe nucleus (Kombian and Colmers, 1992), and almost completely ineffective at eliciting non-Y_2-mediated effects such as the feeding response in the paraventricular or perifornical nuclei (Stanley et al., 1992), or at binding to a cloned Y_1 receptor (Larhammar et al., 1992). Recently, another centrally truncated analogue of NPY has also been developed, in which the central loop from residues 5 to 24 was replaced with 6-aminohexanoic acid (Beck-Sickinger et al., 1992). This manipulation also confers selectivity of action to the Y_2 receptor.

In 1990, it was found that single or multiple-point amino acid substitutions, such as a substitution of proline for glutamine at residue 34 alone to form [Pro^{34}]NPY, or with another substitution of leucine for isoleucine at residue 31, to form [Leu^{31},Pro^{34}]NPY, conferred selectivity for Y_1 receptors, at which they act as full agonists, while being nearly inactive at Y_2 receptors (Fuhlendorff et al., 1990; Krstenansky et al., 1990).

Deamidation of the C-terminus produces a molecule which has no affinity or

activity at any of the NPY receptor subtypes yet characterized. Pancreatic polypeptide is also without activity at known NPY receptors (Schwartz et al., 1990), however, PYY acts as a full agonist at both Y_1 and Y_2 receptors (Wahlestedt et al., 1986).

In addition to Y_1 and Y_2 receptors, there are several lines of evidence for the existence of a third distinct NPY receptor subtype, Y_3. In 1991, Grundemar et al. reported that PYY, when injected into the rat nucleus tractus solitarius (NTS), could not evoke the long-lasting hypotension and bradycardia seen with injection of NPY, [Pro^{34}]NPY, or NPY_{13-36} into the same area (Barraco et al., 1990; Carter et al., 1985; Grundemar et al., 1991; Grundemar et al., 1991a). PP and desamido-NPY were also ineffective at evoking this response. In the adrenal medulla, PYY neither binds specifically (Wahlestedt et al., 1992), nor does it mimic the NPY inhibition of nicotine-induced catecholamine release (Higuchi et al., 1988). Activity or binding profiles matching neither the Y_1 nor Y_2 subtypes also exist in cardiac membranes (Balasubramaniam et al., 1990), bovine chromaffin cells (Li et al., 1990), and isolated rat superior cervical ganglion cells (Foucart et al., 1993).

To summarize, the relative affinities for NPY and various analogues and related peptides at NPY receptor subtypes are:

Y_1: NPY = PYY = [Pro^{34}]NPY $\rangle\rangle$ NPY_{13-36} $\rangle\rangle$ NPY_{26-36}, PP, desamido-NPY

Y_2: PYY $\geq$ NPY $\rangle$ NPY_{13-36} $\rangle\rangle$ [Pro^{34}]NPY, PP, desamido-NPY

Y_3: NPY $\geq$ [Pro^{34}]NPY $\geq$ NPY_{13-36} $\rangle\rangle$ PYY, PP, desamido-NPY.

Very recently, several antagonists (BIBP3226, Rudolf et al., 1994; 1229U91,

Leban et al., 1995) have also been developed, which are selective for the Y_1 receptor. BIBP3226 was tested in the Y_1 expressing SK-N-MC cell line and in the isolated perfused rat kidney preparation. In pithed rats, *in vivo*, BIBP3226 was found to inhibit the NPY-evoked increase in diastolic blood pressure, and did not antagonize the NPY induced inhibition of the electrically stimulated twitch response in rat vas deferens, *in vitro*. 1229U91 was tested in vascular tissue and human erythroleukemia cells, and potently displaces NPY from rat brain membranes (A.J. Daniels, personal communication).

There is evidence that more than three subtypes of the NPY receptor exist. The stimulation of feeding elicited by injection of NPY or PYY into the hypothalamic paraventricular and perifornical nuclei, can be reproduced by both the Y_1 agonist [Pro^{34}]NPY, and the Y_2 agonist NPY_{2-36}, which is usually relatively inactive at Y_1 receptors, and PP, which is ineffective at all three of the NPY receptors which have been characterized (Schwartz et al., 1990). This effect, therefore, is said to be mediated by an "atypical" Y_1 receptor (Stanley et al., 1992).

Anatomical methods

Localization of NPY

Immunocytochemistry involves the use of antibodies (Kachidian and Bosler, 1991) raised against either NPY or the C-terminal flanking peptide of NPY (CPON), which, together with NPY and a three amino acid sequence necessary for C-terminal amidation of NPY, makes up the 69 amino acid proNPY. CPON is cleaved from

NPY during post-translational processing, stored and released with NPY (Allen et al., 1987). Various antibody-conjugated markers, such as fluorescence (Villalba et al., 1988), peroxidase (D'avila et al., 1993; Dobo et al., 1993), and biotin (Milner and Veznedaroglu, 1993) tags have been used to visualize immunological reactions at both light- and electron-microscopic levels. Radioimmunoassays using radiolabelled antibodies have also been used to detect NPY, and are particularly useful in amorphous tissues such as blood (McDonald et al., 1987). When used in conjunction with high performance liquid chromatography, RIA also contributed to the characterization of properties such as molecular weight and solubility (deQuidt and Emson, 1986).

There is an inherent concern with immunological techniques, however, as they do not always correctly identify their targets. For example, it is now believed that several studies claiming to have localized NPY-related peptides, such as avian (Card et al., 1983; Loren et al., 1979; Lundberg et al., 1980; Lundberg et al., 1984b) or bovine (Jacobowitz and Olschowka, 1982; Olschowka et al., 1981) pancreatic polypeptide to regions in the CNS, were actually detecting NPY itself (DiMaggio et al., 1985; Emson and deQuidt, 1984; Hockfield et al., 1983; Moore et al., 1984). Indeed, one of the first cytochemical detections of NPY in rat brain involved the use of antiserum raised against the molluscan tetrapeptide amide, FMRFamide (Muske et al., 1987; Triepel and Grimmelikhuijzen, 1984; Weber et al., 1981). Thus, immunocytochemical studies now usually refer to locations of NPY-like immunoreactivity (NPY-LI).

A complementary technique for localization of NPYergic cells is *in situ* hybridization histochemistry, which uses a radiolabelled complementary RNA probe

to detect NPY or preproNPY mRNA (Ericsson et al., 1987; Terenghi et al., 1987). This method is most useful for detection of somata, where mRNA levels are highest, but it will not label fibres.

NPY receptor localization

Agonist profiles for the NPY receptor subtypes were described above. In this section, I will describe how such information has been used to localize NPY receptors in general, or specific subtypes, to various tissues, regions of tissues, or individual neurons.

Bioassays test the effect of exogenously administered NPY or receptor subtype-specific agonists to evoke or inhibit responses which can be measured *in vivo* (e.g., hypotension and bradycardia, Barraco et al., 1990; Carter et al., 1985; Grundemar et al., 1991; Grundemar at al., 1991a), or *in vitro* (e.g., inhibition of evoked synaptic transmission, Colmers et al., 1985, 1987, 1988; Chapters 2-4).

Radioligand binding assays using radiolabelled NPY or receptor subtype-specific agonists test the ability of various tissues to bind these ligands specifically. In tissue homogenates, dissociation constants, and therefore, agonist affinities can be determined. Autoradiography reveals anatomical locations of specifically-bound radiolabelled ligands, or displacement of radiolabelled NPY by unlabelled receptor-specific ligands.

The cloning of the Y_1 receptor (Eva et al., 1990; Larhammar et al., 1992) has made possible the use of *in situ* hybridization techniques, which allow detection of mRNA encoding it (Ji et al., 1994), and therefore, identification of cells expressing it. To date, no antibodies against the NPY receptor have been successfully generated.

Anatomy and Physiology

NPY in the periphery

NPY has been found extensively in both the sympathetic and parasympathetic nervous systems. It is also found prominently in the adrenal medulla and endocrine cells of the lower gastrointestinal tract (Böttcher et al., 1984; Wahlestedt, 1987). NPY has also been detected in blood cells in some species.

Autonomic nervous system

In the sympathetic nervous system, NPY is present in neuronal somata in sympathetic ganglia (Lundberg et al., 1982a), and in sympathetic nerve fibres, especially those innervating blood vessels (Ekblad et al., 1984; Ekblad et al., 1984a; Sheikh et al., 1988; Sundler et al., 1986). In these somata and fibres, NPY has been found to coexist with dopamine-β-hydroxylase (DBH) (Lundberg et al., 1982a), a synthetic enzyme and marker of noradrenergic neurons (Kaufman, 1966; Viveros et al., 1969). NPY-LI has also been found in some parasympathetic nerves (Sundler et al., 1989).

NPY and noradrenaline (NA) are both released during sympathetic nerve stimulation (Haass et al., 198[illegible]; Haass et al., 1989a; Lundberg et al., 1986a; Lundberg et al., 1987). The release of both is mediated by N-type calcium channels and requires protein kinase C (Haass et al., 1990). However, NA is released at lower frequencies of nerve activity than is NPY, as described in experiments performed in isolated perfused canine spleen (Schoups et al., 1988), and guinea pig heart (Haass et al., 1989),

in perfused pig spleen (Lundberg et al., 1986a; Lundberg et al., 1989a), and *in vivo*, in calves (Allen et al., 1984), guinea pigs (Dahlof et al., 1986), or rats (Zukowska-Grojec et al., 1992). The stimulus requirements for NPY release are similar to those for neuronal release of other peptides (Bicknell et al., 1982) and is consistent with evidence supporting, in general, the concept of differential release of peptides and "classical" neurotransmitters, another example being LHRH and acetylcholine in bullfrog sympathetic ganglia (Peng and Horn, 1991; Peng and Zucker, 1993).

The release of NPY, as well as that of NA, can be inhibited by presynaptic α_2-adrenoceptor agonists (Dahlof et al., 1986; Haass et al., 1989a), or increased by antagonists (Haass et al., 1989a; Lundberg et al., 1984; Lundberg et al., 1984a; Lundberg et al., 1987; Lundberg et al., 1989a). Likewise, NPY can inhibit its own release or that of NA via presynaptic Y_2 receptors (Wahlestedt et al., 1986; Wahlestedt et al., 1987), independently of α_2 receptors (Dahlof et al., 1986; Westfall et al., 1988).

Cardiovascular system

NPY fibres are numerous in the atria of the heart, and occur in more moderate concentrations in the ventricles as well (Lundberg et al., 1982a; Gu et al., 1983, 1984; Uddman et al., 1985). In the heart, most NPY is found in the innervation of the vasculature (Allen et al., 1986), although some intrinsic, non-adrenergic neurons also occur here (Hassall and Burnstock, 1984; Gu et al., 1984).

NPY, NPY_{13-36}, and PYY_{13-36}, but not [Pro^{34}]NPY, have been found to attenuate vagal activity in the heart (Potter et al., 1989, 1991), by inhibiting the release of acetylcholine (Potter, 1985, 1987; Potter et al., 1989; Warner and Levy,

1989, 1989a), suggesting the involvement of Y_2 receptors. Competition binding studies, however, have shown that NPY was more potent than PYY in inhibiting [^{125}I]NPY binding in the rat ventricle (Sheriff et al., 1990; Balasubramaniam et al., 1990), which is not consistent with a typical Y_2 receptor. In atrial tissues, NPY binding studies indicated antagonism by NPY_{18-36}, characteristic of the Y_3 receptor in this tissue (Sheriff et al., 1990).

NPY nerve fibres innervate many vascular beds throughout the body (Edvinsson et al., 1983, 1984; Ekblad et al., 1984, 1984a, 1984b; Lundberg et al., 1982a, 1983, 1985; Uddman et al., 1985), including dense innervation of guinea pig major arteries (Uddman et al., 1985), and small arteries in the respiratory, gastrointestinal, and genitourinary tracts of several species (Wahlestedt, 1987). However, few NPYergic fibres have been found in the liver, spleen, or kidney (Wahlestedt, 1987).

NPY causes constriction of vascular smooth muscle in small coronary arteries (Maturi et al., 1989) and various cerebral and somatic vascular beds (Dacey et al., 1988; Franco-Cereceda et al., 1985; Pernow and Lundberg, 1988; Rioux et al., 1985; Suzuki et al., 1989), via a Y_1 receptor (Wahlestedt et al., 1986), which is likely located on the vascular smooth muscle, as removal of the endothelium does not affect the response (Pernow and Lundberg, 1988; Wahlestedt, 1987). At concentrations lower than those required for vasoconstriction, NPY is able to potentiate the vasoconstriction caused by noradrenaline (Abel and Han, 1898; Dahlof et al., 1985; Ekblad et al., 1984; Wahlestedt et al., 1985). Calcium dependence, and sensitivity to L-type calcium channel antagonists, has been shown for both of these effects in many *in vitro* preparations and *in vivo* (Andriantsitohaina and Stoclet, 1988; Dahlof et al.,

1985; Edvinsson et al., 1983; Ekblad et al., 1984; Franco-Cereceda et al., 1985; Grundemar et al., 1992; Wahlestedt et al., 1985; Zukowska-Grojec et al., 1986).

Although it has previously been thought that NPY expression was confined to cells of neural or neural crest origin (Allen and Balbi, 1993), several reports have been made of NPY in rat mononucleocytes (Ericsson et al., 1991) or platelets (Allen, R. et al., 1991; Ericsson et al., 1991; Myers et al., 1988; Myers et al., 1993; Ogawa et al., 1992), which are of mesenchymal origin (Hopper and Hart, 1985). Platelet or monocuncleocyte-rich fractions of pig, rabbit and human blood show lower levels of NPY-LI (Ericsson et al., 1991; Myers et al., 1993), however, some debate has arisen over the origin of NPY in the blood of these species, since at least one study was unable to detect NPY mRNA in such blood fractions (Ericsson, et al., 1991).

NPY-LI has been found in the plasma of rats, pigs, rabbits and humans. Plasma NPY is likely derived from the adrenal medulla (Allen, J.M. et al., 1983; Corder et al., 1985), as overflow from sympathetic nerve stimulation (Deka-Starosta et al., 1989; Zukowska-Grojec et al., 1992), and, in rats and mice, from platelets or mononucleocytes as well (Ericsson et al., 1987a; Myers et al., 1988; Myers et al., 1993).

Gastrointestinal tract

NPY-LI is widely distributed in the mammalian gastrointestinal (GI) tract. NPY is found in the sympathetic innervation of the entire gastrointestinal tract, and is present in myenteric and submucosal plexus neurons of the enteric nervous system, as well (Ekblad et al., 1984a; Furness et al., 1983; Gershon et al., 1990; Wang et al., 1987). In the stomach (Lee et al., 1985; Tsai and Cheng, 1990;, Wattchow et al., 1987),

it inhibits contractile activity (Suzuki et al., 1983) and gastric emptying (Allen et al., 1984a; Pappas et al., 1986). It is also found in the lower esophageal sphincter where it has a biphasic effect, consisting of contraction followed by relaxation (Parkman et al., 1989), and in the internal anal sphincter (Ferri et al., 1988; Wattchow et al., 1988), where it causes contraction by a direct action on the smooth muscle (Nurko and Rattan, 1990).

NPY-LI is present in perivascular fibres in the porcine pancreas (Sheikh et al., 1988), however, a prominent population of intestinal NPYergic fibres do not contain DBH, and therefore are nonadrenergic (Furness et al., 1983). Both electrical stimulation of the splanchnic nerve (Ahren et al., 1989) and vagal stimulation (Sheikh et al., 1988) result in pancreatic release of NPY, although the latter is much more effective. NPY has been shown to have no effect on basal secretions of insulin or glucagon from rat pancreas (Moltz and McDonald, 1985), however, another study has shown that NPY does stimulate basal secretion of insulin, glucagon, and somatostatin in dispersed islet cells (Milgram et al., 1990). Insulin release stimulated by glucose (Moltz and McDonald, 1985) is also inhibited by NPY.

In the intestine, the highest density of NPY binding sites are in the duodenum and jejunum, where it has been co-localized with vasoactive intestinal polypeptide in secretory vesicles of varicose fibres (Cox and Westbrook, 1994); very little or none is found in the large intestine (Laburthe et al., 1986). NPY has been found to inhibit motility in the cat jejunum and colon (Hellstrom et al., 1985; Lundberg et al., 1982) and to relax guinea pig colon *in vitro* (Wiley and Owyang, 1987). Conversely, it causes contraction of rat duodenum (Krantis et al., 1988), rat colon (Cadieux et al., 1990, 1990a), and dog jejunum (Buell and Harding, 1989).

NPY acts at presynaptic receptors in the GI tract, to modulate the release of acetylcholine in the duodenum and non-adrenergic,non-cholinergic transmitters in the rat ileum (Krantis et al., 1988). In the rat colon, NPY_{13-36} is only an order of magnitude less potent than the full peptide at evoking a response, indicating that Y_2 receptors are probably involved (Cadieux et al., 1990).

NPY alters ion transport in the intestinal epithelium by inhibiting basal electrolyte secretion (Brown et al., 1990; Cox et al., 1988; Cox and Cuthbert, 1990; Saria and Beubler, 1985) via a direct action on epithelial cell receptors (Cox et al., 1988; Friel et al., 1986; Hubel and Renquist, 1986). In the porcine small intestine, this effect is mediated presynaptically, and does not involve opioid neurons, since naloxone had no effect on responses to NPY (Brown et al., 1990). In the rat jejunal mucosa, this effect can be reproduced by NPY_{13-36} (Cox and Krstenansky, 1991), indicating that it is mediated via a Y_2 receptor.

Urogenital tract

In the kidney, NPY-LI has been found in perivascular nerves and in fibres innervating the juxtaglomerular apparatus of several species, including human (Ballesta et al., 1984), with a concentration of the peptide in the cortical areas (Sheikh et al., 1989). [^{125}I]NPY binding is, by contrast, most concentrated in the proximal convoluted tubules (Leys et al., 1987). NPY has been found to decrease glomerular filtration (Allen et al., 1985), inhibit renin secretion (Aubert et al., 1988; Hackenthal et al., 1987; Pfister et al., 1986, Waeber et al., 1990), and increase sodium and water excretion (Smyth et al., 1989). PYY_{13-36} was almost as potent as PYY at effecting these responses, indicating that a Y_2 receptor is involved (Sheikh et al., 1989). Given the

apparent mismatch between NPY-LI and binding sites, it has been suggested that NPY in the circulation may serve as the endogenous ligand at these receptors (Sheikh et al., 1989).

NPY-LI has been colocalized with noradrenaline in male and female urogenital tracts (Jorgensen et al., 1989; Lundberg et al., 1982a; Stjernquist et al., 1987). NPY inhibits the electrically-induced twitch response of the vas deferens in rat (Chang et al., 1985, 1988; Donoso et al., 1988; Forest et al., 1990; Grundemar and Hakanson, 1990; Jorgensen et al., 1990; Lundberg and Stjärne, 1984; Martel et al., 1990a; Minakata and Iwashita, 1990; Wahlestedt et al., 1986; Wahlestedt et al., 1987), mouse (Allen et al., 1982; Serfozo et al., 1986; Stjärne et al., 1986), and guinea pig (Ellis and Burnstock, 1990; Stjernquist et al., 1987), by inhibiting the release of noradrenaline (Lundberg and Stjärne, 1984; Serfozo et al., 1986; Stjärne et al., 1986). This effect is mediated by a Y_2 receptor, as NPY_{13-36} was found to be nearly as potent as NPY in evoking it (Chang et al., 1988; Grundemar and Hakanson, 1990; Wahlestedt et al., 1986).

Respiratory tract

NPY-LI has been found in both sympathetic (Martling et al., 1990 in d) and parasympathetic fibres (Lacroix et al., 1990) innervating the respiratory tract. It has also been described in nerve fibres in the nasal mucosa of several mammalian species, and surrounding seromucous glands of pig and guinea pig, but not rat, cat, or human (Lacroix et al., 1990). NPY-LI has been colocalized with vasoactive intestinal peptide (VIP)-LI in nerve fibres in the tracheobronchial smooth muscle layer and in tracheal periglandular nerve fibres (Martling et al., 1990).

NPY causes relaxation of bronchial contraction evoked by field stimulation (Matran et al., 1989), electrically stimulated tracheal rings (Stretton and Barnes, 1988), and vagally stimulated guinea pig trachea, which is also relaxed by NPY_{13-36} (Grundemar et al., 1988, 1990), indicating that a Y_2 receptor mediates this response. At low concentrations, NPY has also been shown to induce contraction in non-stimulated tracheal tissues, in a manner consistent with a direct, postsynaptic effect on tracheal smooth muscle (Cadieux et al., 1989).

Adrenal medulla

NPY is found in high concentrations in chromaffin cells in the adrenal medulla of several mammalian species, including humans (Allen, J.M. et al., 1983; Corder et al., 1985). Here it has been colocalized with either adrenaline (Fried et al., 1987; Lundberg et al., 1986), noradrenaline (Majane et al., 1985), or enkephalin (Fischer-Colbrie et al., 1986; Fried et al., 1987). Differences in NPY concentrations have been found between species, with the highest concentrations in the mouse and cat, moderate concentrations in the rat, and the lowest concentrations in the pig. In all of these species, adrenal NPY concentrations have been found to increase with the age of the animal (Higuchi and Yang, 1986; Higuchi et al., 1988b).

Little is known of the function of adrenal NPY. However, insulin, which activates splanchnic nerves (Fischer-Colbrie et al., 1988), has been found to cause release of NPY from the adrenal medulla (Allen et al., 1984; Briand et al., 1990; deQuidt and Emson, 1986a), and elevation of NPY mRNA levels there (Fischer-Colbrie et al., 1988).

Patients with pheochromocytoma, a tumour of the adrenal medulla, show

increased plasma levels of NPY-LI (Corder et al., 1985; Pernow et al., 1986; Takahashi et al., 1987). Under normal physiological conditions, however, it is unlikely that secretion of NPY from the adrenal medulla contributes significantly to circulating levels, since, in rats, bilateral adrenalectomy does not affect resting NPY plasma levels (Morris et al., 1987), and may even increase plasma concentrations in response to stress (Zukowska-Grojec et al., 1992).

NPY in the CNS

Spinal cord

Anatomy. In the spinal cord, NPY is confined to large intraspinal neurons in layers II and III of the superficial dorsal horn of several species (Allen et al., 1984c; Gibson et al., 1984; Hunt et al., 1981a), and adrenergic supraspinal neurons of the C1 group (Blessing et al., 1987). In the sacral region, some NPYergic cells have also been reported in the dorsal gray commissure (Sasek and Elde, 1985). No NPY has been detected in the dorsal root ganglia (DRG) (Lewis et al., 1987). In all species studied, NPY immunoreactive fibres are more numerous at sacral than at rostral levels (Allen et al., 1984b; Gibson et al., 1984; Sasek and Elde, 1985), and seem to be densest in the dorsal horn at all levels (Hendry, 1993). At thoracic levels, fibres are particularly dense around preganglionic sympathetic neurons of the intermediolateral cell column (Gibson et al., 1984; Hunt et al., 1981a; Llewellyn-Smith et al., 1990), although only a fraction of the NPYergic terminals here form conventional synapses, and these comprise only a minority of the synaptic contacts in this region (Llewellyn-Smith et al., 1990). Transection of the spinal cord leads to depletion of NPY-LI in fibres caudal

to the lesion, indicating that they probably originate in the brainstem (Hokfelt et al., 1981). This is supported by other studies which show that they originate in the ventrolateral medulla (Morris et al., 1987a; Pilowsky et al., 1987). In the sacral spinal cord, NPYergic fibres have been found to surround parasympathetic neurons (deQuidt and Emson, 1986). In the sacral ventral horn, a dense plexus surrounds motoneurons of the perineal striated muscle in Onuf's nucleus (Gibson et al., 1984). Fibres are also relatively dense in the sexually dimorphic nuclei of the rat lumbar cord (Sasek and Elde, 1985; Newton and Hamill, 1988), where NPY-LI is more intense in males than in females (Newton and Hamill, 1988).

Pharmacology. It has been suggested that NPY innervation of the cremaster nucleus in male rats may control such functions as blood flow and intrascrotal position, thereby controlling the temperature of the testes and influencing reproductive viability (Newton and Hamill, 1988).

Manipulations of the ventrolateral medulla in rabbits result in hypertension and changes to NPY immunoreactivity in the spinal cord, suggesting that NPYergic projections from this area to the intermediolateral cell column may be important in blood pressure control (Morris et al., 1987a; Pilowsky et al., 1987).

Despite the lack of NPY-LI in the DRG, several receptor-mediated responses have been found there. In cultured DRG neurons, NPY inhibits the release of substance P (Walker et al., 1988), probably by its ability to inhibit N-type calcium channel currents, as it does in nodose ganglion neurons (Wiley et al., 1993). It has been suggested that this might represent the mechanism whereby intrathecally injected NPY increases the nociceptive threshold to heat in rats (Hua et al., 1991). This effect is mediated by a Y_2 receptor, as C-terminal fragments also were effective.

The threshold for pain induced by paw pressure was also increased by C-terminal fragments of NPY, however NPY itself was ineffective (Hua et al., 1991), suggesting that a novel receptor subtype may be involved.

Brainstem

Anatomy. Midbrain nuclei have few NPY somata or fibres, however both NPY-LI (deQuidt and Emson, 1986) and mRNA (Morris, 1989) have been described in the rat central gray, interpeduncular nucleus and inferior colliculus. In the inferior colliculus, NPY-positive somata are relatively numerous, are presumed to be interneurons, and are distributed superficially, with lateral somata being generally larger than medial ones (deQuidt and Emson, 1986). NPYergic fibres occur in the caudomedial region of the inferior colliculus and in a relatively dense plexus in the periaqueductal gray matter (deQuidt and Emson, 1986). Other areas which have been found to contain NPY include the dorsal tegmental nucleus of Gudden, the praeposital hypoglossal nucleus, and the lateral superior olive (deQuidt and Emson, 1986).

In the colchicine-treated cat, NPY-IR has been found in many of the "respiratory" brainstem nuclei, including the ventrolateral solitary nucleus, where somata are relatively sparse, in the nucleus ambiguus and Botzinger complex, where somata are densest, and in the nucleus parabrachialis and the Kolliker-Fuse nucleus, which has the greatest fibre density. (Aguirre et al., 1989). However, in experiments in which colchicine is not injected directly into the parenchyma of the brain, NPY-LI is only detectable in the solitary nucleus of several species, including cat (Aguirre et al., 1989), rat (Chronwall et al., 1985; Sawchenko et al., 1985; Wahlestedt et al.,

1990a), rabbit (Blessing et al., 1986) and human (Halliday et al., 1988). It has been suggested that colchicine, in addition to its ability to increase concentrations of NPY in somata by inhibiting axonal transport, may also induce *de novo* synthesis in some groups of neurons (Hendry, 1993). Some NPY-LI preganglionic cells occur in the dorsal motor nucleus of the rabbit vagus which also require colchicine treatment for detection (Blessing et al., 1986). In the rat spinal trigeminal nucleus, NPY-LI is found in small, superficially located cells (deQuidt and Emson, 1986), which are presumed to be interneurons, and which innervate the dorsal layers of this nucleus (Hendry, 1993).

Colocalization. Within the brainstem, NPY has been found in cell groups also containing adrenaline, noradrenaline, or serotonin. NPY has been colocalized with adrenaline in the C1 and C2 groups (Blessing et al., 1986; Halliday et al., 1988; Everitt et al., 1984), and in the solitary nucleus (Everitt et al., 1984; Lundberg et al., 1980). It has been colocalized with noradrenaline in cells of the A1 group in the ventrolateral medulla, in the A4 group of the pons, and in a subpopulation of noradrenergic cells in the A2 group in the dorsal medulla (Everitt et al., 1984; Hunt et al., 1981; Jacobowitz and Olschowka, 1982; Lundberg et al., 1980). In the locus coeruleus, NPY has been reported in some tyrosine-hydroxylase positive (noradrenergic) cells (Everitt et al., 1984; Holets et al., 1988; Moore and Gustaffson, 1989; Smialowska, 1988). Most of these project to the hypothalamus (Blessing et al., 1987; Sawchenko et al., 1985), and possibly to the entorhinal cortex (Wilcox and Unnerstall, 1990). Very few, if any, of the locus coeruleus projections to the spinal cord or neocortex contain NPY. (Blessing et al., 1987). A subpopulation of serotonergic cells have also been found to contain NPY in the nucleus raphe pallidus

of rabbits (Blessing et al., 1986) and the B2-3 group in humans (Halliday et al., 1988). Monoaminergic neurons which clearly do not contain NPY include most noradrenergic cells in the A2 group, all noradrenergic cells in the A5 and A7 groups of the pons, and all dopaminergic neurons of the brainstem, hypothalamus, and forebrain (Everitt et al., 1984).

Pharmacology. In the rat, injections of NPY, [Pro^{34}]NPY, or NPY$_{13-36}$ into the nucleus tractus solitarius (NTS) evoke a long-lasting hypotension and bradycardia (Barraco et al., 1990; Carter et al., 1985; Grundemar et al., 1991; Grundemar et al., 1991a), whereas injections of PYY, PP, or desamido-NPY were ineffective at evoking these responses. Since this binding profile corresponded to neither Y_1 nor Y_2 receptors, this data has been used as evidence of a unique NPY receptor subtype, the Y_3 receptor. It has been found that these effects are sensitive to disruption by pertussis toxin, indicating the involvement of G-proteins in their mediation (Fuxe et al., 1989). In the *in vitro* rat NTS brain slice preparation, NPY has also been shown to suppress forskolin-induced cAMP formation, implicating cAMP as a possible second messenger, as well.

Functional interactions have been shown for NPY, but not PYY, with α_2-adrenoceptors in the NTS (Agnati et al., 1983; Fuxe et al., 1989; Härfstrand et al., 1989). In the medulla oblongata, as well, NPY can specifically increase the number of binding sites for and/or the affinity of α_2-adrenoceptors agonists, but without affecting those of antagonists (Agnati et al., 1983; Fuxe et al., 1984, 1985, 1987; Von Euler et al., 1989).

While [^{125}I]PYY binding sites have been described in the NTS (Martel et al., 1990b), quantitative autoradiography has shown that NPY, but not PYY or PP, can

displace radiolabelled NPY in rat brainstem (Nakajima et al., 1986). Moreover, [^{125}I]NPY binding sites in area postrema are not affected by pertussis toxin treatment, although their density was increased by 75% in the dorsomedial NTS (Von Euler et al., 1989), indicating that there is likely a heterogenous population of NPY receptors in the brainstem.

In the dorsal raphe nucleus, NPY, NPY_{13-36}, and C2-NPY have been shown to inhibit slow, α_1 adrenoceptor-mediated excitatory postsynaptic potentials and $5HT_{1A}$-mediated inhibitory postsynaptic potentials, without affecting fast, amino acid-mediated, synaptic transmission. (Kombian and Colmers, 1992)

NPY has both pre- and postsynaptic effects in the locus coeruleus (LC). In the rat LC, NPY has been found to presynaptically inhibit noradrenergic inhibitory postsynaptic potentials evoked by focal electrical stimulation (Finta et al., 1992). However, it has also been shown to potentiate the hyperpolarization of LC neurons induced by pressure application of noradrenaline. NPY_{16-36} and PYY, but not [$Leu^{31}Pro^{34}$]NPY were also able to produce the latter effect, indicating that it is mediated by a Y_2 receptor (Illes et al., 1993).

