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Neuropeptides, amines and amine receptors in the human spinal cord: The effects of Parkinson's disease

by

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Abstract

The aims of this study were to investigate (i) the levels of catecholamines, indoleamines, substance P and thyrotrophin-releasing hormone (TRH) in the post-mortem spinal cord of subjects who had died with Parkinson's disease and to compare them with those of control subjects (ii) adrenergic and serotonergic receptors in the post-mortem Parkinsonian and control spinal cord and (iii) the effects of subject age and sex and the interval between death and post-mortem (PMI) on the levels of neurotransmitters and neuropeptides and on receptor binding in post-mortem tissue.

To perform these investigations (i) a sensitive radioimmunoassay which is specific for substance P and has low cross-reactivity with other similar peptides and (ii) a common extraction medium for the concomitant extraction of catecholamines, indoleamines, substance P and TRH from CNS tissue were developed.

The main findings were:

There were significant correlations between the levels of 5HT, TRH and α_2-adrenoceptor binding and both subject age and the PMI.

In Parkinson's disease compared with control subjects:
      (i) the levels of noradrenaline were significantly reduced in the thoracic ventral region of the spinal cord,
      (ii) dopamine levels were higher in the thoracic ventral and dorsal spinal cord,
      (iii) in the lumbar spinal cord 5HT levels were significantly reduced in the dorsal horn with an increase in the ratio of 5HIAA/5HT,
      (iv) noradrenaline levels were reduced in both dorsal and ventral horns of the lumbar spinal cord and
      (v) there were no differences between the levels of substance P and TRH in any spinal cord region.

There were no measurable $5HT_{1A}$ or $5HT_2$ binding sites in the human spinal cord under the conditions used. However, specific α_2-adrenoceptor binding was defined in terms of binding affinity and number of receptors in the spinal cord.

Acknowledgements

Errata

1. Throughout the text "et. al." should read "et al.".
2. Page 18: ChAT is an abbreviation of choline acetyl transferase.
3. Page 128: DDD is an abbreviation of deionised double distilled water.

Abbreviations

Abbreviations which appear frequently throughout the text:

NA	noradrenaline
ADR	adrenaline
DHBA	dihydroxybenzoic acid
DOPAC	dyhydroxyphenyl acetic acid
DA	dopamine
HVA	homovanyllic acid
5HT	5-hydroxytryptamine
5HIAA	5-hydroxy indole acetic acid
5HNMO	5-hydroxy-N-methyl oxalate
TRH	thyrotrophin-releasing hormone
HPLC	high performance liquid chromatography
RIA	radioimmunoassay
IR	immunoreactivity
(peptide)-LI	(peptide)-like-immunoreactivity
PMI	post-mortem interval
IML	intermediolateral cell column
MAO	mono-amine oxidase
NIH	nucleus interfasicularis hypoglossi
NG	nucleus gigantocellularis
Pd	Parkinson's disease
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
L-dopa	levodopa
As.	antiserum
U.V.	ultra-violet
PCA	perchloric acid
eth/AA/NaMBS	ethanol/acetic acid/sodium metabisulphite
ANCOVA	analysis of co-variance
BSA	bovine serum albumin
AmiK	7-amino-8-iodo-ketanserin
NSB	non-specific binding
DPAT	8-hydroxy-2-(di-n-propylamino) tetralin
KD	dissociate binding constant
Bmax	maximum number of specific receptor sites
TBZ	tetrabenazine
PYR	pyrilamine
PRZ	prazosine
SEM	standard error of the mean
DDD	deionised double-distilled water

Chapter 1　General Introduction

Introduction

It is now well established, on the basis of animal studies, that a number of neuropeptides co-exist with the classical neurotransmitters in neurones in various regions of the CNS (Hokfelt et.al.,1980; Chan-Palay and Palay, 1984). Reviewed here in the General Introduction is the evidence for co-existence of the neuropeptides substance P and thyrotrophin-releasing hormone (TRH) with 5-hydroxytryptamine (5HT, also known as serotonin) in the spinal cord of several species. Much of the evidence comes from studies involving stereotaxic or chemical lesions of spinal serotonergic pathways. As these experiments are obviously not possible to perform on humans evidence for co-depletion of neurotransmitters and peptides is difficult to attain. However, specific neurotransmitter systems are known to degenerate in certain pathological conditions and one approach is to use such conditions to study the concomitant effects on neuropeptide levels. In this study the neurological disorder, Parkinson's disease, in which spinal cord 5HT levels are depleted and which may represent degeneration of spinal cord serotonergic pathways, is used to investigate whether 5HT depletion is paralleled by depletion of substance P and TRH in the human spinal cord. Parkinson's disease is generally regarded as a condition associated with wide spread loss of dopamine and degeneration of dopaminergic systems in the brain (Ehringer and Hornykiewicz,1960; Javoy-Agid et. al.,1982). However, recent evidence indicates there is also extensive loss of 5HT and noradrenaline in the post-mortem spinal cord from Parkinsonian patients (Scatton et.al.,1986). In this study the levels of the catecholamines, noradrenaline, adrenaline and dopamine, and of 5HT and their metabolites and of the neuropeptides substance P and TRH are determined in the human spinal cord. Comparisons are made between levels in the spinal cord from patients having died with Parkinson's disease and from control subjects to further investigate the reported diminution of 5HT and noradrenaline and to determine whether changes in 5HT levels are paralleled by changes in neuropeptide levels.

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In addition, serotonergic and noradrenergic receptor binding is investigated in the spinal cord from Parkinsonian patients and the results are considered in the light of the changes seen in noradrenaline and 5HT levels.

There is evidence from animal studies that the catecholamines, indoleamines and neuropeptides have roles in the sensory and motor functions of the spinal cord (reviewed below). Moreover, it appears that there may be a great deal of interaction between these neurochemicals in the modulation of these functions. In addition therefore, consideration is given to the roles of catecholamines, indoleamines, substance P and TRH in the sensory and motor functions of the spinal cord and the relevance of these transmitters and peptides to the symptoms of Parkinson's disease. References to amines and peptides measured by immunogenic techniques should for accuracy include the term immunoreactivity, e.g. substance P-immunoreactivity or more precisely substance P-like immunoreactivity, as it is this that is actually measured. However, for fluency of reading this term has been omitted unless it provides additional information or aids the clarity of the sentence.

Background

The Spinal Cord

The spinal cord begins from the caudal end of the brain stem and comprises four main segments on descending the vertebral column, namely the cervical, thoracic, lumbar and sacral cord. On cross-section the cord can be seen to be divided into two regions, the gray and white matter. The more darkly coloured gray matter forms an 'H' shape and is surrounded by white matter. The latter is comprised primarily of myelinated ascending and descending tracts. The gray matter is made up of three areas, the dorsal (posterior), intermediate and ventral (anterior) regions (Fig.1.1). The dorsal horn appears laminated and these areas are termed lamina I (the marginal zone), lamina II (the substantia gelatinosa), and laminae III, IV and V (Fig.1.1). In the thoracic segment of the cord there is an extension of the intermediate region

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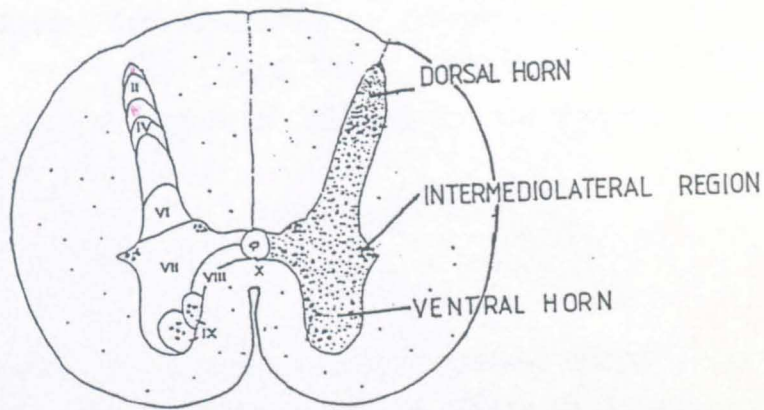
Fig. 1.a Cross-section of the mid-thoracic cord.
The laminae of the gray matter are shown on the left and the
general regions on the right.
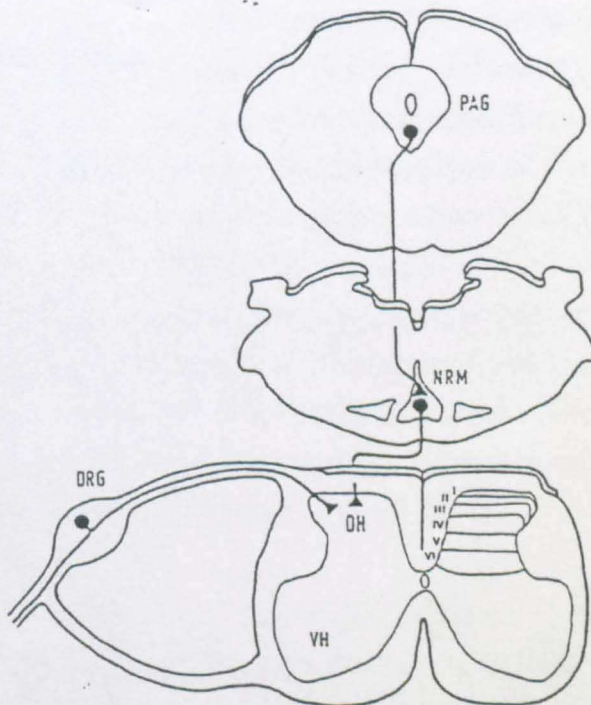
Fig. 1.c Simplified representation of afferent and descending
projections to the dorsal horn of the spinal cord involved in pain
processing. PAG = periaqueductal gray; NRM = nucleus raphe
magnus; DRG = dorsal root ganglion; DH = dorsal horn; VH =
ventral horn. Roman numerals designate the laminar
arrangement of the dorsal horn. (Illustration taken from Jessell,
1982.)

called the sympathetic lateral horn, intermediate horn or intermediolateral cell column (IML). Within each of these regions are several groups of cell nuclei which are associated with particular functions.

Spinal cord function

The dorsal horn is primarily concerned with conveyance of sensory information. High threshold mechanical, thermal and noxious stimuli are mediated via sensory neurones with cell bodies located within the dorsal root ganglia (Fig.1.2). Nearly all of the sensory neurones responding to intense cutaneous stimulation pass into laminae I and II of the dorsal horn in all spinal segments. Neurones conveying information from sensory endings responding to light pressure terminate in the deeper layers of the dorsal horn (Brown, 1981). It has been recently shown that there is somatotopic organization of afferent nerve fibres from the limbs in the dorsal horn (Molander and Grant,1986).

The ventral horn contains the nuclei of the motoneurones. Motoneurones serving the musculature of the limbs are found in the lateral regions of cervical and lumbar ventral horn. Motoneurones serving the muscles of the trunk are located in the medial region of the ventral horn in all segments of the cord (Fitzgerald, 1985). The simple reflex arc illustrated in Fig.1.3 enables a rapid reflex motor response to sensory input from muscles independent of supraspinal control.

The IML of the thoracic cord is the main site of origin of preganglionic sympathetic neurones which innervate the viscera (Strack et.al.,1988) and will not be discussed in detail.

It has been considered for some time that activity in sensory and motor afferent fibres in the spinal cord are influenced by descending pathways from the brain (Eccles,1964; Schmidt,1973). There is also evidence that spinal nocioceptive and motor reflexes are modulated by supra-spinal activity (Fields and Basbaum,1978; McCall and Aghajanian, 1979) for example inhibition of synaptic transmission from nocioceptors to neurones in the dorsal horn may be responsible for the analgesic effects of brainstem

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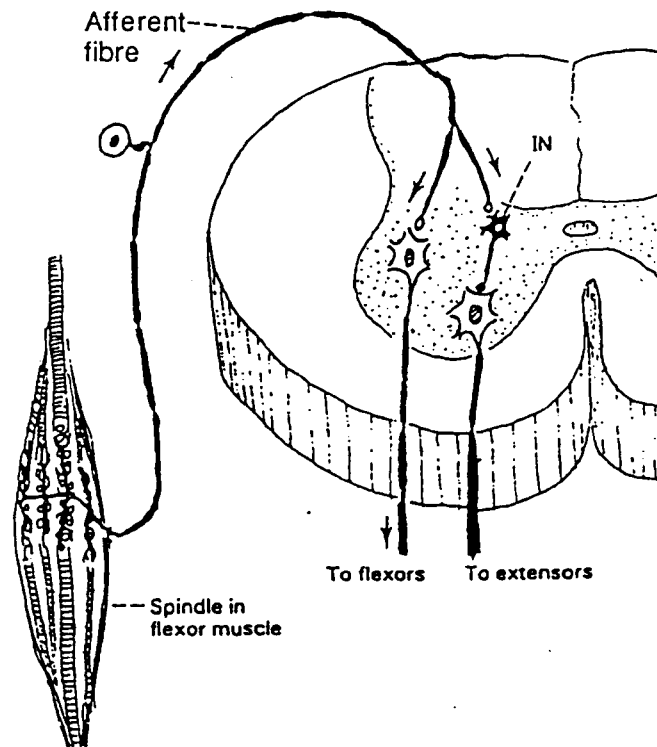
Fig. 1.3 Neurones involved in spinal reflex. This diagram
shows the neurones involved in a reflex action occuring at the
level of the spinal cord. The interneurone (IN) is inhibitory and
the afferent input results in activation of flexor muscles and
simultaneous inhibition of extensor muscles.
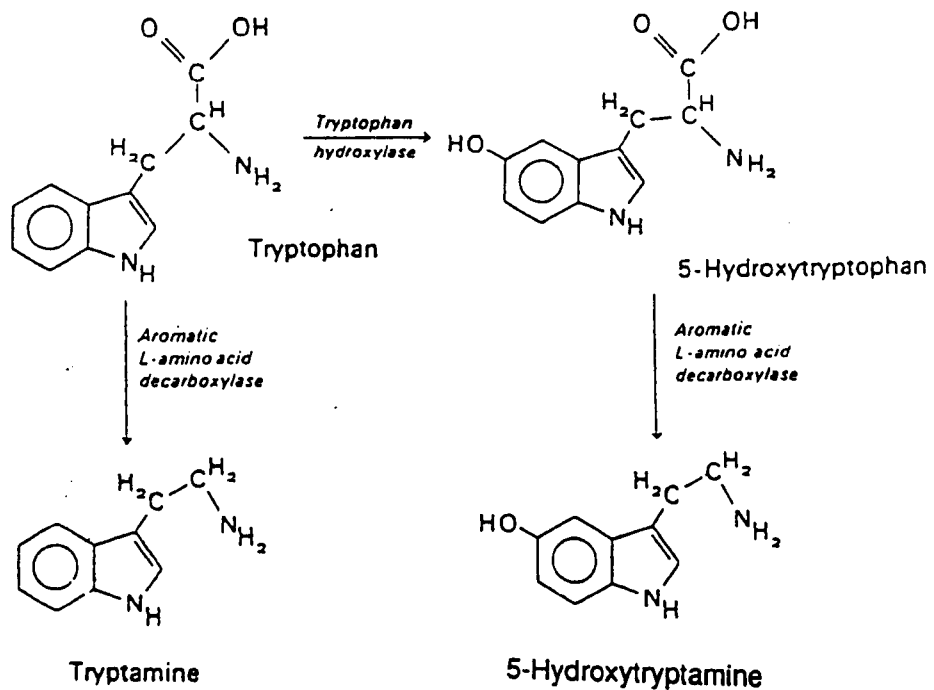
Fig. 1.4 Biosynthesis of 5-hydroxytryptamine.

stimulation (Carstens et.al.,1980; Fields and Basbaum,1978). The possible origins and transmitters of the supraspinal tracts involved in modulation of spinal motor control and sensory function are discussed below.

Neurotransmitters of the spinal cord 1: 5HT, substance P and TRH

5-hydroxytryptamine: synthesis

The biosynthetic pathway of 5HT is illustrated in Fig.1.4. 5HT is synthesized from the essential amino acid tryptophan via the intermediary 5-hydroxytryptophan, the process occurring in the neurones in which 5HT is found. Conversion from tryptophan to 5-hydroxytryptophan is catalysed by the enzyme tryptophan hydroxylase and is the rate limiting step in the synthesis of 5HT. The rate of 5HT synthesis depends on the availability of tryptophan (see Bowman and Rand,1984; Green and Grahame-Smith,1976). After synaptic release a proportion of 5HT is transported back into the pre-synaptic terminal by re-uptake mechanisms. 5HT is metabolised by mono-amine oxidase (MAO) to 5-hydroxyindoleacetic acid (5HIAA) and this may occur both in the synaptic cleft and in the neuronal cytoplasm (Wolf et.al.,1985). Recent evidence indicates that type A MAO (see later page 24) is primarily or exclusively responsible for nerve terminal 5HT metabolism (Fagervall and Ross,1986; Azzaro et.al.,1988).

5HT: distribution

Using immunohistochemical techniques, a serotonergic innervation has been visualized in the dorsal and ventral horns of all segments of the spinal cord and also in the thoracic IML region in close apposition to sympathetic preganglionic neurones in the rat (Hokfelt et.al.,1978; Johansson et.al., 1981; Bowker et.al.,1982; Appel et al.,1987). Similarly, serotonergic innervation of the ventral and dorsal spinal cord of the cat (Holets et.al.,1987) and the ventral horn of the primate (Bowker, 1986) has been demonstrated. In general 5HT immunoreactive fibres are present throughout the

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spinal gray matter but are especially dense in laminae I and II of
the dorsal horn, laminae VIII and IX of the ventral horn (Fig.1.1)
and in the IML. The regions in which the 5HT immunoreactive
terminals are most dense are laminae I and II, the IML and the
ventral motor regions (Bowker et.al.,1983).

In addition, the levels of 5HT have been determined by
HPLC in the ventral and dorsal horns and in the IML of the spinal
cord in the rat (Gilbert et.al 1982; Marsden et al,1982; Lighton
et.al.,1984; Basbaum et.al.,1987) the human (Bennett et.al., 1986;
Scatton et.al., 1986), and the rabbit (Zivon et.al.,1975; Fone
et.al.,1987b). In these studies there is general agreement that
levels of 5HT are 1.1-2 fold greater in the ventral than in the dorsal
horn and that the neurotransmitter is present in all segments of the
spinal cord. In a detailed interlaminae and intersegmental study
of 5HT levels in the rat spinal cord Basbaum and co-workers
(1987) report that 5HT levels increase caudally from cervical to
lumbar cord and that 5HT levels were highest in the region of the
motoneurones (laminae IX). The next highest levels were found
in laminae X followed by the IML and dorsal horn.

5HT: brainstem projections to the spinal cord

In 1965 Dahlstrom and Fuxe (1965) described a serotonergic
projection from the medullary raphe nuclei (the raphe magnus,
raphe pallidus and raphe obscurus) to the spinal cord. Serotonergic
innervation from these nuclei has since been shown to project to the
thoracic IML, and the ventral and dorsal horns of all spinal cord
segments (Kuypers and Maisky, 1975; Amendt et.al.,1979;
Basbaum and Fields,1979) and electrical stimulation of the raphe
cells mimics the effects of direct application of 5HT to the spinal
cord neurones (White and Neuman, 1980). Serotonergic
projections from the raphe nuclei and the nucleus
gigantocellularis (NG) in the adjacent reticular formation to the
spinal cord have been demonstrated in the rat, (Bowker et.al.,1983;
Loewy and McKellar, 1981; Loewy, 1981; Huisman et.al.,1984;
Millhorn et.al.,1987), the monkey (Kneisley et.al.,1978) and the
possum (Martin et.al.,1981) in studies using retrograde transport
with immunocytochemical and immunohistochemical

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techniques. In the cat, raphe projections to the spinal cord have been described (Martin et.al.,1978; Holstege and Kuypers,1982) as has 5HT immunoreactivity (IR) in cell bodies of neurones of the ventrolateral medulla which have been shown to have direct contact with spinal sympathetic areas (Ciriello et.al.,1988) . Furthermore, Bowker and co-workers (1987) determined that more than 85% of the descending raphe neurones from the raphe pallidus and raphe obscurus and 75% of raphe neurones from the raphe magnus contain 5HT. The evidence indicates that the greatest part of the descending raphe magnus innervation is to the dorsal horn whilst that of the pallidus and obscurus is to the IML and ventral horn (Basbaum et al.,1978; Holstege and Kuypers, 1982; Martin et.al.,1978). In addition there is evidence of a serotonergic innervation to the lumbar spinal cord of the cat arising from the locus coeruleus of the pons which contains mainly noradrenergic cells (Leger et.al,1979; Wiklund et.al.,1981; Lai and Barnes, 1985).