Thalamus

Anatomy. The thalamus exhibits some of the lowest concentrations of NPY in the CNS (Allen et al., 1983a; Adrian et al., 1983; Chronwall et al., 1985; Dawbarn et al., 1984). Those cells and fibres which do contain NPY are restricted to specific nuclei or narrow fields of innervation. *In situ* hybridization studies show that preproNPY mRNA is expressed in most somata in the reticular nucleus of monkeys (Hendry, 1993), although very few fibres and no cell bodies demonstrate NPY-LI in

rats or monkeys (Chronwall et al., 1985; Molinari et al., 1987; Smith et al., 1985). Neurons in the reticular nucleus of cats and monkeys also express somatostatin-LI (Molinari et al., 1987; Oertel et al., 1983). Although these cells widely innervate much of the dorsal thalamus, very few fibres expressing NPY-LI can be detected in that region. It is not known why such discrepancies occur, but it has been suggested that either the concentration of NPY is too low for immunocytochemical detection, or that the mRNA being detected is a remnant of transient NPY expression during late prenatal development (Foster and Schulzberg, 1984; Hendry, 1993).

NPY-IR fibres are found in the paraventricular, rhomboid and centromedial nuclei (Hendry, 1993). Cell bodies exhibiting preproNPY mRNA or NPY-LI have also been found in the pretectal complex and are numerous in the lateral geniculate nucleus (intergeniculate leaflet) of several species (Nakagawa et al., 1985; Smith et al., 1985; Ueda et al., 1986) and the pregeniculate nucleus of monkeys (Molinari et al., 1987; Moore, 1989). These cells comprise a subset of projection cells which innervate the suprachiasmatic nucleus of the hypothalamus (Card and Moore, 1982; Card and Moore, 1989; Harrington et al., 1985; Harrington et al., 1987), and may be involved in various circadian rhythms (Card and Moore, 1989; Hendry, 1993).

Pharmacology. Autoradiographic studies indicate that both Y_1 and Y_2 receptors exist in the thalamus (Aicher et al., 1991; Dumont et al., 1990). Y_1 receptor mRNA has also been found here (Eva et al., 1990; Grundemar et al., 1993). To date, however, no studies of NPY's actions in the thalamus have been published.

Hypothalamus

Anatomy. In contrast to the thalamus, the hypothalamus contains some of the

highest concentrations of NPY in the brains of rats (Allen, Y.S. et al., 1983), monkeys (Khorram et al., 1987) and humans (Adrian et al., 1983). Somata positive for NPY-LI or preproNPY mRNA (Gehlert et al., 1987; Morris, 1989) are predominantly present in the arcuate and dorsomedial nuclei, and lateral hypothalamus (Hendry, 1993) in rats (Bai et al., 1985; Card et al., 1983) and other rodents (Reuss et al., 1990; Sabatino et al., 1987). In other species, however, NPY-immunoreactive somata have also been found in other areas, such as the median eminence in golden hamsters (Sabatino et al., 1987), the periventricular, infundibular, and ventromedial nuclei in cats (Hu et al., 1987) and monkeys (Hendry, 1993), and in the monkey supraoptic nucleus (Hendry, 1993; Smith et al., 1985). It may be that NPY expression in this area is influenced by diurnal or seasonal cycles, or by levels of circulating hormones in individual subjects (Abe et al., 1990; Calza et al., 1989; Calza et al., 1990; Hooi et al., 1989; Khorram et al., 1987; Maccarrone et al., 1986). Many studies, however, also use colchicine to arrest axoplasmic transport and thereby enhance immunostaining of cell bodies. As mentioned earlier, however, it has been suggested that this procedure may produce questionable results, and that minor differences in technique may produce such variation as has been reported in the literature (Hendry, 1993).

Neuronal fibres displaying NPY-LI are densest along the midline of the cat (Hu et al., 1987; Leger et al., 1987), monkey (Smith et al., 1985), and human (Pelletier et al., 1984) hypothalamus. Mammillary bodies contain few fibres (Leger et al., 1987; Smith et al., 1985), but are surrounded by many (Leger et al., 1987). Within the suprachiasmatic nucleus (SCN), neuronal fibres form dense plexuses in the ventral and lateral regions, but not in the dorsomedial region. Species differences are evident, as the SCN of rats (Card et al., 1983) is more densely innervated than that of cats

(Cassone et al., 1988), monkeys (Moore, 1989; Smith et al., 1985), or humans (Moore, 1989). NPY-LI fibres originating in the brainstem (Sawchenko et al., 1985) and arcuate nucleus (Bai et al., 1985) innervate the paraventricular nucleus, and are concentrated in the medial regions (Sabatino et al., 1987). Of these, many project from adrenergic cells in the medulla and noradrenergic cells in the A1 cell group. (Sawchenko et al., 1985). In the periventricular nucleus and retrochiasmatic area, NPY-LI fibres are dense and uniformly distributed.

Pharmacology. Differences in reports of NPY concentrations in the hypothalamus are likely due, at least in part, to rapid, state-induced changes in expression. For example, it is known that 48 hours of food deprivation causes increases in NPY levels in the paraventricular (Beck et al., 1990; Sahu et al., 1988) and arcuate nuclei (Beck et al., 1990). Refeeding reduces these to normal levels, and causes increased NPY-LI in the lateral hypothalamus (Beck et al., 1990). Furthermore, injection of NPY into the paraventricular nucleus or perifornical area of rats causes a dramatic stimulation of feeding and drinking behavior (Stanley and Leibowitz, 1985; Stanley et al., 1985; 1985a; 1986; 1989; Morley et al., 1987 Pau et al., 1988), even in satiated animals (Hökfelt et al., 1980; Lewis et al., 1986). This response can also be elicited by injection of [Leu^{31}Pro^{34}]NPY and PYY, but $NPY_{26\text{-}36}$ and C2-NPY are completely inactive at evoking it (Danho et al., 1988; Flood and Morley, 1989; Jolicoeur et al., 1991; Stanley et al., 1992). This is not a typical Y_1-mediated response, however, since PP also elicited this response (Clark et al., 1984), and $NPY_{2\text{-}36}$ was perhaps even more effective than the full peptide (Jolicoeur et al., 1991; Kalra et al., 1990; Magdalin et al., 1989; Quirion et al., 1990; Stanley, 1993). NPY likely acts in concert with other hormonal agents in the control of feeding behavior; CCK reduces

hypothalamic NPY concentrations (Pages et al., 1990), and decreases food intake in rats (Antin et al., 1975; Gibbs et al., 1973).

In the SCN, NPY-LI oscillates diurnally, with gradual increases during light periods and decreases during dark (Calza et al., 1990). NPY innervation from the lateral geniculate nucleus to this area has been implicated in circadian function (Albers et al., 1984; Johnson et al., 1989; Moore and Card, 1985; Pickard et al., 1987).

The release of both luteinizing hormone releasing hormone (LHRH) from the hypothalamus and LHRH-induced release of luteinizing hormone (LH) from the anterior pituitary are modulated by NPY via a Y_1 receptor, in the presence of gonadal steroids, implicating NPY in an intricate control of reproductive functions, such as the pre-ovulatory discharge of LH (Kalra et al., 1990). An involvement in the regulation of stress related hormones, such as corticotropin-releasing hormone (CRH) (and subsequently ACTH)(Wahlestedt et al., 1987a) have also been shown for NPY.

NPY receptors in the hypothalamus seem to comprise a mixture of Y_1 and Y_2 subtypes (Wahlestedt et al., 1990a). However, despite the high concentrations of peptide in the hypothalamus, [^{125}I]NPY binding is extremely low in most areas, except in the SCN (Lynch et al., 1989; Martel et al., 1986; Quirion et al., 1990), pointing to a either a considerable mismatch of peptide and its receptors (Martel et al., 1988), or a technical problem in the detection of receptor binding.

Striatum

Anatomy. Some of the highest levels of NPY-LI in the brain of rats (Allen Y.S., et al., 1983; Nakagawa et al., 1985), monkeys (Khorram et al., 1987), and humans (Adrian et al., 1983) can be found in the caudate nucleus, putamen, and

nucleus accumbens septi. *In situ* hybridization studies in human brain show similar results (Brene et al., 1989). In rat, immunoreactivity for NPY (Vuillet et al., 1989) and the C-terminal flanking peptide of NPY (CPON) have been described in medium-sized, aspiny, neostriatal interneurons (DiFiglia et al., 1976; Dimova et al., 1980; Vuillet et al., 1992; Villalba et al., 1994). Similar neurons have also been described in hedgehog, having fusiform or round perikarya with two to four poorly branched processes (Villalba et al., 1994). In humans, cells are evenly distributed in clusters throughout the striatum, but fibre density is highest in the nucleus accumbens and ventromedial regions of the caudate and putamen. (Kowall et al., 1987). NPY-LI fibres in the hedgehog, by contrast, are evenly distributed throughout the neostriatum (Villalba et al., 1994)

Colocalization. In the rat striatum, NPY, CPON, and somatostatin exhibit a very high degree of colocalization (Gaspar et al., 1987; Kowall et al., 1985; Smith and Parent, 1986; Villalba et al., 1988; Vincent et al., 1983). These neurons also tend to express high levels of NADPH-diaphorase (Aoki and Pickel, 1990). In humans, NPY-LI, Somatostatin-LI, and NADPH diaphorase activity have been described in a single population of medium-sized aspiny intrinsic neurons (Kowall et al., 1987).

NADPH diaphorase is an enzyme involved in the generation of nitric oxide (NO), and is, therefore, also known as NO synthase (NOS). NO has been implicated in many physiological roles, and was originally known as endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980; Garthwaite et al., 1988). It has also been proposed that NO is the putative retrograde signal in long-term potentiation (Schuman and Madison, 1991). The significance of the coexistence of NOS with NPY in the striatum or other areas, such as the olfactory bulb or cortex,

is not known. However, it has been found that neurons containing NADPH-diaphorase seem better able to survive following such insults as excitotoxic lesions (Beal et al., 1986; 1989) or ischemia (Uemura et al., 1990), although this may be due to the calcium binding proteins which may also be present (Nitsch and Leranth, 1991). In human pathological states such as Huntington's chorea, which involves massive neuronal degeneration (Bruyn, 1968), it has been found that NPYergic neurons preferentially survive (Dawbarn et al., 1985). In a model of temporal lobe sclerosis, however, cells which normally express NPY/NOS in the hilus of the dentate gyrus are not spared (Olney et al., 1983).

Experiments using immunoautoradiography have described some cells which contain both NPY and tyrosine hydroxylase (TH), the catecholamine synthetic enzyme. A few terminals in the nucleus accumbens, but not in the dorsal striatum, showed immunoreactivity to TH and also contained dense-core vesicles with NPY-LI. (Aoki and Pickel, 1988)

In the rat, some overlap of NPY with GABA immunoreactivity has also been described (Aoki and Pickel, 1989; Vuillet et al., 1990).

Pharmacology. [^{3}H]NPY binding sites in the striatum have been described as belonging to a single population, based on binding studies showing a single dissociation constant (Unden et al., 1984). Autoradiographic studies show that binding is relatively unaffected by high concentrations of the Y_1 receptor agonist [Pro^{34}]NPY, indicating that these are probably Y_2 receptors (Dumont et al., 1992). NPY has been found to inhibit adenylate cyclase in a manner involving G-proteins (Westlind-Danielsson et al., 1988)

Olfactory bulb

Anatomy. Although some controversy has arisen over the morphological identifications of cell types in the olfactory bulb which contain NPY (Sanides-Kohlrausch and Wahle, 1990), it has been reported to exist in superficial and deep short axon cells (Gall et al., 1986; Matsutani et al., 1988, 1989; Scott et al., 1987), in the deep granule cell layer and subjacent white matter (Gall et al., 1986; Ohm et al., 1988; Sanides-Kohlrausch and Wahle, 1990; Scott et al., 1987). Some somata also occur between the glomerular layer and external plexiform layer (Hendry, 1993). NPYergic olfactory bulb neurons also show somatostatin-LI (Seroogy et al., 1989), and appear to contain NADPH diaphorase (Scott et al., 1987). Projection neurons originating in the olfactory bulb, by contrast, do not seem to contain NPY.

Cerebral cortex

Anatomy. NPY-LI is found in a heterogenous population of non-pyramidal neurons (Hendry, 1993; Hendry et al., 1984, 1984a; Kuljis and Rakic, 1989, 1989a, 1989b; Tigges et al., 1989) having various morphologies and chemical characteristics. The location of somata within the cortex varies somewhat amongst species, however, in rat (Allen, Y.S. et al., 1983), monkey (Beal et al., 1987), and human (Adrian et al., 1983; Beal et al., 1986a; Dawbarn et al., 1984, 1986), the highest concentrations of NPY-LI occur in the cingulate gyrus and association areas of the temporal lobe. The frontal lobe (Adrian et al., 1983) and occipital lobe, by contrast, show much lower concentrations, especially the primary visual area (Beal et al., 1987). Within area 17 of monkey, both somata and fibres are scarce in the regions of the cytochrome oxidase "blobs" which mark the eye dominance columns (Kuljis and Rakic, 1989b),

but are more numerous in surrounding areas (Hendry, 1993). Somata are present in all layers of the cortex, but are relatively more concentrated in layers II, III, V, and VI (Hendry, 1993). In humans but not rodents (Hendry et al., 1984; McDonald et al., 1982), they are especially dense in the subcortical white matter of the parietal lobe, and less so in occipital lobe (Hendry et al., 1984).

Neuronal processes exhibiting NPY-LI are numerous in the cortex, where they often form plexuses in both superficial and deep layers (Hendry, 1993). Generally, the limbic and association areas of cortex exhibit a greater density of NPY-LI fibres than do the primary sensory areas (Kuljis and Rakic, 1989, 1989a). An exception is the primary visual area (area 17), where fibres are relatively dense, even in the middle layers (Hendry et al., 1984; Kuljis and Rakic, 1989, 1989a).

Colocalization. Somatostatin is notable for its degree of co-expression with NPY; studies in rats and primates (including humans) conclude that up to 90% of cortical NPYergic neurons also contain somatostatin (Chronwall et al., 1984; Hendry, 1993; Jones and Hendry, 1986; Vincent et al., 1982) and NADPH-diaphorase (Vincent et al., 1983). Neurons containing both somatostatin and neuropeptide Y generally occur in layer VI and in the subcortical white matter, whereas layers II, III, and V contained neurons labelled with somatostatin alone (Gaspar et al., 1987). One study has shown that cysteamine, which is capable of depleting somatostatin, does not deplete NPY from rat cortical synaptosomes containing both (Chattha and Beal, 1987), indicating that they may exist in separate presynaptic vesicles. In monkey, most of those neurons containing both NPY and somatostatin, also show immunoreactivity for glutamic acid decarboxylase (GAD) which catalyzes the synthesis of GABA (Friauf et al., 1990). Those which do not contain GAD display,

instead, immunoreactivity for substance P and neurokinin A (Jones et al., 1988).

Pharmacology. In the cortex of several species (Inui et al., 1988; Lynch et al., 1989; Martel et al., 1987, 1990, 1990b), [^{3}H]NPY or [^{125}I]NPY binding sites are densest in layers II and III, and moderately dense in layers V and VI. (Lynch et al., 1989; Martel et al., 1986, 1987, 1990, 1990b). Autoradiography suggests that, in rat cortex, Y_1 binding sites are enriched in the superficial layers, while binding in the deeper laminae is much sparser, and seems to be mostly of the Y_2 type. (Dumont et al., 1990). By contrast, binding in human frontal cortex is predominantly of the Y_2 type (Jacques et al., 1994).

Hippocampus

Anatomy. As in the cortex, non-pyramidal cells exhibiting NPY-LI can be found throughout most of the hippocampus, however, *in situ* hybridization for preproNPY shows somata are especially dense in the dentate hilus of rats (Hendry, 1993; Kohler et al., 1986), monkeys (Kohler et al., 1986; Schwarzberg et al., 1990; Smith et al., 1985), and humans (Chan-Palay et al., 1986; Schwarzberg et al., 1990). They are also numerous in stratum pyramidale of the hippocampus proper, and in overlying neocortex (Hendry, 1993). In the dentate gyrus, neuronal types containing NPY include pyramidal basket cells, polymorphic cells, and spheroid cells (Hendry, 1993; Kohler et al., 1986). Interneurons of various morphologies in the hippocampus proper also exhibit NPY-LI, including some basket cells (Hendry, 1993). In rat and monkey, NPY-LI fibres are particularly dense in the outer third of the molecular layer of the dentate gyrus, and the stratum moleculare of all CA subfields of the hippocampus (Kohler et al., 1986). Some laminarity is also found in the subiculum,

where NPY-LI fibres are concentrated in the molecular layer. Such laminarity is not as evident, however, in either the medial or lateral entorhinal cortices. (Kohler et al., 1986a). In the entorhinal cortex, NPY-LI fibre plexuses are more dense than in the hippocampus proper, and include processes of local interneurons, as well as afferent axons from piriform cortex, the lateral nucleus of the amygdala, and locus coeruleus (Kohler et al., 1986a). As in the cortex, human hippocampal white matter also shows considerable NPY-LI. Many NPY-LI somata are found in the fimbria, alveus, and angular bundle in rats, monkeys (Kohler et al., 1986), and humans (Chan-Palay, 1987; Chan-Palay et al., 1986; Lostra et al., 1989).

Inhibition in the hippocampus

Why the hippocampus?

The hippocampus has been the site of extensive study, not only of its anatomy and function, but also as a tool in which to explore the most basic neuronal properties. It is a laminar structure with discrete neuronal populations and complex, but well characterized, synaptic pathways (Amaral and Witter, 1989). The transverse hippocampal slice preparation maintains a much simpler set of these synaptic connections which occur within the plane of the slice, and has been very well described (Andersen et al., 1971; Amaral and Witter, 1989). Briefly, the "trisynaptic pathway" consists of: 1) The angular bundle which enters the dentate gyrus from pyramidal neurons in the entorhinal cortex via the perforant path, branches into the molecular layers of both arms of the dentate gyrus, and forms synapses onto granule cells. 2) Granule cell axons, or "mossy fibres", extend into the hilus of the dentate

gyrus and project in a bundle into the stratum lucidum of area CA3, forming en passant synapses on the proximal dendrites of the CA3 pyramidal cells. 3) CA3 pyramidal cell axons bifurcate to form synapses with other ipsilateral and, via a commissural projection, with contralateral CA3 cells, and also project via the Schaffer collaterals to form synapses onto ipsilateral CA1 pyramidal neurons. 4) CA1 pyramidal axons project, via the alveus, to the subiculum and entorhinal cortex. (cf. Figure 4-6, p. 161)

Connections which are not preserved in transverse slices include CA1 and CA3 pyramidal projections to, and cholinergic projections from, the septal nuclei, noradrenergic projections from the locus coeruleus, serotonergic projections from the dorsal raphe nucleus of the pons, and all septo-temporal projections of the perforant path or Schaffer collaterals (Amaral and Witter, 1989).

Synapses in the trisynaptic pathway, as described above, are glutamatergic, however, a rich population of GABAergic interneurons can be found throughout the hippocampus as well. NPY is present in a subpopulation of GABAergic interneurons, which often also contain somatostatin (Hendry, 1987; Hendry et al., 1984a). The presence of NPY immunoreactive varicosities in immediate apposition to glutamatergic presynaptic terminals in the hippocampus has been determined using electron microscopy. (Milner and Veznedaroglu, 1992).

The anatomy of the hippocampus is very conducive to *in vitro* electrophysiological experimentation. The laminar structure of the hippocampal slice allows accurate visual placement of stimulating or recording electrodes. Also, because the principal cells are aligned with tightly packed cell bodies and orderly dendritic extensions, excitation of even a fraction of the population results in a large electric

field which can easily be measured extracellularly. Moreover, because the hippocampal slice is an *in vitro* preparation, it is possible to control and systematically alter such variables as extracellular ionic and chemical concentrations, temperature, and rate of perfusion.

Many of the phenomena which occur in the hippocampus have been exploited to study various mechanisms of physiology and pathology on levels ranging from molecular, to cellular, to that of the network. One such phenomenon, which has been extensively studied both *in vivo* and *in vitro*, is long term potentiation (LTP). An example of increased synaptic efficacy and plasticity, LTP is widely utilized as a model of learning and memory (Bliss and Collingridge, 1993). While much caution has been urged in the interpretations of data from these studies, it is true that many manipulations which prevent or reduce LTP *in vitro* also result in decreased behavioral learning in *in vivo* animal models (Butelman, 1990; Cox and Westbrook, 1994; Kim et al., 1991). NPY has also been implicated in learning and memory. Flood et al. (1987) found that when injected into the third ventricle or rostral hippocampus in mice, NPY or the weak Y_2-selective agonist NPY_{20-36} cause an improvement in the learning of both passive and active avoidance tasks (Flood et al., 1987). They also found that NPY is able to counteract the effects of agents which cause deterioration of memory, such as the muscarinic antagonist scopolamine and the protein synthesis inhibitor, anisomycin (Heilig, 1993). Conversely, injection of anti-NPY antibodies produces results opposite to those seen with injection of NPY, suggesting that endogenous NPY is playing a physiological role in the formation or retention of memories (Flood et al., 1989).

Limbic structures, such as the hippocampus and amygdala, are often involved

in epileptic seizure activity. They are also often amongst the first to undergo electrographic seizure activity evoked by kindling stimulation of various forebrain structures (Gale, 1993). Repeated electrographic seizures are also associated with hippocampal damage, such as cell death and sclerosis, the most common lesion observed in human epilepsy (Cavazos et al., 1994). The susceptibility of the hippocampus to electrical kindling or chemical convulsants has led to the development of several hippocampal models of epileptiform activity, many of which have also indicated a possible involvement of NPY in the progression of events associated therewith.

Several animal models of epilepsy have been associated with induction of NPY expression (Bellman et al., 1991; Marksteiner and Sperk, 1988; Marksteiner et al., 1989, 1990a). Seizures induced by chemical convulsants such as kainic acid, pentylenetetrazol, or cocaine result in a persistent increase in NPY expression in the dentate gyrus, including expression in the granule cells themselves and NPY immunoreactivity in the mossy fibre terminal field (Goodman and Sloviter, 1993; Marksteiner 1990b; Sperk et al., 1992), which do not occur under normal conditions (Morris, 1989). Electroconvulsive shocks administered *in vivo* are also able to affect NPY expression, causing levels of NPY-LI to double in frontal and parietal cortex and in hippocampus, while NPY-LI in other brain regions, such as striatum, hypothalamus, pons, olfactory bulbs, or cerebellum, and in plasma and adrenal glands, remain unaltered (Wahlestedt et al., 1990). Since NPY has been shown to be inhibitory (Colmers et al., 1985, 1987, 1988), these observations suggest that NPY may play a compensatory role during epileptogenesis.

Presynaptic inhibition in the hippocampus by NPY

In 1985, Colmers et al. demonstrated that application of NPY to a hippocampal slice inhibited extracellularly-recorded postsynaptic potentials in area CA1 (Colmers et al., 1985). Further intracellular experiments showed that this was accomplished without causing any observable changes to postsynaptic properties such as resting membrane potential or input resistance of the postsynaptic cell (Colmers et al., 1987, Haas et al., 1987). Although NPY also inhibited excitatory postsynaptic currents in CA3 cells in response to stimulation of mossy fibre afferents, it had no effect on inward currents caused by iontophoresis of glutamate receptor agonists, further supporting the hypothesis that NPY-mediated synaptic inhibition is a presynaptic phenomenon (Colmers et al., 1987; McQuiston and Colmers, 1992).

Colmers et al. (1988) also demonstrated that while the actions of NPY could be reduced or prevented by prior application of low concentrations of the K^+ channel blocker 4-aminopyridine (4-AP), NPY's effects could also be restored by concomitant reduction of extracellular Ca^{2+} concentrations, in a manner consistent with a mechanism of action for NPY involving modulation of Ca^{2+} influx at the presynaptic terminal (Colmers et al., 1988; Chapter 2). It was also shown that NPY potently inhibited the evoked postsynaptic potentials in the presence of bicuculline, which resemble electrographic seizure events (Colmers et al., 1988).

Elevating extracellular K^+ concentrations causes depolarization of neuronal cell membranes, and can also lead to the gener[illegible]n of electrographic seizures in hippocampal slices (Traynelis and Dingledine, 1988). NPY, acting via Y_2 receptors, is capable of inhibiting K^+-stimulated glutamate release in this preparation (Greber et al., 1994).

Goals of this project

The goals of this project are fourfold:

1) to determine the pharmacology of the NPY-mediated inhibition reported earlier in area CA1. In the absence of selective antagonists, I have made use of peptide fragments, shown earlier to differentiate between NPY receptor subtypes (Wahlestedt et al., 1986, 1987), to determine which receptor subtype mediates the presynaptic inhibition seen in this area (Chapter 2).

2) to determine whether the presynaptic inhibition mediated by NPY is unique, or whether presynaptic modulators share a common mechanism of action. In Chapter 3, I used the methods developed by Colmers et al. (1988) to compare the effects of external manipulations (4-AP and lowered extracellular calcium concentrations) on presynaptic inhibition mediated by NPY via Y_2 receptors, by baclofen via $GABA_B$ receptors, and by 2-chloroadenosine via A_1 receptors.

3) to determine at which hippocampal synapses NPY acts. To do this, I utilized selective electrode placements and pharmacological antagonists to isolate previously well-characterized excitatory and inhibitory synaptic potentials throughout the hippocampus, and tested the ability of NPY to inhibit them specifically. Based on these results, and the assumption that, as a peptide, endogenous NPY may be preferentially released by higher levels of activity, I formulated the hypothesis that the physiological role of NPY, acting on Y_2 receptors, was to control synaptic excitation under conditions when it may be sufficiently elevated to cause NPY release (Chapter 4).

4) to test the ability of NPY to limit excessive synaptic excitation induced by a

number of different manipulations, and thereby to elucidate the physiological role of NPY, in the context of its location and mechanism of action within the hippocampus (Chapter 5).

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CHAPTER 2

PRESYNAPTIC INHIBITION BY NEUROPEPTIDE Y (NPY) IN RAT HIPPOCAMPAL SLICE *IN VITRO* IS MEDIATED BY A Y_2 RECEPTOR †

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INTRODUCTION

Neuropeptide Y (NPY) is an abundant, 36 amino-acid peptide expressed and released by many types of nerve cells in the central (CNS) and peripheral (PNS) nervous systems of mammals (DeQuidt & Emson, 1986, Sundler, et al. 1986). In peripheral smooth muscle tissues, NPY has two sites of action. 1) On the muscle cell itself, relatively high concentrations of NPY induce contractions, and subthreshold concentrations of the peptide markedly enhance the contractile response to other agents, such as noradrenaline and histamine (Wahlestedt, 1987, Wahlestedt, et al., 1986). 2) On the presynaptic sympathetic nerve terminal, NPY inhibits its own release, as well as the release of noradrenaline (Wahlestedt, 1987, Wahlestedt, et al., 1986). In rat hippocampal slice *in vitro*, NPY has been shown to inhibit excitatory synaptic transmission at stratum radiatum-CA1 (glutamatergic) synapses (Colmers, et al., 1985, 1987, 1988, Haas, et al., 1987) by an action at the presynaptic terminals, probably the inhibition of voltage-dependent calcium influx (Colmers, et al., 1988).

To date, no antagonists at NPY receptors have been identified. However, studies which tested agonist fragments and analogues of NPY indicate that at least two subtypes of NPY receptor can be distinguished pharmacologically in peripheral sympathetic neuroeffector junctions (Wahlestedt, 1987, Wahlestedt, et al., 1986). The Y_1 subtype is located on the postsynaptic cell in blood vessels and vas deferens, and requires the intact NPY, or the very closely related Peptide YY (PYY) molecule for its activation (Wahlestedt, et al., 1986). The Y_2 subtype is found on the presynaptic terminal, and can be activated by analogues and C-terminal fragments of NPY as short as NPY 13-36 (Wahlestedt, et al., 1986). The C-terminal desamido form of NPY has very little activity at both receptor subtypes (Wahlestedt, et al., 1986). The two

receptor subtypes in peripheral tissues are also distinguished by the second messenger systems to which they are coupled; Y_1 receptor activation causes phosphatidylinositol hydrolysis, while Y_2 receptor activation inhibits adenylate cyclase (Wahlestedt, 1987, Westlind-Danielsson, et al., 1987, Hinson, et al., 1988, Perney & Miller, 1989.).

To identify which NPY receptor subtype (if either) is responsible for the presynaptic inhibition observed in hippocampus, we compared the response of stratum radiatum-CA1 excitatory synaptic transmission to single concentrations of agonist analogues and fragments of NPY with that to the intact peptide. The results indicate that, in rat hippocampus *in vitro*, NPY inhibits excitatory synaptic transmission via a receptor which is pharmacologically similar in its agonist profile to the peripheral Y_2 subtype.

METHODS

Peptide Synthesis

Fragments of NPY were synthesized by the solid phase method using a manual home-made multireactor synthesizer. The syntheses were carried out with a benzhydrylamine resin (Pietta, et al., 1974) since the peptides bear an amide C-terminal function. All amino acids were coupled via the BOP/DMF method (Fournier, et al., 1988), according to a recently-described protocol (Forest & Fournier, 1989). The Boc amino acids with appropriate side-chain protection were obtained from commercial sources. Completed peptides were cleaved from the resin support and deprotected by a 90 min treatment at 0°C with liquid hydrofluoric acid containing m-cresol and dimethyl sulfide as scavengers (10:1:1 v/v).

After extraction from the resin and lyophilization, the peptides were purified by reverse-phase chromatography on a Waters Deltapak column, using an eluent of A: H_2O (0.06% trifluoroacetic acid, TFA) and B: acetonitrile-H_2O (0.06% TFA). Peptides were eluted with successive linear gradients of solvent B. Analytical HPLC of the individual fractions were carried out and the fractions corresponding to the purified peptide were lyophilized. The purified material was characterized by analytical HPLC, capillary electrophoresis and amino acid analysis.

Electrophysiological studies

Intra- and extracellular recordings were made from area CA1 of rat hippocampal slices as described previously by Colmers, et al. (1987, 1988). Briefly, transverse slices (400 - 450 μm thick) of hippocampus were submerged in a recording chamber continuously perfused (2.5 ml min^{-1}) with saline (Colmers, et al., 1985, 1987,

1988) saturated with 95% O_2/5% CO_2 and heated to 34 ± 0.2°C. Orthodromic stimuli (monophasic, square wave, 100-300 μs, 3-35V) were presented using bipolar electrodes, placed on stratum radiatum of area CA1. Extracellular recordings were made from the stratum pyramidale of area CA1 with glass microelectrodes (3-15 MΩ; 2M NaCl); intracellular recordings were made from CA1 pyramidal neurones in area CA1 with glass microelectrodes (85-150 MΩ; 2M K^+ acetate). An Axoclamp 2A amplifier, used in the bridge current clamp mode was used both for extracellular and intracellular recordings. Current and voltage were displayed continuously using a DC coupled chart recorder (Gould 2200, frequency response DC - 60 Hz), and selected parts of experiments were stored either on FM (Racal) or PCM coded videotape (Vetter). A Nicolet 4094 digital oscilloscope was used to average and store data on-line and analyze data off-line.

Analysis of electrophysiological data was performed as published previously (Colmers, et al., 1985, 1987, 1988). Stimulus amplitudes were chosen to evoke responses between 70 and 85% of maximum, which is on the steepest, and therefore most sensitive, portion of the stimulus-response relationship (Colmers, et al., 1985, 1987). Population spike (PS) amplitude was determined from the peak of the negativity following the stimulus artifact to the peak of the following positivity. Resting membrane potential was determined from chart records; excitatory postsynaptic potentials (EPSP's) were measured as peak amplitudes. EPSP's were evoked 40 msec after the beginning of a hyperpolarizing current pulse applied to the electrode via the bridge circuit to prevent the neurone from achieving action potential threshold. Input resistance was determined by the slope of a least-squares linear regression line fitted to data obtained from families of hyperpolarizing and

depolarizing constant current pulses (125 msec) applied to neurones under each condition.

Peptides and drugs were applied via the superfusate (Colmers et al., 1987, 1988). Native porcine NPY (Richelieu Biotechnologies, Québec, or a generous gift from Dr. T.O. Neild, Monash University, Melbourne) and analogues and fragments (synthesized as above) were prepared just prior to use at a final concentration of 1 μM. All analogues and fragments, except as noted, were porcine NPY sequence, C terminally amidated peptides. Native, intact porcine NPY is referred to here simply as NPY. Fragments are referred to by the number of C-terminal residues.

Data were taken only from preparations where a significant recovery from drug effects occurred upon washout. Preparations were used as their own controls for statistical purposes, and peptide effects relative to control were assessed using a Student's paired-t test. Although we attempted to apply NPY itself to each preparation, it was not always possible; pooled data from fragments and analogues were therefore compared statistically with one another using a Student's t test for 2 means.

RESULTS

Results are based on recordings from 96 separate preparations: extracellular recordings were made in 83 different preparations; intracellular recordings were made of 62 different neurones in some of the preparations where field potentials were simultaneously recorded, and in 10 preparations without simultaneous extracellular recordings.

As we and others have observed previously (Colmers, et al., 1985, 1987, 1988, Haas, et al., 1987), NPY reversibly inhibited excitatory synaptic transmission from stratum radiatum to CA1 pyramidal cells, as measured by a reduction of the amplitude of the extracellular population spike (PS). A concentration-response relationship to NPY indicated the threshold for inhibition of the PS to be about 30 nM; 1 μM inhibited the PS by 89.9 $\pm$ 2.8% (n=16). The EC_{50} for NPY on the PS was about 250 nM, similar to that seen in earlier studies (Colmers, et al., 1985, 1987).

In addition, as reported earlier (Colmers, et al., 1987, 1988, Haas, et al., 1987), NPY also reversibly inhibited the intracellularly-recorded excitatory postsynaptic potential (EPSP) evoked in CA1 pyramidal cells by stratum radiatum stimulation. 1 μM NPY reduced the EPSP by 59.8 $\pm$ 2.3% (n=8); the EC_{50} for NPY effects on EPSP was about 200 nM, in agreement with earlier reports (Colmers, et al., 1987). As reported earlier, NPY had no effects on passive or active membrane properties in CA1 neurones (Colmers, et al., 1987, 1988).

Peptide analogues

All peptide analogues and fragments tested in this study were without any effects on membrane resting potential, input resistance or action potential amplitude or duration, consistent with earlier observations on NPY itself (Colmers, et al., 1987, 1988).

Application of the full-sequence native analogue, PYY, at 1 μM also caused a reduction of both PS and EPSP amplitudes, which reversed upon washout (Fig. 2-1). The inhibition of PS was statistically indistinguishable from that seen NPY (Fig. 2-3) although it appeared greater and persisted longer. However, the effect of PYY on the simultaneously-recorded intracellular EPSP was indistinguishable from that of NPY (Fig. 2-4) although the effect also appeared to persist longer than with NPY.

Human sequence NPY (hNPY), the amino acid sequence of which is identical with rat NPY and differs from that of porcine NPY only at position 17 (Corder, et al., 1984, Minth, et al., 1984, Allen, et al., 1987), was equally effective as NPY in inhibiting synaptic transmission measured both as PS (Figs. 2-1 & 2-3) and EPSP (Figs. 2-1 & 2-4). However, desamido hNPY (hNPYa) had no effects on synaptic transmission (Figs. 2-3 & 2-4).

Peptide fragments

Amidated C-terminal fragments of (porcine sequence) NPY, including NPY2-36, 5-36, 11-36, 13-36, 16-36, and 25-36, were also studied. Data are summarized in Figures 2-2, 2-3 & 2-4.

NPY2-36 was at least as potent as NPY at inhibiting excitatory synaptic transmission in CA1. No significant difference was seen between this fragment's

actions on both PS and EPSP and those of NPY. While NPY5-36 also inhibited excitatory synaptic transmission, it was less active than NPY. At a concentration of 1 μM, this fragment was about 70% as active at inhibiting the PS and about 80% as active at inhibiting the EPSP as was NPY. NPY5-36 was also significantly less active than NPY2-36. NPY11-36 was slightly, but not significantly, less active at the presynaptic NPY receptor than was NPY5-36. However, NPY13-36 was significantly less active than NPY11-36, and was less than half as active as the intact peptide in inhibiting the PS, while its inhibition of the EPSP was somewhat more than 50% of that caused by NPY itself (Fig. 2-3). By contrast, the slightly shorter NPY16-36 fragment did not significantly inhibit PS in CA1, although it had a small, but significant, inhibitory effect on the EPSP recorded intracellularly. NPY16-32 was, on average, 80% as active as NPY13-36 at inhibiting EPSP amplitudes. NPY18-36 was without any significant action on either the PS or the EPSP, and NPY25-36 was without significant effects at 1 μM on PS or EPSP (Fig. 2-4).

DISCUSSION

In this study, there were no effects of NPY, its analogues or fragments on the passive or active membrane properties of the CA1 pyramidal neurones. This is in agreement with other work from this laboratory (Colmers, et al., 1987, 1988) and others (Haas, et al., 1987). Thus, all active peptides tested on stratum radiatum-CA1 synaptic transmission *in vitro* appear to act only at a presynaptic site.

The presynaptic receptor for NPY in the rat hippocampus appears to share an identical agonist profile with the Y_2 receptor first characterized at sympathetic neuroeffector junctions (Wahlestedt, et al., 1986, Wahlestedt, 1987). Thus, the full-sequence analogues PYY and hNPY and are equipotent with NPY, while desamido hNPY was without measurable activity, confirming previous reports that the C-terminal amide is necessary for any activity of the peptide at either Y_1 or Y_2 receptors (Wahlestedt, et al., 1986). The 2-36 fragment of NPY was at least equipotent with NPY, although further reductions in fragment length caused a gradual decline in activity. There was a relatively sharp drop in activity between NPY13-36, which had roughly 50% of the activity of NPY, and NPY16-36, which had no measurable effect on the PS, and a small, but significant effect on the EPSP. Further truncation to NPY18-36 eliminated all activity at the presynaptic receptor in hippocampus.

While NPY16-36 had a small but significant effect on EPSP's, it did not affect PS in this study. Y_2 receptors in peripheral tissue have been shown to be sensitive to fragments as short as NPY23-36 (Grundemar and Håkanson, 1990); NPY18-36 inhibits calcium influx in dorsal root ganglion cells in culture (Colmers, Bleakman, and Miller, unpublished). It therefore seems likely that, at the concentrations tested here, the actions of NPY16-36 were subthreshold for an effect on PS. It is therefore

essential that both pre- and postsynaptic responses be examined in similar studies.

The Y_2 receptor has been shown in peripheral tissues and in homogenates of whole brain to inhibit the activity of adenylate cyclase (Wahlestedt, 1987, Westlind-Danielsson, et al., 1987). However, although the mechanism coupling the presynaptic NPY receptor in hippocampus to its effectors (probably calcium channels in presynaptic terminals; Colmers, et al., 1988) is not known, preliminary results indicate that the inhibition of adenylate cyclase is not responsible, as elevation of intracellular cAMP levels with a membrane-soluble analogue did not affect the inhibition of synaptic transmission brought about by NPY (Klapstein, et al., 1990). The actual mechanism by which NPY exerts its presynaptic action is currently under investigation.

As the results of this and other studies indicate, some presynaptic nerve terminals in both the peripheral and central nervous systems bear NPY receptors which inhibit release of transmitter. Thus far, they all appear to be of the Y_2 subtype. The notable difference is that, in peripheral nerve terminals, the NPY receptor is an inhibitory *autoreceptor*, while in hippocampus, it is an inhibitory *heteroreceptor*. While the inhibition of synaptic transmission by action at a presynaptic terminal appears a common property of the Y_2 receptors studied until now, there appears to be a difference in the role they play in different neuronal systems.

Figure 2-1. **Effects of PYY and hNPY on population spike and EPSP in CA1.** Effect of the full-sequence NPY analogues, PYY (left traces) and hNPY (right traces), on PS (upper traces) and EPSP (lower traces) recorded simultaneously in CA1. Records for PYY and hNPY are from different preparations. Peptides were applied at a concentration of 1 μM. Control and peptide traces are shown superimposed for comparison. The responses recovered after prolonged washout (not illustrated).

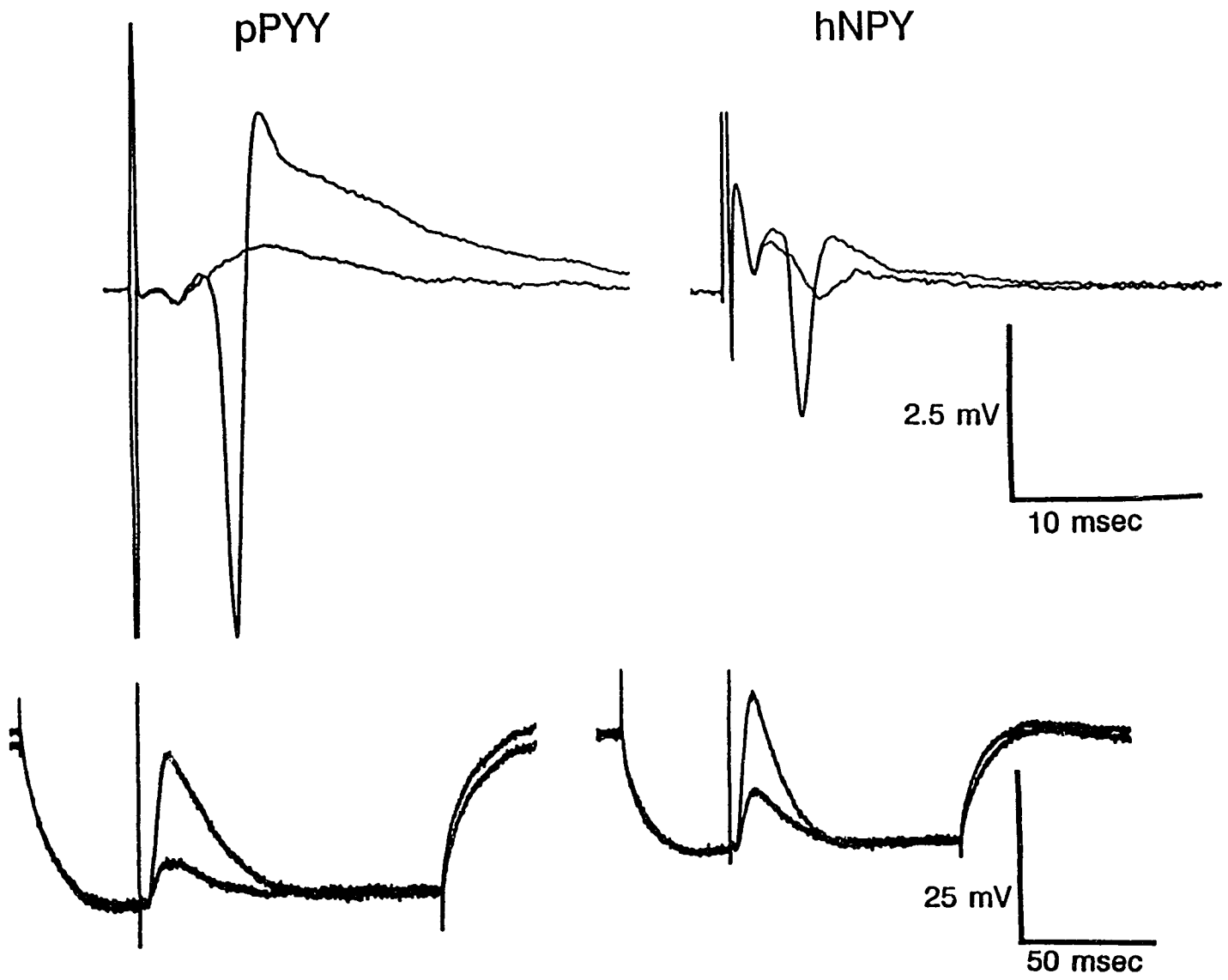

pPYY
hNPY
2.5 mV
10 msec
25 mV
50 msec

Figure 2-2. **Effects of NPY C-terminal fragments on EPSP and population spike in CA1.** Representative effects of different C-terminal fragments of NPY on EPSP (upper traces) and PS (lower traces) applied at 1 μM in CA1. Fragment length is indicated above each trace. Records are all from different preparations. All responses showed recovery upon washout (not illustrated).

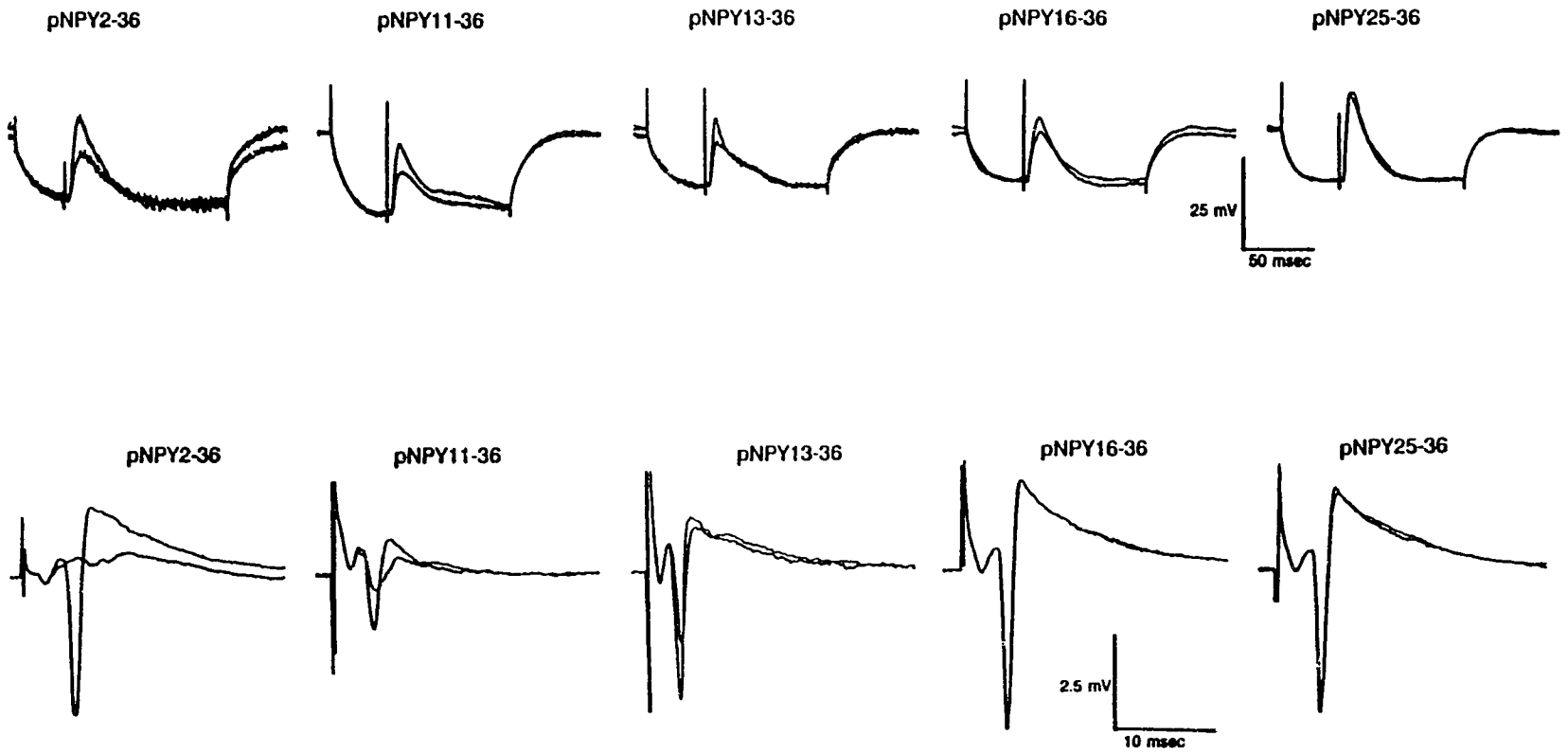

pNPY2-36
pNPY11-36
pNPY13-36
pNPY16-36
pNPY25-36
25 mV
50 msec
pNPY2-36
pNPY11-36
pNPY13-36
pNPY16-36
pNPY25-36
2.5 mV
10 msec

Figure 2-3. **Structure-activity relationship for NPY analogues and fragments on CA1 population spike.** Relative effects of analogues and C-terminal fragments of NPY on PS evoked in CA1 by stimulation of stratum radiatum. Values shown are means ± SEM of effects, expressed as percent of mean NPY (=100%) effect. Analogues are shown on the left of the Figure, while pNPY and its fragments are shown to the right (under bar), listed in order of decreasing length on the X-axis, with spacing along this axis indicating the relative length of the fragments. * - $P < 0.001$ difference from NPY; + - $P < 0.02$ difference from previous fragment; + + - $P < 0.001$ difference from previous fragment. All fragments were tested ≥ 5 times in different preparations. Fragments NPY16-36 and shorter were without significant effect at inhibiting the PS.

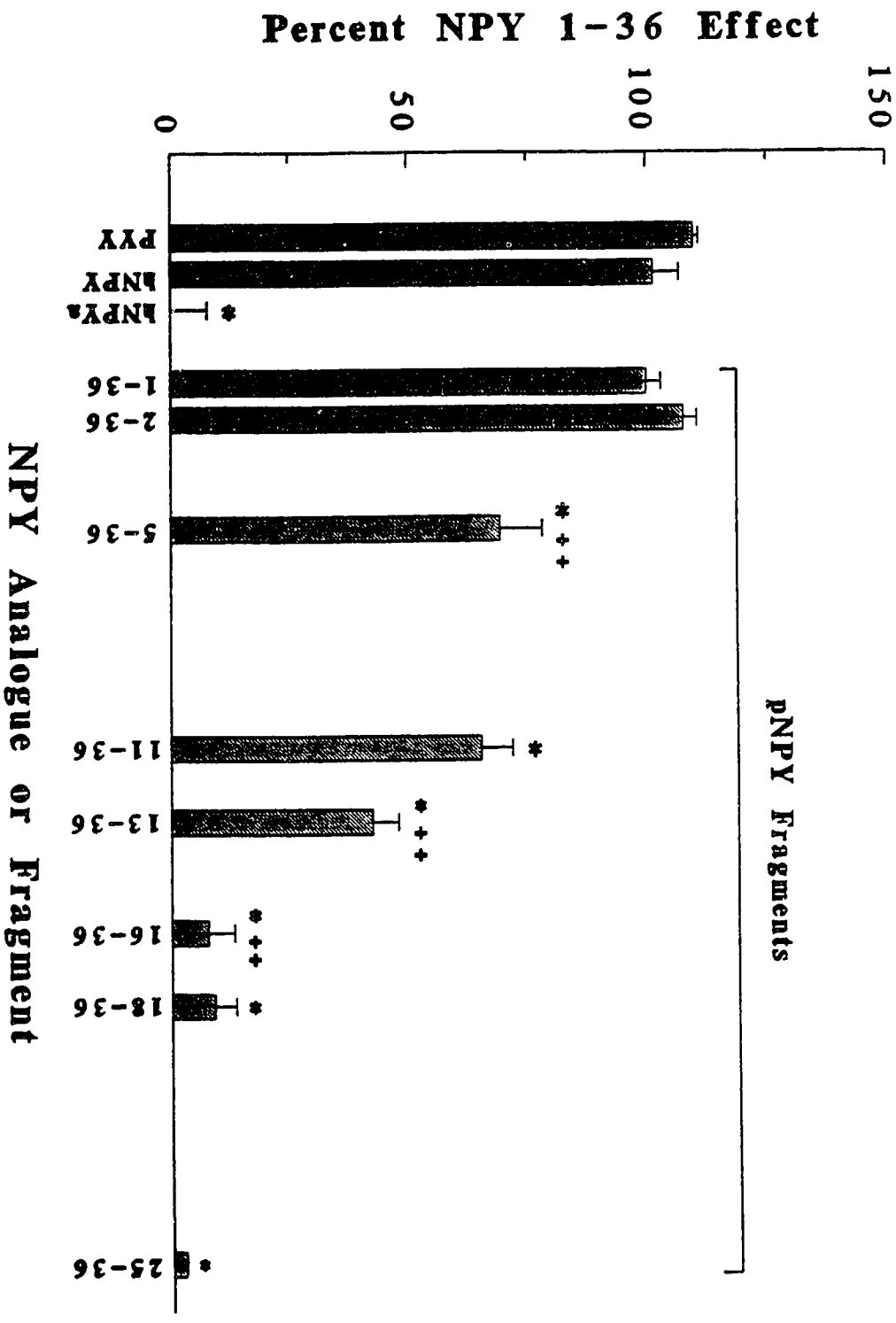
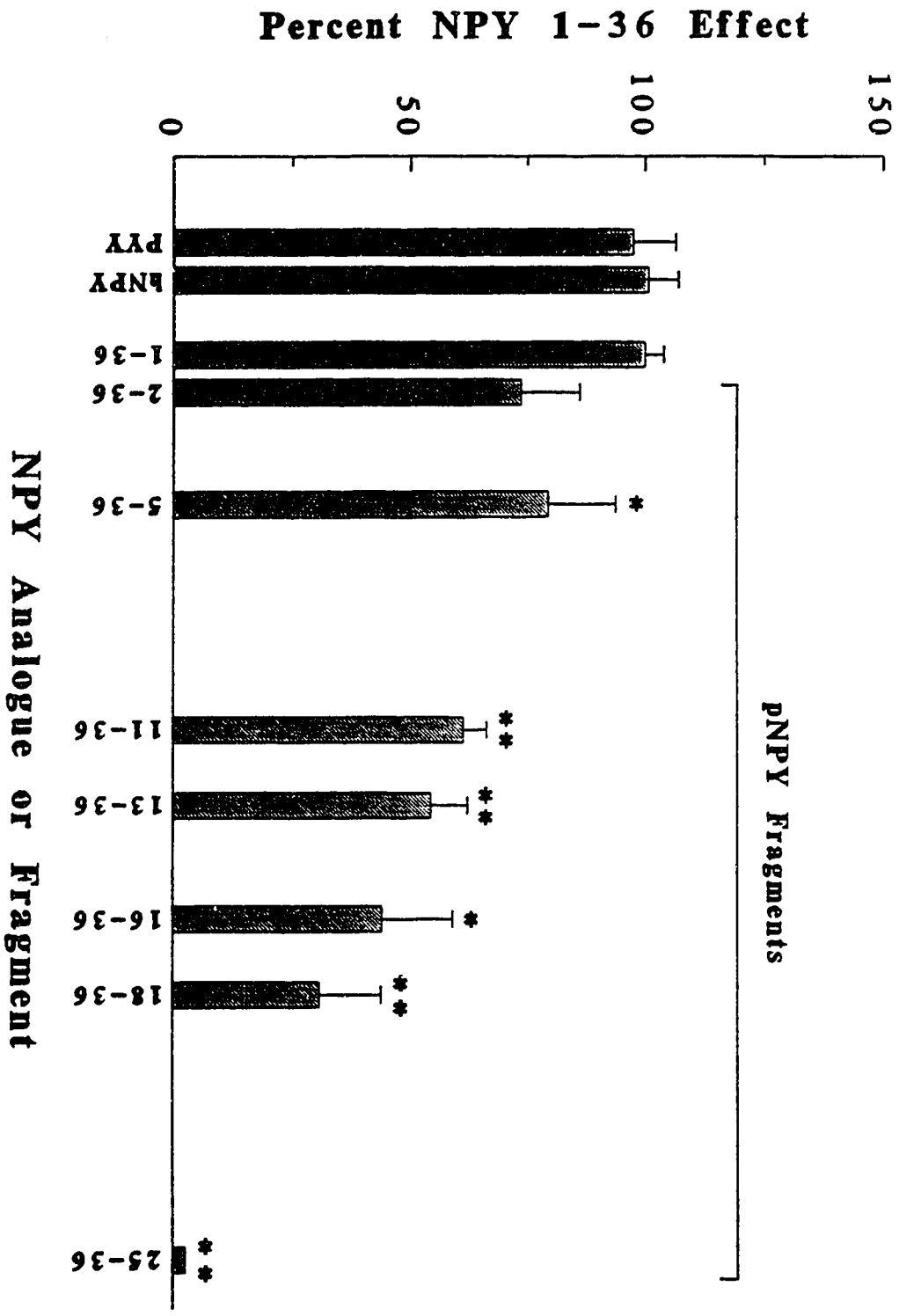

Percent NPY 1-36 Effect
0
50
100
150
NPY Analogue or Fragment
pNPY Fragments
pYY
bNPY
bNPYa
1-36
2-36
5-36
11-36
13-36
16-36
18-36
25-36

Figure 2-4. **Structure-activity relationship for NPY analogues and fragments on EPSPs in CA1 pyramidal neurons.** Relative effects of analogues and C-terminal fragments of NPY on EPSP evoked in CA1 pyramidal neurons by stimulation of stratum radiatum. Values shown are means ± SEM of effects, expressed as percent of mean NPY (=100%) effect. Analogues and fragments are distributed as in Fig. 3. * - $P < 0.005$ difference from NPY; ** -$P < 0.001$ difference from NPY. Immediately neighboring fragments were not statistically different from one another. All fragments were tested on preparations ≥ 5 times on different preparations. Fragments NPY18-36 and shorter were without effect on the EPSP.


Percent NPY 1-36 Effect
0
50
100
150
NPY Analogue or Fragment
pNPY Fragments
pYY
hNPY
1-36
2-36
5-36
11-36
13-36
16-36
18-36
25-36
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CHAPTER 3

4-AMINOPYRIDINE AND LOW CA^{2+} DIFFERENTIATE PRESYNAPTIC INHIBITION MEDIATED BY NEUROPEPTIDE Y, BACLOFEN AND 2-CHLOROADENOSINE IN RAT HIPPOCAMPAL CA1 *IN VITRO.* †

† A version of this chapter has been published. Klapstein & Colmers 1992. British Journal of Pharmacology. 105: 470-474.

INTRODUCTION

Several neurotransmitters and neuromodulators are known to mediate the presynaptic inhibition of neurotransmitter release in the mammalian CNS. In hippocampal area CA1, at least 5 receptors are known to mediate the inhibition of excitatory amino acid neurotransmitter release, presumably glutamate, from the terminals of stratum radiatum fibres that form synaptic connections with dendrites of CA1 pyramidal neurones. These receptors include: 1) a γ-aminobutyric acid ($GABA_B$) receptor (Ault & Nadler, 1982); 2) a muscarinic receptor (Hounsgaard, 1978); 3) an unusual glutamate receptor, at which L-2-amino-4-phosphonobutyrate (AP4) is an agonist (Forsythe & Clements, 1990); 4) an adenosine receptor (A_1-like; Dunwiddie & Fredholm, 1989) and 5) a neuropeptide Y (NPY) Y_2 receptor (Colmers et al., 1988, 1991). Of the known endogenous substances mediating presynaptic inhibition in area CA1 of hippocampus, NPY is the only one that has no postsynaptic effects (Colmers et al., 1987).

Since the first description of presynaptic inhibition, a debate has continued as to the mechanism by which transmitter substances prevent release of transmitter (cf. Starke, 1981). The precise mechanism of presynaptic action in hippocampus is not known for any of these substances, although there is evidence that they all activate receptors coupled to GTP binding proteins. Nearly all of these substances have been shown to inhibit calcium influx into cultured or acutely dissociated neurones (Dolphin & Scott, 1986; Wanke, 1987; Macdonald, et al., 1986; Walker, et al., 1988), and most are known to activate potassium conductances in cell bodies (Newberry & Nicoll, 1984; Egan & North, 1986; Greene & Haas, 1985; Zidichouski, et al., 1990). Because of technical limitations, it has not been possible to examine the actions of

these substances on evoked transmitter release by direct measurements on presynaptic terminals of CNS. The question of how NPY acts at the presynaptic terminal has been indirectly addressed by the use of 4-aminopyridine (4-AP), which blocks most major potassium conductances at presynaptic terminals (Buckle & Haas, 1982). 4-AP at concentrations well below those needed to affect the somatic "A" current has been shown to block the presynaptic actions of NPY in hippocampal CA1 (Colmers, et al., 1988); higher concentrations block presynaptic inhibition mediated by adenosine in guinea pig olfactory cortex (Scholfield & Steel, 1988). Lowering extracellular calcium concentrations in the presence of 4-AP restored NPY's inhibition, suggesting the inhibition of a presynaptic terminal calcium conductance by NPY (Colmers et al., 1988).

To examine whether the presynaptic inhibition mediated in hippocampus by NPY, baclofen and adenosine could be via the same mechanism, we compared the effects of 4-AP and low calcium on the concentration-response relationships for all three substances. If inhibition of transmitter release by all three receptors is mediated by the same mechanism, the inhibition by all three should be equally affected by 4-AP and low Ca^{2+}, since neither manipulation should alter the binding of the agonists to their receptors. The data indicate that, although baclofen and NPY are similar in their response to these manipulations, adenosine differs from the others.

METHODS

Transverse hippocampal slices (450 μm thick) were prepared from male Sprague-Dawley rats (75-150g), and incubated at 34 ± 0.5 °C, submerged and constantly perfused (2.5 - 3 ml/min) with carbogenated saline (pH 7.35) (Colmers, et al. 1988, 1991). Composition of the saline was (in mM) NaCl, 124, KCl, 1.8, $MgSO_4$, 2, KH_2PO_4 1.25, $CaCl_2$, 1.5, glucose, 10. Low Ca^{2+} solutions were prepared from Ca^{2+}-free stock solution to which appropriate amounts of $CaCl_2$ were added. Stimuli were delivered to stratum radiatum of area CA1 via a shielded bipolar, etched-tungsten electrode connected to a stimulus isolation unit (AMPI). Orthodromic population field potentials evoked by stimuli (3 - 40V, 50 - 200μs, 0.1 Hz) were recorded in stratum radiatum of CA1 with a glass micropipette (4-15 MΩ, 2M NaCl; Figure 3-1) connected to the headstage of an Axoclamp 2A amplifier. For each data point, three successive field potentials were digitized at 50 μs point^{-1}, and averaged using a virtual averaging paradigm by a digital storage oscilloscope (Nicolet 4094; Model 4562 plug-in) and were stored on diskette. The slope of the initial, linear portion of the population excitatory postsynaptic potential (pEPSP) was measured as the difference in voltage and time between the extremes of the linear portion (determined by inspection) of the pEPSP, using the oscilloscope. Once stable responses had been obtained, stimulus-response curves were constructed, and a stimulus intensity was chosen to elicit a response on the linear portion of the stimulus-response curve (Andersen, et al., 1978). Generally, representative responses were chosen which were of similar size (± 20%) to those observed in control for each preparation; however, they were always on the linear portion of the stimulus-response curve. Stimulus-response curves were constructed under each basal condition,

and stimulus intensities chosen using the same criteria as for control conditions. In most cases, the responses evoked in a given preparation by a given stimulus in control and in the presence of 4-AP/low Ca^{2+} were similar in slope and amplitude, while 4-AP at both concentrations caused a much greater response, requiring lower stimulus intensities to elicit the same responses. Stimulus-response curves were also obtained during drug effects and upon washout.