Substance P

Substance P was detected as a peptide with vasodilator properties present in the brain as earlier as 1931 (von Euler and Gaddum, 1931) but its chemical composition was not identified until much later (Chang and Leeman,1970). The undecapeptide structure of substance P is illustrated in chapter 2 (Fig.2.2). Small biologically active peptides are generally believed to be synthesized from larger precursors and analysis of DNA sequencing has demonstrated the presence of substance P precursors (α- and ß-preprotachykinin) in bovine brain and in human carcinoid tumour from which the substance P structure is cleaved (Nawa et.al.,1983; Harmar et al.,1986). Inactivation of substance P appears to be due to degradation by membrane bound peptidases rather than re-uptake though substance P metabolites may have biological activity of their own (Iversen et.al.,1976; Arzumanyan et.al.,1985; Chapter 2, page 61). A wide and heterogeneous distribution of substance P within the CNS has been described from radioimmunoassay and immunocytochemical studies (Cuello and Kanzawa,1978; Hokfelt et.al.,1976; Strabanek

and Powell,1977) and its role as a neurotransmitter or more probably as a neuromodulator has been established by satisfaction of the necessary basic criteria (Pernow,1983 for review; Otsuka and Konishi,1983; Frenk et.al.,1988a,b).

An early study of the distribution of substance P in the rat spinal cord reported an extremely dense network of substance P containing fibres and cell bodies in the substantia gelatinosa and a less dense distribution in the ventral horn (Hokfelt et.al.,1975). Subsequent studies have confirmed that substance P is located in the ventral and dorsal horns in all cord segments and in the IML but its dense distribution within the dorsal roots and the dorsal horn of the spinal cord in particular indicate a role in mediation of sensory information. Immunohistochemical and radioimmunological studies have described the presence of substance P in the spinal cord and dorsal root ganglia of rat (Chan-Palay and Palay,1977; Cuello and Kanzawa,1978; Ljungdahl et.al.,1978), cat (Hokfelt et.al,.1975b; Takahashi and Otsuka, 1975), pig (Holzer et.al.,1981), monkey (DiFiglia et al.,1982; Lanerolle and La Motte,1982), and humans (Cuello,1976; Anand et.al.,1983; Przewlocki et.al.,1983; Bennett et.al.,1986). The brainstem origins of the substance P terminals in the spinal cord have been demonstrated to be the raphe nuclei and nuclei of the adjacent retricular formation (Bowker et.al.,1983; Gilbert et.al.,1981; Menetry and Basbaum,1987). As such, there is a great deal of overlap of the descending pathways containing substance P and 5HT and the evidence for co-existence of substance P with 5HT is discussed below.

TRH

Original interest in TRH was due to its role as a hypothalamic releasing factor and this lead to its chemical characterization (Boler et.al., 1969). TRH is a small peptide of only 3 amino-acids (pyroGlu-His-ProNH2). There is evidence that it is derived from a much larger peptide, prepro-TRH, which is processed to 5 molecules of TRH via an intermediary sequence, pro-TRH (Lechan et.al.,1986; Wu and Jackson, 1988). TRH is rapidly degraded by metabolism at both terminals by neuronal

peptidases (Prasad and Peterkofski, 1976; Griffiths et.al.,1980). A
wide distribution of TRH throughout the CNS has been described
(Brownstein et.al.,1974; Winokur and Utiger, 1974) and
experimental evidence supports the principle that TRH has a
neuromodulator or neurotransmitter role in the CNS (Brown,1981;
Reichlin,1986 for reviews).

TRH has been located in the ventral and dorsal horns and
in the IML region of the spinal cord using immunocytochemistry
and immunofluorescence (Hokfelt et.al.,1975; Gilbert et.al.,1981;
Johansson et.al.,1981; Harkness and Brownfield,1985). Radio-
immunoassay studies indicate that levels of TRH are greater in
the ventral than in the dorsal horn in the rat (Marsden et.al.,1982;
Lighton et.al.,1984), rabbit (Fone et.al.,1987b), monkey (Lechan
et.al., 1984), and human (Yates et.al.,1983; Mitsuma et.al.,1984;
Bennett et.al.,1986). The brainstem origins of spinal cord TRH
include the medullary raphe nuclei, and the nuclei of the
retricular formation (Johansson et.al.,1981; Bowker et.al.,1983)
and the evidence for co-existence of TRH with 5HT in the
descending raphe neurones is discussed below.

Substance P and TRH: Brainstem origin of the neuropeptides and
evidence of co-existence with 5HT

Co-existence of neuropeptides with classical
neurotransmitters within the CNS is now well established (Hokfelt
et. al.,1980; Chan-Palay and Palay,1984). There is evidence that
the neuropeptides substance P and TRH co-exist with 5HT in the
descending raphe (bulbospinal) pathway. This evidence comes
from localization of TRH and substance P within the raphe cell
bodies, within descending serotonergic fibres and in nerve
terminals. This evidence is reviewed here.

' Cell bodies containing substance P were located in the
ventral medulla with a similar distribution to that of 5HT cell
bodies (Ljungdahl et.al,.1978). Subsequently, the distribution of
substance P in the serotonergic raphe nuclei and in the nucleus
gigantocellularis and the nucleus interfasicularis hypoglossi
(NIH) of the retricular formation has been confirmed
(Johansson et.al.,1981; Bowker et.al.,1983; Chan-Palay, 1979;

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Menetry and Basbaum,1987). Substance P and 5HT were also demonstrated to have a similar distribution in nerve terminals in the spinal cord (Hokfelt et.al.,1978; Johansson et.al.,1981; Gilbert et.al.,1981; Gilbert et.al.,1982). Substance P containing fibres from the medullary cell bodies were demonstrated to project to the spinal cord (Bowker et.al.,1983; Gilbert et.al.,1981; Menetry and Basbaum,1987) and substance P and 5HT were shown to have an overlapping distribution in spinal cord nerve fibres (Johansson et.al.,1981; Gilbert et al.,1982). Destruction of the serotonergic fibres resulted in loss of substance P at the level of the spinal cord and it was proposed that approximately 40-50% of the serotonergic projections to the ventral horn and IML also contain substance P though the extent of the supraspinal origin of dorsal horn substance P is much lower (Johansson et.al.,1981; Gilbert et.al., 1982; Marsden et.al.,1982; Bowker et.al.,1983).

Similarly, TRH has been located in the raphe nuclei of the medulla and of the nuclei of the retricular formation (Johansson et.al.,1981; Bowker et.al.,1983) and in nerve terminals in the ventral and dorsal horns and the IML of the spinal cord in a similar distribution to that of 5HT and substance P (Johansson et.al.,1981; Gilbert et.al.,1982; Lechan et.al.,1984).

The results of neurochemical and electrothermal lesions of the raphe nuclei, the NIH, the NG and the spinal pathways indicate that TRH containing fibres project from these nuclei to the ventral and dorsal horns of the spinal cord and the IML and that the proportion of TRH projections to the IML and ventral horns which are also serotonergic may be as much as 90% (Marsden et.al.,1982; Gilbert et.al.,1981; Gilbert et.al.,1982; Helke et.al., 1986; Appel et.al.,1987).

There has been a search for evidence that substance P, 5HT and TRH measured in cell nuclei, fibres and terminals are located within the same cells. By combining immunofluorescence histochemistry and elution/restaining techniques with serial sections Johansson and co-workers (1981) were able to demonstrate the co-existence of 5HT, TRH and substance P within cells of the medulla and retricular formation. In addition, using the technique of simultaneous double labeling, TRH and 5HT or TRH

and substance P have been co-localized in the fibres and terminals of the spinal cord (Bowker et.al.,1986; Appel et.al.,1987). Furthermore, recently 5HT, TRH and substance P have been visualized concomitantly in descending bulbospinal neurones using a triple labelling method (Staines et.al.,1987).

In summary, there is evidence that in the rat 80-90% of the projections from the raphe nuclei to the spinal cord are serotonergic and some of these fibres also contain substance P and/ or TRH. There are also descending serotonergic and peptidergic pathways from the adjacent retricular formation. The origins and distribution of the serotonergic and peptidergic fibres of the spinal cord are not identical but overlap so that various proportions of these pathways contain 5HT alone or with one or both of the neuropeptides substance P and TRH (Johansson et.al.,1981; Bowker et.al.,1983; Helke et.al.,1986).

Co-storage of 5HT, substance P and TRH

Further to the investigation into co-existence of TRH and substance P with 5HT, there have been some studies into the intraneuronal storage and release of these neurochemicals. Substance P and 5HT have been located in the same dense core vesicles in nerve terminals of the raphe nuclei and dorsal spinal cord using serial sections. No more than 20% of serotonin containing nerve terminals were also positive for substance P (Pelletier et.al.,1981). Depletion of spinal cord 5HT, by compounds known to cause neuronal depletion of 5HT by interfering with its storage, is accompanied by depletion of substance P and TRH which is less marked and of a different time course (Gilbert et.al.,1981). As proposed by the authors these results do not conclusively indicate a direct action of the drugs on vesicles co-storing 5HT with substance P and /or TRH but may reflect a secondary effect on peptide release due to depletion of 5HT stores. Furthermore, that 5HT releasing agents p-chloroamphetamine (PCA) and fenfluramine have different effects on 5HT and TRH release and also affect TRH release in brain areas where 5HT and TRH are not known to co-exist indicates that it is likely that there

is no co-storage of 5HT and TRH (Marsden et.al.,1982). In addition, using gradient centrifugation of isolated synaptic vesicles and synaptosomes a distribution of substance P and 5HT has been demonstrated which may indicate that at least one population of 5HT containing vesicles does not contain substance P (Fried et.al.,1984). It therefore appears that in the main at least, co-existent 5HT, substance P and TRH are stored separately. Further experimental data supports this concept as both low frequency stimulation and PCA in low doses are capable of releasing [^{3}H] 5HT from loaded slices of ventral and dorsal spinal cord without substantial release of substance P (Bartfai et.al.,1986). However, although substance P and 5HT storage and release mechanisms may be separate there is evidence of interaction between them. Rats were treated with pharmacological agents which block 5HT uptake at nerve terminals and which are known to result in a reduction in the firing rate of serotonergic neurones. As a result tissue levels of 5HT fell by 10-20% in the hypothalamus, cortex and ventral spinal cord. Tissue levels of substance P were unchanged in the brain areas where the peptide is not co-existent with 5HT but were significantly increased in the dorsal and ventral horn spinal cord, thus indicating that substance P builds up either because of reduced 5HT axonal firing or through a receptor mediated effect brought about by the alteration in 5HT release (Bartfai et.al.,1986 for review). Substance P (and other neuromodulators) may be stored in large dense core vesicles which appear to secrete their neurochemical contents at non-specialized areas of the membrane whereas classical transmitters have been visualized in small synaptic vesicles. This indicates that exocytosis involving large vesicles may serve to deliver neuropeptide modulators into a wider area and on to appropriate receptors than release into a specialized synaptic cleft would allow (Zhu et.al.,1986)

Evidence for functional significance of co-existence of 5HT, substance P and TRH

There is strong evidence for physiological interaction between 5HT, substance P and TRH in the spinal cord and for the

importance of interaction between these neurochemicals in spinal cord function. TRH appears to enhance the stretch reflex through interaction with a site closely associated with the 5HT receptor (Barbeau and Bedard, 1981) . Additionally, substance P causes a concentration dependent inhibition of TRH receptor binding in the spinal cord, especially in the ventral horn and possibly through a discrete population of low affinity substance P receptors (Sharif and Burt, 1983). Furthermore, co-depletion of 5HT and TRH in spinal neurones by neurotoxins is accompanied by a 30-40% increase in the number of TRH binding sites which is apparently dependent not on TRH modification but on the loss of serotonergic neurones (Sharif et.al.,1983). Also, 5HT can increase the K^+-stimulated release of substance P from nerve terminals in the ventral spinal cord and that this may be effected via $5HT_2$ receptors (Bartfai et.al.,1986 for review). These effects appear to be mediated through post-synaptic receptors. Other evidence implicates interaction of 5HT, substance P and TRH at pre-synaptic receptors. Substance P has been shown to antagonise inhibition of 5HT release caused by high 5HT concentration in the lumbar ventral spinal cord (Mitchell and Fleetwood-Walker,1981) and long-term intrathecal administration of TRH analogues elevate levels of 5HT in the ventral spinal cord (Fone et.al.,1988a) These effects are possibly mediated through interaction with pre-synaptic 5HT autoreceptors. In summary, these reports indicate that 5HT, substance P and TRH interact physiologically in the spinal cord at the level of both pre- and post-synaptic receptors and that substance P and TRH both tend to enhance 5HT transmission though by different mechanisms.

Substance P and TRH also demonstrate interactions independent of 5HT. Substance P antagonists can cause necrotic damage to the gray matter of the spinal cord when administered intrathecally (Post and Paulsson,1985; Freedman et.al.,1986). It has been observed that repeated treatment with TRH before and after administration of the substance P antagonist prevents the inducement of necrosis (Post and Paulsson,1985; Freedman et.al.,1986). Hokfelt and co-workers (1986) suggest that the protective effect of TRH in the descending bulbospinal pathways is mediated by blockade of the substance P receptor, thereby

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preventing interaction between the receptor and the substance P antagonist.

The evidence indicates that the concept of an autoreceptor includes regulation of transmitter release not only by the transmitter itself but also by release of co-existent peptides. Substance P and TRH may therefore act as modulators of the effects of 5HT by acting both pre- and post-synaptically, thereby incorporating a greater degree of flexibility into regulation of CNS functions mediated by 5HT. Separate, but interactive storage and release mechanisms for 5HT, substance P and TRH gives rise to the possibility of differential release of these neurochemicals by different patterns of physiological stimulus. It has been proposed that the functional significance of co-existence of substance P and TRH with 5HT may be to 'push through' an important message travelling in a small system by concomitant release of multiple transmitters and the authors provide a hypothetical model for such a mechanism (Hokfelt et.al.,1984a). Co-existent peptides may also be important in regulating the sensitivity of receptors to 5HT and thereby providing a flexible regulation of the effect of stimulation of the serotonergic system. It has been proposed that co-existing peptides change the characteristics of the receptors for monoaminergic transmitters at the post-synaptic level without at the same time inducing compensatory responses at the pre-synaptic level (Fuxe et.al.,1983). Reviewed here is the evidence that (i) the interaction between 5HT and the co-existent peptides substance P and TRH in the ventral horn is important in motor control and possibly supra-spinal modulation of spinal cord motor function and (ii) 5HT, substance P and TRH may play interactive roles in nociception.

The role of 5HT, substance P and TRH in spinal cord motor control

5HT

Early histofluorescence studies indicated projections from the brainstem raphe nuclei to the motonuclei of the ventral horn of the spinal cord (Dahlstrom and Fuxe, 1965). It has since been confirmed using retrograde labeling that the more caudal nuclei,

the raphe pallidus and raphe obscurus and nuclei of the adjacent
retricular formation including the NIH and NG project directly
onto the motoneuronal cell groups in the spinal cord and that the
bulbospinal projections from the raphe nuclei terminate in the
ventral horn, closely apposed to the nuclei of motoneurones (Hayes
and Rustioni,1981; Huisman et.al.,1980; Huisman et.al.,1984;
Martin et.al.,1981). Immunohistochemical studies have revealed
5HT in synaptic boutons apposing motoneurones (Atsumi
et.al,.1985; Takeuchi et.al.,1983). In addition, by combining
anterograde transport of leucine from the brain stem nuclei and
retrograde transport of horse-radish peroxidase (HRP) from the
hind leg muscles direct contacts between descending neurones
and lumbar motoneurones have been demonstrated (Holstege and
Kuypers,1987a). These findings have been confirmed recently
using a different method, anterograde transport of wheat germ
agglutin in combination with a chromagen which can be
visualized by electron microscopy (Holstege,1987). Physiological
evidence also indicates an interaction between 5HT and spinal
cord motor function as 5HT increases the excitability of
motoneurones when applied systemically or by bath application to
isolated tissues (McCall and Aghajanian, 1979; White and
Neuman,1980; White,1985). Similarly electrical stimulation of
the raphe nuclei also produced facilitation of motoneurone
excitability which can be blocked by 5HT antagonists (Cardona
and Rudomin,1983). Recently, it was demonstrated that stimulus
induced plateau potentials in spinal motoneurones were abolished
after transection of the spinal cord and then re-appeared after
injection of a 5HT releasing agent (Hounsgaard et.al.,1988). In
general the evidence supports the principle that activation of the
bulbospinal pathway and release of 5HT facilitates the excitability
of the motoneurones (McCall and Aghajanian, 1979). The results
of injecting fluorescence markers into different levels of the
spinal cord and then investigating the brainstem nuclei to which
they are transported indicate that projections from the raphe nuclei
have collaterals which terminate diffusely at different levels of the
spinal cord (Huisman et.al.,1984). A diffuse system, as the
authors suggest, may subserve generalized motor functions such
as the overall responsiveness of motoneurones. This may be

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mediated via supra-spinal modulation of muscle afferent fibres in the spinal cord (Eccles,1964; Schmidt,1973). There is evidence that a post-synaptic 5HT$_2$ receptor is implicated in mediation of motor behaviours including head twitches, fore-paw treading and wet-dog shakes (Leysen, 1989; Fone, 1989a). In summary, the spinal cord serotonergic system appears to be involved in motor function both at the level of spinal cord reflexes and supra-spinal modulation of reflexes.

Substance P and TRH

There has now been amassed a catalogue of data supporting the proposal that substance P and TRH have similar effects on the motoneurones as 5HT itself. Firstly, substance P and TRH are found in the ventral horn and IML in the vicinity of motoneurones (Johansson et.al.,1981; Hokfelt et.al.,1975b: Appel et.al.,1987). Substance P-immunoreactivity in synaptic boutons has been observed in close apposition to motoneurones in the ventral spinal cord of the rat (Barber et.al.,1979; Vacca et.al.,1982) and in the monkey and human spinal cord (Lanerolle and La Motte,1982). Also, TRH has also been observed in synaptic boutons (Johansson et.al.,1980). In the rat autoradiographic studies have demonstrated anterograde transport of [^{3}H] leucine from the raphe nuclei to terminals of spinal motoneurones which morphologically resemble terminals which contain 5HT and / or substance P or TRH (Holstege and Kuypers,1987a). In a recent study 5HT, TRH and substance P containing axonal boutons and varicosities were observed in close contact and forming synaptic contact with dendrites of motoneurones in the motor nucleus of the spinal cord (Ulfhake et.al.,1987). Secondly, substance P and TRH have a similar excitatory effect on motoneurones as observed for 5HT (Barasi and Roberts,1974; Myslinski and Anderson, 1978; Clarke and Stirk,1983; Oka & Fukuda,1984; Nicoll,1978; Clarke et.al.,1985). Substance P directly depolarizes the spinal motoneurones of newborn rats (Konishi and Otsuka,1974a; Suzue et. al.,1981) and certain motor behaviour in the rat (rearing) has been attributed to specific amino-terminal fragments of the substance P structure (Hall et.al.,1987). Similarly, TRH

administered intrathecally or systemically produces clearly defined motor behaviours such as wet-dog shakes and fore-paw licking (Wei et.al.,1975). Furthermore, like 5HT, iontophoretically applied substance P and TRH enhance both glutamate and aspartate induced excitation of spinal cord motoneurones (White and Neuman, 1983; White,1985). The effect of substance P on motoneurones may be mediated predominantly by the direct action on the motoneurone rather than via synaptic transmission (Konishi and Otsuka,1974a,b; Nicoll,1978). The above evidence indicates that substance P and TRH have direct effects on motoneurones. However other evidence also indicates that interactions between serotonergic and peptidergic systems are important in motor function. TRH activation of the stretch reflex may be mediated through 5HT receptors (Barbeau and Bedard, 1981). Additionally, the sensitivity of motoneurones to 5HT increases after destruction of pre-synaptic bulbospinal neurones (denervation supersensitivity). It has been demonstrated that this supersensitivity is dependent not on the absence of 5HT but possibly on the loss of substance P and/or TRH in the destroyed terminals (Tremblay et.al.,1985). It therefore appears that substance P and TRH are involved in spinal cord motor function from their location in the CNS and from their direct and indirect motor effects.

There is also evidence from clinical studies that TRH may be important in motor co-ordination in humans. TRH was demonstrated to have beneficial effects on the motor deficiencies which are symptoms of amyotrophic lateral sclerosis (ALS) (Engel et.al.,1983). ALS is a neurological condition which affects motoneurones. There is no known cause or cure for ALS and the condition progresses relentlessly resulting in the death of the sufferer after 1-5 years (Engel et.al.,1983). Intravenous. administration of TRH in high doses to ALS sufferers was originally demonstrated to improve symptoms of muscle weakness and spasticity which are related to motoneurone function (Engel et.al.,1983). Since then a number of clinical studies have been carried out but the benefits of TRH in ALS therapy have not been consistently proven. The ability of TRH

therapy to prevent the progression of ALS and its value in acute motor improvement is still controversial (Caroscio et.al.,1986; Brooke et.al.,1986; Imoto et.al.,1984; Brooks et.al.,1987; Mitsumoto et.al.,1986).

A trophic function of TRH may be important to its effects on motoneurones. Cell cultures of ventral horn neurones treated with TRH are more numerous, more healthy appearing neurones and have thicker bundles of long processes compared with controls (Schmidt-Achert et.al.,1984). The authors proposed these effects were a result of a trophic effect of TRH on lower motoneurones. In addition, the TRH analogue CG 3509 (orotyl-L-histidyl-L-Pro-NH$_2$) significantly increases rat ventral horn ChAT activity indicating a trophic-like function on mature motoneurones (Fone et.al.,1988a).

The role of 5HT, substance P and TRH in spinal cord sensory function

5HT

Many studies have indicated that the bulbospinal pathway is also involved in sensory processing and have implicated the serotonergic system descending from the raphe nuclei to the dorsal horn in analgesia (Hentall and Fields,1979; Basbaum and Glazer, 1979; Basbaum,1981; Yaksh and Wilson,1979). It has been established that activation of the raphe nuclei is involved in the analgesia produced by narcotics or electrical stimulation of the mid-brain (Basbaum and Fields,1984). Furthermore, analgesia is produced by stimulation of the raphe (Oliveras et.al,.1975; Oliveras et.al.,1979) and can be inhibited by lesions or anaesthesia of the ventral medulla (Duggan and Morton,1983; Lovick,1985; Sandkuhler and Gebhart, 1984). In addition, depletion of the descending serotonergic neurones reduces the nociceptive threshold of spinal nociceptive neurones (Proudfit and Yaksh, 1980). Stimulation of the raphe nuclei in the rat modulates spinal nociceptive reflexes as illustrated by stimulation-induced inhibition of the heat-evoked tail-flick (Hentall and Fields,1988) and other withdrawal responses (Hentall et.al.,1987; Sandkuhler and Gebhart, 1984). There is evidence that this effect is mediated

through release of 5HT. Intrathecal application of 5HT inhibits the excitatory effects of noxious stimuli on neurones in the superficial layers of the dorsal horn (Jordon et.al.,1978) and inhibits reflex withdrawal responses elicited by noxious stimuli (Berge et.al.,1985). Furthermore, axon terminals that take up [^{3}H] 5HT and are assumed to originate from the brainstem synapse with dendrites in the dorsal horn (Ruda and Gobel, 1980). The receptors through which the antinociceptive effects of 5HT are mediated in the dorsal spinal cord are reported to be the 5HT$_1$ receptor with different effects being mediated through 5HT$_1$ subtypes (El-Yassir,1988). However, further studies are required to either confirm or refute this proposal. In the human the 5HT$_2$ receptor has been implicated in 5HT induced spinal analgesia (Sandrino et.al.,1986). This effect however may be partly or wholly supra-spinally mediated. In summary, the evidence indicates that the 5HT is involved in the analgesia both at a spinal and supra-spinal level.

Substance P and TRH

Substance P itself may also be a transmitter in afferent pain pathways and evidence for this comes from many lines of investigation: (i) substance P and substance P receptors are present in the dorsal horn and dorsal root ganglia of the spinal cord in high concentrations (Chan-Palay and Palay,1977; Cuello and Kanzawa,1978; Ljungdahl et.al.,1978; Quirion et.al.,1983) (ii) substance P is released from rat spinal cord with stimulation that activates nociceptive neurones (Jessell et.al,.1979), (iii) iontophoretically applied substance P activates nociceptive neurones in the dorsal horn of the spinal cord (Henry,1976; Randic and Miletic,1977) (iv) depletion of substance P from the dorsal horn is accompanied by attenuation of pain sensation (Hayes and Tyers,1980) and (v) substance P elicits behavioural responses thought to be expressions of perceived pain (Piercey et.al,.1981). However, in a series of experiments Frenk, Bossut, Mayer and co-workers (Frenk et.al.,1988a: Frenk et.al.,1988b; Bossut et.al.,1988) provided evidence that a number of responses assumed to be related to nociception were not necessarily so, indicating that

the role of substance P as a neurotransmitter in nociceptive afferents has not been validated and that it is more likely that substance P acts as a neuromodulator in the perception of pain.

A model which is in accordance with the evidence is of a modulatory role for substance P in spinal cord nociception which can modify bulbospinal regulation of spinal reflexes. Other evidence supports this interaction. The in vivo release of 5HT in the spinal cord can be evoked by exogenous substance P (Tsai .,1984). Additionally, using the tail pinch as a noxious stimulation 5HT release in the spinal cord was demonstrated in vivo and this release was inhibited by administering a substance P antagonist (Tsai ,1984). Furthermore, substance P attenuates the inhibitory effects of a 5HT agonist on the tail flick test (Eide and Hole,1988).

The inhibitory effects of the raphe neurones may be mediated by release of 5HT via post-synaptic receptors at which substance P modifies the effects of 5HT and bulbospinal control of spinal nociceptive reflexes. It is also possible that TRH in the dorsal horn also plays a part in modulation of bulbospinal input. TRH has been located in the dorsal horn of the spinal cord in a number of species (Marsden et.al.,1982; Fone et.al.,1987b; Lechan et.al.,1984; Bennett et.al.,1986). Furthermore, there is evidence of a TRH system in the dorsal horn which is separate from the descending bulbospinal pathway (Harkness and Brownfield, 1986) which may be part of a sensory afferent pathway though TRH has not been localized in the dorsal root ganglia.

Neurotransmitters of the spinal cord 2: Catecholamines

Noradrenaline

Dahlstrom and Fuxe (1964) first demonstrated that the neurones of the locus coeruleus in the pons are noradrenergic. Later, noradrenergic projections from the locus coeruleus to the spinal cord were demonstrated using retrograde transport of HRP (Kuypers and Maisky,1975; Mason and Fibiger,1979; Basbaum and Fields,1979). In the rat the retrogradely labelled cells were confirmed to be noradrenergic by immunocytochemical staining

techniques combined with HRP histochemistry (Westlund et.al.,1983). The results indicated that the nuclei of the pons including the locus coeruleus (A6 cell group), the subcoeruleus and lateral and Koller-Fuse nuclei (A7) and the superior olivary nucleus (A5) were the sole source of descending noradrenergic innervation. Quantitative analyses demonstrated that 86% of the spinal noradrenergic neurones project from the locus coeruleus and the sub-coeruleus in the rat (Westlund et.al.,1983). The locus coeruleus was confirmed as the major source of spinal cord noradrenaline in histochemical and biochemical studies (Commissiong et.al.,1978a; Karoum et.al.,1980; Nygren and Olson,1976). More recently, the pontine source of descending noradrenergic innervation in the cat has been located as the more caudal locus coeruleus pars ∝ and the sub-coeruleus rather than the locus coeruleus proper (Nakazato,1987).

Noradrenergic terminals in the spinal cord are observed throughout the spinal gray matter and are most heavily concentrated in the superficial layers of the dorsal horn (laminae I, II and III), in the ventral horn, in the IML and around the central canal (Anden,1965; Carlsson,1964; Lackner,1980; Westlund,1983). Reported levels of noradrenaline in the ventral and dorsal horn of the rat spinal cord detected by radio-enzymatic, gas chromatography- mass spectrometric (GC-MS) or HPLC techniques are ~~in good agreement~~ consistent (Commissiong et.al.,1978a; Mouchet et.al.,1982; Basbaum et.al,.1987) and are similar to those in the cat (Fleetwood-Walker and Coote,1981).

Dopamine

Dopamine levels in the spinal cord are at least ten fold lower than those of noradrenaline (Commissiong and Neff,1979; Mouchet et.al.,1982; Basbaum et.al,.1987) and in the past it was thought that dopamine in the spinal cord was present solely as a precursor of noradrenaline (Anden et.al.,1966). However, an independent transmitter role for dopaminergic innervation of the cord is being established on the basis of evidence from spinal lesions, retrograde axonal tracing and immunohistochemistry of dopamine synthesizing enzymes (Commissiong et.al.,1978a,b;

Commissiong and Neff,1979; Bjorklund and Skagerberg,1979; Magnusson,1973; Skagerberg and Lindvall, 1985). The sole source of spinal cord dopaminergic innervation is the diencephalic A11 catecholamine cell group (Bjorklund and Skagerberg, 1979; Hokfelt et.al.,1979; Skagerberg and Lindvall, 1985; Skagerberg et.al.,1988; Takada et.al.,1988). This cell group, first defined by Dahlstrom and Fuxe (1964) is located in the dorsal and posterior hypothalamus, the caudal thalamus and the mesencephalic periaqueductal gray. This diencephalo-spinal dopaminergic system projects mainly to the dorsal horn, the IML and the central gray matter at all spinal segments (Bjorklund and Skagerberg,1979; Hokfelt et.al.,1979; Skagerberg et.al,1982; Skagerberg et.al.,1988). Using GC-MS, HPLC or enzymatic methods dopamine has been measured in the dorsal and ventral horns in all spinal segments and in the thoracic IML (Commissiong et.al.,1978; Commissiong and Neff,1979, Mouchet et.al.,1982; Basbaum et.al,.1987). The distribution of dopamine reported is in agreement in general with the studies of dopaminergic innervation although dopamine terminals in the ventral horn are reported to be sparse or negligible (see Basbaum et.al.,1987). Dopamine levels are highest in the dorsal horn, IML and around the central canal and ventral horn levels of dopamine are approximately one third of dorsal horn levels (Commissiong et.al.,1978; Commissiong and Neff,1979, Mouchet et.al.,1982; Basbaum et.al.,1987).

Adrenaline

The distribution of adrenaline in the spinal cord is less well documented and adrenergic fibres are concentrated almost exclusively in the thoracic (Coote et.al.,1981; Ross et.al.,1984; Kohno et.al.,1988) and to a lesser extent in the sacral (Hokfelt et.al.,1984b) intermediolateral region. The spinal adrenergic innervation originates solely from the ventrolateral medulla (Hokfelt et.al.,1974; Hokfelt et.al.,1984b).

There is also growing evidence for catecholaminergic neurones which are not supra-spinal in origin but intrinsic to the

spinal cord in both lower vertebrates (Parent and Northcutt, 1982; Wolters et.al.,1984) and in the rat (Mariani et.al,.1986).

CNS synthesis and metabolism of catecholamines

Independent noradrenergic, dopaminergic and adrenergic systems are possible due to specific enzymes within the common pathway for synthesis of the catecholamines. In addition a description of this pathway will facilitate understanding of the mechanism of action of the drug L-dopa which is administered in the treatment of Parkinson's disease and which is discussed later (pp.29). Furthermore, the enzyme systems responsible for catabolism of the catecholamines may play a part in the cause of Parkinson's disease. For these reasons the catecholamine synthetic and catabolic pathways are outlined here.

The main pathway in the synthesis of catecholamines in the CNS is illustrated in Fig.1.5. Catecholamine synthesis starts with two essential amino acids phenylalanine and tyrosine, and tyrosine can be synthesized from phenylalanine by the enzyme phenylalanine hydroxylase. The enzyme tyrosine hydroxylase catalyses the conversion of tyrosine to dopa and this step is important in regulation of catecholamine synthesis. Tyrosine hydroxylase is produced in the cell body of the adrenergic neurones and its rate of synthesis is dependent on the level of neuronal activity and the rate of release of catecholamines. When the rate of catecholamine release is high tyrosine hydroxylase synthesis is enhanced. Furthermore, the conversion of tyrosine to dopa is relatively slow so that this process is the rate limiting step in the pathway. Tyrosine hydroxylase activity is inhibited by noradrenaline through competition for the cofactor tetrahydrobiopterin which is essential for the conversion (Kaufman and Fisher,1974). Tyrosine hydroxylase is affected by neuronal depolarisation (Alousi and Weiner,1966; Roth et.al.,1966) and during in situ nerve stimulation tyrosine hydroxylase appears to maintain the stores of noradrenaline (Weiner et.al,1978). Once formed, dopa is rapidly converted to dopamine by dopa decarboxylase which requires pyridoxal phosphate as a co-enzyme. Dopa decarboxylase acts only on the L-

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Phenylalanine → (Phenylalanine hydroxylase) → **Tyrosine**

Tyrosine → (Tyrosine hydroxylase) → **Dopa (dihydroxyphenylalanine)**

Dopa (dihydroxyphenylalanine) → (Dopa decarboxylase) → **Dopamine**

Dopamine → (Dopamine β-hydroxylase) → **Noradrenaline**

Noradrenaline → (Phenethanolamine-N-methyl transferase) → **Adrenaline**
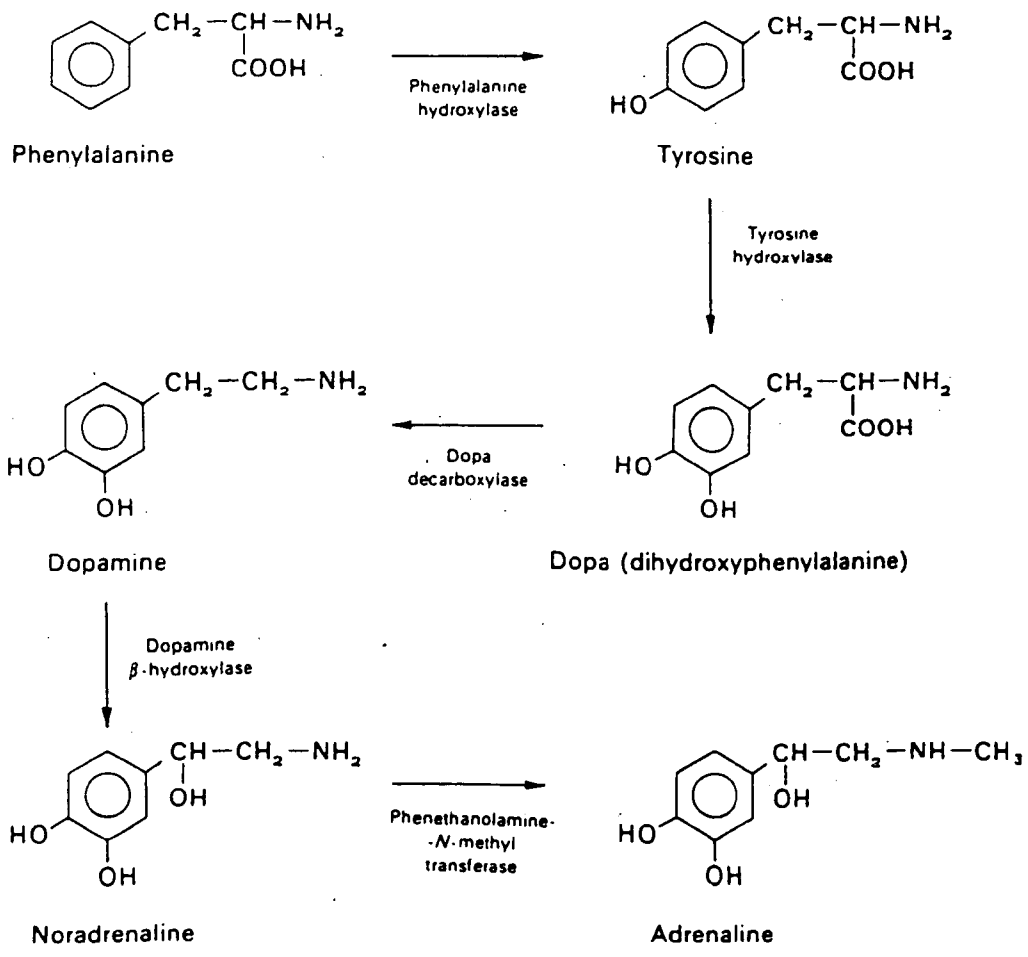
Fig 1.5 The main pathway in the synthesis of catecholamines.

isomer of dopa and this isomer is used in the treatment of Parkinson's disease in the form of Levadopa (L-dopa). In the dopaminergic system described above the synthetic pathway stops at the formation of dopamine as these neurones lack the enzyme, dopa ß-hydroxylase, necessary for the formation of noradrenaline. Similarly, in noradrenergic systems conversion of noradrenaline to adrenaline is prevented by the absence of the necessary enzyme (phenylethanolamine-N-methyl transferase).

Catecholamines are catabolised by two main enzyme systems. Two enzymes catecholamine-O-methyl transferase and mono-amine oxidase act concomitantly. Catechol-O-methyl transferase (COMT) acts on dopamine, noradrenaline and adrenaline and the 3-methoxy products of its actions inhibit the re- uptake of the catecholamines. The main products of metabolism of noradrenaline and adrenaline are 3-methoxy-4-hydroxyphenyl-ethyleneglycol (MHPG) and 3,4-dihydroxyphenyl-ethyleneglycol (DHPG) and those of dopamine are 3,4-dihyroxyphenylacetic acid (DOPAC) and homovanyllic acid (HVA). Monoamine oxidase (MAO) is the major enzyme responsible for degradation of amines and exists in two forms, MAO A and MAO B. The MAO enzymes catalyse oxidation of the catecholamines to the deaminated state. The difference between these two enzymes is their specificity for different substrates, their inhibitor specificity, their distribution and immunological characteristics (Denney et.al.,1982, 1983 for review). However, in vivo, the effects of released catecholamines are terminated to a large extent by their rapid up-take into pre- and post-synaptic terminals and the exact physiological function of the MAOs has not been determined. Roles proposed for MAO include regulation of pre-synaptic neurotransmitter levels (Von Korff,1979 for review).

The role of the catecholamines in spinal cord motor function

It has been proposed that the noradrenergic innervation of the ventral spinal cord originating in the locus coeruleus is involved in regulation of spinal motor activity (Grillner,1975). The terminals of noradrenergic spinal pathways are found in the ventral horn in the areas where motoneurones are concentrated

and have been observed to be in close contact with motoneurones (Anden,1965; Lackner,1980; Westlund et.al.,1983; Holstege and Kuypers,1987). In addition, electrical stimulation of the locus coeruleus facilitates activity of the spinal motoneurones (Fung and Barnes,1981). This effect is mediated at least partly by the large noradrenergic component of the locus coeruleus as facilitation of lumbar flexor and extensor monosynaptic reflexes produced by stimulation of this nucleus can be inhibited by adrenoceptor antagonists (Strahlendorf et.al.,1980). Furthermore iontophoretic application of noradrenaline, adrenaline and dopamine into the ventral horn induces facilitation of motoneurone activity or motoneurone depolarization (Parry and Roberts, 1980; White and Neuman,1980; White and Neuman,1983; Kitazawa et.al.,1985). The site at which depolarisation of spinal neurones by catecholamines is effected may be the cell bodies or dendrites rather than the axons of the motoneurones (Kitazawa et.al.,1985). Furthermore, modulation induced by noradrenaline may be mediated through α2-adrenoceptors in the spinal cord (Kitazawa et.al.,1985; Conway et.al.,1988; Clarke et.al.,1988). It has been demonstrated that intrathecal administration of the α2-adrenoceptor agonist clonidine reduces tonic activity of the hind limb muscles but allows walking movements (Tremblay and Bedard,1986) indicating a role for α2-adrenoceptors in locomotor function. It appears therefore that spinal noradrenergic pathways facilitate spinal motoneurone activity as do the serotonergic-peptidergic pathways but that the effects of these two systems are mediated through different receptors (see Conway et.al.,1988).