Test substances were made freshly in saline of appropriate composition. Synthetic porcine NPY (Richelieu Biotechnologies), was made up at 100 μM in distilled water and kept frozen until just prior to use. 2-Chloroadenosine (2-CA; Research Biochemicals Inc.) and (±) baclofen (Lioresal: gift of Ciba-Geigy) were made up as concentrated stock solutions (dilutions of 1:100 or greater in final use) and kept frozen or refrigerated, respectively, in the dark until prior to use.

Data are expressed as percent inhibition of control values. Preparations served as their own controls; all data are from preparations which showed significant recovery upon washout. Statistical comparisons were performed using a Student's t test. Statistical differences were considered significant at $P \leq 0.05$. Concentrations of drugs somewhat above the EC_{50} were selected for comparison in the Figures, and for representative statistical comparisons. In addition, ligand concentrations over which significant differences between treatments were observed are given, where appropriate.

RESULTS

Data were obtained from 56 preparations, with a total of 361 drug applications. Initial pEPSP slope measurements in control conditions varied from preparation to preparation, and ranged from 0.12 to 1.26 mV/msec. Although we attempted to perform concentration-response curves on each preparation under each condition, this was not always possible. When more than one concentration of a drug was applied to a given preparation, lowest concentrations were always applied first; the order of application of different drugs was randomized between preparations.

Bath application of 1 μM NPY reversibly reduced the pEPSP slope in CA1 by 63.15 $\pm$ 1.94% (n = 4; Figure 3-2). NPY was significantly effective at concentrations above 300 nM (Figure 3-3); concentrations of NPY higher than 3 μM were not tested. Baclofen (10 μM) also reduced the pEPSP slope, by 71.53 $\pm$ 2.88 (n = 6; Figure 3-2). Baclofen significantly inhibited pEPSP slope at concentrations above 300 nM; (Figure 3-3). 1 μM 2-CA inhibited pEPSP slope by 65.86 $\pm$ 6.03% (n = 6; Figure 3-2). 2-CA was effective at concentrations of 100 nM or greater (Figure 3-3).

Application of 30 μM 4-AP caused a significant increase in orthodromic synaptic responses (Figure 3-2). At initial stimulus intensities, pEPSP's were prolonged compared with control, and often demonstrated multiple late components. Recording in the cell body layer revealed multiple population spikes and often spontaneous epileptiform discharges were observed. To study the actions of the presynaptic inhibitors under conditions similar to those of control, stimulus-response relationships were again constructed, and a stimulus amplitude chosen which evoked a pEPSP of approximately the same slope and amplitude as in control (see Methods). Once stable responses had been obtained, dose-response curves to the inhibitory

substances were again constructed.

As reported previously for population spike and intracellularly recorded EPSP (Colmers et al., 1988), 4-AP also caused a significant ($P < 0.05$) reduction in the inhibition of pEPSP slope by NPY at concentrations above 300 nM. At 30 μM, 4-AP reduced the inhibition of pEPSP by 1 μM NPY from 63.15 $\pm$ 1.94% (n=4) to 30.60 $\pm$ 6.66% (n=3) of control. The inhibition of pEPSP slope by 10 μM baclofen was reduced from 71.53 $\pm$ 2.88% (n=6) to 38.78 $\pm$ 8.69% (n=4) of control (Figure 3-2); the 4-AP effect was significant for baclofen concentrations above 1 μM. The inhibition by 1 μM 2-CA was reduced from 65.86 $\pm$ 6.03% (n=6) to 34.82 $\pm$ 6.81% (n=6) of control; 4-AP effects were significant at 2-CA concentrations above 100 nM.

A higher concentration (100 μM) of 4-AP caused only a slight, and generally insignificant, further reduction in pEPSP inhibition by all three drugs, indicating that 30 μM 4-AP was near but slightly below the maximal effective concentration for 4-AP at these terminals. However, an interesting effect of increasing concentrations of 4-AP was a significant, progressive depression of the maximal inhibition of pEPSP by 2-CA (Figure 3-3). The maximal inhibition by baclofen was also significantly depressed by 30 μM 4-AP, but was not further reduced by 100 μM 4-AP. It was not possible to observe this effect with NPY, as maximal concentrations could not be tested.

As has been previously observed (Colmers, et al., 1988), reduction of extracellular Ca^{2+} in the presence of 4-AP caused a reduction in evoked synaptic responses. Thus, in 30 μM 4-AP/ 0.75 mM Ca^{2+}, pEPSP slopes were reduced, as expected from the lower levels of extracellular Ca^{2+} available to the presynaptic

terminals. Once responses had stabilized, and the stimulus intensity was re-adjusted so responses were comparable with controls (see Methods), drug effects were again tested. For both NPY and baclofen, low Ca^{2+}/4-AP significantly restored the ability of the ligands to inhibit transmitter release. Indeed, no significant difference was observed between control saline and low Ca^{2+}/4-AP for inhibition mediated by NPY or baclofen. For 2-CA, by contrast, low Ca^{2+}/4-AP caused no significant restoration of inhibition at drug concentrations less than 10 μM (Figure 3-2). Experiments could not be conducted in the presence of 0.75 mM Ca^{2+} alone since, in the absence of 4-AP, this Ca^{2+} concentration did not support synaptic transmission (not illustrated).

To test whether there was a significant postsynaptic component to the observed reduction in pEPSP slope, we applied a supramaximal concentration of the serotonin (5-HT) agonist 5-carboxamidotryptamine (5-CT; Beck, 1989). 5-CT activates the postsynaptic $5\text{-}HT_{1A}$ receptors in hippocampus (Beck, 1989) that cause an increase in the same potassium conductance as does baclofen (Andrade, et al., 1986). At 1 μM, 5-CT had no significant effect on pEPSP slope (n = 5; Figure 3-4).

DISCUSSION

NPY, baclofen and 2-CA all inhibit evoked glutamatergic synaptic transmission in area CA1 of the rat hippocampus by presynaptic actions in stratum radiatum. While all of the receptors mediating presynaptic inhibition in hippocampus have also been shown to inhibit Ca^{2+} influx into neuronal cell bodies, an unequivocal demonstration that this is the mechanism whereby they inhibit transmitter release at presynaptic terminals remains elusive. We have asked a related but somewhat different question: does the presynaptic inhibition mediated at the same population of terminals by different receptors respond in the same manner to two manipulations which should only indirectly affect processes inhibiting transmitter release? Because neither of the manipulations should influence the binding of the ligands to their receptors or significantly affect the coupling of the receptors to their G-proteins, these manipulations should have equal effects on responses to all three ligands if they share a common mechanism. The evidence presented here indicates that this is not entirely the case.

All three substances tested inhibit synaptic transmission in hippocampus, with EC_{50}'s of between 500 nM and 2.8 μM. The physiological measure chosen, the slope of the pEPSP, is relatively insensitive to the postsynaptic actions of neuromodulators (Harrison, 1991). However, it has been demonstrated that postsynaptic $GABA_B$ receptors reduce synaptic NMDA conductances evoked at higher stimulus levels (Morrisett, et al., 1991). Here, application of a supramaximal concentration of 5-CT, an agonist at the $5HT_{1A}$ receptors (Beck, 1989) that activate the same postsynaptic K^+ conductances in CA1 pyramidal cells as are activated by $GABA_B$ receptors (Andrade et al., 1986), had no significant inhibitory effect on pEPSP slopes when applied either

alone or in the presence of 30 μM 4-AP, although a later component of the response was somewhat reduced (Figure 3-4). The absence of an effect of 5-CT on the slope of the pEPSP is in agreement with other observations (Harrison, 1990), and suggests that the postsynaptic potassium conductance activated by both 5-HT_{1A} or $GABA_B$ receptors (Andrade et al., 1986) has no significant effect on the modest levels of excitatory transmission elicited in these experiments. Furthermore, NPY has no demonstrable postsynaptic actions in hippocampus (Colmers et al., 1987, 1988). Therefore, we are confident that the only significant inhibition measured in the present experiments is at a presynaptic site.

The action of 4-AP suggests, at first glance, that the three compounds cause presynaptic inhibition by increasing a K^+ conductance at the presynaptic terminal, as has been hypothesized for the action of adenosine in guinea pig olfactory cortex (Scholfield and Steel, 1988). However, the effect of the low-Ca^{2+} /4-AP treatment on NPY and baclofen responses are not consistent with an increase in K^+ conductance. The inhibition by all effective concentrations tested of both substances was restored to control levels by lowering extracellular Ca^{2+} in the presence of 4-AP. This restoration militates against either 4-AP or low Ca^{2+} affecting the binding of the drugs to their receptors or the coupling of the receptors to their effectors. In fact, the observed decrease in receptor - G-protein coupling caused by lowered Ca^{2+} concentrations (Gilman, 1987) would be expected to produce the opposite of the results observed here. Because the restoration of inhibition by low Ca^{2+} occurs throughout the concentration-effect curve to both substances, it also argues against a significant component of the observed inhibition by NPY and baclofen being mediated directly by another mechanism, such as an increase of K^+ conductance in

the presynaptic terminal (Colmers, et al., 1988). Thus, the present evidence is entirely consistent with an inhibitory action by presynaptic NPY and $GABA_B$ receptors on Ca^{2+} influx underlying the effect on synaptic transmission.

However, the response of the 2-CA-mediated inhibition to 4-AP/low Ca^{2+} is not as clearly consistent with an exclusive effect at presynaptic Ca^{2+} channels. Most importantly, lowering extracellular Ca^{2+} did not significantly restore the inhibition, except at concentrations of 2-CA greater than 10 μM, (a supramaximal concentration in control; Figure 3-3). Clearly, this differs significantly from the response of NPY and baclofen; the lack of response to low Ca^{2+} is also not consistent with a mechanism involving predominantly the reduction of Ca^{2+} influx, at least at concentrations of 2-CA lower than 10 μM. At the neuromuscular junction, it has been proposed that adenosine inhibits transmitter release at a step subsequent to the entry of Ca^{2+} (Silinsky, 1985). Recent evidence indicates that, in rat hippocampal cultures, adenosine inhibits quantal release of glutamate by a mechanism independent of Ca^{2+} influx (Scholz and Miller, 1991). Although it is difficult to reconcile a mechanism involving a decrease in Ca^{2+} sensitivity by the release process with the present data, it appears that adenosine receptors are capable of controlling the release process in more than one fashion (Silinsky, 1985). The data admit of the explanation that, at low concentrations, adenosine inhibits transmitter release largely by a process independent of voltage-dependent Ca^{2+} influx, although there may be an additional effect on Ca^{2+} influx at higher concentrations.

The present results suggest that there are at least two different mechanisms of presynaptic inhibition mediated by the three receptors studied here. We consider it unlikely that all three receptors utilize three altogether different mechanisms.

Although the nature of the present and similar experiments limits the conclusions about presynaptic processes which can be obtained (Milner, et al., 1986), it is nevertheless possible to demonstrate under identical experimental conditions that the events which underlie presynaptic inhibition are not identical for all receptors.

The results indicate that presynaptic inhibitions mediated by NPY and baclofen in stratum radiatum of hippocampal area CA1 *in vitro* respond in a comparable manner to the application of 4-AP and reduced extracellular Ca^{2+} in the presence of 4-AP. The response is consistent with a mechanism of presynaptic inhibition involving the inhibition of Ca^{2+} influx into the presynaptic terminals that release glutamate. However, the inhibition mediated by 2-CA does not respond in the same manner, suggesting that it may thus act by a predominantly different mechanism to inhibit transmitter release.

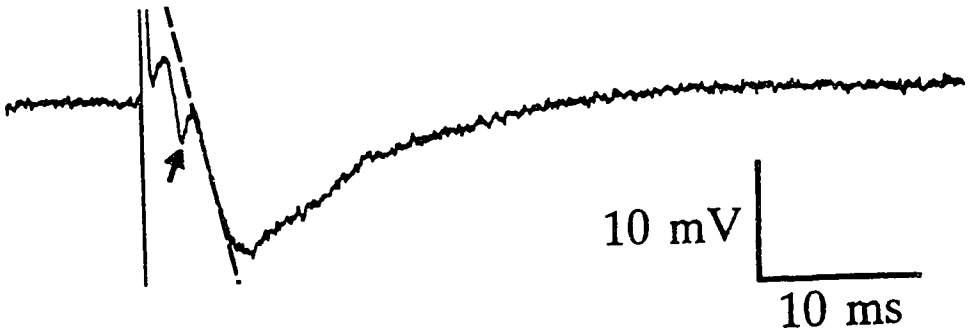


Figure 3-1. **CA1 stratum radiatum population EPSP showing measurement of initial slope.** Population EPSP (digital average of three traces) recorded in stratum radiatum of area CA1. Dashed line indicates slope of pEPSP, arrow indicates presynaptic volley, which was resolved in most, but not all preparations.

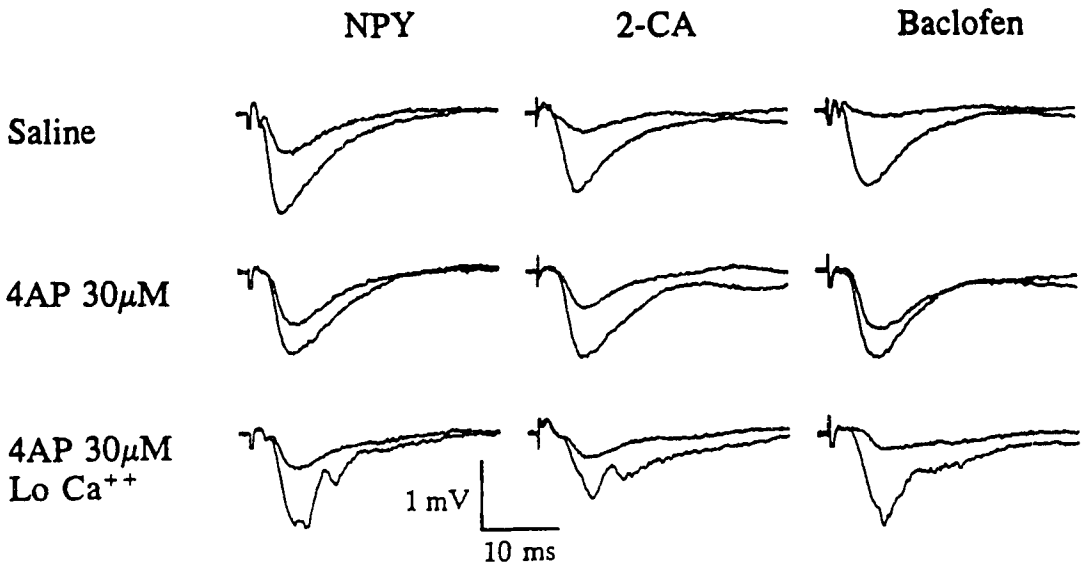
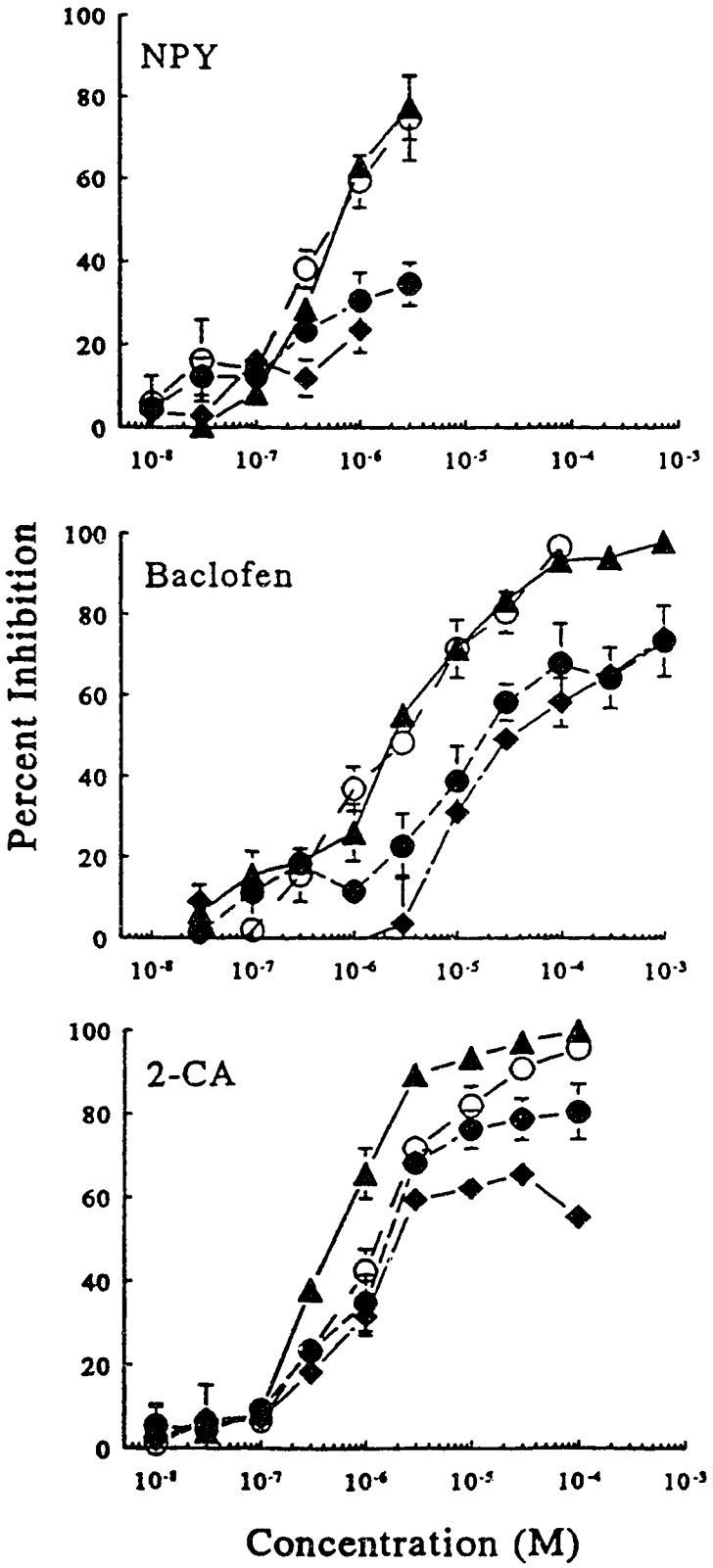


Figure 3-2. **Effects of 4-aminopyridine (4-AP) and 4-AP/low Ca^{2+} on inhibition of pEPSP by representative concentrations of Neuropeptide Y (NPY), baclofen and 2-chloroadenosine (2-CA).** Upper row shows superimposed traces in control and during peak drug effects in normal saline (1.5 mM Ca^{2+}) for 1 μM NPY (left column), 10 μM baclofen (centre column), and 1 μM 2-CA (right column). Centre row shows the effects of these drugs, superimposed on controls, in the presence of 30 μM 4-AP. Bottom row shows the effects of these drugs, relative to controls, in 30 μM 4-AP with 0.75 mM Ca^{2+}. Data illustrated for a given drug were obtained from the same preparation. All preparations recovered from inhibition upon washout (not illustrated). All traces shown are digital averages of three successive field potentials (0.1 Hz). In all cases, the smaller response represents the effect of the drug tested.

Figure 3-3. **Dose-response relationships for inhibition of pEPSP by NPY, Baclofen and 2-CA.** Dose-response relationships for inhibition of pEPSP by NPY, Baclofen and 2-CA in control (1.5 mM Ca^{2+}) saline (▲), and in the presence of 30 μM 4-AP (●), 100 μM 4-AP (◆), and 30 μM 4-AP, 0.75 mM Ca^{2+} (○). Data shown are means ± s.e.m. for n ≥ 3 for each point.


NPY
Baclofen
2-CA
Percent Inhibition
Concentration (M)
100
80
60
40
20
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}

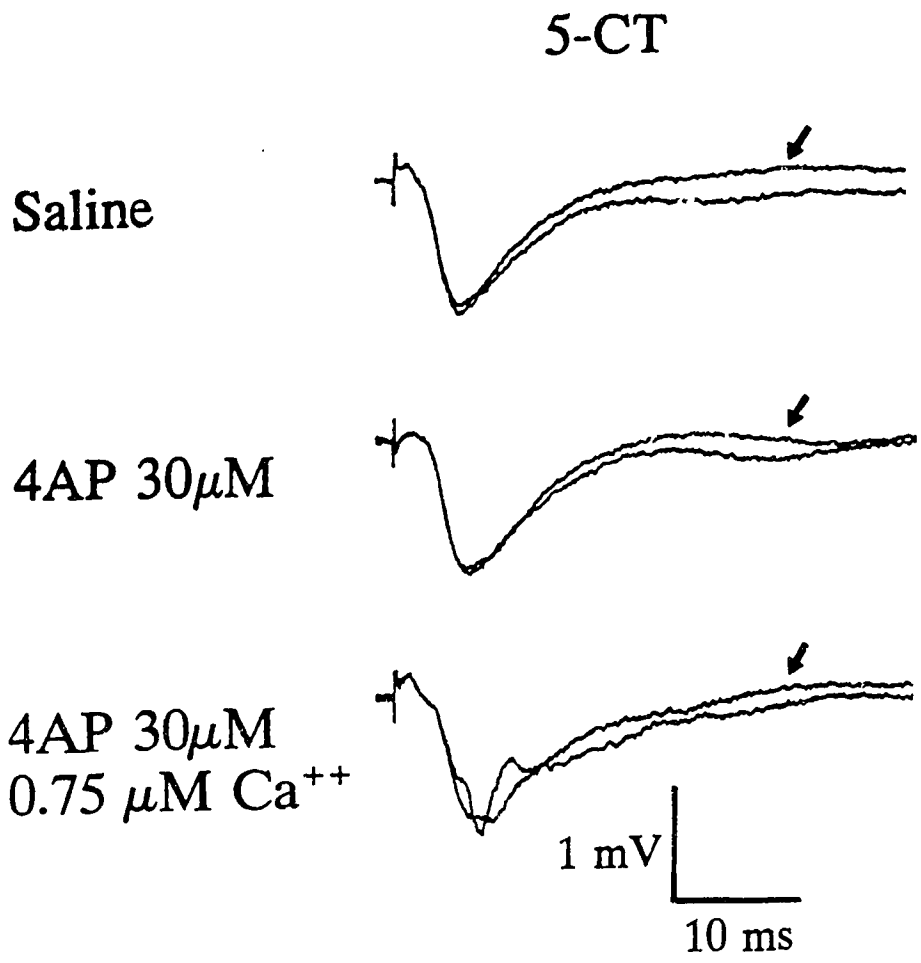


Figure 3-4. **pEPSP slope is not affected by the postsynaptic inhibitor 5-CT.** 5-Carboxamidotryptamine (5-CT; 1 μM) does not affect pEPSP slope in control (upper traces), in 30 μM 4-AP (middle traces) or in 30 μM 4-AP, 0.75 mM Ca^{2+}. Traces in control and during 5-CT application are shown superimposed. Arrows indicate traces obtained during 5-CT application. Data are all from the same preparation. All traces are digital averages of three successive field potentials.

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CHAPTER 4

ON THE SITES OF PRESYNAPTIC INHIBITION BY NEUROPEPTIDE Y (NPY) IN RAT HIPPOCAMPUS *IN VITRO*. †

† A version of this chapter has been published. Klapstein & Colmers 1993. Hippocampus. 3: 103-112.

INTRODUCTION

Neuropeptide Y (NPY), an abundant neuronal peptide in mammals (DeQuidt and Emson, 1986; Sundler et al., 1986) is present in interneurons of hippocampus, where it is co-expressed with γ-aminobutyric acid (GABA) and/or somatostatin (Hendry et al., 1984; Hendry, 1987). Autoradiographic studies have shown a great concentration of NPY binding sites in stratum radiatum of hippocampal area CA1 (Martel et al., 1986, 1990). NPY has been shown to inhibit excitatory transmission at the glutamatergic stratum radiatum-CA1 synapse by a presynaptic mechanism, probably by inhibiting calcium influx into the presynaptic terminal (Colmers et al., 1988; Klapstein and Colmers, 1992). No effects of NPY were observed on postsynaptic cell properties (Colmers et al., 1987, 1988).

Binding sites for NPY have been demonstrated autoradiographically in the dendritic fields of other areas of hippocampus, although few, if any, have been found in the cell body layers (Martel et al., 1986, 1990; Köhler et al., 1987). NPY binding sites are highly enriched in stratum radiatum of CA3, and in stratum oriens of CA1 and CA3, while a much lower concentration of binding sites were found in the dentate gyrus, where they were restricted to the inner one-third of the molecular layer (Martel et al., 1986, 1990; Köhler et al., 1987). Because these layers represent the synaptic input regions to a number of neuronal types, largely the pyramidal neurons of areas CA1 and CA3, and the granule cells of the dentate gyrus, as well as to several subtypes of interneuron, we examined several identifiable synaptic responses in neurons of these hippocampal regions to elucidate the specific sites of action of NPY. The results suggest that NPY acts as a modulatory influence in sequential synaptic processing of hippocampal information, by selectively inhibiting feed-

forward excitation and inhibition within the hippocampus proper, while apparently not restricting excitatory input into the dentate gyrus. Some of the data have already been reported in abstract form (Colmers, 1989, Colmers and Klapstein, 1989)

METHODS

Details of most methods used in this study have been published earlier (Colmers et al., 1987, 1988). Briefly, 450 to 500 μm thick transverse slices were prepared from midtemporal regions of hippocampi of male Sprague-Dawley rats (75 - 200g), and incubated, submerged at 34 $\pm$ 0.2°C in a small chamber (approximately 300 μl volume; Colmers, 1990) and perfused constantly at 2 - 3 ml min^{-1} with saline bubbled continuously with 95% O_2, 5% CO_2. Composition of the saline was (in mM): NaCl 124, $NaHCO_3$ 26; $MgSO_4$ 2, KCl 1.8, $CaCl_2$ 1.5, KH_2PO_4 1.25, glucose 10. For some of the tight-seal, whole-cell recordings, $CaCl_2$ was elevated to 2.0 mM. Etched, glass coated tungsten electrodes (either monopolar or bipolar) were placed in the pathways of interest (see Results); in some cases they were moved independently of one another (to optimize stimuli) by mounting each bipolar electrode (when two pathways were stimulated), or each pole (for focal stimuli) on separate micromanipulators.

Intracellular recordings were made with glass microelectrodes (2M K^+-acetate, 70-150 MΩ), which were lowered into the cell body layer of the region of interest until a neuron was successfully impaled. Responses were recorded using an Axoclamp 2A amplifier (Axon Instruments, Foster City, California). Neurons were identified on the basis of standard electrophysiological criteria, including resting potential and action potential waveform and amplitude (Brown et al., 1981). Neurons were selected whose resting membrane potential and response to synaptic stimulation were stable for at least 20 min. In most circumstances, a pulse of hyperpolarizing current (125 ms, 100 - 400 pA) was passed via the bridge circuit of the amplifier beginning 40 ms prior to stimulation of synaptic inputs. This prevented neurons from achieving action

potential threshold, and provided an estimate of input resistance (Colmers et al., 1987). Once stable recording conditions had been achieved, synaptic responses were evoked at 0.1 Hz by brief (50 - 200 μs, 6 - 40 V) pulses applied via a stimulus isolation unit (A.M.P.I., Jerusalem, Israel) to the stimulating electrodes. The excitatory postsynaptic potential (EPSP) amplitude was measured from the membrane potential after the shock artifact had subsided to the peak amplitude of the EPSP. To establish a stimulus-response relationship for a cell, the stimulus voltage was varied stepwise from near threshold for the EPSP to the maximum possible without eliciting an action potential. A representative stimulus was then chosen whose response lay on the linear portion of the stimulus-response curve, and monitored during the experiment. In most experiments, such stimulus-response curves were repeated under each of the different experimental conditions. For recordings of inhibitory postsynaptic potentials (IPSP's), hyperpolarizing steps were omitted, but stimulus-response relationships were established as with EPSP's. In most experiments, families of hyperpolarizing and depolarizing constant current pulses were applied via the electrode to estimate neuronal input resistance under each condition.

Extracellular recordings from the granule cell layer of dentate gyrus were made as described earlier (Colmers et al., 1987, 1991), using glass micropipettes (2M NaCl, 3-10 MΩ) which were connected to the headstage of the Axoclamp. Stimulus-response relationships were established as for intracellular recordings.

In some experiments, tight-seal, whole cell recordings were made from neurons in hippocampal slices ("slice-patch" recordings: Blanton et al., 1989; Staley et al., 1990; McQuiston and Colmers, 1992). Patch-type micropipettes (1 μm tip aperture; 4 - 7 MΩ resistance), were filled with a solution containing (in mM): K^+ gluconate, 145;

$MgCl_2$, 2; N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 5; Ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 1.1; $CaCl_2$, 0.1; adenosine triphosphate Na^+ salt, 5; guanosine triphosphate Na^+ salt, 0.3; pH 7.20, 270 - 280 mOsm. Pipettes were lowered with slight positive pressure into the bathing medium, and advanced into the cell body layer of interest with small, sudden movements until a change of resistance was observed. Release of positive pressure, and sometimes gentle suction, generally resulted in seal formation; seal resistance was typically 2 GΩ or greater (McQuiston and Colmers, 1992). Access to the cell was gained by suction in current clamp. Criteria for cell identification with whole cell recordings were identical with those used with sharp microelectrodes. Synaptic potentials were evoked as above; generally, IPSP's were the subject of these whole-cell experiments.

Synthetic porcine NPY (Richelieu Biotechnologies Ltd., St-Hyacinth, Québec, or the generous gift of Dr. T.O. Neild) stock solutions were prepared at 10^{-4}M in distilled water and kept frozen at -20°C until immediately prior to use. Concentrated stock drug and peptide solutions were diluted with carbogenated saline immediately prior to use, and delivered to the slice via the perfusate using a 4-way HPLC valve (Hamilton); care was taken to ensure that flow rate through the bath during drug delivery did not vary significantly from control flow rates. As in earlier studies, (Colmers et al., 1985, 1987, 1988; Klapstein and Colmers, 1992), 10 ml of the NPY solution was applied to the preparation in each experiment. In some experiments, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM; Tocris Neuramin, U.K.), L-2-amino-5-phosphonovalerate (APV, 50 μM; Sigma Chemical company, St. Louis, MO, U.S.A.), and/or picrotoxin (50 μM; Sigma Chemical company) were applied to block

quisqualate/kainate, N-methyl-D-aspartate (NMDA) excitatory amino acid receptors, or $GABA_A$ receptor-mediated synaptic potentials, respectively. In these experiments, the action of NPY was tested only after the responses to these antagonists had stabilized. (-)-Bicuculline methobromide (bicuculline, 50 μM; Sigma Chemical company) was used in some experiments to confirm that depolarizing post-synaptic potentials observed during pharmacologic isolation of IPSP's in dentate granule cells were due to activation of $GABA_A$ receptors.

Data from intracellular recordings were collected on a d.c.-coupled pen recorder (Gould 3200; filter cutoff: 100 Hz), which continuously recorded cell membrane potential and current injected throughout the duration of the experiment. Critical parts of the experiment were recorded in digital form on videotape (A.R. Vetter 200T), for subsequent analysis. In addition, a digital storage oscilloscope (Nicolet 4094) was used to observe, average and record on floppy diskettes individual responses. This instrument was also used to analyze signals from tape.

All numerical data are presented as means $\pm$ standard error of the mean. Reductions or increases are expressed as percent of control values. Neurons were used as their own controls for statistical purposes. Statistical comparisons were performed using a Student's paired-*t* test, unless otherwise noted. Statistical differences were considered significant at $P \leq 0.05$. All data are from preparations which showed a recovery from the effects of NPY application, or (for dentate gyrus or isolated IPSP's) showed stable responses for at least 20 minutes after initiation of NPY washout.