The role of the catecholamines in spinal cord sensory function

Noradrenaline is thought to be involved in mediation of nociception in the dorsal spinal cord (Dennis et.al.,1980; Kuraishi et.al.,1979,85; Reddy et.al.,1980). Ionotophoretic application of noradrenaline increases the threshold of A- and C- nociceptive fibres in the dorsal horn (Curtis et.al.,1983; Jeftinija et. al., 1981; Carstens et.al.,1987) and noradrenaline inhibits the responses of dorsal horn nociceptive neurones to noxious stimuli or electrical stimulation (Belcher et.al.,1978; Fleetwood-Walker et.al.,1985).

Intrathecal application of noradrenaline produces antinociception in a number of pain reflex tests (Kuraishi et.al.,1985). Furthermore, the analgesic action of noradrenaline is exploited clinically and both α-adrenoceptor agonists and adrenergic re-uptake blockers are used in the treatment of pain (Coombes et.al.,1986; Feimann,1985). The analgesic action appears to be mediated through α-adrenoceptors (Reddy et.al.,1980; Howe et.al.,1983) and is mimicked by clonidine (Schmitt et.al.,1974; Reddy et.al.,1980; Fielding et.al.,1978). There is evidence that analgesia to different types of pain may be mediated independently through α_1- and α_2-adrenoceptors (Paalzow and Paalzow,1982; Tasker and Melzack,1989). There is strong evidence indicating a role for descending noradrenergic pathways in the antinociceptive effects of noradrenaline. Electrical stimulation of the locus coeruleus produces an inhibitory effect on pain transmission in the spinal cord (Jones and Gebhart,1986; Mokha et.al.,1986). In addition neurotoxin depletion of spinal noradrenaline in the cat is accompanied by supersensitivity to the antinociceptive effect of intrathecally administered noradrenaline (Howe and Yaksh,1982). This result and others were taken to indicate that the effect was mediated through post-synaptic adrenoceptors, and in part at least through α_2-adrenoceptors (Howe and Yaksh,1982; Jones and Gebhart,1986; Fleet-wood Walker et.al.,1985). However, as the antinociceptive effect of clonidine was unaffected by lesion of the locus coeruleus (Sawynok and Reid, 1986) and as α_2-adrenoceptors were subsequently demonstrated to be unaffected by noradrenaline depletion in the spinal cord (Howe et.al.,1987) the mechanism of supra-spinal noradrenergic modulation of nociception is undetermined. There is also one report that noradrenergic modulation of the tail flick (pain/heat) reflex is mediated through a noradrenergic pathway descending from the lateral reticular nucleus (LRN) in the ventral medulla independent of the locus coeruleus, though the presence of an adrenergic LRN projection to the cord is still controversial (see Janss and Gebhart,1988).

The dopamine agonist apomorphine has also been shown to exert an anti-nociceptive effect in the spinal cord (Jensen and Smith,1983).

It may appear from this discussion that the spinal serotonergic-peptidergic and adrenergic systems are independent of each other but in fact there is evidence that the effects of the two systems are highly interactive. In the CNS in general there is regulation of α- and ß-adrenoceptors by serotonergic activity. Serotonergic denervation results in modification of α1-, α2- and ß-adrenoceptor binding and modulation of noradrenergic activity affects 5HT mediated behaviour (Manier et.al.,1983; Rappaport et.al.,1985; Manier et.al,.1987). Serotonergic control of locus coeruleus activity may be effected through post-synaptic 5HT2 receptors in the locus coeruleus (Gorea and Adrien,1988). In addition, the anti-nociceptive action of dopamine in the spinal cord may be under the tonic inhibitory control of descending serotonergic and noradrenergic systems (Jensen and Smith,1983). Furthermore, there is evidence that TRH elicited motor effects are mediated through dopaminergic or noradrenergic mechanisms. Motor behaviour including wet-dog shakes and forepaw licking induced by intrathecal TRH is attenuated both by compounds which deplete monoamine stores and by adrenoceptor antagonists (Fone et.al.,1989b). Nociception also appears to be modulated by interaction between serotonergic and noradrenergic systems. Spinal noradrenaline depletion attenuates 5HT agonist-induced analgesia in hot plate or tail flick measurements of pain sensitivity (Archer et.al.,1986) and intrathecal 5HT reduces the antinociceptive potency of intrathecal noradrenaline (Clatworthy et.al,1988).

Parkinson's disease

Neurochemical changes and symptoms

One in one thousand people suffer from Parkinson's disease (Calne, 1970). This condition was described as early as 1817 (Parkinson, 1817) on the basis of its classic symptoms but it wasn't until the 1960's that the discovery of the deficit of nigrostriatal dopamine in the post-mortem brains of Parkinsonian patients was reported (Ehringer and Hornykiewicz,1960). This discovery lead to great improvements in the control of the symptoms of this condition by treatments to reverse the deficit (Birkmayer and Hornykiewicz, 1961). The most apparent symptoms of Parkinson's disease are those relating to loss of motor coordination. Patients suffer bradykinesia (slowness in initiating and executing voluntary movements), resistance to muscle movement (rigidity), tremor and postural instability (Birkmayer and Reiderer,1983; Parkinson,1817). However, more recently sensory symptoms in Parkinson's disease are also being appreciated, especially the pain associated with this condition (see , pp.183-184). Confirmation of the loss of nigro-striatal dopamine and of pigmented dopamine containing cells of the substantia nigra in Parkinson's disease (Bernheimer et.al.,1973; Fahn et.al.,1971; Lloyd and Hornykiewicz,1970) has stimulated extensive investigation in the biochemistry of the CNS in attempts to find the causes and cures of a whole variety of neurological, and in particular basal ganglia disorders (see Marsden, 1981; Rossor and Emson, 1982) . It has been subsequently confirmed that neurochemical changes in the CNS of Parkinsonian patients extend further than that of the dopaminergic striatal cells. In the post-mortem Parkinsonian brain there are lower levels of dopamine in the mesolimbic and mesocortical dopamine systems and in the lateral hypothalamus (Hornykiewicz, 1982; Javoy-Agid and Agid,1980) and these changes are accompanied by cell loss and Lewy bodies (Javoy-Agid and Agid, 1980; Javoy-Agid et.al.,1983). These changes may be related to the psychiatric and cognitive disturbances associated with Parkinson's disease (Mayeux, 1982). In the Parkinsonian brain regions of

noradrenaline loss are widespread including the locus coeruleus, nucleus accumbens, amygdala, hippocampus and the cortical regions (Farley and Hornykiewicz,1976; Scatton et.al.,1983). Deficits of serotonin in the Parkinsonian brain have also been reported in a number of regions including the basal ganglia, hypothalamus, hippocampus, raphe nuclei and frontal cortex (Bernheimer et.al.,1961; Scatton et.al.,1983). In addition, loss of cells of both the raphe nuclei and locus coeruleus have been reported (Greenfield and Bosanquet, 1953). Furthermore, lower levels of noradrenaline and serotonin but not dopamine have been described in the post-mortem spinal cord of Parkinsonian patients (Scatton et.al.,1986). In addition, modifications in the GABA (γ-aminobutyric acid) and peptide systems including CCK (cholcystokinin) and substance P have been implicated in Parkinson's disease (Barbeau and Kastin,1976; see Agid and Javoy-Agid,1985).

Treatment of Parkinson's disease

Discovery of the loss of striatal dopamine lead to treatment by replacement therapy in the form of L-dopa which is taken up by dopaminergic neurones and converted to dopamine (pp. 23). L-dopa can also be taken up by adrenergic neurones in which it is converted to noradrenaline and dopamine can be formed from L-dopa in serotonergic cells which also have decarboxylase enzymes. However, studies indicate that dopaminergic cells contain 80-90% of decarboxylase activity and that these cells are primarily responsible for the decarboxylation of L-dopa (Melamed et.al.,1980). L-dopa is administered in conjunction with dopa decarboxylase inhibitors which do not cross the blood brain barrier (benserazide, carbidopa) and so ensure that increased dopamine synthesis from L-dopa is prevented in the periphery and restricted to the CNS. In addition dopamine agonists such as bromocriptine and dopamine releasing agents are also useful in the treatment of Parkinson's disease (Reynolds and Reiderer, in press; Hoehn, 1981) as are anticholinergic agents which redress the imbalance between the central dopaminergic and cholinergic systems (Reynolds and Reiderer, in press). Treatment with L-

dopa has improved the quality of life of many sufferers immensely by controlling the symptoms and evidence indicates that it may slow the progression of the disease and increase life expectancy (Hoehn, 1985). However, L-dopa is not a cure for Parkinson's disease. L-dopa therapy in particular reduces bradykinesia and rigidity but results in end of dose akinesia and fluctuations between symptoms of deficiency and of over-activation of the dopaminergic system resulting in abnormal involuntary movements (dyskinesias) (Hornykiewicz, 1973; Reynolds and Reiderer, in press).

Causes of Parkinson's disease

The cause of Parkinson's disease has not yet been proven. The main possible causes which have been considered are heredity, environmental factors, a virus or an effect of aging of the nervous system. It now appears unlikely that the condition is transferred genetically (Duvoisin,1984). In only 5% of cases are both individuals from a pair of twins likely to develop Parkinson's disease and this is irrespective of whether the twins are monozygotic or dizygotic (Duvoisin et.al.,1981). There has been some difficulty in differentiating environmental influences on affected and unaffected twins (Bharucha et.al.,1986) because of similarities in their backgrounds. However, environmental toxins have been implicated in a number of selected populations of people exhibiting high incidence of parkinsonian symptoms (Martilla and Rinne,1981; Gajdusek and Salazar,1982; Spencer and Schaumburg,1980). As an epidemic of encephalitis lethargica in the 1920's resulted in a clinical syndrome resembling Parkinson's disease (post-encephalic parkinsonism) there has been a search for evidence of a viral cause of Parkinson's disease. However, viral infection suffered by people developing Parkinson's disease has not been confirmed (Duvoisin,1981) and no viral cause has been discovered. Many of the symptoms of Parkinson's disease appear to be exagerations of changes occurring with age and in normal aging dopamine levels in the striatum fall by approximately 13% per decade (see Snyder and D'Amoto,1986).

The basal ganglia in the human appears to be specifically sensitive to neuronal degeneration. A number of markers of basal ganglia dopaminergic activity decline with age including the levels of dopamine and tyrosine hydroxylase, dopaminergic neurones in the substantia nigra and striatal D_2 receptors (reviewed by Morgan et.al,.1987). Similarly there are age related declines in substance P and neurotensin in the putamen and substantia nigra of the human brain which are not apparent in other regions (Buck et.al.,1981). Furthermore, cellular state in human brain determined by wet weight, total protein and ganglioside NeuNAc content is unaffected by age except in the caudate nucleus where all three factors are diminished with age (Allen et.al.,1983). It has been proposed therefore that Parkinson's disease occurs due to aging of the nervous system and would develop in more people if they lived longer though most evidence is against this theory (see Calne and Langston, 1983). A popular hypothesis at the present time is that Parkinson's disease develops with age as a result of an earlier exposure to substances toxic to the CNS. This may be a viral exposure (see above) or exposure to an environmental chemical. There are toxins which can cause irreversible Parkinsonism which is alleviated by L-dopa (see Calne and Langston, 1983). This hypothesis is supported by the Parkinsonian syndrome apparent in subjects who have been exposed to the substrate MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine).

MPTP

Discovery of the effects of MPTP may prove important in elucidating both the causes of Parkinson's disease and the mechanisms resulting in specific neuronal degeneration. The Parkinsonian symptoms induced by MPTP were first observed in a group of people in California who had self-administered this chemical believing it to be synthetic heroin (Langston et.al.,1983). These subjects developed Parkinsonian motor symptoms including bradykinesia, rigidity and tremor in a matter of days and the symptoms were found to be alleviated by L-dopa (with similar complications to those experienced by Parkinsonian

patients) and by dopamine agonists (Langston and Ballard,1984; Langston et.al.,1984a). This produced excitement in the research world as it appeared that MPTP could be used as a tool to produce animal models of parkinsonism to aid the investigation of drugs to control the symptoms. Furthermore, it appeared that MPTP produces selective permanent degeneration of the dopaminergic nigro-striatal system in the monkey (Burns et.al.,1983; Langston et.al.,1984a) and in one person who committed suicide 18 months after injecting MPTP (Davis et.al.,1979). This indicated that the symptoms could be elicited by degeneration in this region only and that MPTP had specific neurotoxic effects. Furthermore, MPTP produces a good animal model of Parkinsonism as symptoms are observed only after a massive reduction (more than 80%) of striatal dopamine as seen in clinical Parkinson's disease (Chiueh et.al.,1985; Hornykiewicz,1982) and in addition the symptoms respond to L-dopa therapy (Burns et.al.,1983; Chiueh et.al.,1984). The effects of MPTP are species specific and cause degeneration of dopamine neurones, loss of striatal dopamine and related motor symptoms in primates (Burns et.al.,1983; Langston et.al.,1984a; Crossman et.al.,1985; Jenner et.al.,1984; Chiueh et.al.,1983) and in mice at much higher doses and with prolonged treatment (Heikkila et.al.,1984; Tadano et.al.,1987) but not in other species (Chiueh et.al.,1983). Subsequently much effort has been expended to illucidate the mechanism of action of MPTP.

The active form of MPTP appears to be the oxidized derivative MPP+ (1-methyl-4-phenylpyridine) to which MPTP is converted by MAO enzymes via the intermediate MPDP+ (1-methyl-4-phenyl-2,3-dihydropyridinium ion) (Langston,1985). This idea is consistent with the finding that the neurotoxic effects of MPTP can be prevented by the prior administration of MAO-inhibitors which prevent the conversion to MPP+ (Heikkila et.al.,1984; Langston et.al.,1984b). The transformation may occur in elements other than the dopamine neurones such as glia and serotonergic terminals surrounding dopamine neurones (Uhl et al.,1985; Langston et.al.,1985). MPP+ accumulates in the brain, is taken up by dopaminergic neurones (Javitch and Snyder,1985) and is detectable for some time after administration of MPTP (Lewin,1984; Markey et.al.,1984). Selectivity of the effects of

MPTP between species and brain regions may be dependent on the differential retention of MPP+ (Johannesson et.al.,1985; Markey et.al.,1984; Langston et.al.,1984b) and the heterogeneous distribution of the MPTP receptor. There is strong evidence that MPP+ binding site is the MAO-B enzyme (Lewin,1984; Javitch et.al.,1984). The accumulation of MPP+ in the substantia nigra may be related to the specific suseptibility of the neurones in this region to the degeneration caused by toxins and age. Additionally, it appears that MPTP may have a specific affinity for melanin (Lyden et.al.,1983).

More recently the neurotoxic specificity of MPTP to striatal dopaminergic neurones has been contradicted. MPTP has been reported to induce metabolic but not degenerative effects on the serotonergic system in the mouse CNS (Hara et.al.,1987) and degenerative effects on dopaminergic, noradrenergic and serotonergic systems in the rat CNS have been reported (Namura et.al.,1987). It has been demonstrated that in addition to the dopaminergic systems MPTP produces damage to the ventral tegmentum and locus coeruleus in the primate CNS producing a breadth of neurochemical changes similar to that seen in Parkinson's disease (Mitchell et.al.,1985). This evidence indicates that MPTP may produce a better model of parkinsonism than first anticipated.

The evidence, in general, demonstrates that MPTP induced parkinsonism is very like the clinical syndrome and this indicates that one of the causes of Parkinson's disease may be exposure to substances which can be metabolised to the neurotoxic entity MPP+ or other pyridines, many of which are present in the environment. For example, 4-phenylpyridine is present in peppermint tea and has been shown to result in tyrosine hydroxylase depletion in the striatum when administered to mice (Snyder and D'Amato,1986). Symptoms may appear several years after a neurotoxic insult due to the further degenerative effects of aging.

Factors considered and procedures used in this study

Factors considered in the use of human tissue

To determine the effects of a neurological condition on neurotransmitter levels or receptor binding in the human CNS the effects on these parameters of a number of variables must be considered including the age and sex of the subject, the interval between death and post-mortem dissection of the tissue (post-mortem interval, PMI) and drug treatment before death. The effects of these factors must be determined as it is unlikely that the group under investigation could be matched exactly with controls. Generally speaking, the levels of neurotransmitters in the brain fall in the first 24 hours post-mortem whereas peptide levels are relatively stable (see Rossor,1986 for review). However, when the effects of these factors on specific neurotransmitters and peptides are reviewed carefully it is obvious that the picture is more complicated. Studies have also been conducted in animal tissue in order to control the variables more closely. In investigations using rat spinal cord and mouse brain (Mitsuma et.al., 1984 and Emson et.al., 1981b) TRH levels were reported to increase and to remain constant respectively in similar post-mortem time periods. Although this may reflect species variation it is likely that it is also dependent on regional variation, a factor which appears to be very important in the consideration of the effects of both PMI and age on CNS transmitter levels. For example, the basal ganglia appears to be specifically sensitive to neuronal loss of both dopamine and peptides with age as discussed above (Morgan et.al,.1987; Buck et.al.,1981; Allen et.al.,1983). Similarly, Siddique et. al., (1988) reported that post-mortem noradrenaline, 5HT and dopamine levels vary from control values in a differential manner depending on the brain region analysed and the exact time post-mortem. Moreover Severson and co-workers (1985) proposed that the relationship between imipramine binding (an indication of brain 5HT uptake) and age in humans depended largely on the exact age range of the subjects selected. The evidence indicates that the effects of PMI and age on CNS transmitter levels vary depending on the CNS region studied

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and furthermore that the effects depend on the exact ranges of age or PMI under study. Ante-mortem drug therapy has also been reported to affect post-mortem studies. One of the most conclusive findings is the increase in dopamine receptor number and affinity in the brains of schizophrenics which has been related to ante-mortem neuroleptic medication. This finding is supported by studies in animals treated with neuroleptics (see Rossor,1986).

Monoamine metabolites as indicators of monoamine turnover

In this study it was intended to use monoamine metabolites as indicators of monoamine turnover. The monoamines noradrenaline, dopamine and 5HT are metabolised after release from nerve terminals and, as their metabolites do not rejoin the monoamine synthetic pathway, the concentrations of the metabolites are taken as an indication of the rate of release of these neurotransmitters. The final product of 5HT metabolism is 5HIAA and CNS 5HIAA concentration is frequently used to determine 5HT activity and release (turnover). However, use of metabolites in this way has been questioned. There is convincing evidence for an intracellular supply of the degradative enzyme MAO so it is possible that 5HT could be metabolised before release. In that case increased levels of 5HIAA may indicate increases in intracellular 5HT metabolism by the degradative enzyme MAO unrelated to release (Wolf et.al.,1985; Commissiong,1985). In the intact untreated animal it is considered that 5HIAA concentration is indicative of 5HT release and metabolism (turnover) and DOPAC and HVA levels are indicative of dopamine turnover (Commissiong, 1985). However, in the treated animal these assumptions only hold if the drugs used do not (i) induce synthesis of the monoamine which is metabolised before becoming part of the releasable pool or (ii) stimulate monoamine synthesis in elements other than the monoaminergic neurones (Commissiong, 1985). In this study therefore some consideration will be given to the effect of L-dopa administered to Parkinsonian patients on monoamine metabolism. The major metabolites of noradrenaline metabolism were not determined in the present study.

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The present work utilises the technique of radio-ligand binding. The in vitro method of radio-ligand binding is well established and has been used extensively to investigate the receptors in the CNS through which the endogenous catecholamines and indoleamines are effective (Rouot and Snyder, 1979; Giron et .al., 1985; Petrash and Bylund, 1986; Mann et.al., 1986; Brown et. al 1988). Primarily this technique is used to define the sites at which ligands bind in terms of the number of receptors and the affinities of the ligand for the site. Using Scatchard and Hill plot analysis of the data the saturability, reversibility and homogeneity of the sites can be assessed. Such studies may provide evidence for subtypes in the classification of these receptors. For example, the endogenous compound 5HT has varying affinities for different subclasses of the receptor at which it binds and with the use of specific ligands these have been classified into $5HT_{1A}$, $5HT_{1B}$, $5HT_{1C}$, $5HT_{1D}$, $5HT_2$ and $5HT_3$ receptors (Peroutka and Snyder,1979; Leysen et. al., 1982; Gozlan et. al., 1983; Hoyer et al., 1985b; Heuring and Peroutka, 1987; Peroutka and Hamik, 1988). Furthermore, with the development of specific ligands investigations into the anatomical location of the various binding site subtypes have been made. For instance pre- and post-synaptic locations of serotonergic receptors, their heterogeneity throughout brain regions and species variation have been studied (Leysen et. al., 1982; Leysen.et.al., 1983; Pazos et al., 1984b). However, for the binding site to be considered as a receptor there must be evidence for correlation between the site and some physiological function. In the case of serotonergic binding sites functional correlates have been obtained from the physiological and behavioural responses induced or antagonised by the administration of specific ligands (Doods et .al., 1985; Middlemiss, 1985; Benkirane et. al., 1986). For $5HT_{1A}$ sites in particular multiple correlates have been established between its pharmacological characteristics and its specific physiological effects. There is also evidence for association of the $5HT_2$ site with hallucination (Glennon, 1987), phosphoinositide turnover (Kendall and Nahorski, 1985; Roth et al.,1984) and depolarization of neuronal membranes (VanderMaelen and Aghajanian, 1980). It is agreed however that a definite classification

for serotonergic receptor subtypes will have to wait until a clearer correlation between binding sites and functional significance has been achieved (Bradley et. al., 1986; Peroutka, 1988).