RESULTS

Results are based on intracellular recordings from 36 CA1 pyramidal neurons, 19 CA3 pyramidal neurons and 23 dentate granule cells, and extracellular recordings were made from dentate gyrus in a further 12 preparations. Impalements of pyramidal neurons with sharp microelectrodes were considered acceptable if the resting membrane potential was more negative than -58 mV and action potential amplitude was greater than 90 mV. Acceptable whole-cell recordings included these criteria, but in addition required initial formation of a high-resistance (>2 GΩ) seal prior to entering whole-cell mode. CA1 pyramidal neurons were generally quiescent, but because CA3 pyramidal cells were sometimes spontaneously active, injection of DC hyperpolarizing current was required to maintain the neuron below action potential threshold. Impalements of dentate granule cells were considered acceptable if the resting membrane potential was more negative than -75 mV.

EPSP's in area CA1

Stimulation of stratum radiatum (see inset, Figure 4-1A) has previously been shown to elicit a short latency EPSP in CA1 pyramidal neurons. Application of 1 μM NPY has been reported (Colmers et al., 1987, 1988, 1991) to reduce the amplitude of this response by between 40 and 60 percent, with an average reduction of 47.18 ± 3.73% ($n = 19$, $P < 0.001$) from control levels. (Figure 4-1A). When the stimulating electrode was placed on stratum oriens (Inset, Figure 4-1B), a short latency EPSP could also be elicited in CA1 neurons. NPY 1 μM also inhibited this response by 46.91 ± 4.21% ($n = 4$, $P < 0.001$; Figure 4-1B). As has been previously observed (Colmers et al., 1987, 1988), the NPY-mediated inhibition of both synaptic potentials

gradually abated upon washout, often requiring 60 minutes or more for complete recovery.

EPSP's in area CA3

Three major excitatory inputs impinge upon CA3 pyramidal neurons: 1) mossy fibres, from the ipsilateral dentate granule cells; 2) commissural fibres, arising from the contralateral hippocampus; and 3) collaterals from ipsilateral CA3 neurons themselves (Amaral and Witter, 1989). We studied the actions of NPY on all three synaptic potentials, using selective placement of stimulating electrodes to discriminate between them (Figure 4-2, insets).

Mossy fibre EPSP. Intracellular recordings were generally made from the middle of area CA3 (area CA3B, cf. Bayer, 1985). Stimulating electrodes were placed in the molecular layer of dentate to selectively elicit a mossy fibre response (Inset, Figure 4-2A). Stimuli evoked a short latency EPSP in the CA3 neurons, which generally had a very steep initial slope. Application of 1 μM NPY caused a reduction in the amplitude of this EPSP by 48.84 $\pm$ 4.09% ($n = 16$, $P < 0.001$; Figure 4-2A). This inhibition also gradually reversed upon washout, with approximately the same time course as in CA1.

Commissural EPSP. When stimulating electrodes were placed on stratum oriens of CA3, which contains largely contralateral fibres which synapse onto the basal dendrites of CA3 neurons (Swanson et al., 1978), a short latency EPSP could be elicited in nearby pyramidal cells. Application of 1 μM NPY reduced this EPSP by 43.66 $\pm$ 1.37% ($n = 3$, $P < 0.001$; Figure 4-2B). As with other synaptic responses, this inhibition reversed upon washout.

Stratum radiatum EPSP. Stimulation of stratum radiatum in area CA2 also elicited an EPSP in CA3 pyramidal neurons, originating with fibres which are in part ipsilateral CA3-CA3 projections (Swanson et al., 1978; Bayer, 1985). Experiments were conducted in which stimulating electrodes were placed both on stratum radiatum and on the mossy fibre input to CA3, and both pathways were stimulated alternately at 0.05 Hz for the same neuron. NPY reversibly attenuated both EPSP's by 50.64 ± 4.86% and 55.09 ± 3.58%, respectively ($P < 0.001$ and < 0.001, respectively, $n = 9$; Figure 4-2C).

In all recordings from CA3 neurons, NPY had no measurable effect on passive or active cell membrane properties, consistent with earlier findings (Colmers, et al., 1988; Colmers, 1989).

EPSP's in Dentate gyrus

Recordings were made from dentate granule cells near the crest of the dentate (terminology from Amaral and Witter, 1989). Stimulating electrodes were placed on the perforant path near the cell (Inset, Figure 4-3A), or on the commissural input to dentate (Inset, Figure 4-3B; Amaral and Witter, 1989). In some experiments stimulating electrodes were placed on both pathways and stimulated alternately at 0.05 Hz for the same neuron. Intracellular recordings from dentate granule cells confirmed their distinctive properties. Compared with pyramidal neurons, dentate granule cells had quite negative resting potentials (-83 ± 2 mV, $n = 18$), and their action potential waveforms were distinct, showing a pronounced afterhyperpolarization relative to that seen in pyramidal cells consistent with observations by others (Brown et al., 1981). Stimulation of perforant path elicited a

short-latency, sharply rising EPSP in these cells which application of NPY did not affect (increase by 0.54 ± 4.56%, n = 4, P > 0.25; Figure 4-3A). At the same time, NPY had no effect on passive or active properties of these neurons.

Autoradiographic studies indicated that the receptors for NPY are relatively concentrated in the inner one-third of the molecular layer of dentate (Köhler et al., 1987), where the commissural inputs synapse with the granule cells, while the perforant path makes its synapses in the middle third of the molecular layer (Laatsch and Cowan, 1967; Hjorth-Simonsen and Jeune, 1972) Therefore, similar experiments were performed on the short-latency EPSP elicited in granule cells by stimulation of commissural inputs (Inset, Figure 4-3B). Again, application of 1 μM NPY was without significant effect (increase by 6.13 ± 11.81%, P > 0.1, n = 3, Figure 4-3B).

Because the effects of NPY in CA1 are less pronounced on the intracellularly-recorded EPSP than on the population spike (PS) response (Colmers, et al., 1985, 1987), we hypothesized that a subtle effect of NPY might not be seen in single intracellular recordings of dentate granule cells. Therefore, extracellularly-recorded population responses in the dentate granule cell layer were evoked by stimulation of either the perforant path or commissural fibres. Population responses typically consisted of a sharp negative PS superimposed on a slow positive wave. The amplitude of the PS was measured from the bottom of the negative spike to the peak of the positive wave following it. Application of 1 μM NPY had no significant effect on granule cell population responses to stimulation of either the perforant path (inhibition by 3.60 ± 2.12%, P > 0.05, n = 13, Figure 4-3A2) or the commissural input (inhibition by 3.53 ± 2.81%, P > 0.1, n = 4 Figure 4-3B2). These responses were, however, reduced by baclofen, indicating that they are synaptic in nature (not

illustrated).

IPSP's in area CA1

Because NPY has such a potent effect on feedforward excitation in hippocampus, we tested whether there were consequences of its action on the IPSP's evoked by orthodromic stimulation (Alger and Nicoll, 1982, LaCaille et al., 1989). Stimulation of stratum radiatum without the hyperpolarizing current pulse often revealed an IPSP that followed the EPSP in CA1 cells. Application of NPY reduced the EPSP by 39.74 ± 4.51%, while reducing the IPSP by 38.80 ± 3.87% of control ($n = 7$, $P < 0.01$, for both; Figure 4-4A). In the same neurons, an IPSP was also evoked by stimulation of alveus (Alger and Nicoll, 1982; Inset, Figure 4-4B). This response was not significantly affected by NPY (Figure 4-4B). Orthodromically-evoked EPSP's and IPSP's were equally inhibited by NPY in individual cells (Figure 4-4C).

To address the question of whether NPY affected IPSP's evoked in the absence of any excitatory synaptic transmission, we applied a mixture of excitatory amino acid antagonists to block all excitatory synaptic transmission (see Methods), then elicited an IPSP by stimulating the interneurons themselves focally (Davies et al., 1990, inset, Figure 4-5A). These IPSP's sometimes, but not always, exhibited clearly resolved fast ($GABA_A$) and slow ($GABA_B$) mediated components (Newberry and Nicoll, 1985; Dutar and Nicoll, 1988). As the slow component was smaller than, and usually rendered unmeasurable by, the decay phase of the fast component, it was not studied under these conditions. Furthermore, this component often "ran down" spontaneously under these conditions, especially when patch clamp recording

techniques were used. Application of NPY to the pharmacologically isolated IPSP had no significant effect on the fast component (reduction by 8.34 ± 4.13%, n = 6, $P > 0.05$; Figure 4-5A). When the slow component of the IPSP was pharmacologically isolated with CNQX, APV, and picrotoxin (to block the $GABA_A$ response), we found that it also was not significantly affected by 1 μM NPY (increase by 0.08 ± 3.37%, n = 3, $P > 0.25$; Figure 4-5A2).

IPSP's in area CA3

IPSP's were elicited in CA3 pyramidal neurons by focal stimulation in the presence of the CNQX and APV (inset, Figure 4-5B). As was observed in CA1, NPY had no significant effect on the IPSP in CA3 (reduction by 7.09 ± 4.50%, n = 5, $P > 0.05$; Figure 4-5B). The EPSP-IPSP complex evoked by mossy fibre stimulation in 2 CA3 cells was inhibited by NPY; however, this complex response was not systematically studied here.

IPSP's in Dentate gyrus

As with CA1 and CA3, pharmacological blockade of the EPSP unmasked an IPSP in dentate granule cells. Since the resting membrane potential for these cells is negative relative to the chloride reversal potential, the $GABA_A$-mediated IPSP (which involves a Cl^- conductance) is normally depolarizing. 1 μM NPY has no effect on this IPSP (increase by 0.35 ± 2.33%, n = 5, $P > 0.1$); however, 50 μM bicuculline, a $GABA_A$ receptor antagonist, reduces it by 90.96 ± 3.13% (n = 4, $P < 0.05$) (Figure 4-5C2). When a cell was held depolarized to rest at approximately -60 mV, a hyperpolarizing IPSP was evident (Lacaille et al., 1989; Scharfman and Schwartzkroin,

1990). The effect of 1 μM NPY on these pharmacologically isolated IPSP's was variable, but not significant (reduction by 17.58 $\pm$ 16.16%, n = 5, $P > 0.10$; Figure 4-5C1).

DISCUSSION

Figure 4-6 summarizes the results of the present study in schematic form. The results demonstrate a very consistent pattern of action by NPY on synaptic transmission within the hippocampus proper. As has been seen in CA1, NPY's actions throughout the hippocampus appear to be presynaptic in nature, since there were no effects observed on postsynaptic neuronal properties, such as resting membrane potential, input resistance, or action potential parameters. Excitatory connections between principal neurons are all inhibited by NPY, while others, such as the collaterals that excite recurrent inhibitory interneurons, are not affected by the peptide. Interestingly, all the excitatory connections within CA3 were susceptible to NPY's presynaptic action, despite an earlier preliminary report that responses evoked in CA3 from stratum radiatum were insensitive to NPY (Colmers, 1989).

It is somewhat puzzling that dentate gyrus synaptic responses are essentially insensitive to NPY's action. Although there are relatively fewer NPY binding sites in the dentate than in Ammon's horn, there nevertheless appear to be substantial numbers of binding sites there (Martel et al., 1990, Köhler et al., 1987), and there is an extraordinarily high concentration of NPY in fibres that innervate the molecular layer of dentate (Köhler et al., 1986; Milner and Veznedaroglu, 1992). One possible explanation for the present results could be that the NPY receptors in dentate are desensitized, due to chronic exposure to elevated levels of NPY in the region. However, experiments in cultured dorsal root ganglion cells indicate that the Y_2 receptor (also present in hippocampal CA1; Colmers et al., 1991) does not appear to desensitize (Bleakman et al., 1991). A further possibility is that, due to the high concentrations of NPY in dentate, the receptors there are nearly always fully

occupied. A test of this hypothesis awaits the development of an antagonist to the NPY receptor in the dentate. Finally, it is possible that the NPY binding site in dentate represents a different NPY receptor subtype from those found within the hippocampus proper (Aicher et al., 1991)

NPY affects neither IPSP's elicited antidromically or in pharmacological isolation, suggesting that it has no postsynaptic actions on interneurons themselves. It did, however, inhibit IPSP's evoked by orthodromic stimulation, but only by the same amount as the EPSP. This suggests that the reduction in orthodromic IPSP is indirect, and is caused by NPY's action at feedforward excitatory synapses that 1) have direct feedforward input onto inhibitory interneurons mediating the feedforward IPSP, and/or 2) excite neighbouring pyramidal cells, whose axon collaterals drive the basket cells in a recurrent fashion (Lacaille et al., 1989), although these two possibilities cannot be distinguished here. The action of NPY is therefore also highly selective, as the terminals of recurrent pyramidal cell axon collaterals which drive the recurrent IPSP (Andersen et al., 1969) do not appear to respond to high concentrations of NPY. A recent electron microscopical study indicates the presence of NPY immunoreactive varicosities in immediate apposition to glutamatergic presynaptic terminals in the hippocampus (Milner and Veznedaroglu, 1992). This suggests that NPY may normally be released onto terminals exhibiting receptor-mediated NPY responsiveness, and may provide an anatomical correlate to the physiological observations described herein.

On the basis of our observations, the net effect of endogenous NPY release within the hippocampus would presumably be to progressively attenuate excitation as it passes through the hippocampus from principal cell to principal cell. Neither

recurrent inhibition nor postsynaptic responses within hippocampus are affected by the peptide's action. Responses by hippocampal circuits to the attenuated excitatory input will therefore not be altered. The action of NPY in hippocampus could thus be likened to the volume control of a high quality amplifier, where no distortions are introduced by attenuation (Colmers, 1990).

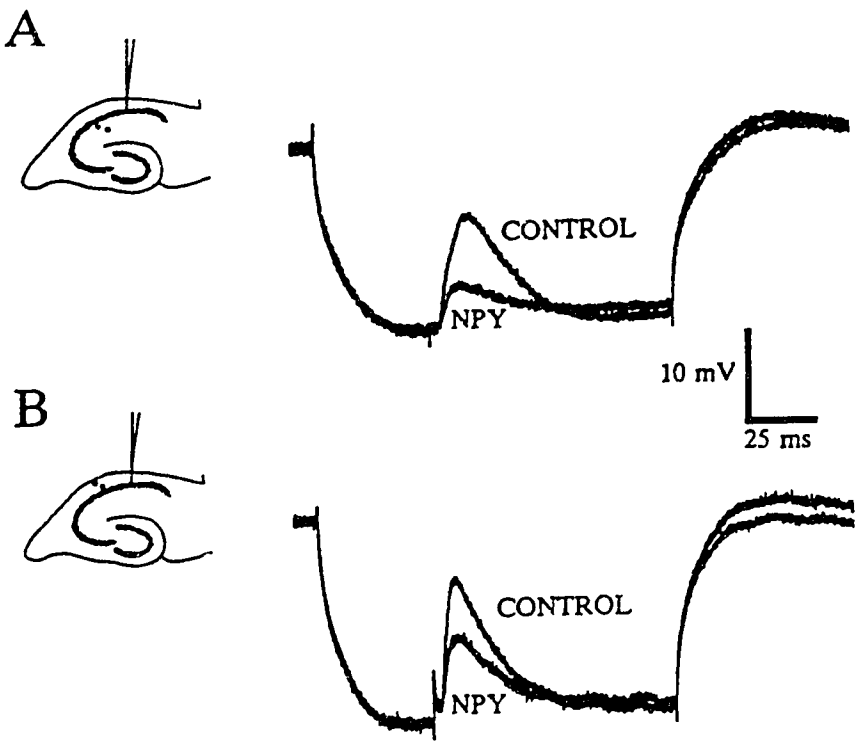


Figure 4-1. **Representative traces showing the effect of 1 μM NPY on excitatory post-synaptic potentials (EPSP's) recorded intracellularly from CA1 pyramidal neurons.** Responses were evoked with a bipolar stimulating electrode (shown by asterisks, insets) placed either in stratum radiatum (A) or stratum oriens (B). Controls and peak NPY effects are superimposed. Responses recovered on washout (not shown). Resting potential of cell in A was -64 mV, in B, -62 mV. A hyperpolarizing pulse of -300 pA, 125 ms was applied to cells in both A and B. Traces in this and all other data figures are the digital averages of three successive responses.

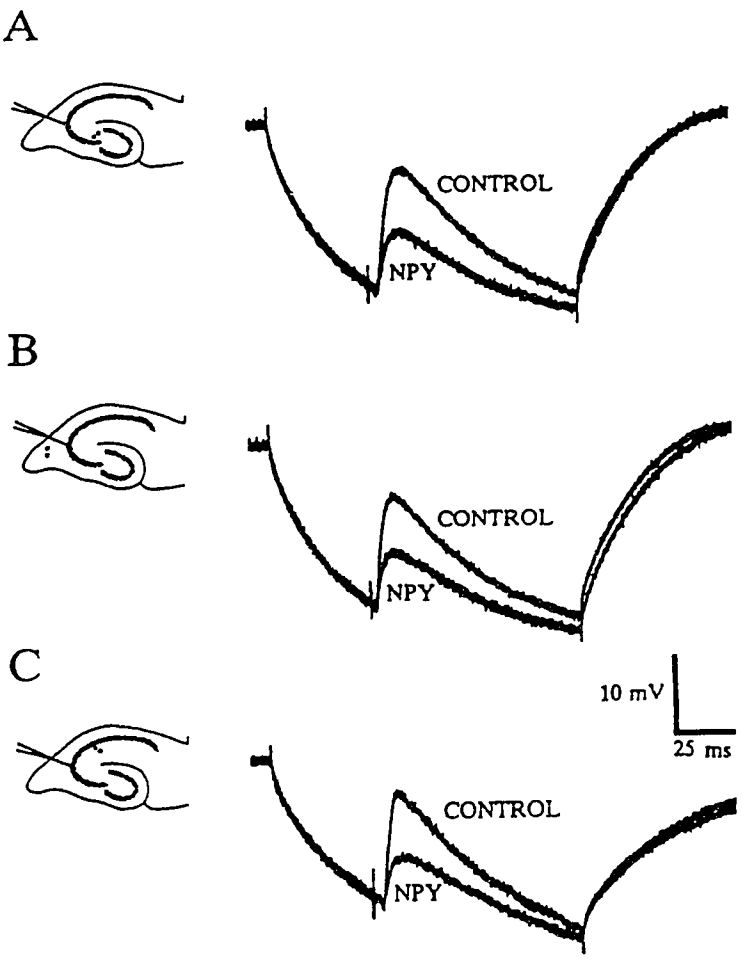


Figure 4-2. **The effect of 1 μM NPY on EPSP's recorded intracellularly from CA3 pyramidal neurons.** Stimulating electrodes (asterisks, insets) were placed on the mossy fibers (A), the commissural fibers (B) or the ipsilateral CA3 collateral fibers (C). Cell in A and B is the same, pathways were stimulated alternately at 0.05 Hz for each pathway during the same application of NPY. Controls and peak NPY effects are superimposed. All responses reversed on washout. Resting potentials were -67 mV (A, B), -64 mV (C). Hyperpolarizing pulses were -400 pA, 125 ms in A,B and C.

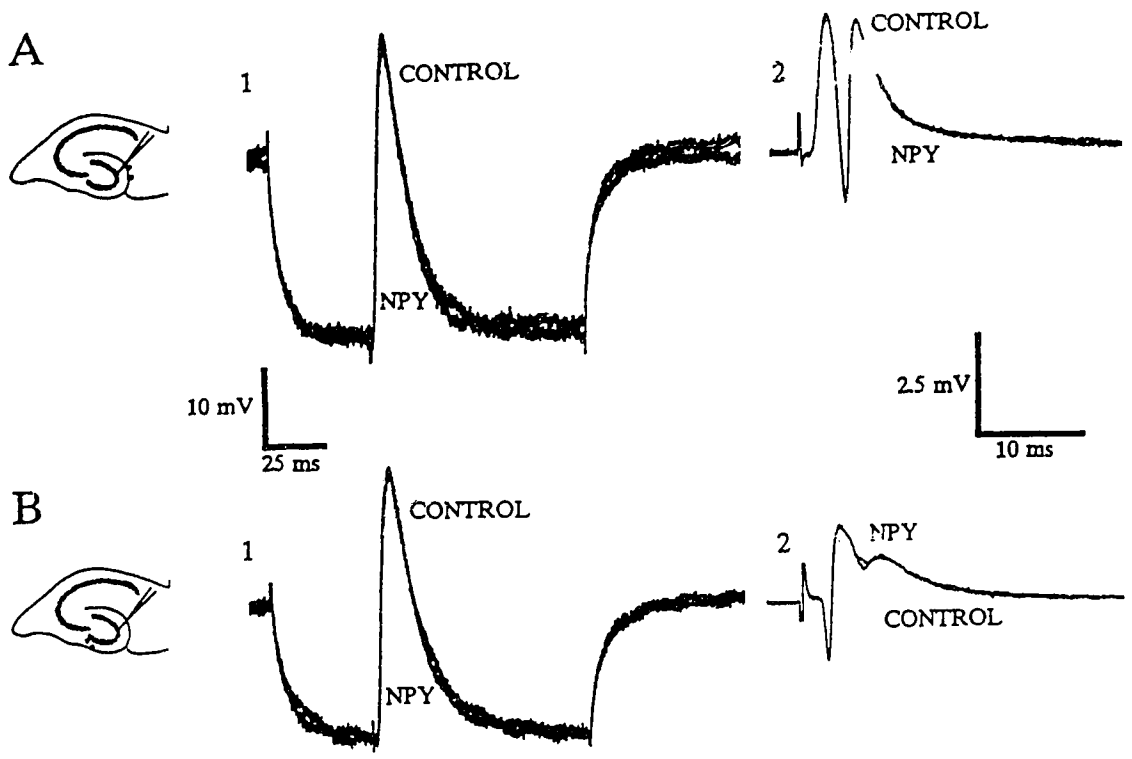


Figure 4-3. **The effect of 1 μM NPY on synaptic potentials recorded in the dentate gyrus.** Stimulating electrodes were placed on the perforant path (A) or on the commissural fiber pathway (B). Left panel shows intracellular recordings from dentate granule cells (different cells in A and B); right panel shows evoked population field responses from dentate granule cells. Control responses and those taken at the same time as peak NPY effects were observed in CA1 and CA3 are shown superimposed. Synaptic responses were sensitive to CNQX (not illustrated). Resting potential of cells were -92 mV (A), -80 mV (B). Hyperpolarizing pulses were -300 pA, 125 ms in A and B.

Figure 4-4. **The effect of 1 μM NPY on complex post-synaptic potentials recorded intracellularly in a CA1 pyramidal neuron.** Stimulating electrodes were placed in stratum radiatum (A), and on the alveus (B). Stimuli were presented alternately to each pathway (0.05 Hz). Controls and peak NPY effects are superimposed. The positive wave preceding the IPSP in B w. insensitive to NPY; the larger response was obtained in NPY, at the same time as the stratum radiatum response was reduced (A). The stratum radiatum response recovered upon washout; there was no change in the response from alveus. Resting potential -62 mV. (C). Plot of inhibition by 1 μM NPY of synaptic responses to orthodromic stimulation recorded in seven individual neurons. Line represents the best-fit linear regression to the data (slope = 0.988).

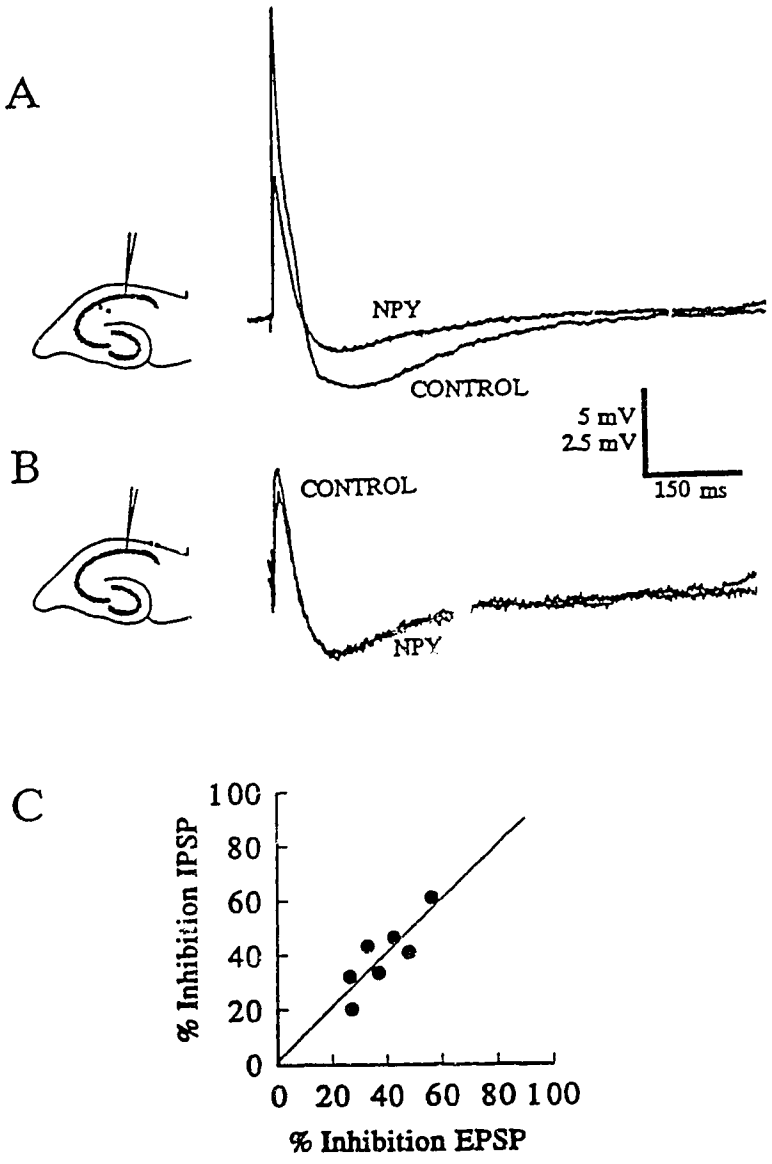
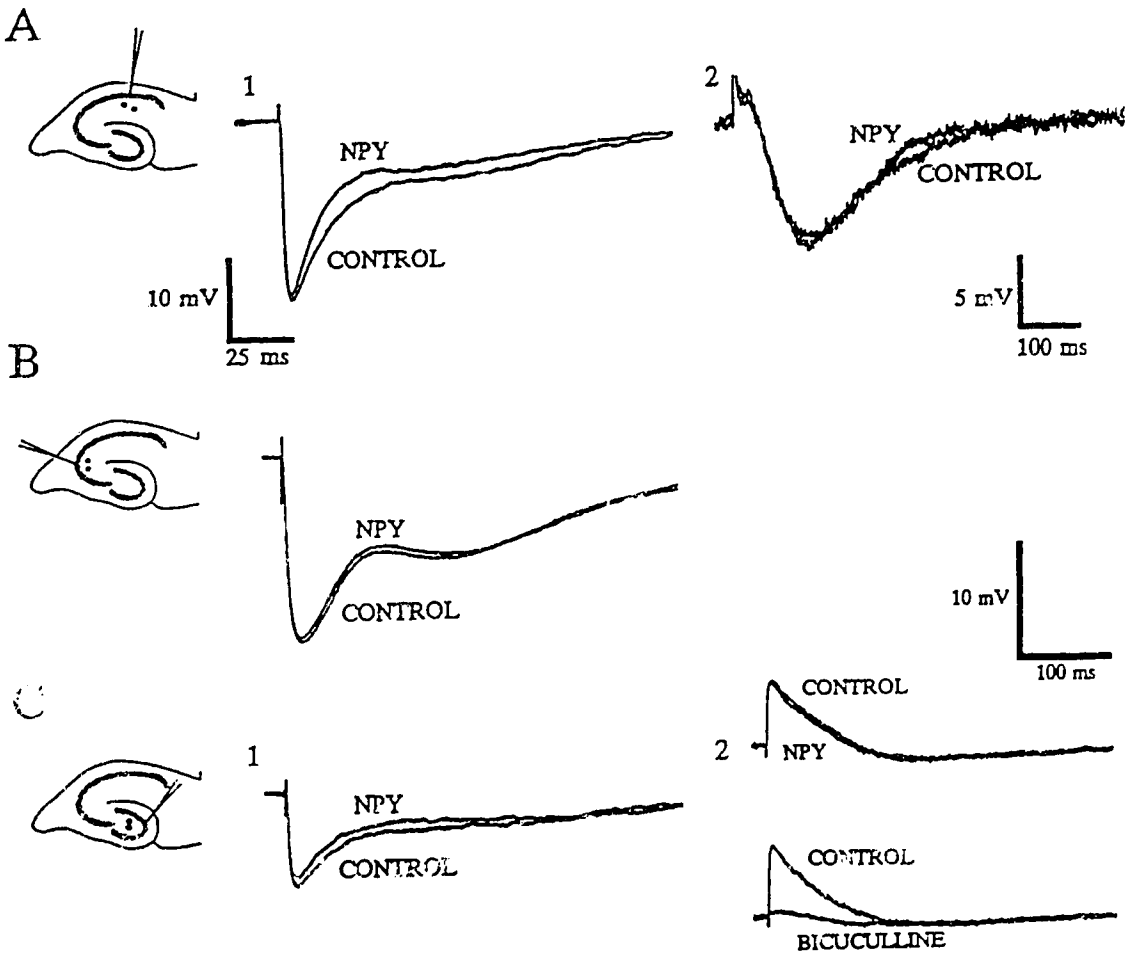

A
NPY
CONTROL
5 mV
2.5 mV
150 ms
B
CONTROL
NPY
C
100
80
60
40
20
0
0 20 40 60 80 100
% Inhibition IPSP
% Inhibition EPSP

Figure 4-5. **NPY does not directly inhibit IPSPs.** 1 μM NPY has no significant effect on pharmacologically isolated (CNQX 10μM, APV 50μM) inhibitory post-synaptic potentials recorded intracellularly from a CA1 pyramidal neuron (A), a CA3 pyramidal neuron (B), or a dentate granule cell (C). Focal stimuli were delivered via a bipolar stimulating electrode (see Insets). A2. The slow, $GABA_B$-mediated, focally-evoked IPSP recorded from a different CA1 pyramidal neuron (resting potential = -63 mV) in pharmacological isolation (CNQX 10 μM, APV 50 μM, picrotoxin 100 μM) is also not affected by 1 μM NPY. C2. Pharmacologically isolated (CNQX 10 μM, APV 50 μM) $GABA_A$-mediated inhibitory post-synaptic potentials recorded from a different dentate granule cell are depolarizing at normal resting potentials (-80 mV), and are not affected by 1 μM NPY (upper trace) but are dramatically reduced by 50 μM bicuculline (lower traces). Control responses and those recorded during peak drug effect or expected time of peak effect are superimposed in all traces.


A
1
NPY
CONTROL
10 mV
25 ms
2
NPY
CONTROL
5 mV
100 ms
B
NPY
CONTROL
10 mV
100 ms
1
NPY
CONTROL
2
CONTROL
NPY
CONTROL
BICUCULLINE

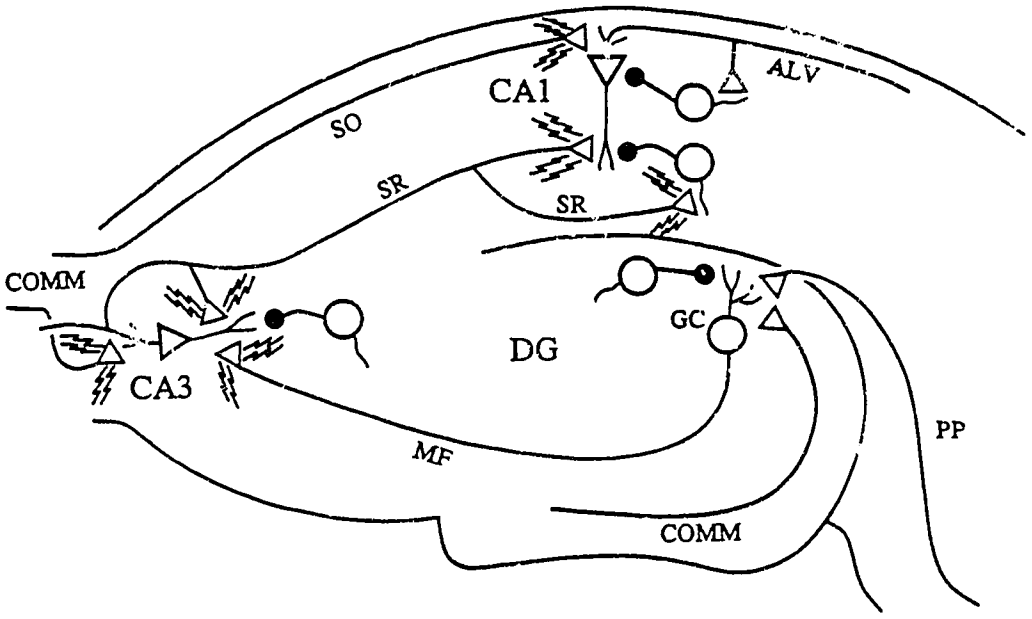


Figure 4-6. A schematic diagram of a transverse hippocampal slice, showing the synaptic connections tested, and summarizing the results. Open triangles represent excitatory connections, while closed circles represent inhibitory synapses. Pyramidal cells are stylized in areas CA1 and CA3, while granule cells are represented in dentate. Other open circles represent interneurons. "Zigzags" indicate synaptic connections that are sensitive to NPY's action. Abbreviations: ALV, alveus; CA1, CA3, pyramidal cells of areas CA1 and CA3; COMM, commissural fibres entering dentate or CA3 from contralateral hippocampus; DG, dentate gyrus; GC, dentate granule cell; MF, mossy fibres; PP perforant path; SO, stratum oriens; SR, stratum radiatum.

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CHAPTER 5

NEUROPEPTIDE Y SUPPRESSES EPILEPTIFORM ACTIVITY IN RAT HIPPOCAMPUS *IN VITRO* †

† A version of this chapter has been submitted for publication.

Introduction

The inappropriate synchrony of electrical activity that is the hallmark of epilepsy has eluded a simple explanation. Several models of epilepsy have been developed to study the synchronous activity of neurons, both *in vivo* and *in vitro* (McNamara, 1994). The *in vitro* models utilize several fundamentally different means, including a reduction of endogenous inhibition, an increase of excitation, or both, to produce synchronized electrical discharges resembling those recorded during (ictal) or between (interictal) seizures in human epilepsy patients. Much of the synchrony observed in these models is thought to arise from collateral excitatory synaptic interactions within area CA3 of the hippocampus (Wong and Traub, 1983). Therefore, our studies have been concentrated in this region.

We have been interested in the biological role in the hippocampus of the endogenous peptide, neuropeptide Y (NPY). We have previously shown that NPY selectively reduces feedforward synaptic excitation within the hippocampus by a presynaptic mechanism (Colmers et al., 1987, 1988; Klapstein and Colmers, 1993), involving a receptor subtype which has been identified in the CA1 region to be Y_2 (Colmers et al., 1991; Dumont et al., 1993). Activation of this receptor has no other demonstrated effects on these neurons (Colmers et al., 1987, 1988; Bleakman et al., 1992). We have hypothesized that NPY may normally play a role in the control of excitability within the hippocampus, (Bleakman et al., 1993; Klapstein and Colmers, 1993). A definitive test of this hypothesis awaits the availability of a Y_2-selective antagonist. Therefore, here we test a corollary hypothesis, namely, that exogenously-applied NPY can inhibit epileptiform activity produced by three different *in vitro* models of epilepsy using the hippocampal slice preparation.

Two of these models involve alterations in the activity of endogenous transmitters. In the 0 Mg^{2+} bursting model (Anderson et al., 1986), Mg^{2+} is removed from the perfusate, relieving the voltage-dependent block of NMDA receptors, and thereby potentiating the depolarizing effect of endogenous glutamate (Sah et al., 1989). In the picrotoxin bursting model, $GABA_A$ receptors are blocked, thereby reducing the effect of endogenous GABA (Hablitz, 1984). Both of these treatments result in the development of spontaneous, synchronous population bursts, resembling interictal events in epilepsy. The third model is stimulus train induced bursting (STIB; Stasheff et al., 1985), in which a series of high frequency stimuli applied to the stratum radiatum of CA3 results in a widespread, primary ictal afterdischarge in addition to delayed, spontaneous, secondary ictal and interictal discharges (Stasheff et al., 1985; Rafiq et al., 1993).

Together, the three models produce a wide range of epileptiform electrographic behaviours by fundamentally different mechanisms. They are therefore ideally suited as models on which to test the inhibitory effects of NPY.

Methods

Male Sprague-Dawley rats (15-40 days old) were decapitated according to guidelines of the University of Alberta Health Sciences Laboratory Animal Care Committee, and brains were removed into cold (4°C) artificial cerebrospinal fluid (ACSF), bubbled continuously with 95% O_2, 5% CO_2 (carbogen). Hippocampi were removed and sliced transversely using a McIlwain tissue chopper. Slices were kept in carbogenated ACSF at room temperature, then incubated, submerged at 34° ± 0.5°C in a perfusion chamber (flow rate 2.5 - 3.5 ml/min) for at least 15 minutes prior to the beginning of experiments.

$0Mg^{2+}$ and picrotoxin bursting

Composition of ACSF used for dissection and storage of hippocampal slices (500μm thick), and baseline control recordings was (in mM) 120 NaCl, 3.3 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2 \bullet 2H_2O$, 1.23 NaH_2PO_4, 25 $NaHCO_3$, 10 dextrose. Slices were transferred to the perfusion chamber, and extracellular glass recording electrodes (3 - 15 MΩ), filled with ACSF, were placed in stratum pyramidale of area CA1 or CA3. In some experiments bipolar stimulating electrodes were placed in the stratum radiatum of CA2 or the dentate hilus, respectively, to deliver single stimuli for optimization of recording electrode placement. Stimuli were not given during test conditions. The perfusion medium was then changed to either $0Mg^{2+}$ ACSF (identical to ACSF except for the omission of $MgSO_4$) or ACSF to which Picrotoxin (100μM) and KCl (1.7mM, such that $[K^+]_{out}$ = 5mM) was added (picrotoxin ACSF), and the slice was monitored for development of spontaneous epileptiform activity. Experiments were only initiated once such activity had reached a relatively stable

frequency. Concentrated stock solutions of NPY and other drugs were diluted in the appropriate ACSF immediately prior to bath application. Agonists were applied at concentrations approximating the EC_{50} for inhibition of the pEPSP in area CA1, i.e., 3μM for the centrally truncated analogue [ahx$^{5\text{-}36}$]NPY and 1μM for all others (Klapstein and Colmers, unpublished observations).

STIB

Methods were adapted from Stasheff et al., 1985. Briefly, hippocampal slices (600μm thick) were dissected and maintained in ACSF containing (in mM) 120 NaCl, 3.3 KCl, 0.9 $MgSO_4$, 1.1 $MgCl_2$, 1.8 $CaCl_2 \bullet 2H_2O$, 1.23 NaH_2PO_4, 25 $NaHCO_3$, 10 dextrose. ACSF used for perfusion was identical except for the omission of $MgCl_2$ (final $[Mg^{2+}] \approx 0.9$mM). Stimuli were delivered through a bipolar stimulating electrode placed in the stratum radiatum of CA3a. Extracellular glass recording electrodes (3 - 15 MΩ) or intracellular patch pipette recording electrodes (3 - 15 MΩ) were placed in stratum pyramidale of CA3a, CA3b, or CA3c. In some extracellular experiments, single stimuli were delivered to facilitate placement of recording electrodes.

Extracellular electrodes were filled with ACSF. Electrodes used for whole-cell slice patch recordings were filled with a solution containing (in mM) 130 K^+-gluconate, 10 HEPES, 11 EGTA, 1 $CaCl_2$, 2 MgATP, 0.3 NaGTP, buffered to pH 7.2 with KOH and adjusted to 270-280 mOsm. Seal resistances were routinely > 2 - 5 GΩ prior to break-in (Hamill et al., 1981; Staley et al., 1992). Lucifer Yellow (0.5% w/v) was included in some electrodes used for whole cell interneuron recording.

Stimulus trains were delivered every 4 or 5 minutes via the bipolar stimulating

electrode and consisted of 4 stimuli (30V,100-200μsec) at 100 Hz, repeated 15 times at 5 Hz (cf. Larson et al., 1986). For some NPY analogue experiments, the number of repetitions was gradually reduced from 15 until the afterdischarge failed to occur, then raised gradually until at least 3 consecutive normal afterdischarges occurred. Because of time constraints during whole cell recordings, this stimulus threshold determination was not conducted.

Hippocampal slices from which interneurons had been recorded remained in the perfusion chamber for 0.5 - 1 hour after the recording electrode was removed, for diffusion of Lucifer Yellow to occur. They were then fixed overnight in 4% paraformaldehyde in phosphate buffered saline consisting of (in mM) 96 NaOH and 122 NaH_2PO_4, then transferred sequentially every 8-12 hours through increasing concentrations of ethanol (30, 50, 70, 90, 100% in H_2O). Slices were then equilibrated in 30% sucrose overnight, embedded in Tissuetek, subsectioned to 60μm on a cryotome, put on slides, and viewed under a fluorescence microscope.

Experiments were recorded on chart paper (Gould RS3200) and pcm tape and analyzed offline. Burst frequency and afterdischarge length were measured from chart recordings. Individual bursts were played back from tape into Fetchex (Axon Instruments) and exported to Axum (Trimetrix). The linelength measurement program, written in Axum, measures the sum of the voltage differences between adjacent points on the digitized trace, and provides an estimate of activity within a burst (Korn et al., 1987). To account for possible artifacts due to changes in electrical noise during experiments, linelengths were calculated for recording segments showing no burst activity (noise), which were of identical length and adjacent to those analyzed with bursts (total). Statistics were calculated on the difference between the

linelength of total and noise traces. Simultaneous dual patch clamp recordings were captured from tape in Axotape (Axon Instruments) and plotted to show synchronicity of events.

Results

Experiments consisted of 199 drug applications to a total of 96 hippocampal slice preparations. Whole cell recordings were made from 36 pyramidal cells and 4 interneurons.

0 Mg^{2+} and Picrotoxin bursting

Following a change of perfusate to 0 Mg^{2+} ACSF or picrotoxin ACSF, synchronous population bursts appeared with a latency of 424 ± 72 and 294 ± 99 seconds, respectively. When recorded from the pyramidal cell body layer, bursts generally consisted of multiple, sharply negative population spikes superimposed upon a slow, positive going wave, approximately 50 to 250 milliseconds in duration (Figure 5-1 insets). The morphology of individual discharges was not remarkably different between the first and subsequent bursts, however the frequency of these events generally tended to increase over a period of 0.5 to 1.5 hours to rates between 6 and 36 per minute for 0 Mg^{2+} and between 3 and 18 per minute for picrotoxin. Peptides were applied only once the burst frequency appeared to be stable (ie, not changing significantly over a period of 10 - 20 minutes). For statistical purposes, frequency was calculated by counting the number of population bursts for a 60 second period under control conditions and again during the peak peptide effect. Data were only included if the frequency of the events recovered significantly upon washout.

NPY (1μM) reduced the frequency of 0 Mg^{2+}-evoked spontaneous population bursts recorded extracellularly in stratum pyramidale (SP) of area CA1 by 71.3 ± 6.8% (n=12) and, in a separate group of experiments recorded in SP of area CA3 by 81.4 ± 4.7% (n=14, Figure 5-1). Analogues of NPY were also tested on bursts

recorded in SP of area CA3. PYY_{3-36} and [ahx^{5-36}]NPY, which are selective agonists of the Y_2 receptor, inhibited the frequency of bursts by 63.8 ± 12.7% (1μM, n=4) and 43.0 ± 6.9% (3μM, n=6), respectively. The Y_1 selective agonist, $Leu^{31}Pro^{34}$ NPY was less potent, but also inhibited burst frequency by 28.5 ± 5.3% (1μM, n=8).

Linelength measurements of individual discharges were performed as described in the Methods section, to provide a quantitative comparison of activity between discharges under different conditions. Linelengths were calculated for 4 - 6 randomly chosen bursts recorded in CA1 under control conditions and in the presence of 1 μM NPY for each of 6 experiments. NPY did not cause any consistent change in the morphology of individual discharges measured in this way (Figure 5-1, insets). Immediately following the peak NPY effect, the average linelength was significantly and reversibly decreased in one experiment, significantly and reversibly increased in 2 experiments, and not significantly affected in 3 experiments, compared to controls.

Spontaneous bursts observed upon perfusion of the hippocampal slice with ACSF containing 100μM picrotoxin were similar in morphology to those observed in $0Mg^{2+}$ ACSF. NPY reduced the frequency of these bursts (recorded extracellularly in SP of area CA3) by 88.8 ± 8.3% (n=5; Figure 5-1), without causing any obvious change in their morphology, as was the case in the $0Mg^{2+}$-induced bursts. The Y_2-selective agonists PYY_{3-36} and [ahx^{5-36}]NPY, inhibited the frequency of bursts by 100.0 ± 0.0% (1μM, n=6) and 81.9 ± 11.7% (3μM, n=7), respectively. The Y_1 selective agonist, $Leu^{31}Pro^{34}$ NPY, also inhibited burst frequency by 93.9 ± 3.7% (1μM, n=10), although this effect persisted in the presence of the Y_1-selective antagonist 1229U91 (1nM), where $Leu^{31}Pro^{34}$ NPY caused a 96.8 ± 3.3% reduction in burst frequency (1μM, n=4).

STIB

The STIB model produces several morphologically-distinct electrographic events. Within 2 seconds following the end of a stimulus train applied to stratum radiatum of CA2 (see Methods), large amplitude, spontaneous afterdischarges (ADs) were evident in both extracellular and whole cell patch recordings made from area CA3. These ADs were generally clonic or tonic-clonic in nature, and ranged from 5.2 to 58.4 seconds in duration.

In extracellular field recordings, bath application of NPY (1μM) reduced the duration of the ADs by 100 ± 0.00% (n = 4). AD duration was also dramatically reduced by bath application of agonists which act at the Y_2 receptor, such as PYY (1 μM, Y_1 and Y_2 selective) which reduced AD duration by 99.7 ± 0.3% (n=4). Similarly, AD duration was inhibited by the Y_2-selective agonists NPY_{13-36} (3μM; n=3) by 100 ± 0.00%, [ahx$^{5-24}$]NPY (1μM; n=5) by 97.6 ± 2.1%, and PYY_{3-36} by 100.0 ± 0.0% (1μM, n=2). By contrast, the Y_1 selective agonist Leu^{31}Pro^{34}NPY (1 μM) reduced AD duration in only 4 of 7 experiments, increasing it in the remaining 3, with an average inhibitory effect of 12.9 ± 22.93% (n=7, Figure 5-2A). The time course of action differed between agonist analogues, with the full peptides (NPY, PYY) having the longest duration of action, and the centrally-truncated analogue, [ahx$^{5-24}$]NPY, having both fastest onset and recovery (Figure 5-2B). NPY's effects on afterdischarges were not systematically studied in whole cell patch clamp recordings.

In extracellular field potential recordings in the STIB model, interictal bursts resembling those seen in the 0Mg^{2+} and picrotoxin models, often began to occur spontaneously after several stimulus trains had been applied to a preparation. The frequency of these interictal bursts was highly variable both within and between

preparations, making statistical comparisons difficult. However, when they did occur, their frequency was noticeably or entirely inhibited by bath application of NPY (1μM, n=6), PYY (1μM, n=2), NPY_{13-36} (3μM, n=3), and [ahx$^{5-24}$]NPY (1μM, n=7). Leu^{31}Pro^{34}NPY (1μM, n=5) also inhibited these events, even when it had little or no effect on the afterdischarges (Figure 5-2A).

SRSEs

In all whole cell patch clamp recordings from CA3 pyramidal neurons in slices which exhibited ADs in the STIB model, spontaneous, rhythmic, synchronous events (SRSEs, terminology from Schwartzkroin and Haglund, 1986) also usually appeared after several stimulus trains had been given, and preceded the development of the interictal bursts. The SRSEs appeared as outward currents when the membrane potential was held at -56 mV in voltage clamp, reversed polarity near the chloride equilibrium potential, (E_{Cl}) (-63mV - -68mV), and were inhibited by the $GABA_A$ antagonist picrotoxin (100μM) (Figure 5-3).

Once SRSEs were observed in a preparation, discontinuation of stimulus trains permitted the appearance and development of inward currents superimposed upon the outward currents. These inward currents generally increased in amplitude and duration over time until they were no longer constrained by the voltage clamp amplifier, and appeared to be unclamped interictal bursts (Figure 5-4), which could also be observed in extracellular field recordings from the same area (data not shown). Frequently, but not always, these bursts would periodically increase in frequency to form clusters resembling ictal discharges. After a period of relative quiescence following the spontaneous end of a cluster, SRSEs reappeared and inward currents once again developed. Simultaneous whole cell patch clamp recordings from well

separated pairs of pyramidal neurones within area CA3 (n=4, Figure 5-4) show all of these events to be very highly synchronized throughout that population.

The SRSEs recorded in CA3 pyramidal cells were not measurably affected in frequency or amplitude by bath application of 1μM NPY (n = 7; Figure 5-4B). In testing a possible involvement of a hyperpolarization-activated cation current (I_h) in the coupling between the SRSEs and the interictal bursts, we found that neither the development of the inward currents nor their coincidence with the SRSEs was prevented by 2mM CsCl (n = 2; Figure 5-5). Likewise, no consistent change in SRSEs was caused by bath application of 50 μM APV (NMDA receptor antagonist, n = 4), however they were completely and reversibly inhibited by the AMPA glutamate receptor antagonist, NBQX (1μM, n=2) (Figure 5-6). In order to determine the origin of the SRSEs, whole cell patch clamp recordings were made from interneurons in stratum oriens or stratum radiatum of area CA3 using the STIB model (n=4). These cells were identified by their electrophysiological properties, and additionally by either filling them with lucifer yellow (n=1) or by applying the μ opiate agonist, DAGO (n=3; Madison and Nicoll, 1983; Lambert and Wilson, 1993) and observing a hyperpolarizing response. We observed rhythmic events in interneurons which occurred with the same time course as those observed in pyramidal cell recordings, including the appearance of ADs, SRSEs and paroxysmal depolarizations (Figure 5-7). The most noticeable difference between these and pyramidal cell recordings was that, in interneurons, SRSEs were inward currents at holding potentials of -56 mV, with E_{rev} estimated to be near -9 mV (Figure 5-7C,D). In current clamp mode, when the interneurons were hyperpolarized below action potential (AP) threshold, these events rhythmically depolarized the cell membrane by 20 - 30 mV. As injected

hyperpolarizing current was reduced, allowing the membrane potential to depolarize, each event became capable of initiating action potentials (Figure 5-7B).

SRSEs recorded in interneurons were not measurably affected by bath application of NPY (1μM, n = 4), APV (50μM, n = 2) or MCPG (100μM, n = 1). As in pyramidal cell recordings, however, these rhythmic events were completely and reversibly inhibited by bath application of 1μM NBQX (n = 3) (Figure 5-7F).

Discussion

In this study we used three different *in vitro* models of epileptiform activity to test whether NPY was capable of controlling the synchronous population discharges produced by different mechanisms. NPY was effective in reducing excitability in all three models.

0 Mg^{2+} and picrotoxin models

In the 0 Mg^{2+} model, omission of Mg^{2+} from the extracellular medium removes its physiological blockade of NMDA receptors, increasing the excitation mediated by ambient glutamate (Sah et al., 1989). In the picrotoxin model, $GABA_A$ receptors are blocked, reducing endogenous inhibition (Hablitz, 1984). Both models exhibit spontaneous, synchronous activation of neurons which can easily be observed with extracellular recording electrodes placed in stratum pyramidale of area CA3. The complex waveform of these synchronous electrical discharges is similar in time course and shape to those observed to occur between seizures in human epilepsy patients and in animal models of epilepsy. These events have therefore been used as an *in vitro* model of interictal discharges (McNamara, 1994). Neuropeptide Y reduced the frequency of these events without affecting their electrographic morphology. Both Y_2 and Y_1 receptor selective agonists are also capable of reproducing this effect. Interestingly, though, the potent and selective Y_1 receptor antagonist 1229U91 was not able to block the inhibition caused by the reportedly selective Y_1 receptor agonist $Leu^{31}Pro^{34}NPY$, suggesting that in this preparation, either 1) 1229U91 is not active at the Y_1 receptor, or 2) a different NPY receptor subtype is being activated by $Leu^{31}Pro^{34}NPY$.

1229U91 is related to another compound which causes potent functional antagonism at Y_1 receptors (Leban et al., 1995). It potently displaces NPY binding from rat brain membranes with an IC50 of 1 pM. Furthermore, it potently inhibits the $Leu^{31}Pro^{34}$NPY induced reduction in Ca^{2+} influx into dissociated dentate granule cells, and it does not inhibit Y_2 mediated responses in area CA1 of hippocampus (Colmers, unpublished observations), suggesting that its effects are selective for the Y_1 receptor in the rat hippocampus. $Leu^{31}Pro^{34}$NPY has been shown to have a relative potency less than 1:1000 compared to NPY in Y_2 receptor containing tissues such as pig spleen (Krstenansky et al., 1990) and human SK-N-BE2 cells (Wahlestedt et al., 1990; Wahlestedt et al., 1992). Thus, it is unlikely that $Leu^{31}Pro^{34}$NPY is acting via a Y_2 receptor in this preparation. As in dentate granule cells, NPY also causes an inhibition of N-type Ca^{2+} channels in acutely dissociated rat superior cervical ganglion (SCG) cells. The agonist profile in these cells suggests that it does so via activation of receptors which are neither Y_1 nor Y_2 (Foucart et al., 1993). Therefore, it is most likely that $Leu^{31}Pro^{34}$NPY inhibits burst frequency in these models by acting on a receptor which does not fit the classic Y_1 or Y_2 characteristics. While it is possible that a Y_3 receptor is mediating the burst frequency reduction caused by $Leu^{31}Pro^{34}$NPY, it must also be noted that no distinct Y_3 mediated response has yet been observed in the rat hippocampal slice preparation.

A significant difference in the effects of NPY was seen between the bursts caused by $0Mg^{2+}$ and picrotoxin. All agonists tested show greater inhibition of burst frequency in the picrotoxin model. This difference might be accounted for by the difference in burst frequencies seen in the two models. Since the measurement used involves counting the number of bursts in a 60 second interval, it is more likely that

a slight inhibition of burst frequency will produce a burst-free 60 second interval in a preparation which had fewer bursts per minute under control conditions.

STIB model

Afterdischarges

The STIB model has been widely used as an acute, *in vitro* model of electrographic seizure and epileptogenesis (Stasheff et al., 1985; Slater et al., 1985; Rafiq et al., 1993). A series of high frequency trains of stimuli (such as those used in kindling studies), when applied to the stratum radiatum of area CA2, excite axons in that region, including the recurrent CA3 collateral projections which are thought to be important in the synchronization of activity seen in this region (MacVicar and Dudek, 1980; Wong and Traub, 1983).

The afterdischarge which immediately follows the stimulus trains is similar in appearance to the electrographic activity which occurs during seizures in human epilepsy patients and *in vivo* animal epilepsy models (McNamara, 1994). The length of the AD could be taken to indicate the duration of a seizure. Here we show that the afterdischarge is sensitive to inhibition by NPY, and that this inhibition is mediated through the Y_2 receptor, as evidenced by the fact that NPY, PYY, [ahx$^{5-36}$]NPY and NPY_{13-36} are all able to drastically shorten or completely inhibit the AD, whereas the Y_1-selective agonist Leu^{31}Pro^{34}NPY is unable to do so.

Interictal bursts in STIB

Like the ADs, the interictal bursts which occur in the STIB model are also sensitive to Y_2 receptor activation. As with $0Mg^{2+}$ and Picrotoxin-induced bursts,

they are also sensitive to Y_1 receptor activation, as they are inhibited by $Leu^{31}Pro^{34}$NPY, suggesting that they have a distinct underlying mechanism from that which causes the AD. This is, to our knowledge, the first means by which these interictal bursts and the ADs can be pharmacologically differentiated, and may be useful in further studies of the pathways and mechanisms responsible for ictal and interictal behavior. A clue to how this occurs may be found in the distribution of NPY receptor subtypes. Rafiq et al. (1993) found that secondary interictal bursts occurred first in the dentate gyrus, then progressed through CA3, CA1, and the entorhinal cortex. Y_1 receptors are particularly numerous in the inner third of the molecular layer of the dentate gyrus, however they do not affect evoked postsynaptic potentials anywhere in the hippocampus. Activation of Y_1 receptors causes a reduction in Ca^{2+} influx through N-type Ca^{2+} channels in the soma and dendrites of dentate granule cells (McQuiston et al., 1994, and submitted). Whether this is how Y_1 agonists inhibit interictals is unknown. It may be that the difference between interictals and ADs is one of magnitude, i.e., that Y_1 agonists are sufficient to inhibit the occurrence of interictals, which seem to occur as an all-or-none response, but are overwhelmed by the magnitude of activity present during a stimulus train and subsequent afterdischarge. Alternately, $Leu^{31}Pro^{34}$NPY, the Y_1 agonist used in this study, may be acting at a receptor type other than Y_1, as it seems to be in the picrotoxin bursting model. In any case, the differential distribution of the NPY receptor subtypes suggests that the development of interictals and ADs may involve completely different neuronal populations and projection pathways within the hippocampus.

SRSEs

Events similar to those we describe here were reported to occur in epileptic human and normal monkey temporal lobe (Schwartzkroin and Haglund, 1986). Whether or not they are associated with the pathology of epilepsy was unclear from that report, however, since the "normal" monkey temporal lobe reported was actually tissue contralateral to an epileptic focus, and may therefore not have been entirely normal (Wyler et al., 1975).

SRSEs have not previously been well described or systematically studied, as is now possible with patch clamp recording techniques in conjunction with an accelerated model of epileptogenesis. Ours is the first report of this activity occurring in a rat hippocampal slice preparation. Several factors may contribute to the development or observation of SRSEs in this model. For example, our experimental protocol differs from others in several ways. The longevity of NPY effects prevented their reversal following prolonged applications. To accommodate a shorter agonist application period, stimulus trains were delivered at either 4 or 5 minute intervals, rather than 10 minute intervals used by others (Stasheff et al., 1985; Rafiq et al., 1993). The time course for development of spontaneous interictaform bursts was not different from that in which stimulus trains were delivered every 10 minutes, although robust ADs were typically evident following even the first stimulus train. Perhaps more importantly, the whole cell patch clamp recordings used in this study allowed us to observe events which may be undetectable in extracellular field or intracellular sharp electrode recordings (Hamill et al., 1981).

In this study we observed that SRSEs, recorded in both interneurons and pyramidal neurons, are dependent on AMPA receptor activation. Greber et al. (1994)

observed that NPY inhibits high [K^+]-stimulated glutamate release from hippocampal slices. Furthermore, NPY has also been shown to inhibit glutamatergic transmission in CA3 by 45% to 55% (Klapstein and Colmers, 1993), and yet the present data clearly show that NPY does not inhibit SRSEs in any measurable way. Therefore, the glutamatergic requirement for SRSE expression must come from afferent inputs which are insensitive to inhibition by NPY, and therefore, different from, or a subset of, those terminals which innervate the pyramidal cell population. The question remains as to where this afferent input originates. It may be relevant that synaptic inputs to dentate granule cells are also insensitive to the actions of NPY (Klapstein and Colmers, 1993).

Rhythmic inward currents persist in interneurons under voltage-clamp conditions, and their frequency is unaltered even during paroxysmal depolarizing shifts in the interneuron. They are also insensitive to the mGluR antagonist MCPG, clearly differentiating them from similar events seen in CA1 interneurons in rat (McBain et al., 1994) and guinea pig hippocampal slices (Taylor and Wong, 1993) which show the interneurons to have intrinsic oscillations mediated by metabotropic glutamate receptors. Together, these observations demonstrate that the rhythmic behavior of the interneurons is synaptic in origin, and furthermore, suggest that the origin of the synaptic input is unaffected by local paroxysms. In current-clamp, SRSEs rhythmically depolarize the interneuron. In at least one cell, each of these depolarizations was able to elicit an action potential, suggesting that they may be causing the GABAergic events recorded in pyramidal neurons.

The amplitude of the rhythmic excitatory events recorded in the interneurons is large; in one cell recorded in current clamp, each event triggered an action

potential, in another, they caused nearly 30 mV depolarizations from membrane potentials near -70 mV (Figure 5-7B,F). In voltage clamp, the rhythmic events were approximately 60 pA at -60 mV. Based on recent observations of quantal synaptic excitation in CA3 interneurons (Arancio et al., 1994), the data suggest that the rhythmic events result from the synchronous input of 4 to 10 presynaptic neurons, none of which are affected by locally-recorded bursts. This suggests that the synaptic excitation may arise from a source distant to area CA3, and may indicate an association between these and the NPY-insensitive inputs to the dentate granule cells, as noted earlier.

The present results clearly show that exogenous NPY is capable of reducing the synchronous excitatory discharges in the hippocampus mediated by three different mechanisms. The question remains whether this is the biological role of the peptide in the hippocampus. In addition to the present data, several observations support this concept. NPY is found in varicosities, thought to represent release sites, next to glutamatergic presynaptic terminals in the hippocampus (Milner and Veznedaroglu, 1992). The peptide and its receptors are thus matched to the task of inhibiting glutamatergic transmission. Interneurons containing NPY receive collateral excitation from the axons of principal cells (Lacaille et al., 1989), indicating that their activity would be regulated by that of the principal cells, and could provide feedback inhibition of local glutamatergic excitation in an activity-dependent manner. This hypothesis can be tested directly upon the availability of a Y_2 receptor selective antagonist.

Figure 5-1. **Neuropeptide Y (NPY) reduces spontaneous burst frequencies.** AC-coupled chart records of extracellular field potentials from the pyramidal cell layer of area CA3 show spontaneous bursts occurring in the presence of Mg^{2+}-free artificial cerebrospinal fluid ($0Mg^{2+}$ ACSF; top trace) and ACSF containing 100μM picrotoxin (bottom trace). Insets show enlarged detail of individual bursts in the $0Mg^{2+}$ model under control conditions (left trace) and during NPY effect (right trace). Neither the structure nor the amplitude of these events is affected by application of 1μM NPY.