Autoradiographic localization of serotonergic and adrenergic receptors

Autoradiography is also employed in this study to measure and locate serotonergic and adrenergic receptor binding sites. A knowledge of the anatomical distribution of receptor sites within the CNS is important in order to characterize the sites. Their anatomical location can be correlated with the physiological significance of the area indicating a possible functional role for the receptors. Also pharmacological characterization of receptors in binding studies can be facilitated using CNS areas enriched in one specific subtype. Autoradiography provides a high degree of anatomical resolution for mapping the distribution of receptors and can be used quantitatively to estimate receptor concentrations and affinities (Young and Kuhar, 1979; Pazos and Palacios, 1985; Stephenson and Summers, 1985.)

Effects of transmitter changes on receptor number and affinity

Long-term denervation of transmitter systems can be expected to be accompanied by changes in receptor number and /or affinity for the transmitter and such changes may be an indication of receptor location. Loss of pre-synaptic receptors and supersensitivity in post-synaptic receptors could be expected to parallel pre-synaptic neuronal degeneration. For example in the Parkinsonian brain it has been reported that there are reduced dopamine (D_1) receptor sites in the pars compacta of the substantia nigra indicating that D_1 receptors may be located on the terminals of striato- or pallidonigral neurones which have been reported to be destroyed in Parkinson's disease (Cash et.al.,1987). Alternatively, these results may indicate that D_1 receptors are located on the nigral dopaminergic cell bodies which are lost in Parkinson's disease (Cash et.al.,1987). In addition, there is an increase in D_2 receptor binding in the MPTP lesioned striatum

indicating upregulation of post-synaptic D2 receptors (Leenders et.al.,1988). Similarly, up-regulation of α_1- and α_2-adrenoceptors in the rat cerebral cortex after treatment with the neurotoxin 6-hydroxydopamine indicates that these receptors are mainly located on structures other than noradrenergic nerve terminals (Dausse et.al.,1982). Receptor adaptation and developed tolerance also occurs in the TRH system as demonstrated by progressive attenuation of the motor behaviour elicited by TRH after its long-term administration (Simasko and Horita,1985). Consideration is given to receptor adaptation in evaluation of spinal transmitter system changes in Parkinson's disease

Aims of this thesis

Catecholamine, indoleamine, substance P and TRH levels in the human spinal cord

The initial aim of this thesis was to compare the levels of catecholamines, indoleamines, TRH and substance P in the human spinal cord from patients who had died with Parkinson's disease and from control subjects. This was performed to investigate (i) the report of reduced 5HT and noradrenaline in the spinal cord in Parkinson's disease and (ii) whether, in the light of co-existence of 5HT, substance P and TRH in the rat spinal cord, substance P and TRH depletion parallels 5HT depletion in the human spinal cord. In order to accomplish this a sensitive assay for detection of substance P was required and the development of a sensitive radioimmunoassay specific for substance P and with low cross-reactivity for other peptides which may be present in the spinal cord is described in chapter 2 part 1. Secondly, comparison of the changes in the levels of 5HT, substance P and TRH in the Parkinsonian cord necessitated an extractant in which 5HT, substance P and TRH could be extracted concomitantly from tissue samples. The development of such an extractant and rigorous analysis of its use in extraction of catecholamines as well as indoleamines and peptides from rat spinal cord tissue is described in chapter 2 part 2. Rat spinal cord tissue was employed in order (i) to spare the limited supply of human tissue; (ii) to validate

the methods by comparing results with previously published data and (iii) to extend the study of catecholamine, indoleamine and peptide levels in the rat spinal cord as this region has been neglected somewhat for all species.

There have been few reports of the distribution of catecholamines, indoleamines and substance P in the human spinal cord possibly because of the difficulty in dissection of this tissue. In chapter 3 the levels of these neurotransmitters and peptides in post-mortem cervical, thoracic and lumbar spinal cord from control human subjects are given. This is accompagnied by a full analysis of the effects of a number of factors, such as subject age, sex and the interval between death and post-mortem which may affect post-mortem levels of amines and peptides.

Considering these factors the levels of noradrenaline, adrenaline, dopamine, DOPAC, 5HT, 5HIAA, substance P and TRH are compared in the post-mortem spinal cords from Parkinsonian patients and control subjects. The results are discussed with reference to the effects of Parkinson's disease on indoleamines, catecholamines and peptides in the spinal cord and consideration of co-existence of 5HT, substance P and TRH in the human spinal cord.

Serotonergic and adrenergic receptor binding

To illucidate further the neurotransmitter changes occurring in the spinal cord in Parkinson's disease an investigation of the adrenoceptors and serotonergic receptors in this condition was undertaken. The aims of this study were to look at α_1- and α_2-adrenoceptors, and $5HT_{1A}$ and $5HT_2$ receptors using selective ligands in both ligand binding and autoradiographic studies. The protocols were first carried out using rat brain tissue to validate the methods as there have been a number of reports of the specific binding of the selected ligands to rat brain for comparison. A number of modifications to improve the ligand binding protocol for use with human spinal cord tissue were also tested using rat brain homogenate and these are all

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reported in chapter 4. The final experimental chapter reports on a comparison of α_2-adrenoceptor binding in spinal cord from Parkinsonian and control subjects with reference to the effects of Parkinson's disease and noradrenergic depletion on α_2-adrenoceptor binding. In chapter 5 the effects of subject age, sex and the PMI on α_2-adrenoceptor binding are also analysed and discussed.

Chapter 2 Methods used in the measurement of indoleamine, catecholamine, substance P and TRH levels in rat and human spinal cord.

Introduction

This chapter describes the development and validation of a number of methods which were subsequently used to measure catecholamines, indoleamines, substance P and TRH in human post-mortem spinal cord. The availability of the human spinal cord material was limited and so it was important to use it efficiently. For this reason all the methods which were to be used were first applied and refined using rat spinal cord material. The methods were validated by comparing the levels of neuroamines (catecholamines and indoleamines in neuronal tissue) and peptides measured in rat spinal cord with those previously reported (Marsden et. al., 1982; Mouchet et. al., 1982; Lighton et.al.,1984; Basbaum et.al.,1987). The various methods used are discussed in this chapter in two parts. The first part covers the substance P radioimmunoassay and the production of an antiserum with high specificity for substance P. In the second part the development of a common extractant for the neuroamines and peptides and the methods utilised in that study are described. The practical application of the methods is demonstrated by analysis of the catecholamine, indoleamine and peptide levels in rat spinal cord.

Part 1 Substance P Radioimmunoassay

Introduction

Radio-immunoassay (RIA) is the only method suitable for routine quantitation of endogenous substance P levels in animal tissue samples and is well established as such (Powell et.al.,1973; Iversen et.al.,1976; Di Giulio et.al.,1985). Firstly this method is sensitive enough to measure the relatively low (pM) levels of substance P in the CNS. A limit of detection of less than 0.1 fmols substance P has been reported using RIA to measure substance P in dialysates collected from rat brain (Brodin et.al.,1983). Secondly RIA can, potentially, offer the degree of specificity required to differentiate between substance P and various other peptides with similar structures which may be present in CNS tissue. The assay

is based on displacement by substance P of a tracer, radio-labelled substance P, bound by antibodies. The concentration of endogenous substance P in samples is determined by comparing the displacement it produces with the displacement caused by known concentrations of synthetic substance P (standards).

The quality of the assay, with respect to sensitivity, specificity and precision, depends on obtaining a high titre antiserum (As.) and a specific high activity tracer. A good antiserum is one that will bind the tracer with high avidity yet allow displacement of the tracer by substance P. The avidity of binding of the antiserum to substance P must be in the region of more than 100 fold better than that for other similar peptides or substance P fragments in order to be highly specific for substance P. Ideally, non-specific binding (NSB) to the buffer and assay vials should also be low and less than 10% of total tracer binding. Working with a high antiserum dilution ensures a low concentration of other components of the serum which may interfere with the antiserum-tracer interaction and so final assay antisera dilutions of 1 in several thousand are preferable for RIA.

The sensitivity and precision of the antisera and the optimal assay conditions including antisera and tracer dilutions can be assessed using antisera titration and displacement (standard) curves (Fig.2.1). Primarily the tracer must be diluted such that its activity in the assay is at the level of maximum counting efficiency of the gamma counter (usually, approximately 10,000 cpm). The proportion of the total amount of tracer that is bound by antibodies, the binding coefficient, depends on the dilution of tracer and antiserum used and in practice dilutions and conditions are selected such that (i) the binding coefficient is 30-40% (ii) the range of substance P concentration over which the tracer is displaced is more than ten fold and (iii) the displacement curve is steep in order to produce a high precision assay.

In addition to substance P many of the antisera used in substance P assays have been shown to bind substance P fragments or peptides containing amino-acid sequences present in substance P. Furthermore this cross-reactivity is often specific in that an antiserum may selectively recognize either the N- or C-terminal of the substance P molecule and any peptide containing
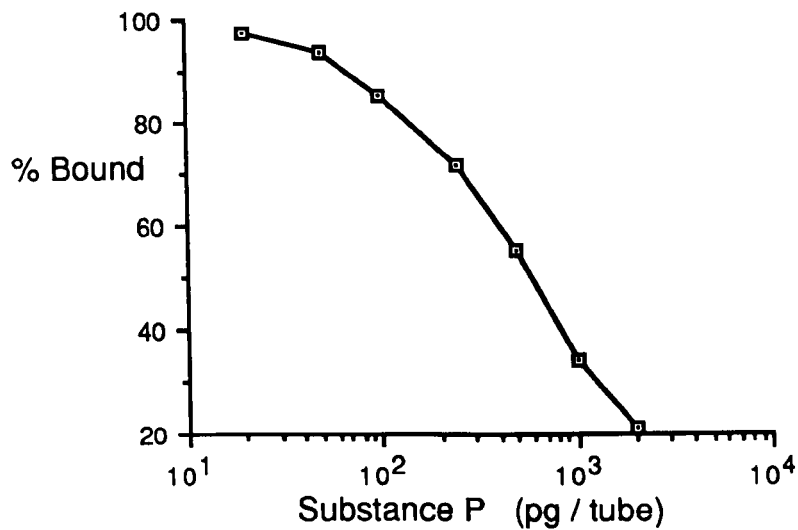
Fig.2.1 Substance P displacement curve

This figure shows a typical substance P displacement or standard curve. Log. substance P concentration is plotted against %Bound; where % bound = B/B_0 expressed as a percentage; B = tracer bound in the presence of substance P and B_0 = tracer bound in the absence of substance P. Each point is determined from the mean of four replicates. This format is used for all the displacement curves of substance P and other peptides shown in this thesis.

either or both of these elements (Lee et al., 1980; Toresson et al., 1988). For this reason the RIAs are described as detecting substance P-like-immunoreactivity (substance P-LI). Using an antiserum with low substance P specificity would result in the substance P-LI measured being greater than the substance P concentration in the sample.

This cross-reactivity of antisera with substance P-like peptides is especially important to consider in the light of the evidence for the presence of multiple forms of substance P in CNS tissue including (i) peptides with similar structures to substance P (i.e. other tachykinins), (ii) substance P or tachykinin metabolites and (iii) tachykinin precursor peptides.

(i) Substance P is one of a group of peptides known as tachykinins which share the C terminal amino-acid sequence, Phe-X-Gly-Leu-Met-NH2 which is responsible for the aspects of biological activity that the tachykinins have in common. Recently tachykinins, other than substance P, including neurokinin A (also known as substance K) and neurokinin B (or neuromedin K) have been reported to be present in the mammalian central nervous system (Kimura et al., 1983; Kangawa et al., 1983; Tatemoto et.al.,1985) and may be responsible for effects previously accorded to substance P. Functionally, substance P, neurokinin A and neurokinin B are closely associated in that they produce similar effects in the periphery (Hua et. al., 1984) though they interact preferentially with different receptor sites (Quirion, 1983; Buck et. al.,1986). Furthermore both neurokinin A and substance P are derived from the same precursor polypeptide, preprotachykinin, in bovine brain and human carcinoid tumour though there are minor differences in the amino acid make up of the precursor in the two species (Nawa et al.,1983; Harmar et.al.,1986). The structures of these tachykinins are shown in Fig.2.2.

(ii) Recent evidence also indicates the presence of membrane bound enzymes isolated from central nervous tissue which act to cleave substance P into smaller fragments (Arzumanyan et.al.,1985). Furthermore, Sakurada and co-workers (1985) have reported that levels of C-terminal fragments in the areas of rat brain rich in substance P are 1-2% of those of the

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Fig.2.2   The structure of substance P and some related peptides

Substance P

       ARG-PRO-LYS-PRO-GLN-GLN-PHE-PHE-GLY-LEU-MET-NH$_2$

Neurokinin A

       HIS-LYS-THR-ASP-SER-PHE-VAL-GLY-LEU-MET-NH$_2$

Neurokinin B

       ASP-MET-HIS-ASP-PHE-PHE-VAL-GLY-LEU-MET-NH$_2$

Substance P fragments

1-4   ARP-PRO-LYS-PRO

1-7   ARG-PRO-LYS-PRO-GLN-GLN-PHE

7-11   PHE-PHE-GLY-LEU-MET-NH$_2$

Tyr-8-substance P

       ARG-PRO-LYS-PRO-GLN-GLN-PHE-TYR-GLY-LEU-MET-NH$_2$

Residues common to substance P are under-lined.

parent compound. While in the spinal cord levels of substance P 1-7 were reported as 18% of those of substance P (Sakurada et.al., 1985). Substance P may also be broken down into smaller fragments during extraction from tissue samples in the process of measuring tissue levels.

(iii) In addition to reports of a tachykinin precursor present in bovine brain, substance P fragments extended at the N-terminal or both the N- and C- terminal of substance P have been described in extracts of rat CNS tissue and in human cerebrospinal fluid (Nawa et.al.,1983; Harmar et.al.,1984; Toresson et al.,1988).

It is therefore vital that the antiserum used in the substance P RIA is substance P specific and can distinguish between substance P and other similar peptide structures. Moreover, antisera known to be directed towards the N- or C- terminal of substance P can be valuable in assessing whether substance P-LI measured comprises non-substance P components. For example Harmar and co-workers (1984) used C- and N- terminally directed antisera in combination with enzymatic degradation of tissue extracts to identify a substance P sequence which is extended at the N-terminal and present in rat hypothalamus. Many of the substance P fragments discussed above contain the C-terminal sequence of substance P and there would be little or no recognition of these peptides by an N-terminally directed substance P antiserum. This chapter therefore describes an attempt to raise antisera specifically directed against either the N- or C- terminal of substance P. A number of antisera were raised in sheep and subsequently evaluated for their specificity, titre and tracer binding capacity as well as for their use in RIA detection of substance P with respect to sensitivity and precision of the assay.

For in vivo production of antisera against a peptide the immunogenicity of the peptide is enhanced by conjugation with a large immuno-reactive carrier protein such as haemocyanin before injection into the animal. A cross-linking agent is required to link the peptide and protein and the product of the linking reaction depends on the conjugating agent, the protein and the reaction mechanism (Williams and Chase, 1967). Different conjugating agents involve different residues of the peptide in the

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peptide-protein link leaving the remaining residues free against which the immunogenic reaction is directed. Carbodiimide and glutaraldehyde used in peptide protein linkage tend to produce C- and N- terminal linked protein conjugates respectively (Goodfriend et.al.,1964; Sternberger,1979) and were therefore used as conjugating agents because of this property.

The antisera produced were then tested for cross-reactivity with the tachykinins neurokinin A and neurokinin B and with the substance P fragments 1-7, 1-4 (N-terminal) and 7-11 (C-terminal) as well as with tyr-8-substance P to evaluate their specificity.

HPLC with RIA

HPLC separation of peptides used in conjunction with RIA is a well established technique for determining the number of components of a sample contributing to the total immunoreactivity measured by RIA (Hua et.al.,1985; Toresson et.al.,1988). HPLC with U.V. detection is not very sensitive compared to RIA and is therefore not used for the routine measurement of tissue peptide levels. However, HPLC is invaluable for separating the large number of similar peptides and peptide fragments present in samples. By analysing the separated components for immunoreactivity using RIA the specificity and cross-reactivity of the assay can be assessed. Detailed in this chapter is an attempt to use HPLC with U.V. and RIA detection to separate substance P and other peptides which may be present in spinal cord extracts in order to determine the specificity of the antiserum used.

Methods

Radio-active labelling of substance P

Substance P was radio-labelled with [125]iodine by the chloramine T method as described by Powell and co-workers (1973). Tyr-8-substance P (5µg) was incubated with 1mCi sodium [125]I (10µl) in phosphate buffer (0.5 M, pH 7.4) and 50µg of chloramine T. The reaction was stopped by the addition of 150µg of sodium metabisulphite after 15 seconds followed by 1ml of human plasma. Quso glass (10mg) previously activated by heating to 600°c was added to the reaction mixture to adsorb the radio-active peptide. After centrifugation (1000g, 5minutes) the remaining free iodine was discarded in the supernatant and the pellet was resuspended and centrifuged in 1ml of distilled water (1000g, 5mins). The labelled peptide was eluted from the second pellet in 60% acetone /1% acetic acid (1ml) and the supernatant of the third spin (1000g, 10 mins.) containing the ^{125}I labelled tyr-8-substance P was stored at 4°c. For RIA the tracer was diluted such that 50µl produced approximately 10,000 cpm on the gamma counter (LKB Clinigamma) and was used at an assay dilution of at least 1 in 4000.

Production of substance P antisera

A. Substance P conjugation to haemocyanin

Substance P was conjugated with haemocyanin using a modification of the method of Goodfriend (Goodfriend et.al., 1964). Either 0.5 mls of 0.1 M glutaraldehyde or 0.5 mls of 50% 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide (referred to here as carbodiimide) were added slowly to 1.5 mg substance P, 10mg whelk haemocyanin and one drop of undiluted tracer in 1ml of deionised double distilled water. After 18 hours incubation at room temperature the reaction was terminated using dialysis by suspending the reagents in dialysis tubing (size 2-18/32") in 3 litres of continuously stirred water at 4°c for 48 hours. Twenty four and 48 hours after initation of dialysis samples of water were

tested for radio-activity which was compared with activity in the original conjugate. The percentage of total peptide which was conjugated to haemocyanin was calculated from the amount of labelled peptide retained in the tubing on the basis of two assumptions. These were firstly that the conjugated peptide (labelled or unlabelled) was retained within the dialysis tubing and thereby separated from the unconjugated peptide which passed through it. Secondly, it was assumed that the proportion of substance P conjugated to haemocyanin was the same for the labelled and unlabelled forms. Aliquots of the conjugate were stored at -80°c.

B. Ovine immunization

For the production of antisera to substance P in young ewes a procedure similar to that used in rabbits by Bassiri and Utiger (1972) was employed. Each conjugate (750µl) was mixed with 4.5 mls of Freund's complete adjuvant, a general immuno-potentiator, and the total volume divided amongst six two year old ewes (sheep numbers 312C, 308C, 314C, 304G, 306G, 310G). Three ewes each received one intramuscular and multisite subcutaneous injections of 1.6 mls of either carbodiimide or glutaraldehyde conjugate. Subsequently five subcutaneous booster immunizations with either conjugate (300µl) emulsified in Freund's incomplete adjuvant were given at approximately monthly intervals.

At 18 and 22 weeks after the initial immunization blood samples were taken from the jugular vein for determination of substance P antiserum titre. Serum production was discontinued in sheep no.s 312C and 308G after 18 weeks as the titre was very low. After 22 weeks sheep 304G and 308C were killed and exanguinated while the remainder received a booster of 0.5 mg substance P in Freund's incomplete adjuvant. These two were killed and bled 10 days later.

Collected blood was allowed to clot and retract overnight at 4°c. The serum was centrifuged (2000g, 30minutes, 4°c.) and stored at 4°c in 0.1% sodium azide as preservative.

A. Titration Curves

Eight antisera (As. 8A, 8B, 8i, 8ii,8iii, 9/80, 65/66, SP8) raised in rabbits (MRC, Cambridge) were tested with those raised in sheep as described above for use in RIA. Initially the optimal antisera dilutions for RIA, those producing a value of 30-40% for the ratio of antibody bound tracer to total tracer (binding coefficient) were determined from antiserum titration assays.

A series of dilutions from 1 in 100 to 1 in 10,000 of each antiserum was made in RIA buffer containing 0.825% sodium barbital, 0.5% bovine serum albumin. Fifty µl of each antiserum dilution was incubated with 100µl RIA buffer and 50µl diluted tracer overnight at 4^0c. The antibody-tracer conjugate was precipitated by addition of 1ml ice cold ethanol and separated by centrifugation (1000g, 4^0c, 15 mins.) The bound tracer was determined by counting on a LKB gamma counter for 90 seconds.

The ratio of antibody-bound tracer to total tracer in a 50µl standard was calculated for each antiserum dilution and plotted against antiserum dilution. From the titration curves optimal antiserum dilutions resulting in binding coefficients of 30-40% were selected for use in substance P displacement curves.

B. Displacement curves

The ability of the antibody bound tracer to be displaced by substance P was determined using a radioimmunoassay based on a modification of the method of Powell and co-workers (1973) with antiserum dilutions producing a binding coefficient of 30% , or greater dilutions if reproducible displacement curves could be still obtained. Antiserum dilutions used in displacement curves were as follows: As. 314C, 1 in 100; As. 308C, 1 in 100; As. 304G, 1 in 1000; As. 306G, 1 in 750, As. 8A, 8C, 8ii, 9/80, 65/66 SP8, 1:1000; As.8iii, 1:2500.

Fifty microlitres of antiserum were incubated with 50µl of diluted tracer and 100µl of unlabelled substance P standard (10-2000pg) or buffer overnight at 4^0c. Bound tracer was separated

from unbound using ethanol and measured as above. The ratio of bound tracer in the presence and absence of substance P (B/B_0) was calculated for each concentration of substance P. Displacement curves were obtained by plotting B/B_0 against the log. of substance P concentration (Fig. 2.1).

Specificity of the new antisera

Six batches of antisera of which the displacement curves covered at least a ten fold substance P concentration range with a maximum displacement of approximately 70% were selected for testing for cross-reactivity with other peptides. Four of the six batches As. 306G, 304G, 308C and 314C comprised sera collected at the end of the immunization period. Batches were also selected from blood samples collected at 18 weeks from 2 of the same sheep (As.306G2 and 304G2). All six batches of antisera were tested for cross-reactivity with the substance P fragments substance P 7-11, 1-7 and 1-4 and the substance P-like peptides neurokinin A, neurokinin B and tyr-8-substance P (see Fig.2.2 for structures). The peptides were incubated within a range of 10-2000 pg/tube with tracer and antiserum diluted as described for substance P displacement curves. A higher concentration (20ng/tube) of peptide was used for those producing insignificant displacement at 2ng/tube. Percentage cross-reactivity of the antisera with the non-substance P peptides was calculated as the ratio of the concentration of substance P producing 50% displacement to that of non-substance P peptides and expressed as a %. Where the greatest displacement obtained was less than 50% the respective concentrations producing maximum displacement were used. This ratio was determined as the mean value from duplicate assays. The cross-reactivity of the antisera to substance P was taken as 100%. Displacement assays with the peptides at a concentration of 200 ng/tube were performed in quadruplicate using As.304G.

Radio-immunoassay modifications

The effect of the following treatments on the sensitivity and precision of the substance P assay was assessed by comparing the displacement curve profiles and NSB in treated and control assays.

1. Filtering antisera through 0.45 μm millipore glass fibre filter before use.

2. Twenty four hour incubation of antiserum and substance P standards followed by a further 24 hour incubation period after addition of the diluted tracer instead of overnight incubation of standards, tracer and antisera.

Antisera 306G and 8A were used at 1 in 750 and 1 in 1000 dilutions respectively following the RIA methods described above.

Assay Reproducibility

Inter-assay and intra-assay variation was determined as a measure of reproducibility of the substance P assay using a 1 in 1000 dilution of As. 304G (final dilution, 1 in 4000).

Inter-assay variation was calculated as the coefficient of variation (standard deviation/mean) of 6 identical measurements made in separate assays using different batches of tracer over a period of 5 months. The measurement made was the substance P-LI of a substance P standard (500 pg/tube) selected as the mid-point of the steep portion of the substance P displacement curve.

Intra-assay variation was determined as the coefficient of variation of values obtained for the substance P-LI of 4 identical standards (500 pg/tube) determined in the same assay. The coefficient was calculated as the mean± SEM of 9 assays.

Evaluation of antisera specificity using HPLC

HPLC with U.V detection was used to separate substance P from other peptides present in human spinal cord extracts. To find the optimal wavelength at which to detect the peptides within the HPLC mobile phase solutions of tachykinins and substance P fragments (100 μg/ml) were scanned to determine the wavelength at which

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their absorbance of light was greatest. A number of potential constituents of the mobile phase including methanol, ethanol, propan-1-ol, propan-2-ol and phoshate buffer were also scanned to determine the wavelength of peak light absorbance. Absorbance over the range 190-340nm of solutions of peptides and solvents in glass cuvettes were made against water as a reference using a CE 500 scanning spectrophotometer.

Synthetic substance P, neurokinin A and neurokinin B and substance P fragments 1-4, 1-7 and 7-11 (10 µg each) were separated by high performance liquid chromatography (HPLC) using a mobile phase of 55% HPLC grade methanol, 0.3% trifluoroaceticacid, pH 2.0 delivered at a rate of 1.5 mls/minute to a Waters Z module employing a 10 µm Bio-Rad ODS (250x 4mm) cartridge. Peptides were detected by a Beckman 160 ultra-violet detector set at 0.1 A.U. using a 240 nm filter and time taken for samples to travel through the system was determined by the interval between sample injection and detection of the solvent front.

An extract of control human spinal cord in ethanol and acetic acid (prepared as described in chapter 3, pp. 94-95) was dried by evaporation under vacuum at 60°c, resuspended in 1ml of mobile phase, centrifuged (1300g, 3mins.) and filtered through an ACS acrodisc before injection onto the HPLC system. With the detector disconnected 1 minute fractions of mobile phase were collected from the end of the cartridge for sixty minutes starting 2 minutes after injection. Cocktails of substance P, substance P fragments 1-7, 1-4 and 7-11 and neurokinin A and neurokinin B at concentrations of either 10 µg or 40 ng/injection were separated and collected in the same manner. The system was washed with mobile phase overnight between injections of high and low concentration peptides. Fractions were dried and stored at -80°c.

Fractions were assayed for substance P-LI by RIA after resuspension in 500 µl of RIA buffer. Those of the high concentration cocktails were diluted 1 in 1000. Substance P displacement curves using As. 304G were produced as described except that standards were made in dried down mobile phase resuspended in RIA buffer to be comparable with the samples of

HPLC fractions. Standard curves were also produced in dried mobile phase diluted 1 in 1000 with RIA buffer. To detect substance P-LI in the fractions 100μl of each resuspended fraction (in 4 replicates) were incubated with antiserum and tracer and assayed alongside the displacement curves. The ratio of bound tracer in the presence of the sample to that in the absence of substance P (B/B_0) was calculated. The concentration of substance P-LI in the sample was determined by interpolation from the appropriate (diluted or undiluted) displacement curve.

Drugs

The peptides substance P, neurokinin A, neurokinin B and tyr-8-substance P and the substance P fragments 1-4, 1-7 and 7-11 were obtained from Peninsula Laboratories Europe Ltd. Antisera 8A, 8B, 8i, 8ii, 8iii, 65/66, 9/80 and SP8 were obtained from Dr. P Emson, MRC Neurochemical Pharmacology Unit. Cambridge.

Results

Antisera production

After 24 and 48 hours respectively of dialysis against water there was 58 and 31% recovery of the tracer from the glutaraldehyde conjugate and 10 and 8% recovery from the carbodiimide conjugate.

Although antiserum titre itself was not measured directly, an indication of titre was determined from antisera titration curves. Larger antisera dilutions producing a binding coefficient of 30% are indicative of higher serum antibody content. Therefore the antisera dilution factor producing a binding coefficient of 30% determined from titration assays using consistent conditions and tracer dilution was used to represent serum titre. This value is the final dilution of the antisera as it is used in the assay. Eighteen weeks after initial immunization serum samples from the six sheep showed a wide variation in antiserum titre defined in this way (Fig.2.3) and the titres from the sheep receiving the glutaraldehyde conjugate (no.s 304G, 306G and 310G) were higher than of those receiving carbodiimide conjugate (314C, 308C and 312C). The titres in the latter case being less than 1 in 400. Antiserum production was discontinued in the sheep (no.s 312C and 310G) producing the lowest titre for each of the two treatments. Four weeks later although the titres of the antisera raised against carbodiimide conjugate (carbodiimide-antisera) had increased moderately they were still both less than 1 in 600. Sheep no. 304G had a stable titre but that of 306G had fallen to 1 in 800. Ten days after the final booster immunization with substance P the antiserum titre of sheep no.s 306G and 314C had increased moderately but were still low with values of approximately 1 in 1400 and 1 in 400 respectively.

Evaluation of antisera

The antibody titre and/or binding coefficient determined using 1 in 4000 final antiserum dilution are given in Fig.2.3 for

Fig. 2.3   The titre* and binding coefficients of a number of antisera

Rabbit Antisera

	8A	8B	8i	8ii	8iii	9/80	65/66	SP8
*Titre	4000				24000			
Binding Coefficient (%)	30	20	20	18	55	6.2	7.5	12

Sheep Antisera

Interval after immunization (weeks)	306G	304G	310G	308C	312C	314C
*Titre 18	2800	4000	400	<400	<400	<400
22	800	4000	-	520	-	<400
23.5	1400					400
Binding Coefficient (%)	22	28	-	23	-	11

*Titre is expressed as the dilution factor producing a binding coefficient of 30%. The binding coefficient given is that produced by a final assay serum dilution of 1 in 4000.

the rabbit antisera, As. 8A, 8B, 8i, 8ii, 8iii, 9/80, 65/66, and SP8 .
Only As.8A and 8iii had titres of 1 in 4000 or more. The
displacement curve of As.8iii was relatively flat with a maximum
displacement of only 30% (Fig.2.4) whereas that of As. 8A was
sigmoidal with a maximum displacement of 85%. The straight
part of this curve was steep and covered a substance P
concentration range of 100-600 pg/tube (Fig.2.4).

Reproducible displacement curves over the substance P
concentration range 30-2000 pg/tube were obtained for sheep
antisera As. 304G, (Fig.2.9), 306G, 308C and 314C with maximum
displacements of over 80% for each at the highest substance P
concentration.

Antisera specificity

There were no differences in peptide cross-reactivity
between the batches of serum collected from the same sheep at 18
and 22 weeks after initial immunization for either sheep 306G or
304G despite differences in titre and they will therefore be
discussed as As. 306G and 304G only.
The % cross-reactivity of the carbodiimide- and
glutaraldehyde-antisera with neurokinin B, neurokinin A, tyr-8-
substance P and substance P 1-7, 1-4 and 7-11 is shown in Fig.2.5.
With respect to the antisera raised against the
glutaraldehyde conjugate (glutaraldehyde-antisera) As.306G and
304G, there was no discernible displacement of the antibody bound
tracer by the substance P fragments 1-4, 7-11 and 1-7 or by the
peptides neurokinin A and neurokinin B in the concentration
range 10-2000 pg/tube, the concentration range of the substance P
standard curve. There was still no discernible displacement of the
As. 304G bound tracer by any of these peptides at concentrations of
20ng and 200ng/tube (See Fig.2.6). In the case of As.306G there
was displacement by substance P 7-11 (17%) and neurokinin B
(14%) only at 20ng/tube but no displacement by the other peptides.
Peptide cross-reactivity of the carbodiimide-antisera
differed from that of the glutaraldehyde-antisera with respect to
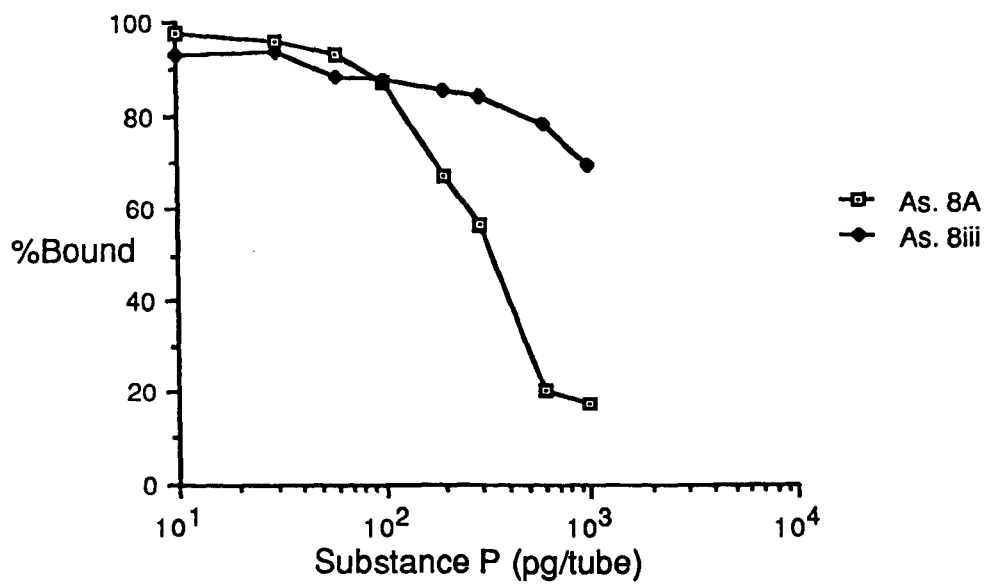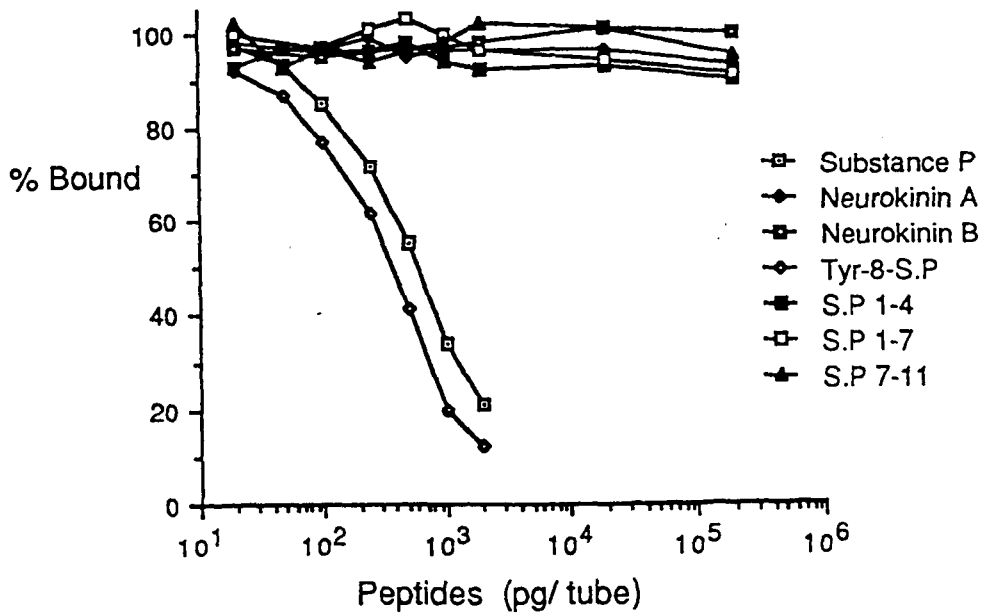displacement of the bound tracer by substance P 7-11, neurokinin B

Fig. 2.4 Substance P displacement curves
using two different antisera 8A and 8iii.

Fig.2.5 %Cross-reactivity of anti-sera with peptides

	Anti-sera			
	306G	304G	314C	308C
Peptides				
Substance P	100	100	100	100
Neurokinin A	0	0*	0	1.0
Neurokinin B	0.5	0*	1.0	1.2
Tyr-8-substance P	109	122	-	185
Substance P fractions				
1-4	0	0*	0	0
1-7	0	0*	0	0
7-11	0.6	0*	19.6	28

Cross-reactivity of an antiserum with a peptide was calculated as the ratio of the concentration of substance P producing 50% displacement of binding to that of the peptide and is expressed as a %. Cross-reactivity of substance P was defined as 100%. For further details see methods. A value of zero is given where there was no displacement of the antisera bound tracer with 20 or 200* ng/tube peptide concentration.

A. Cross-reactivity of anti-serum 304G

% Bound

	Substance P
	Neurokinin A
	Neurokinin B
	Tyr-8-S.P
	S.P 1-4
	S.P 1-7
	S.P 7-11

Peptides (pg/ tube)

B. Cross-reactivity of antisera 308C
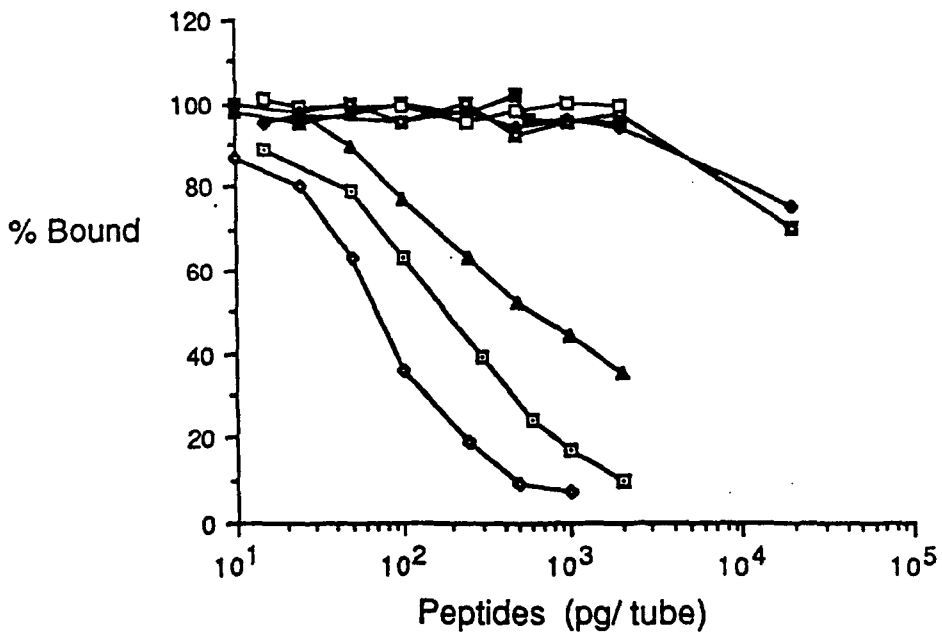

% Bound

Peptides (pg/ tube)

Fig.2.6 Cross-reactivity of antisera 304G and 308G with 7 peptides

This figure compares the displacement of antisera 304G (A) and 308C (B) bound tracer by substance P and 6 other peptides named in the key. The major differences between the two antisera is in the displacement by the C-terminal substance P fragment 7-11 and by neurokinin A and neurokinin B.

and neurokinin A. There was no discernible displacement with the N-terminal fragments substance P 1-7 and 1-4 at any concentration up to 20ng/tube. In contrast the C-terminal fragment substance P 7-11 displaced the carbodiimide antisera bound tracer in a concentration dependant manner as illustrated in Fig.2.6 for As.308C. The degree of displacement at each concentration was less than that by substance P. Neither neurokinin A or neurokinin B displaced carbodiimide-antisera bound tracer at concentrations up to 2ng/tube. However at 20ng/tube neurokinin A displaced As. 308C only by 25% and neurokinin B produced 29% and 24% displacement respectively of As.308C and 314C bound tracer (Fig.2.6).