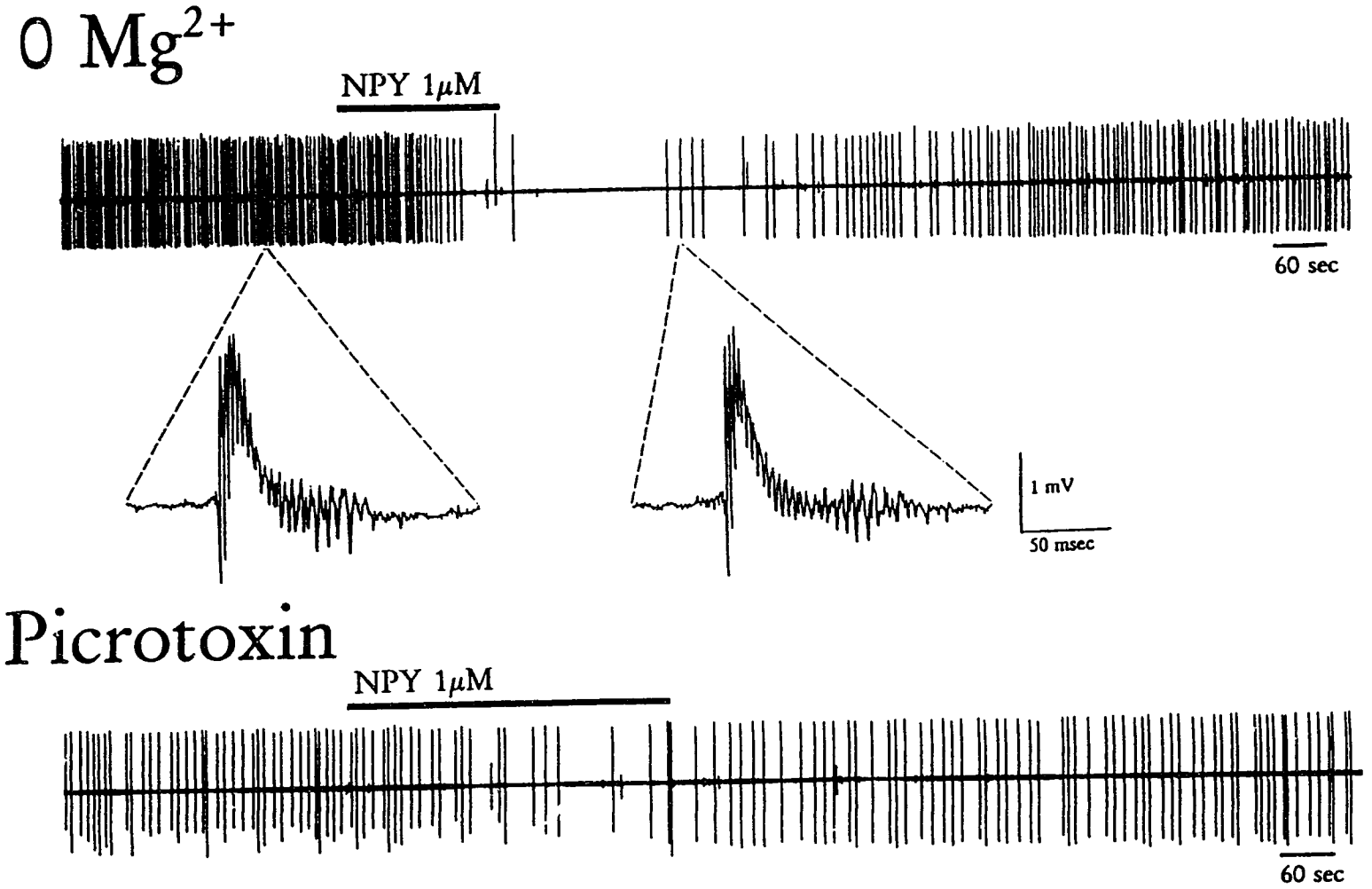
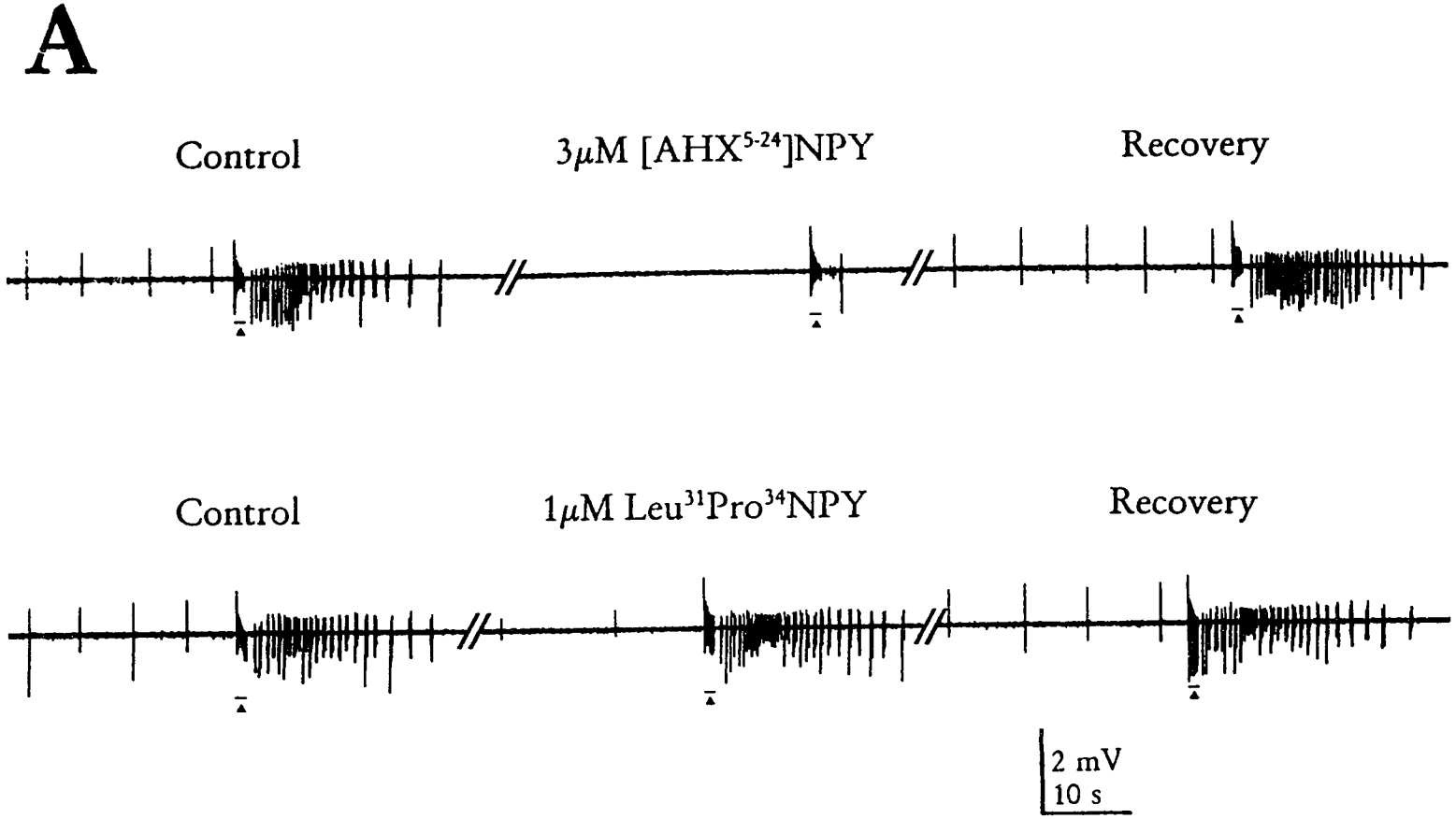

0 Mg^{2+}
NPY 1μM
60 sec
1 mV
50 msec
Picrotoxin
NPY 1μM
60 sec

Figure 5-2. **Effect of NPY receptor agonists on afterdischarges (ADs) and interictal bursts in the stimulus train-induced bursting (STIB) model.** A: Extracellular recordings from a single preparation exhibit tonic-clonic (top trace, control) or tonic (top trace, recovery) ADs immediately following stimulus trains (arrows) applied to stratum radiatum of area CA2. Spontaneous interictal bursts, resembling those seen in $0Mg^{2+}$ and picrotoxin models, occur between stimulus train applications, and can be seen here preceding the stimulus trains. Bath application of the Y_2-selective agonist [ahx^{5-24}]NPY (3μM, top trace, centre) eliminates the AD and interictal bursts. 1μM $Leu^{31}Pro^{34}$NPY, a Y_1-selective agonist (bottom trace, centre), does not inhibit the ADs but does inhibit interictal bursts. Diagonal lines represent interruptions in traces. B: Time course of inhibition of AD. AD duration was normalized to the length of the AD immediately preceding agonist application, which is indicated by a bar. Points represent averages ± standard error of the mean.


A
Control
3μM [AHX5-24]NPY
Recovery
Control
1μM Leu31Pro34NPY
Recovery
2 mV
10 s

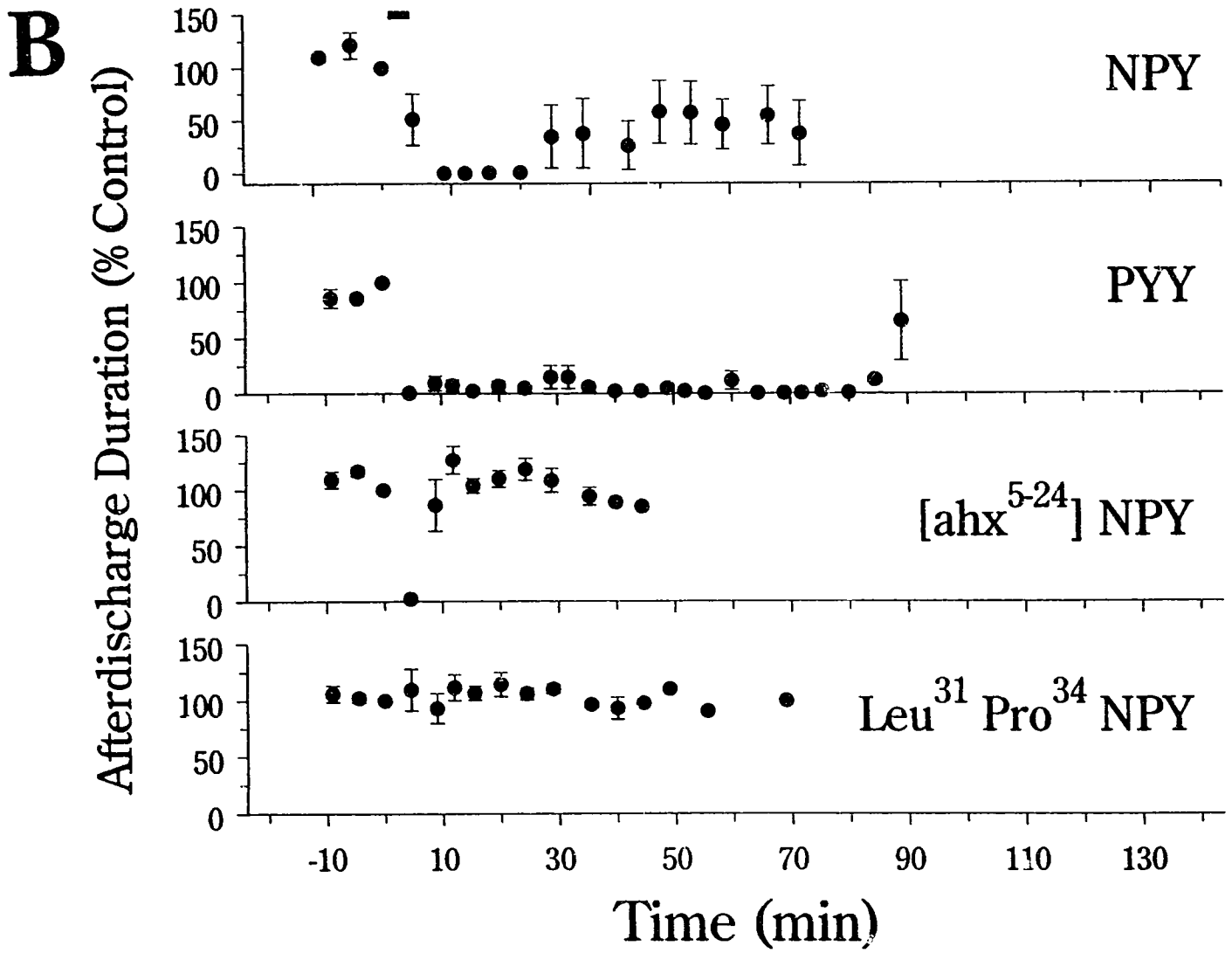

B
Afterdischarge Duration (% Control)
150
100
50
0
NPY
PYY
[ahx5-24] NPY
Leu31 Pro34 NPY
-10
10
30
50
70
90
110
130
Time (min)

Figure 5-3. **Spontaneous, rhythmic, synchronous events (SRSEs) in pyramidal neurons are $GABA_A$-ergic.** Whole cell voltage-clamp recording from a CA3 pyramidal neuron in a stimulated slice following application of 1μM NPY to prevent the occurrence of interictal bursts. The top four traces show the reversal of SRSEs near the chloride equilibrium potential. The bottom trace shows the absence of SRSE's in the presence of 100μM picrotoxin.

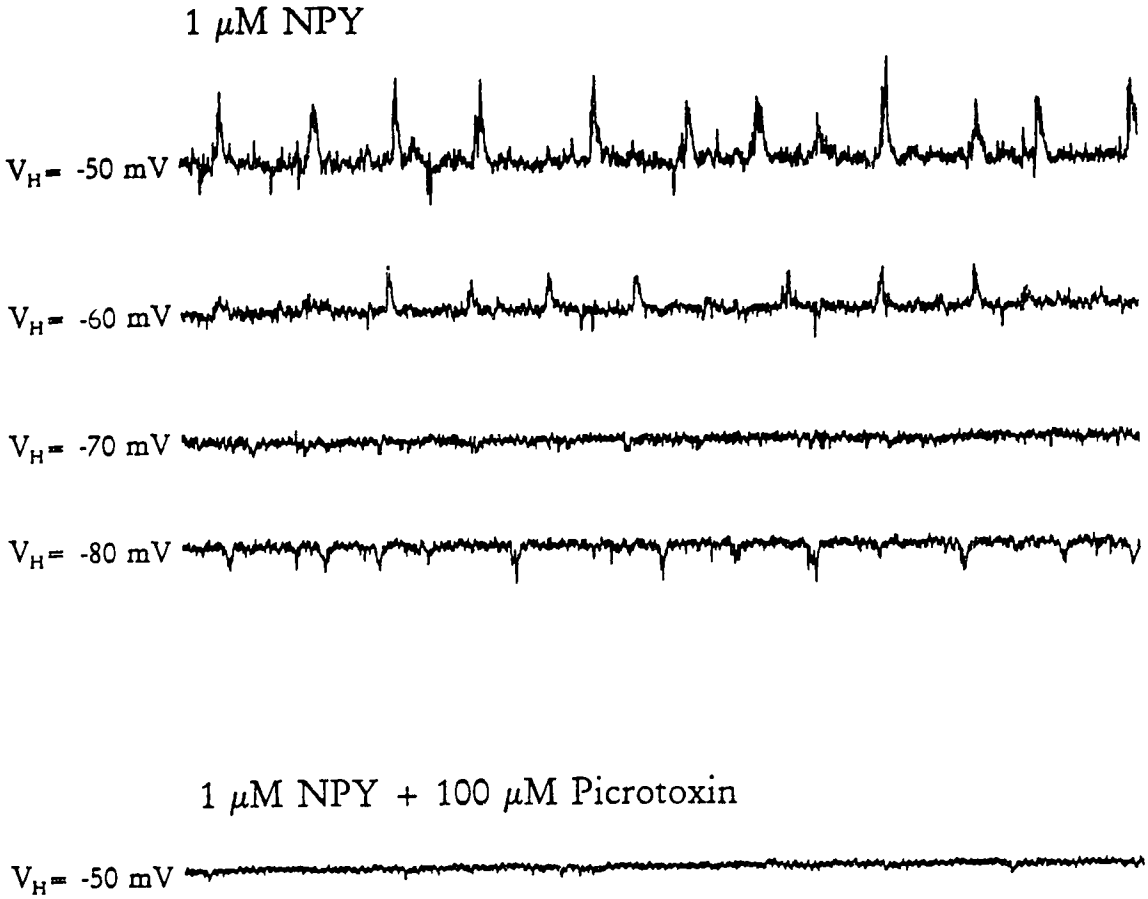
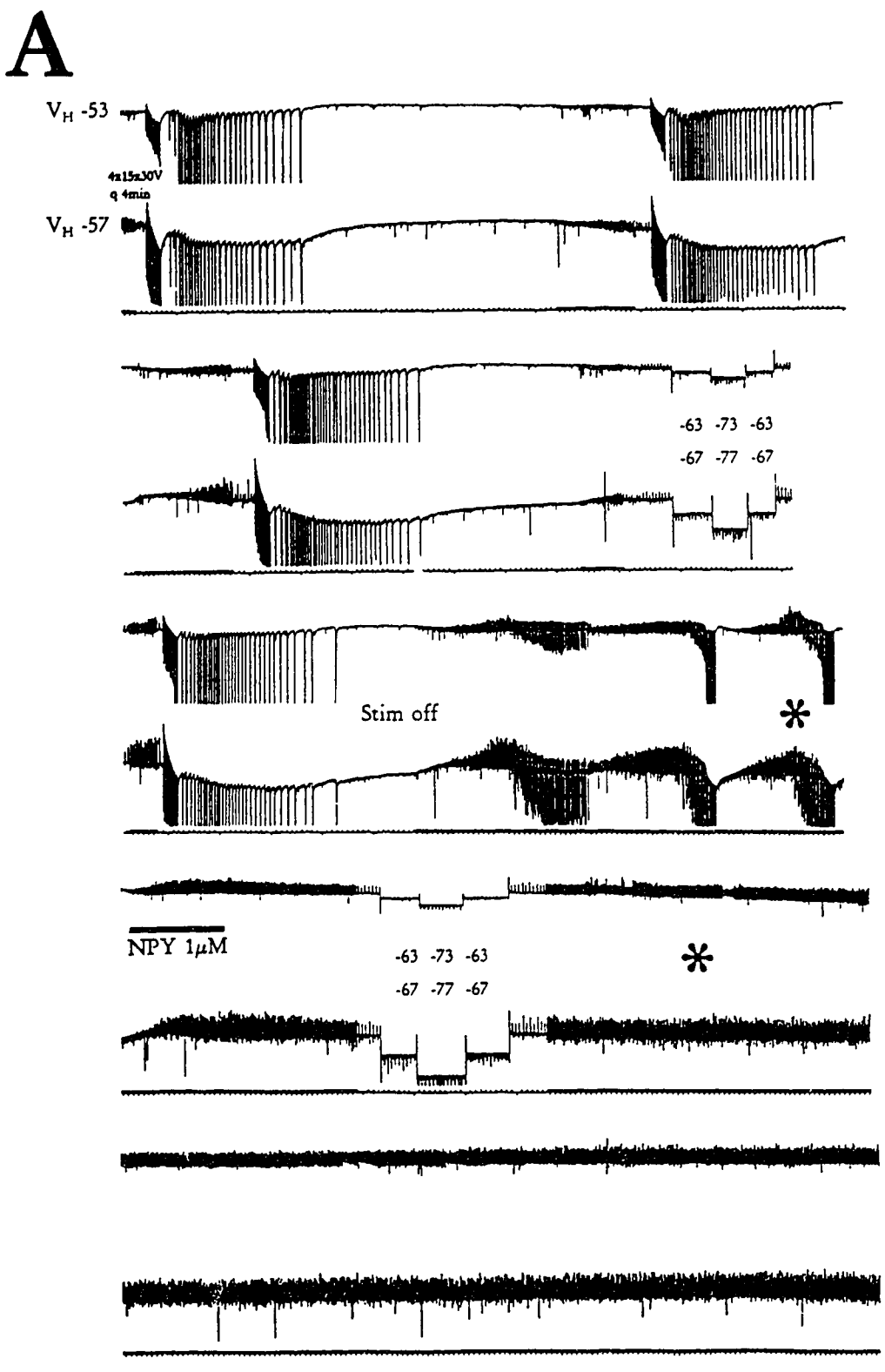

1 μM NPY
V_H= -50 mV
V_H= -60 mV
V_H= -70 mV
V_H= -80 mV
1 μM NPY + 100 μM Picrotoxin
V_H= -50 mV

Figure 5-4. **Simultaneous whole cell voltage clamp recordings of 2 pyramidal neurons in CA3a/b and CA3b/c, showing synchronicity of events throughout the CA3 region.** A: Once the spontaneous, rhythmic, synchronous outward currents (SRSEs) appear, the stimulus trains are discontinued and interictal bursts are allowed to develop. 1 μM NPY abolishes the appearance of the interictal bursts without affecting the frequency, morphology or reversal potential of the outward currents. Upward deflections on time bars occur every second, downward deflections every 10 seconds. B: Expanded view of recording segments obtained at time points indicated by asterisks in (A). Note that the gradual development of inward currents superimposed on or closely following outward currents is absent in the presence of 1 μM NPY.


A
V_H -53
4x15x30V
q 4min
V_H -57
-63 -73 -63
-67 -77 -67
Stim off
*
NPY 1μM
-63 -73 -63
-67 -77 -67
*

B

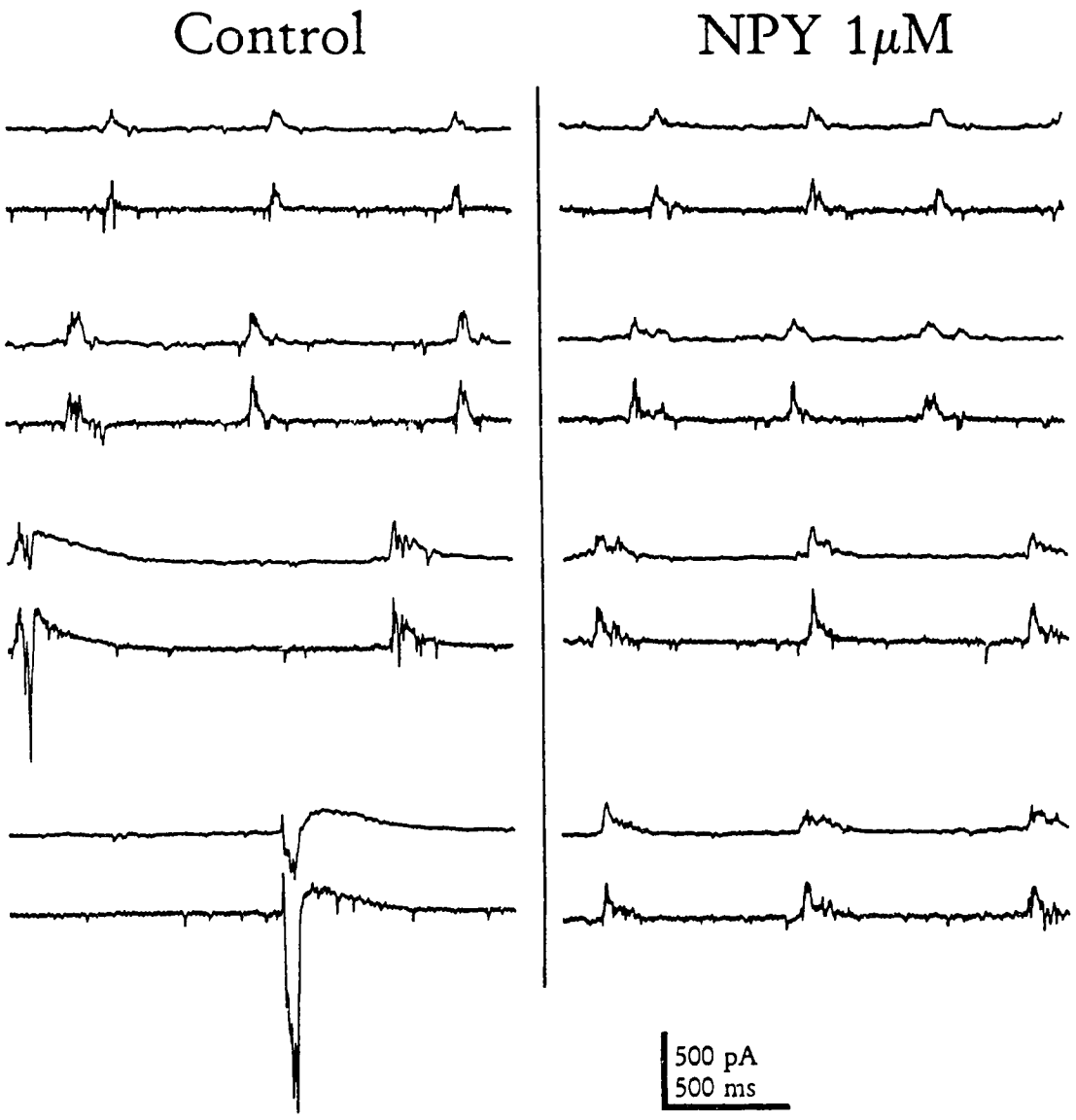


Figure 5-5. **Interictal bursts are not coupled to SRSE's by activation of I_h.** Voltage clamp recording from CA3 pyramidal neuron showing inward currents and interictal bursts superimposed upon and led by outward currents develop similarly in the absence (top trace) and presence (bottom trace) of 2mM CsCl. Diagonal lines represent interruptions in traces.

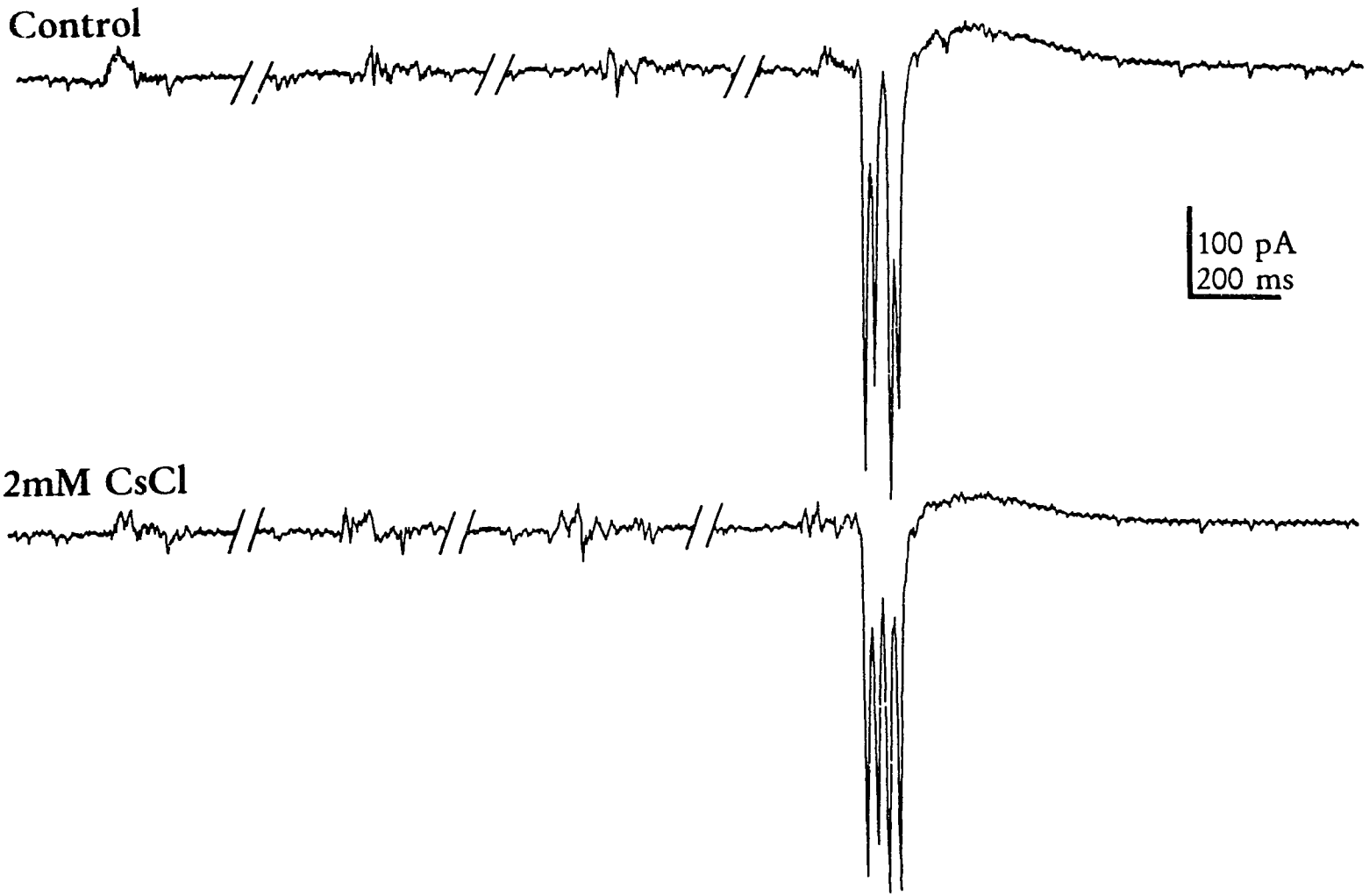

Control
100 pA
200 ms
2mM CsCl

Figure 5-6. **Whole cell voltage clamp CA3b pyramidal cell recording of NPY-isolated rhythmic outward currents.** Expansions of indicated points on trace are shown below; V_H = -55 mV. 1) Outward currents isolated by 1μM NPY. 2) DL-2-Amino-5-phosphonovaleric acid (APV, 50μM) causes a slight decrease in the duration and increase in the frequency of these events. 3) Further application of 6-Nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione (NBQX, 1μM) causes a reversible inhibition of the outward currents.

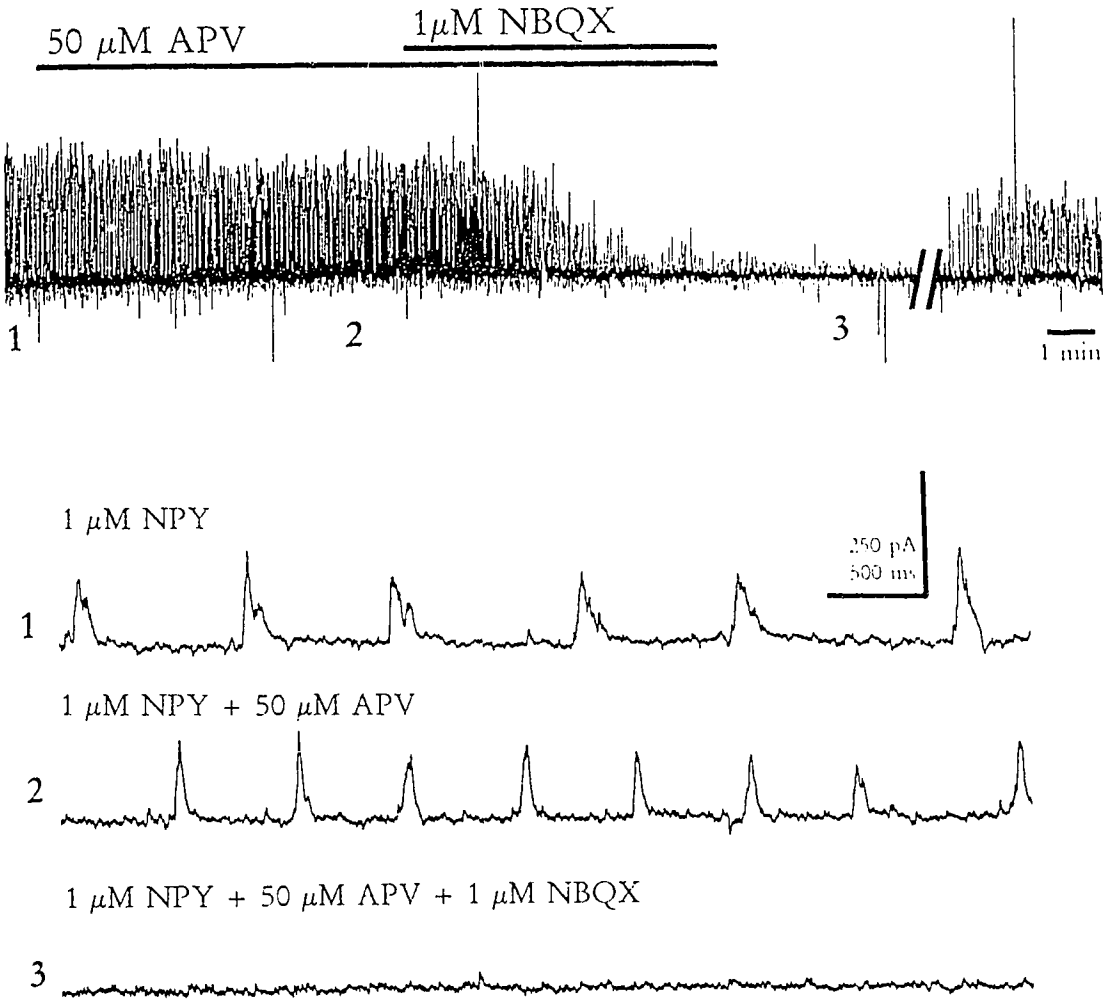
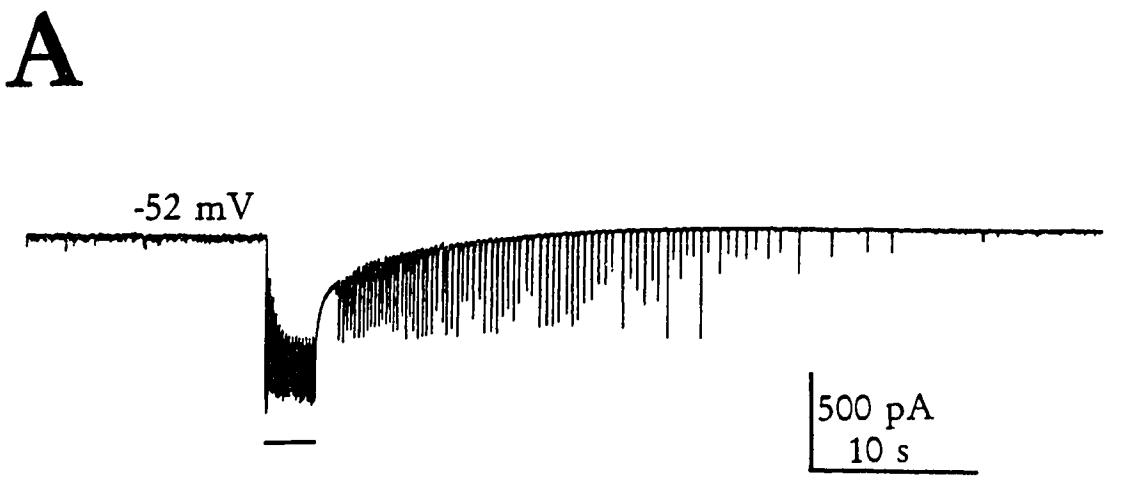
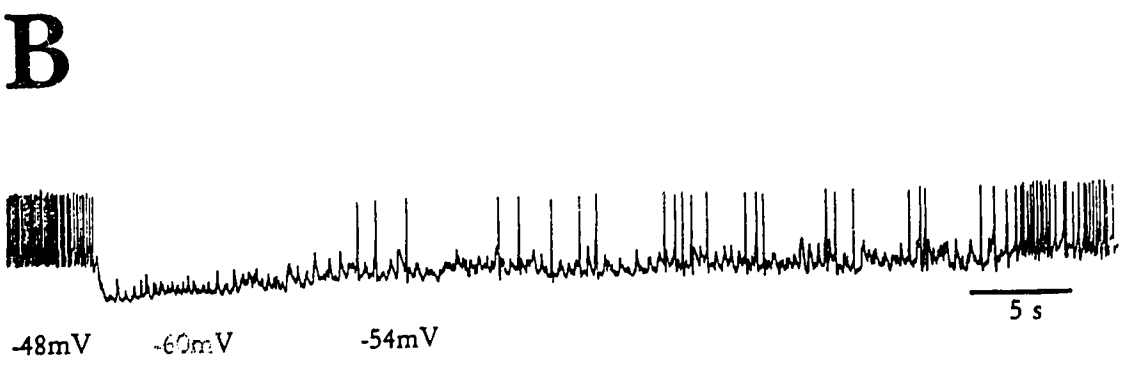
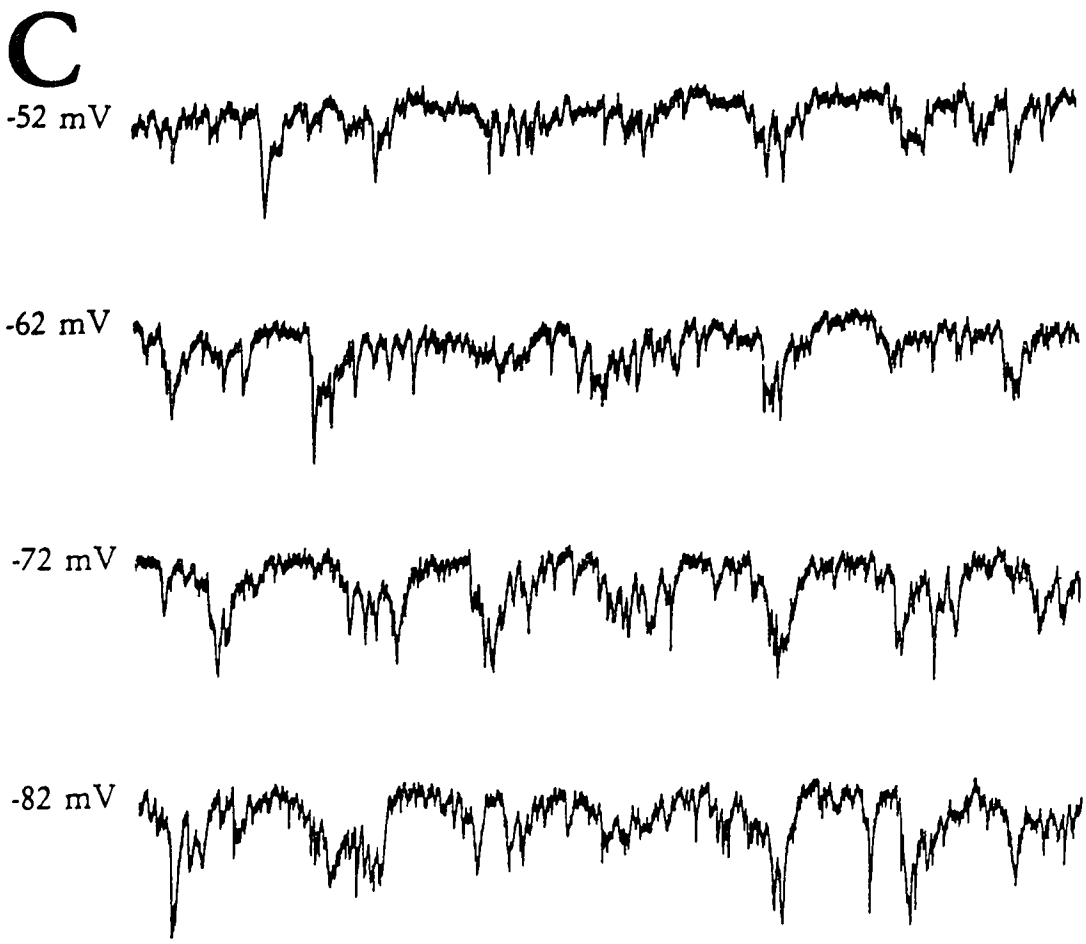

50 μM APV
1μM NBQX
1
2
3
1 min
1 μM NPY
250 pA
500 ms
1
1 μM NPY + 50 μM APV
2
1 μM NPY + 50 μM APV + 1 μM NBQX
3

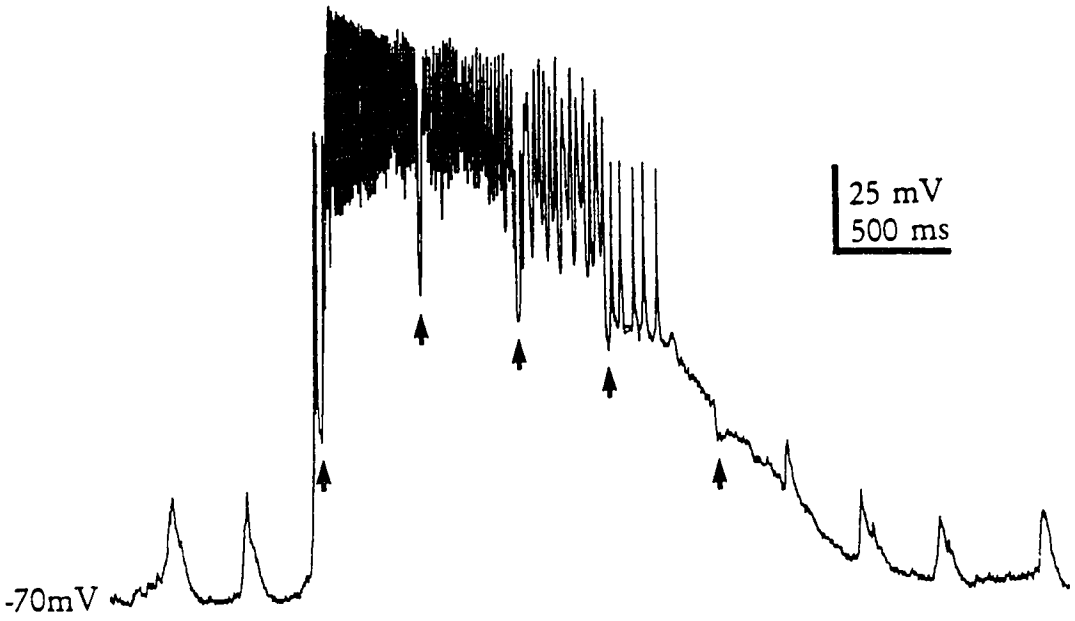
Figure 5-7. **Whole cell voltage and current clamp recordings from interneurons in area CA3 in the STIB model.** (A),(C),(D),(E), and (F) are from the same neuron. A: An afterdischarge following a stimulus train (bar) is similar in appearance to those recorded from pyramidal neurons. B: At resting membrane potential (V_R = -48 mV), an interneuron fires action potentials (AP) at a frequency similar to SRSEs recorded from pyramidal neurons. Hyperpolarization below AP threshold reveals rhythmic depolarizations at the same frequency. C and D: In voltage clamp, these rhythmic events appear as inward currents with E_{rev} near -9 mV. E: Paroxysmal depolarization shift (PDS) recorded in current clamp. During the PDS, the rhythmic events reverse polarity (arrows). F: Rhythmic excitatory events are insensitive to bath application of 1μM NPY, 50μM APV, or the metabotropic glutamate receptor antagonist (+)-α-Methyl-4-carboxyphenyl-glycine (MCPG, 100μM). NBQX (1μM) completely inhibits them.


A
-52 mV
500 pA
10 s

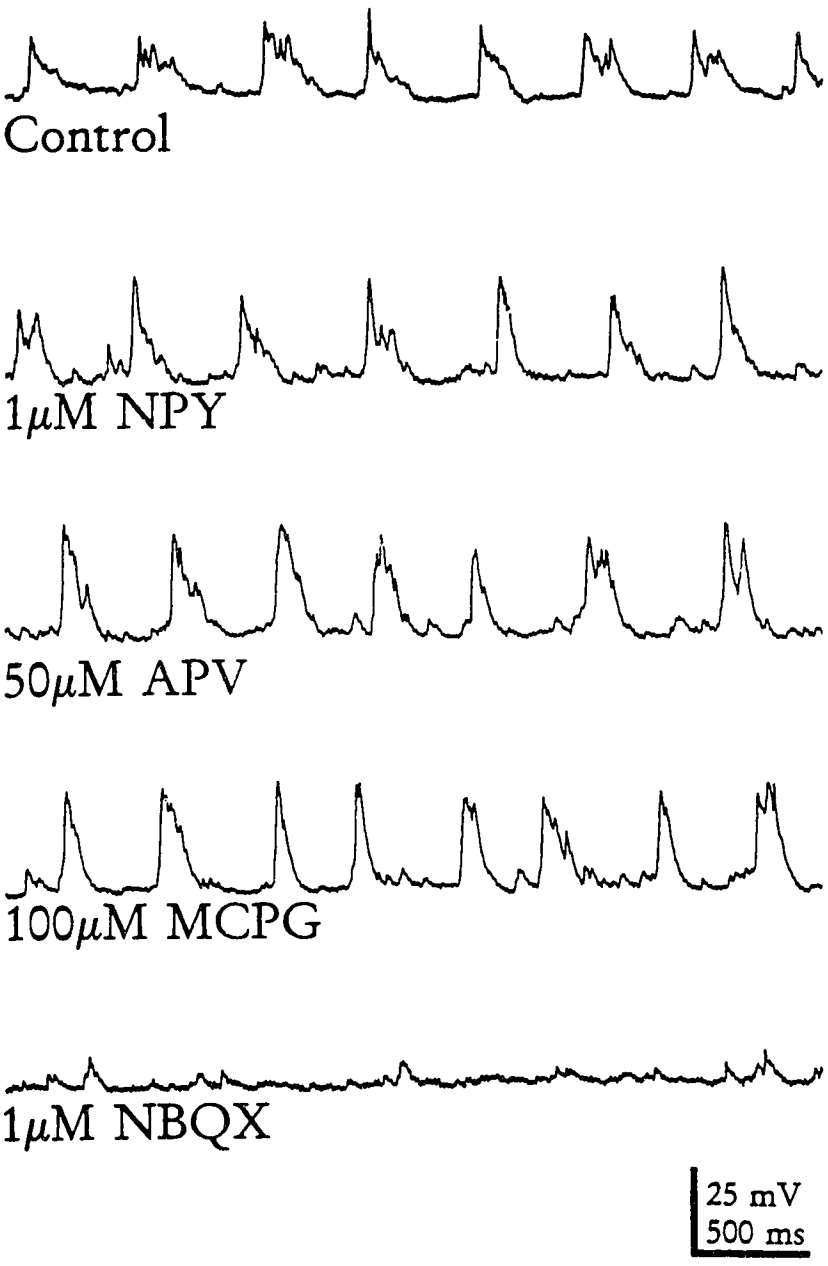

B
5 s
-48mV
-60mV
-54mV


C
-52 mV
-62 mV
-72 mV
-82 mV
D
50pA
400ms
SRSE amplitude (pA)
40
0
-40
-80
V_M (mV)
-80
-40
0
40

E



F



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CHAPTER 6

GENERAL DISCUSSION

Neuropeptide Y is the most abundant neuropeptide in the mammalian central nervous system (Adrian et al., 1983; Allen et al., 1983; Tatemoto et al., 1982), and is widely distributed throughout the periphery, as well (Sundler et al., 1986). Since its discovery in 1982 (Tatemoto et al., 1982), thousands of studies of its anatomy, evolution, physiology, and pharmacology in diverse tissues and species have been published. The physiological role that NPY plays in most tissues, however, has yet to be adequately explained. The papers in this thesis contribute to our understanding of the anatomical, pharmacological, and physiological aspects of NPY, as they appear in the *in vitro* rat hippocampal slice preparation.

In chapter 2, the pharmacology of the NPY-mediated inhibition in area CA1 was studied. It had been previously reported that NPY inhibited the excitatory synaptic transmission mediated by glutamate at stratum radiatum-CA1 synapses (Colmers et al., 1985; 1987; 1988; Haas et al., 1987). Since NPY receptor antagonists have been unavailable until just recently, NPY, analogues, and C-terminal fragments of NPY were used to construct an agonist profile of this response. It was found that porcine sequence NPY (NPY), human and rat sequence NPY (hNPY), PYY, and $NPY_{2\text{-}36}$ were equipotent at inhibiting both extracellularly-recorded population spikes and intracellularly-recorded evoked postsynaptic potentials in CA1. Shorter fragments, from $NPY_{5\text{-}36}$ to $NPY_{25\text{-}36}$ were less potent than NPY, and had decreasing effect with decreasing length, while desamido hNPY was without effect. This agonist profile is identical to that of the presynaptic Y_2 receptor which was characterized by Wahlestedt and coworkers at sympathetic neuroeffector junctions (Wahlestedt et al., 1986).

Both central and peripheral Y_2 receptors have been shown to inhibit the

formation of cAMP. Whether or not the Y_2 receptor does so in this preparation has not been tested. Experiments testing the possible involvement of various second messenger pathways showed, however, that the inhibition mediated by NPY in the hippocampus was not affected by application of 8(4-chlorophenylthio)cAMP, a membrane permeant cAMP analogue (Klapstein et al., 1990). Although this finding does not preclude the possibility that Y_2 receptors inhibit adenylate cyclase in this tissue, it does indicate strongly that this is not the mechanism mediating the presynaptic inhibition of synaptic transmission.

None of the agonists tested affected the passive or active properties of the CA1 neurons. In intracellular recordings, NPY changed neither the resting membrane potential nor the voltage response to injection of polarizing current. This is in agreement with previously published reports (Colmers et al., 1988), and suggests that, like the Y_2 receptors at the sympathetic neuroeffector junction, the Y_2 receptors mediating this response are also presynaptic. Of course, the possibility exists that NPY is acting postsynaptically, in a manner which does not affect recordings from the cell body. For example, if NPY were to induce even a small conductance in the distal dendrites of CA1 pyramidal neurons, the reduction in resistance may be sufficient to shunt a dendritic postsynaptic potential, and the conductance itself may be small enough or electrotonically distant enough from the recording electrode to elude detection. The experiments in the present study did not test this possibility, however, other investigators have presented data which argue against NPY's effects here being postsynaptic. It has been shown that 1μM NPY does not affect the postsynaptic response to glutamate or NMDA, applied iontophoretically to the dendrites of CA1 or CA3 pyramidal cells (Colmers et al., 1987; McQuiston and

Colmers, 1992); in one set of experiments it was shown that NPY at the same time inhibited evoked mossy fibre-CA3 synaptic currents (McQuiston and Colmers, 1992). Furthermore, NPY has been shown to inhibit the release of glutamate from rat hippocampal slices treated with elevated extracellular K^+ (Greber et al., 1994). Together, these observations strongly suggest that NPY inhibits glutamatergic synaptic transmission in area CA1 (and perhaps elsewhere) of the rat hippocampus by acting at a presynaptic Y_2 receptor.

Neuropeptide Y is not the only agonist known to inhibit glutamate release in the hippocampus. Others include GABA, acting via a $GABA_B$ receptor (Ault and Nadler, 1982), acetylcholine, acting via a muscarinic receptor (Hounsgaard, 1978), glutamate, acting via a presynaptic, AP4-sensitive metabotropic receptor (Forsythe and Clements, 1990, Koerner and Cotman, 1981), and adenosine, acting via an A_1-like receptor (Dunwiddie and Fredholm, 1989). The exact mechanisms whereby these neurotransmitters effect an inhibition of glutamate release is not known, nor is it known whether they share a common mechanism within the presynaptic terminal. Indeed, it is not clear that any given terminal actually possesses receptors for all five agents; it has been demonstrated only that each agent is independently capable of completely inhibiting evoked synaptic transmission at a population of synapses onto a given population of postsynaptic cells, suggesting that there is a certain overlap.

Since direct recordings from presynaptic terminals in the hippocampal slice preparation are, at the moment, not technically feasible, chapter 3 made use of indirect manipulations to address the question of whether presynaptic modulators share common mechanisms. NPY (shown in chapter 2 to act at Y_2 receptors), baclofen, an agonist at $GABA_B$ receptors and 2-chloroadenosine (2-CA), an agonist

at A_1 receptors, were tested for their ability to cause a decrease in the initial slope of the evoked population EPSP recorded in stratum radiatum of area CA1 under different conditions, a response which is particularly sensitive to presynaptic modulation. It was found that the concentration-response curves of all three agents were shifted to the right in the presence of the K^+ channel antagonist 4-aminopyridine (4-AP), suggesting that these agents might mediate presynaptic inhibition by activating a 4-AP-sensitive K^+ channel. However, by lowering extracellular Ca^{2+} from 1.5 mM to 0.75 mM, the concentration-dependent effects of both NPY and baclofen, but not 2-CA, could be restored, which is inconsistent with these agonists activating a 4-AP-sensitive K^+ channel, but which is consistent with an action of NPY and baclofen to reduce Ca^{2+} influx through voltage-dependent calcium channels. This study clearly differentiates purinergic A_1 receptors from the other two, since lowering extracellular Ca^{2+} was only able to restore the inhibition caused by very high ($>10\mu M$) concentrations of 2-CA, suggesting that it does not mediate synaptic inhibition by decreasing Ca^{2+} influx at lower concentrations, but that, at high concentrations, it may do this as well.

The results in chapter 3 can be interpreted in the following manner. 4-AP mediated block of presynaptic K^+ channels, by prolonging the depolarization of the nerve terminal membrane, may have resulted in an increased Ca^{2+} influx there, which was sufficient to overwhelm the effects of reduced Ca^{2+} caused by NPY or baclofen on transmitter release. However, when extracellular Ca^{2+} concentrations are reduced in the presence of 4AP, such that the transmitter release, and therefore presumably the Ca^{2+} influx, following a stimulus is similar to that under control conditions, a further decrease in Ca^{2+} influx mediated by NPY or baclofen may once more be

sufficient to affect transmitter release.

Experiments using co-cultured sympathetic neurons and atrial myocytes have indicated that NPY inhibits Ca^{2+} influx into presynaptic terminals through ω-conotoxin GVIA-sensitive N-type Ca^{2+} channels (Toth et al., 1993). Studies which test the ability of NPY to inhibit Ca^{2+} influx into hippocampal synaptosomes, however, have had mixed results. It has been reported that NPY failed to modify either basal or K^+-stimulated Ca^{2+} influx through Cd^{2+}-sensitive Ca^{2+} channels (Lundy and Frew, 1991). However, others have observed that NPY does inhibit K^+-stimulated $^{45}Ca^{2+}$ influx in the same preparation (Colmers et al., unpublished observations). Therefore, although the evidence for NPY's mediation of presynaptic inhibition, in the hippocampus, by reduction of Ca^{2+} influx is strong, this issue remains to be solved incontrovertibly.

The present study was not able to differentiate the presynaptic inhibitory mechanisms used by NPY and baclofen. Another set of experiments using synaptosomes prepared from rat cortex, striatum, or hippocampus have indicated that activation of a variety of presynaptic receptors, including $GABA_B$ and A_1, promotes K^+ efflux through dendrotoxin-sensitive K^+ channels (Zoltay and Cooper, 1993; 1993a; 1994; 1994a). The ability of NPY to do the same was not tested in these experiments. Whether or not these findings are of relevance to the inhibition of synaptic transmission is not known. They do not preclude the possibility that baclofen inhibits glutamatergic transmission via an action at Ca^{2+} channels. Since dendrotoxin blocks many of the same K^+ channels as does 4-AP, this could be tested by simultaneously lowering extracellular [Ca^{2+}], as in Chapter 3. It is possible that K^+ efflux through these channels is merely coincidental with a decrease in Ca^{2+}

influx, or that it subserves a different purpose.

In contrast to the presynaptic modulators examined in chapter 3, inhibition of hippocampal glutamatergic transmission by muscarine, acting at presynaptic muscarinic acetylcholine receptors, was not reduced in the presence of 4-AP (Klapstein et al., 1994). Rather, the effects of muscarine were antagonized by tetraethylammonium, an antagonist at several different K^+ channels (Storm, 1990), and were potentiated by Ba^{2+}, which blocks the inward rectifier and calcium-activated K^+ channels (Williams et al., 1988, 1988a); these results are consistent with activation of a presynaptic K^+ channel by muscarine, and lend further evidence that not all presynaptic modulators share common mechanisms.

Chapter 4 addresses the question of where in the hippocampus NPY acts. Previous experiments had shown that NPY inhibited glutamatergic synaptic transmission in CA1 and CA3 (Colmers et al., 1985; 1987; 1988; Haas et al., 1987; McQuiston and Colmers, 1992; Chapters 2 and 3), but pyramidal neurons in these areas receive input from several different afferent pathways. Whether these all respond identically to application of NPY had not been tested. In this chapter, specific afferent pathways to CA1 or CA3 pyramidal neurons or dentate granule cells were isolated either by selective placement of the stimulating electrode and/or by selective pharmacological antagonism. The ability of NPY to inhibit synaptic transmission was tested at each pathway separately. It was found that NPY inhibited all excitatory synapses onto principal cells tested in CA1 and CA3. However, NPY did not inhibit either the perforant path or the commissural inputs to dentate granule cells, nor did it directly inhibit GABAergic transmission in any of these areas. NPY also had no effect on feedback inhibitory transmission in area CA1 evoked by

antidromic stimulation of CA1 pyramidal cell axons, although it reduced feedforward inhibition in this area by the same amount as it reduced feedforward excitation, suggesting that it does so by reducing the synaptic drive to GABAergic interneurons by reducing synaptic excitation onto the somata of the cells innervating the interneurons. As was previously reported for area CA1, no effects by NPY were detected on resting membrane potential or input resistance of neurons. Interestingly, it has been shown that NPY, by activating post-synaptic Y_1 receptors, inhibits the influx of Ca^{2+} through N-type Ca^{2+} channels in the soma and dendrites of acutely dissociated granule cells (McQuiston and Colmers, 1994), however, no changes in whole cell recordings from granule cells were detected in these experiments, under conditions which did not isolate I_{Ca}. Together, these results suggest that, while NPY does not affect synaptic information entering the hippocampus through the dentate gyrus, it is able to attenuate such information as it passes through the rest of the hippocampus, and is thus capable of playing a physiological role under conditions of high activity which might cause its release (Lundberg et al., 1989).

How NPY affects the ongoing physiology of the hippocampus remains unknown. The hippocampus is clearly involved in memory storage, as bilateral removal in humans leads to a complete deficiency in the storage of long term memories (Scoville and Milner, 1957). Long-term potentiation, a model of learning and memory at the synaptic level, was first described in the hippocampus (Bliss and Lomo, 1973). Behavioural tests of memory formation or retention in mice have implicated NPY in these functions. In one study, injection of NPY into the rostral hippocampus of mice improved performance in an active avoidance task (Morley and Flood, 1990). Interestingly, similar injections into the caudal hippocampus had the

opposite effect. The authors suggested that the memory enhancement produced by NPY may be mediated by an inhibition of the release of GABA, thus facilitating excitatory, glutamatergic transmission. The results outlined in chapter 4 indicate that this is clearly not the case in rat hippocampus.

In addition to its role in learning or memory storage, the hippocampus is also frequently involved in pathological states, such as epilepsy. In chapter 5, the ability of NPY to inhibit epileptiform activity was tested in three *in vitro* models. It was found that NPY could inhibit ictaform afterdischarges in the stimulus train-induced bursting (STIB) model and spontaneous, synchronous, interictaform discharges in the $0Mg^{2+}$, picrotoxin, and STIB models. As might be expected from the findings of chapters 2, 3, and 4, Y_2 agonists such as NPY_{13-36} and [ahx$^{5-24}$]NPY and PYY were able to mimic the effects of NPY. Surprisingly, it was found that the Y_1 agonist Leu^{31}Pro^{34}NPY was also effective at inhibiting interictaform activity in all three models, although it had no effect on afterdischarges in the STIB model. Whether or not Leu^{31}Pro^{34}NPY mediates this effect by an inhibition of Ca^{2+} influx into dentate granule cells, as described above, is not known. Since this effect is insensitive to antagonism by the potent and selective Y_1 receptor antagonist, 1229U91, whereas the Y_1-mediated inhibition of Ca^{2+} currents in dentate granule cells is highly sensitive to this antagonist (McQuiston and Colmers, unpublished observations), it is unlikely that they are related. There are two possible explanations for these results. The first possibility is that Y_3 receptors, at which [Leu^{31}Pro^{34}]NPY is an agonist (Grundemar et al., 1991), are also involved. However, no evidence for Y_3 receptor binding has yet been found in the hippocampus. Moreover, since Y_3 receptors would constitute a fraction of the receptors mediating this response, one might expect that PYY, which

is not an agonist at this receptor, would be less effective than NPY, which activates both Y_2 and Y_3 receptors. This was not observed. On the contrary, PYY was perhaps even more potent than NPY. The second possibility is that [Leu^{31}Pro^{34}]NPY mediates inhibition of interictal bursts by activating a different NPY receptor subtype, such as that in the hypothalamus which mediates the feeding response (Stanley et al., 1992). This hypothesis could be tested by application of PP to one of the *in vitro* slice epilepsy models.

A novel finding in chapter 5 is that hippocampal slices which have undergone stimulus train-induced bursting also exhibit spontaneous, rhythmic, synchronous events (SRSEs), which are evident in whole-cell slice patch recordings from CA3 pyramidal neurons. SRSEs in CA3 pyramidal cells are mediated by $GABA_A$ receptors, (but also require AMPA receptor activation); in interneurons, they are mediated directly by AMPA receptors. These events are similar in appearance and frequency to those previously described in epileptic human hippocampus (Schwartzkroin and Haglund, 1986). As expected from the findings of chapter 4, these events are not sensitive to inhibition by NPY.

Why these events occur or where they originate is not known. The observations that SRSEs, recorded in either cell type, are sensitive to the AMPA receptor antagonist, NBQX, and that their frequency is not altered by local paroxysms indicate that they are driven by a relatively distant, rhythmically active, glutamatergic source. Experiments to locate this source have not been conducted, however, one series of such experiments might involve the lesioning of selective afferent pathways following establishment of these events, to determine which pathways are necessary for their maintenance. Gap junction decouplers, such as

propionate, might be used to determine whether the afferent pathway drives many interneurons synchronously but independently, or whether a single interneuron receives rhythmic excitation and subsequently excites a syncytium of interneurons. This experiment would not preclude the possiblity that a single interneuron, making thousands of synaptic contacts throughout CA3 (Gulyas et al., 1993) is the source of SRSEs observed in pyramidal cells throughout this area. Since SRSEs were observed in four out of four interneurons in both stratum radiatum and stratum oriens, this possibility is very highly unlikely. Whether SRSEs contribute to the pathology of epilesy, or whether they constitute a compensatory mechanism is not known. Therefore, the implications of NPY's failure to inhibit them are also not known. The implications of NPY's ability to inhibit epileptiform activity are somewhat more obvious. That NPY is able to do so, suggests that it may play a role in reducing the high levels of excitation present during epileptic events *in vivo*. A test for this hypothesis awaits the development and availability of a selective Y_2 antagonist.

In summary, the data presented here have shown that presynaptic modulators are not all alike. In the rat hippocampal slice, NPY is unique, in that it potently and selectively inhibits feedforward glutamatergic synaptic transmission, without directly affecting GABAergic inhibition. The consequences of this are not entirely clear, however, NPY has been shown here to limit epileptiform activity in several different *in vitro* models, by acting at Y_2 receptors, and possibly a different NPY receptor subtype. Whether or not this is its primary activity *in vivo* is not yet known. Further experiments are required which test NPY's actions under more physiological conditions.

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