Tyr-8-substance P displacement curves exhibited a similar profile to the substance P displacement curves for the three antisera tested with this peptide (As. 306G, 304G and 308C). The gradients of the two curves were similar but tyr-8-substance P generally produced greater displacement of tracer at each point than substance P (Fig.2.6).

RIA Modifications

Neither filtering the antisera nor varying the incubation period of the RIA affected either non-specific binding or the displacement profile of As. 304G or 8A as illustrated in Figs. 2.7 and 2.8.

As.304G Displacement curve

The binding coefficient of the tracer using As. 304G at a final dilution of 1 in 4000 was between 20-30%. Variation within this range occurred using different batches of tracer. Reproducibility of the assay was good with inter- and intra-assay variability of 8.0% and 12.0±2% respectively calculated as described in the methods. The steep part of the curve covered a 10 fold concentration range from 100-1000 pg/tube and the maximum displacement was approximately 82%. See Fig.2.9. Sensitivity of the assay was 74 fmols/tube.

Fig.2.7 Effect on substance P RIA of filtering antisera

Substance P displacement curves obtained using antisera 306G. Each point is the mean from two assays. The antisera was either filtered (□) or unfiltered (◆) before use. Non-specific binding (NSB) was the same in each case
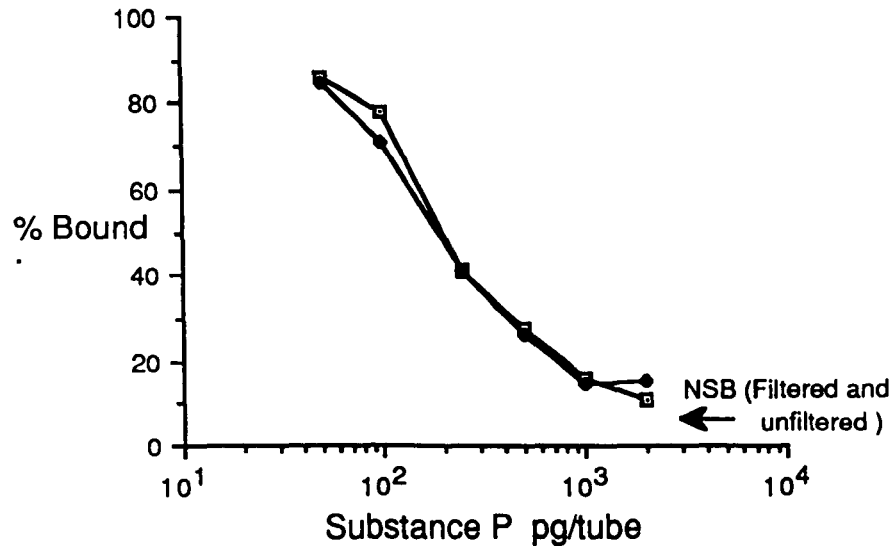
Fig.2.8 Effect on substance P RIA of prolonged incubation period

Substance P displacement curves obtained using antisera 8A and two treatments.

◆ Antisera standards and tracer were incubated overnight.

□ Antisera and standards were pre-incubated for 24 hours and the incubation period was extended for a further 24 hours after addition of tracer. Each point is the mean of two assays.
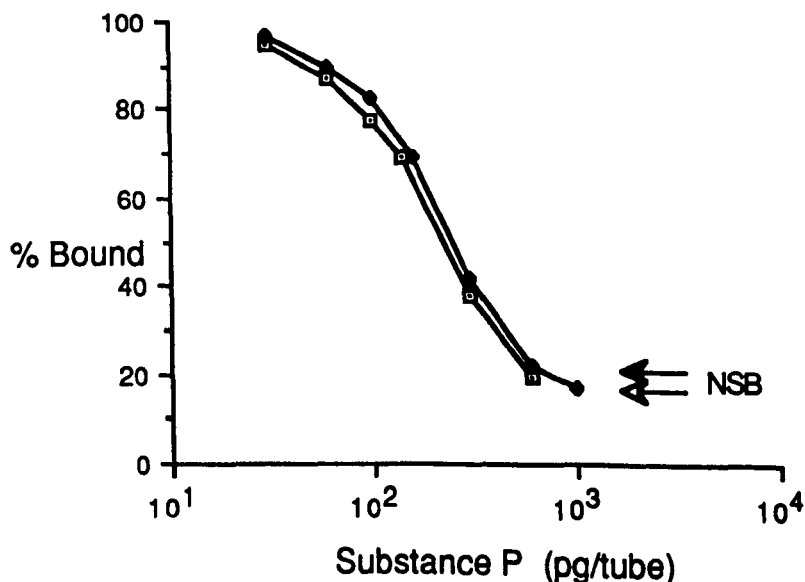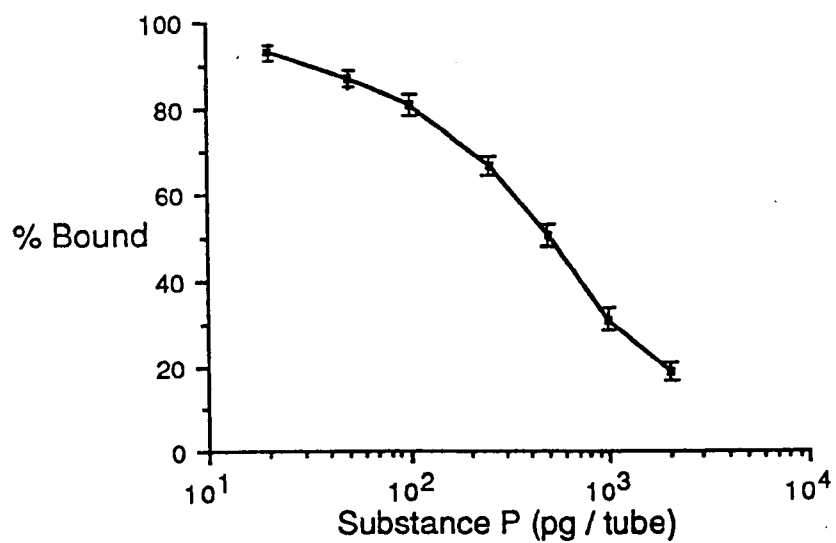
Fig. 2.9
Substance P displacement curve using antisera 304G

This curve shows the mean and SEM of 9 substance P displacement curves performed on different occasion using antisera 304G at a dilution of 1 in 4000. [^{125}I] substance P dilution varied with the batch used and was no less than 1 in 4000.
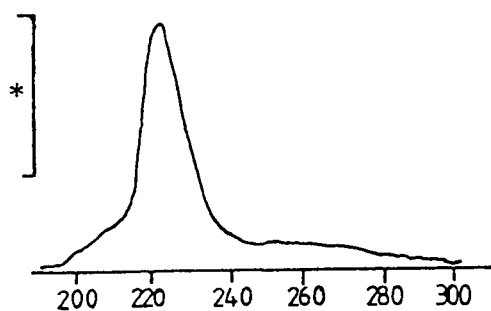
The absorbance of light by substance P, neurokinin B, neurokinin A and substance P fragments 1-4, 1-7 and 7-11 as measured with a scanning spectrophotometer increased sharply over the range 210-240nm with maximum absorbance at 225-230 nm compared with the reference cell. Typical scan profiles of 3 peptides are given in Fig.2.10. Maximum light absorbance by ethanol, methanol, propan-1-ol and propan-2-ol occurred between 190-240nm with the peak at 190-220nm whereas for phosphate buffer it occurred at 190-200nm and fell to zero compared with the reference cell above 205nm.

Substance P fragments 7-11, 1-7 and 1-4 eluted within 5 minutes after sample injection (Fig.2.11). Neurokinin A, neurokinin B and substance P eluted over the intervals 3-5, 5-7 and 10-16 minutes after injection respectively (Fig.2.11). The limit of U.V. detection was approximately 600ng for substance P and as low as 60ng for the other peptides depending on their elution time. The later the peptides eluted from the column the wider and smaller were the corresponding chromatographic peaks thereby resulting in less sensitive detection levels.

After injection of the high concentration (10 µg/injection) cocktail of peptides substance P-LI was detected in fractions 10-15 but not in any other fraction (Fig.2.11). Fractions 10-15 corresponded to the time interval over which substance P was eluted from the column. These HPLC fractions were diluted 1 in 1000 before RIA. There was, however, very little binding in any of the fractions which were not diluted before assay. This included those collected after injection of low concentration (40ng/injection) peptide standards and samples of spinal cord extract. The binding in the standard curve made using dried undiluted mobile phase was also very poor and maximum displacement by synthetic substance P was approximately 20%. For this reason substance P-LI in the extract of human spinal cord was not quantifiable though there was some displacement of binding in fractions 10-15 only.

Fig. 2.10 Ultraviolet scan profiles of substance P, neurokinin A and substance P 7-11.

substance P
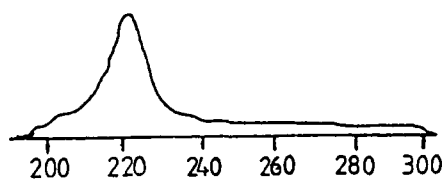
substance P 7-11
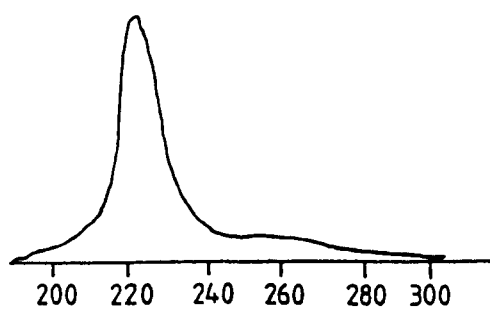
neurokinin A

wavelength (nm)

Absorbance of light by substance P, substance P 7-11 and neurokinin A (100μg/μl) over the wavelength range 190-300nm. Peptides were dissolved in water and absorbance was measured against a reference cell of water. *Scale is 0.2 Absorbance Units

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Fig. 2.11 HPLC separation of peptides with U.V. and R.I.A detection

(i) HPLC separation of synthetic neurokinin A (A, 10μg), neurokinin B (B, 3μg) and substance P (SP, 8μg) in a mobile phase of 55% methanol, 0.3% TFA, pH 2.0 delivered to a 10μm Bio-Rad ODS cartridge at 1ml/min. Peptides were injected at time zero and detected by U.V. absorbance at 240nm. The scale is in absorbance units (A.U).

(ii) U.V detection of substance P fragments 1-7 (2μg), 1-4 (2μg) and 7-11 (1μg) injected separately on to the HPLC system at time zero. Each fragment eluted within 5 mins of injection.

(iii) Substance P-like immunoreactivity in one minute fractions of eluate collected from the HPLC system after injection of a cocktail of 10μg neurokinin A, neurokinin B, substance P and substance P fragments 1-4, 1-7 and 7-11. Fractions were diluted 1:1000 before RIA.

(i)

.001 A.U.

A

B

SP

Elution time
(minutes)

(ii)

.001AU

1-7

1-4

7-11

Elution time
(minutes)

(iii)

substance P-IR (µg /fraction)

3.0

2.5

2.0

1.5

1.0

0.5

0.0

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Fraction
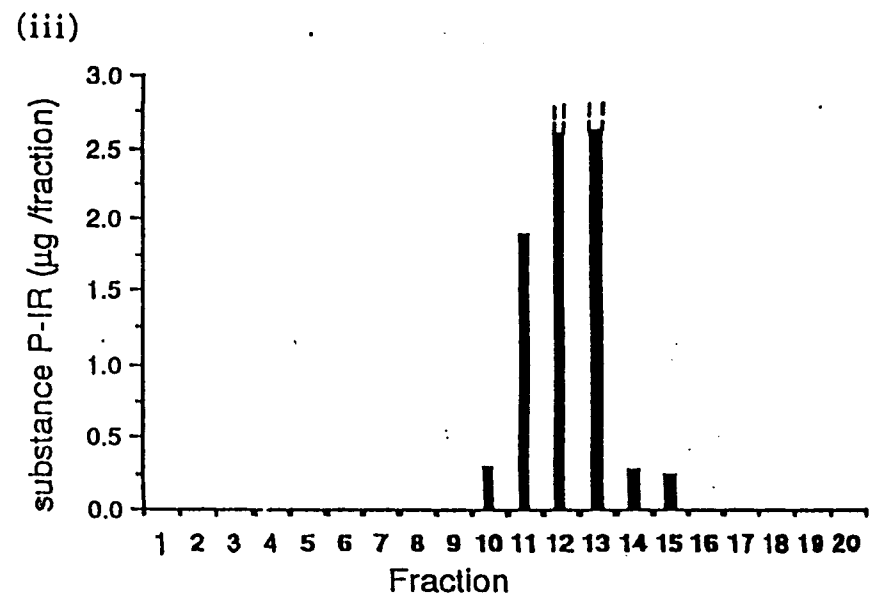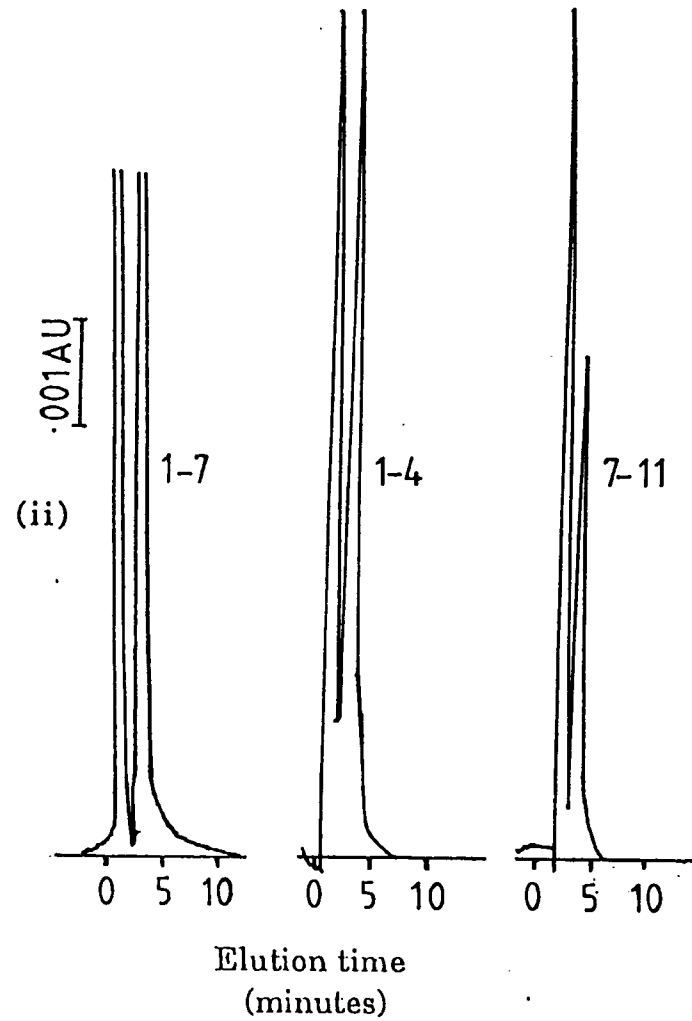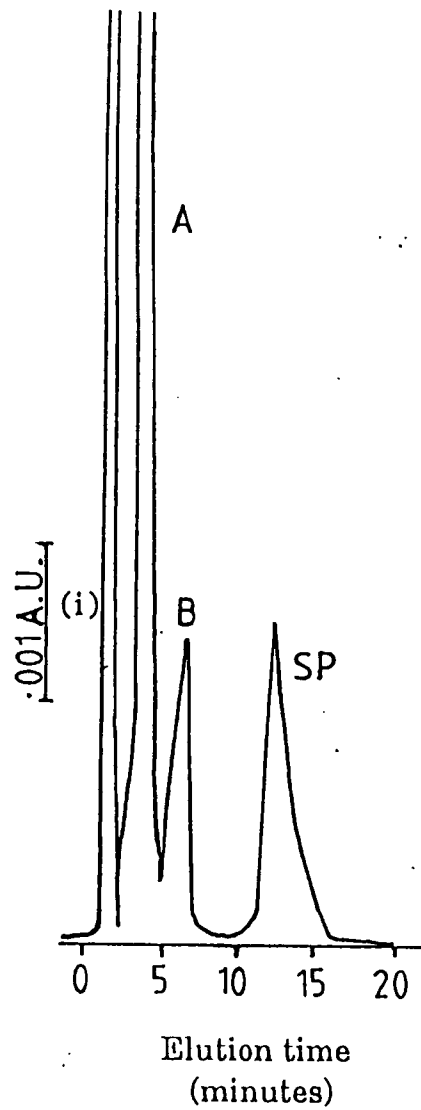
Discussion

The sensitivity of a radioimmunoassay is dependent as much on the properties of the radio-labelled peptide used as on the antiserum. The tracer must be as pure as possible to ensure low levels of non-specific binding. Synthetic substance P itself cannot be labelled directly with radio-active iodine because of the absence of either a tyrosine or histidine residue so a substance P analogue with a tyrosine residue at position 8, tyrosine-8-substance P, was used as described in the methods. The products of the chloramine T method of iodination of tyr-8-substance P are labelled substance P, tyr-8-substance P, damaged peptide and free ^{125}I. Even after purification with quso glass the tracer may be contaminated with free ^{125}I and a proportion of the tracer may also degenerate during storage. It is therefore important that the tracer can be used in high dilutions to minimize the effects of components other than tyr-8-substance P on the antisera -tracer binding. For this reason only batches of tracer which produced 10-15,000 cpm at dilutions of 1 in 4000 or more were used and batches of this standard were produced in 4 out of 5 attempts. The level of non-specific binding varied with different antisera but was less than 10% with As. 304G. Substance P iodinated using Bolton Hunter reagent is available commercially (Amersham International plc.) but is more expensive to use. The tracer was kept at 4°c and used within six weeks of production after which time the binding coefficient fell to less than 10%. Storage of the tracer in frozen aliquots did not improve its viability after 6 weeks.

Of the eight antisera raised in rabbits only two, As.8A and 8iii, had antibody titres which were reasonably high enough for RIA enabling use of 1 in 4000 antisera dilutions. With As.8iii there was little displacement of bound tracer by the highest substance P concentration resulting in an insensitive assay. In contrast the assay using As. 8A was more sensitive with a steep substance P displacement curve displacing 85% of total binding within less than an 8 fold concentration range. An assay with a steep displacement curve is very precise as small changes in peptide levels produce large changes in % displacement. However, such a precise assay

can be less practical to use as it provides a very small concentration range within which the endogenous substance P content of samples must fall if it is to be measured. It is more practical to use an assay of which the steep part of the displacement curve covers more than a ten fold concentration range.

Comparison of glutaraldehyde and carbodiimide as conjugating agents

An immunogenic response was stimulated in all sheep injected with the substance P conjugates used in this study but the antisera varied markedly in their titre and specificity to substance P.

The highest titre of antisera raised against the carbodiimide conjugate was less than 1 in 600. The failure to produce an antiserum of high titre against the carbodiimide conjugate is probably due to the low yield of conjugate produced (indicated by the substance P tracer loss of more than 90% by the end of dialysis). It appears that this method is not appropriate for conjugation of carbodiimide with substance P in the production of antisera for RIA as a repeated attempt produced the same result. The method was adapted from that used by Goodfriend (Goodfriend et.al.,1964) who raised antibodies to bradykinin and angiotensin using carbodiimide hydrochloride to conjugate the haptens with rabbit serum albumin. An adaptation of this method was used successfully by Lee and co-workers (Lee et.al., 1980) to produce substance P antisera by conjugating the peptide to bovine serum albumin using carbodiimide. The presence of whelk haemocyanin in place of serum albumin may not facilitate the covalent bonding of carbodiimide to substance P. Haemocyanin was initially selected as the immunogenic protein as it is a foreign protein to the sheep further removed than BSA or thyroglobulin and was therefore expected to produce a stronger antigenic response. It is also an inexpensive peptide to obtain.

The conjugation of substance P to haemocyanin using glutaraldehyde was more successful than using carbodiimide and though tracer loss indicated that only 30% of the peptide had been conjugated with the hapten this is a reasonable level for practical purposes. The variation in immunogenic response between sheep is

illustrated by the difference in titre of the antiserum produced in sheep receiving the same glutaraldehyde conjugate. The immunogenic response to the glutaraldehyde conjugate varied widely from one sheep to another and after 18 weeks exposure only moderate increases in titre could be stimulated by further injections of the conjugate.

Low specificity of substance P antisera may account for variations in CNS substance P-LI levels reported by different workers (See Toresson et.al.,1988 for review). For this reason carbodiimide and glutaraldehyde were selected for conjugation of substance P to an immunogenic protein in an attempt to produce antisera which specifically recognized either the N- or C-terminal of substance P respectively. The results of the cross-reactivity study indicate that there was some evidence of selective recognition of the C- terminal residues by the carbodiimide antisera but not by the glutaraldehyde antisera raised.

Antisera specificity

The carbodiimide-antisera had 20-30% cross-reactivity with the C-terminal amino acid sequence substance P 7-11, and a smaller degree of cross-reactivity with neurokinin B and neurokinin A which have a similar C-terminal to substance P. This is a clear indication that these antisera are directed towards the C-terminal of the substance P structure. The residues towards the N-terminal of neurokinin B and neurokinin A may interfere with the antiserum binding with the C-terminal residues, this would explain why the cross-reactivity with substance P 7-11 is greater than that of neurokinin A and neurokinin B (Fig.2.6). The results also indicate that antisera recognition of the residues must be partly dependent on configuration as there was no discernible recognition of substance P fragments 1-4 or 1-7 yet bound-tracer displacement by substance P was greater than that by fragment 7-11 alone (Fig.s 2.6).

Cross-reactivity of the glutaraldehyde antisera with peptides other than substance P was much lower than that of the carbodiimide antisera. Antiserum 304G showed less than 0.5% cross-reactivity with neurokinin A, neurokinin B and substance P fragments 1-7,

1-4 or 7-11 producing a sensitive and reproducible substance P assay covering a ten fold concentration range. Exact values for cross-reactivity could be determined by repeating the assays using the peptides at higher concentrations until the bound tracer was displaced.

Antisera recognition of tyr-8-substance P was expected to be great because of the close structural similarity to substance P. In fact the cross-reactivity with tyr-8-substance P was greater than that with substance P itself. The reason for this may be that tyr-8-substance P resembles the substance P-glutaraldehyde conjugate, against which the antiserum was raised more strongly than substance P itself. The hydroxyl group on the substituted tyrosine residue may produce similar configurational changes in the substance P structure as conjugation to the carrier protein. The ability to differentiate between such similar analogues is an indication of the high specificity of the antiserum.

In this study, which attempted to use glutaraldehyde as a linking agent at the C-terminal of substance P, an antiserum (As.304G) has been produced which appears to have low recognition of the C-terminal residues. Antiserum 304G appears to be directed against the whole of the substance P molecule. Lack of cross-reactivity with C- or N-terminal fragments or with the tachykinins neurokinin A or neurokinin B support this claim as does the high degree of cross-reactivity with tyr-8-substance P. This antiserum was therefore selected for use in all subsequent substance P assays in this project. The other glutaraldehyde antiserum 306G, which also showed low cross-reactivity to the peptides tested, was retained for possible use in immuno-histochemical techniques which do not require such high antisera titre.

It has been proposed that substance P itself is inactive within the CNS and that its effects are produced by its degradation into active components (Stewart et. al., 1982). Nyberg and co-workers (1984) have characterized an endopeptidase in human CSF which degrades substance P to residues 1-7, 1-8, 8-11 and 9-11 and there is also evidence that N-and C- terminal fragments of substance P may produce opposite effects in behavioural paradigms such as isolation induced fighting and passive avoidance behaviour (Hall et.al.,

1987; Gaffori et.al.,1984). A recent publication reports the effects of intrathecal administration of selected substance P fragments compared to substance P itself in the rat tail flick test. Substance P 1-7 produced similar effects to that of substance P. Administration of both peptides resulted in a large transient decrease in reaction time whereas fragments 8-11 and 1-9 had no effect and 7-11 produced a small prolonged decrease in reaction time (Cridland and Henry, 1988). These results support the claim that the effects of substance P in the CNS may occur after degradation into smaller fragments and that the resulting peptides may have different effects. The availability of radioimmunoassays specific for selected amino acid sequences could therefore be important in clarifying the relative roles of substance P and other tachykinins and substance P fragments in mediating CNS effects previously associated with substance P.

Antisera specific for N- and C- terminal substance P fragments are useful experimental tools and have been used to identify precursors and metabolites (Harmar et.al.,1984; Toresson et.al.,1988). Many attempts to produce substance P antisera result in the production of C-terminally directed antisera which cross-react with C-terminal fragments and tachykinins (Lee et.al., 1980; Brodin et.al.,1983; Harmar et.al.,1981; Sakurada et.al.,1985). Carbodiimide can be used as the linking agent between hapten and carrier protein. Carbodiimide can couple with a number of functional groups including carboxylic acids, amines and alcohols. Although the exact reaction mechanism with substance P is not known it probably proceeds through more than one intermediary to produce an amide link between hapten and protein (Goodfriend et.al.,1974). Antisera raised against carbodiimide conjugated substance P have been shown in the present study to cross-react with C-terminal residues of substance P, indicating that the carbodiimide tends to form N-terminal linkages. This is obviously a problem for accurate measurement of substance P in systems containing a number of C-terminal substance P metabolites and precursors. The production of an antiserum in this study which has low cross-reactivity with substance P fragments and other tachykinins is therefore a great advantage. Although the reaction mechanism for conjugation of substance P with

glutaraldehyde is not clear it seems likely that a number of different bonds are made in different molecules. In this way consistent masking of specific amino acid sequences is avoided and the antiserum produced recognizes the whole of the substance P structure. However, whatever the nature of the reaction mechanism the advantage of glutaraldehyde over carbodiimide as a conjugating agent in the production of specific substance P antisera has been demonstrated here.

Development of the RIA

A solid phase RIA for quantitation of substance P has been reported by Oblin and Zivkovic (1983) as an improvement of the standard substance P assay. Solid phase RIA is based on the partition of substance P antisera between free substance P and insoluble substance P immobilized in a polyacrylamide gel. [125]Iodinated protein A (a cell wall component) replaces ^{125}I-tyr-8-substance P for use in quantifying the amount of antibody bound substance P-insoluble complex. Cross-reactivity of the assay is still dependent on the antisera employed and is no different from that in a standard assay using the same antisera. The solid phase assay is however marginally more sensitive than the assay described in this chapter and does not require overnight incubation. Therefore its use in combination with As.304G may prove to be an improvement on the present assay.

All antisera were stored at 4°c but some samples were found to have formed growths despite the presence of 0.1% sodium azide. It was decided that the sera should be filtered to remove the growth. Small aliquots of antisera were first filtered to test whether this produced any adverse or advantageous effects on the RIA with respect to sensitivity or precision. Filtering these samples through 0.45 μm filters removed the growths as well as other sediment which had settled out during storage without affecting displacement profiles or NSB (Fig 2.7). The remaining sera were then also filtered though this was a time consuming process as the filters were very rapidly saturated with particles. In some cases the growths returned in time and as the future effects of these are not known it

may be necessary to store some aliquots at -16°c as described by William and Chase (1976).

In the substance P assay generally used in our laboratory diluted tracer and antiserum are added to substance P standards or samples and incubated overnight. Many workers however incubate antiserum and samples for 24 hours before addition of the tracer and/or incubate the three components together for up to 72 hours (Powell et.al.,1973; Brodin et.al.,1983). Comparison was made between two assays set up simultaneously and incubated either overnight or for 24 hours before addition of tracer with a further 24 hours afterwards. There were no differences in displacement profile or NSB of either assay (Fig.2.8). Therefore in all further assays our original protocol was adopted.

Monoclonal Antibodies

A major problem arising in the use of RIA methods is the lack of specificity of the antisera as discussed here. Antibodies to an antigen raised in an animal are not homogeneous as they are produced from a large population of lymphocytes and are therefore polyclonal. There is now the possibility of obtaining greatly improved antiserum specificity with the recent development of monoclonal antibodies. Monoclonal antibodies are obtained from a single clone of cultured cells. The principles of immunizing the animal with the antigen are essentially the same as described in this chapter. However, after the immunization period spleen cells containing lymphocytes which are producing antibodies are fused with myeloma cells. Then individual hybrid myeloma/spleen cells are cloned so that a cell line producing homogeneous antibodies to the antigen is established. The binding properties of monoclonal antibodies are identical and thus highly specific. They can also be reproduced indefinitely so that the radio-immunoassay can be standardized on an international level. However problems of biological specificity may arise from the use of monoclonal antibodies in RIA since the antigenic determinant of a monoclonal antibody consists of only approximately 5 amino acid residues. As every antibody in a monoclonal culture is identical this means the

monoclonal antisera are directed very specifically to a short amino acid sequence. In the case of small peptides such as thyrotrophin-releasing hormone this could result in a very specific antiserum directed against the whole molecule. Unfortunately in the case of larger peptides such as substance P the result would be an antiserum directed specifically at one short section of its structure. This is illustrated by a report from Courand et.al., (1987). These authors raised 5 monoclonal antibodies to substance P which were all directed to the C-terminal pentapeptide of substance P. Accordingly they were reported to cross-react with neurokinin A and neurokinin B. Therefore as yet the development of monoclonal antibodies can not improve the specificity of antisera to larger peptides such as substance P.

Evaluation of antiserum specificity by HPLC with RIA detection

The isocratic HPLC method for peptide separation was adapted from that used by Bennett and co-workers (1989). These authors measured electro-active peptides using HPLC with electro-chemical detection. However, as substance P does not contain oxidizable tyrosine, cysteine or tryptophan residues it is not electro-active so U.V. detection was employed. A number of factors determined the HPLC conditions.

The mobile phase had to be volatile, to allow eluate concentration for subsequent RIA , and have a low absorbance within the peptide absorbance range for sensitive U.V. detection of peptides. The phosphate mobile phase of Bennett et. al.(1989) left a white residue after evaporation and so was replaced by a TFA/methanol mixture. Using the scanning spectrophotometer the peptides were found to have a peak absorbance at 225-230nm but to obtain a greater differential between the sample absorbance and baseline absorbance a 240nm filter was used. At this wavelength both the absorbance of solvents in the mobile phase (of methanol in particular) and the corresponding absorbance offset were lower. The sensitivity of the system to the peptides may however have been improved by detection at 220nm. TFA was chosen as a mobile phase component as it has a low absorbance at 220-240nm and is routinely used in HPLC separation with U.V. detection of peptides.

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For HPLC separation of peptide standards high concentrations were used because the U.V. detector sensitivity was low compared to RIA. After separating the peptides and collecting the column eluate in fragments it was diluted 1 in 1000 before RIA. However when samples were not diluted some component of the eluate badly disrupted the antiserum-tracer binding. RIA could only be performed accurately with samples collected in the eluate and diluted 1 in 1000 though a number of lower dilutions were tested. This effect may have been caused by disintegration of the cartridge at the low pH used or by the NaOH in the mobile phase when it was concentrated. It was not practical to dilute spinal cord extracts 1 in 1000 as such large amounts of tissue would be required.

Difficulties arose when trying to modify the mobile phase as it had to be appropriate to HPLC separation as well as both U.V. and RIA detection of peptides. A number of peptides including substance P were separated by HPLC with U.V. detection by Meek (1980). He reports however that NaCLO4 is an essential component of the mobile phase to produce sharp peaks of substance P and as it is not volatile the eluate can not be concentrated for RIA. Peptide separation in the present study was poor and the peaks were not sharp (Fig.2.11). Although substance P was separated from neurokinin A, neurokinin B and the substance P fragments because of the width of the chromatographic peaks the latter peptides could not be distinctly identified when injected in a cocktail (Fig.2.11). The best separation was obtained using a mobile phase of pH 1.6 but this was not employed as the manufacturers suggest that cartridge damage may occur at levels less than pH 2.

Due to the problems described we were unable to fully validate by HPLC the substance P specificity of the assay using antiserum 304G. However, standards of substance P, substance P 1-4, 1-7 and 7-11, and neurokinin A and B were separated by HPLC, collected in one minute fractions and assayed by RIA using antiserum 304G. Substance P-LI was detected only in HPLC fractions 10-15, the fractions which corresponded to the time interval over which substance P eluted from the column. In support of the results from the RIA cross-reactivity studies, no substance P-LI was detected in the HPLC fractions in which the tachykinins or substance P fragments eluted.

On injection of the extract of human spinal cord onto the HPLC column we were unable to detect any peptides by U.V absorbance because the levels were lower than the limit of detection. Substance P-LI in the collected HPLC fractions could not accurately be determined due to the poor binding caused by assaying the samples without dilution of the mobile phase. There was however some indication of displacement in fractions 10-15 without displacement in the other fractions but this was not conclusive evidence of antiserum specificity. As the binding was disrupted the assay was not representative of the substance P RIA under normal conditions. If normal binding had been achieved and substance P-LI was detected only in fractions 10-15 this would be evidence that the assay is specific for substance P. The results obtained using standards do not account for antiserum recognition of any other tachykinins, metabolites or precursors which may be present in the spinal cord samples. For complete validation of specificity of the antiserum, for substance P HPLC separation of peptides in spinal cord samples with RIA of the HPLC fractions needs to be accomplished.

Separation of peptides including substance P by HPLC has been reported using a linear gradient of mobile phase eluting different peptides from the column at increasing concentrations of, for example, acetonitrile (Hua et. al.,1985; Toresson et. al., 1988). This technique is more flexible than isocratic HPLC as the gradient can be easily modified to obtain the optimal resolution of the peaks and it has been shown that peptide retention times can be predicted from their amino acid composition (Meek,1980). The retention time depends on the sum of the hydrophobicity of the constituent amino acids and 'retention coefficients' for 100 peptides have been determined and found to correspond closely to values estimated from amino acid composition (Meek and Rossetti,1981).

Linear gradient HPLC with U.V. detection is therefore the appropriate method for separation of peptides. Using an isocratic system is impractical although it can be used successfully with sensitive electro-chemical detection of certain electro-active peptides. The major problem using the isocratic system was probably that peptide separation depended on achieving a very low

pH. Using a linear gradient system peptide retention times can easily be manipulated by changing the organic solvent content of the mobile phase. As methanol is volatile even high concentrations would not affect RIA binding in the HPLC fractions after evaporation.

In conclusion, an antiserum (304G) has been raised in sheep using a substance P-glutaraldehyde-haemocyanin conjugate. The antiserum is specific for substance P with less than 0.5% cross-reactivity with neurokinin A, neurokinin B or the substance P fragments 1-4, 1-7 and 7-11. Antiserum 304G appears to recognize the whole of the substance P structure and can be used for RIA of substance P at a 1 in 4000 assay dilution. The resultant assay is sensitive, precise and reproducible. Unfortunately the specificity of the antiserum has not been fully validated using HPLC separation of peptides in conjunction with RIA. However, the results obtained so far using this technique support those obtained in RIA antisera cross-reactivity studies.

Part 2   A common extraction method for the measurement of indoleamines, catecholamines, substance P and TRH

Introduction

The supply of post-mortem human spinal cord material available for the measurement of its neuroamine and neuropeptide content as described in the next chapter was limited. This was due partly to the necessity that the donor subjects fulfilled a number of criteria and secondly to the difficulty and time involved in dissecting the spinal cord. In addition, different protocols were reported for the extraction of substance P, TRH and the neuroamines from tissue samples (Gilbert et.al., 1982; Lighton et.al., 1984; Bennett et.al.,1986). As part of the intention of the investigation was to study the inter-relationship of the indoleamines with substance P and TRH known to co-exist in rat spinal cord neurones (Gilbert et.al.,1982) as discussed in the general introduction an extraction process common to the peptides and amines was required. A common method for extracting neuroamines and neuropeptides from one sample of tissue would therefore enable the measurement of their levels and the investigation of their inter-relationship, whilst simplifying the methodology and making most effective use of the material available. To satisfy these aims a method described in this chapter was developed by which 5HT, 5HIAA, noradrenaline, adrenaline, dopamine, DOPAC, substance P and TRH can be extracted using a common medium and measured using HPLC (indoleamine and catecholamines) and RIA (TRH and substance P).

In this chapter the levels and percentage recoveries of substance P, TRH and indoleamines and catecholeamines extracted from rat spinal cord in the common extractant, a mixture of ethanol, acetic acid and sodium metabisulphite (NaMBS) and in a number of more generally used solutions are compared. The effect of the common extractant on the HPLC chromatography of the amines is also discussed.

All the methods which were to be used to measure peptide, indoleamine and catecholeamine levels in the human spinal cord

including the common extraction method, substance P and TRH radioimmunoassay, HPLC separation and detection of neuroamines and the protein assay were first performed using rat spinal cord material. This enabled problems arising in the methodology to be rectified without wasting any human tissue.

Radioimmunoassay was used for the detection of TRH because it is sensitive enough to measure the pg/mg levels found in rat and human CNS tissue. The TRH is well established and has been used for such analysis since its development (Jeffcoate et. al.,1973) and subsequent improvement (Lighton et.al.,1984). The antiserum used in the TRH radio-immunoassays was raised in sheep in Nottingham (Lighton, 1985) and the RIA using this antiserum was sensitive and reproducible (Lighton, 1985) .

Similarly, HPLC with electrochemical detection (ED) has been used to measure catecholeamines and indoleamines for a number of years and has a limit of detection of 0.1-10 pmols though lower limits can be achieved.

Principles of HPLC

Separation of indoleamines by reverse phase column HPLC depends on separation of the analytes by partition between the hydrophobic stationary phase of the column and the aqueous mobile phase. Elution is in the order of increasing hydrophobicity. As catecholamines are highly positively charged at the pH range of the mobile phase (pH 2-8) they are hydrophilic and eluted rapidly and are therefore both inseparable and indistinguishable from the solvent front. However, with the addition of ion pair reagents to the mobile phase (e.g. sodium octyl sulphate) the hydrophobic side chain and negatively charged group selectively retards the elution of the catecholeamines enabling their separation. Ion pair reagents in the mobile phase make the retention time for 5HT very long and so separate systems are described in this chapter for the separation of indoleamines and catecholeamines.

Electrochemical detection of noradrenaline, adrenaline, dopamine and 5HT and their metabolites is achieved by oxidation at a carbon based electrode. They oxidize at relatively low

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potentials of 0.25- 0.65V, their ring hydroxyl groups producing orthoquinone derivatives and two free electrons which are detected by auxilliary and reference electrodes and recorded as an amplified current. The use of both glassy carbon and carbon paste working electrodes have been recommended (Marsden and Joseph, 1986). The carbon paste electrode can achieve greater sensitivity than the glassy carbon but is more suseptible to surface damage and degeneration. Both types were used here to assess which was the most appropriate for the detection of neuroamines at levels present in the rat spinal cord.

Methods

Sample Preparation

Male wistar rats between 220-280g were killed by
decapitation after stunning. The spinal column was dissected out
and the spinal cord removed on a cool tray after cutting away the
dorsal lamina of the vertebrae and the nerve roots (Fig.2.12). The
first 2.5cm and the last 2cm of the cord were removed as cervical
and sacral cord and the remainder was separated into thoracic
and lumbar regions by identifying the enlargement and
coloration (distinct white spinal roots) of the lumbar region. The
cord was cut longitudinally in two along the ventral median
fissure and then into ventral and dorsal (lumbar cord) or dorso-
intermediolateral (thoracic cord) areas by bisecting the exposed
gray matter. The tissue was snap frozen in liquid nitrogen and
stored at -80°c until extracted later the same day.

Extraction

The tissue was sonicated (NSE Soniprep 150) in small tubes
containing 2 mls of extraction medium (see later) with the
indoleamine and catecholamine internal standards added (see
amine recovery) and placed within a beaker of ice. After
centrifugation (1000g, 4°c, 15 minutes) the supernatant was
decanted and divided into aliquots. A half ml was taken for
indoleamine detection and 0.65 mls each for substance P and TRH
analysis. For catecholamine detection tissue was extracted in 0.5
mls of extraction medium and the whole sample was used because
of the low concentrations in the rat cord. The pellets were assayed
for protein content with Folin-phenol reagent after alkaline copper
treatment according to the method of Lowry et. al., (1951) (See
Appendix 1).

The supernatant from the aliquots for indoleamine analysis
was filtered using ACRO LC35 filters from Gelman Sciences and
injected onto the HPLC column.

1.

Spinous process

Transverse Process

Body of vertebrae

Dorsal aspect

Ventral aspect

M

2. Remove dorsal process

M

3. Invert the vertebrae

Ventral

Dorsal

4. Transfer spinal cord from central canal to a cool surface for dissection
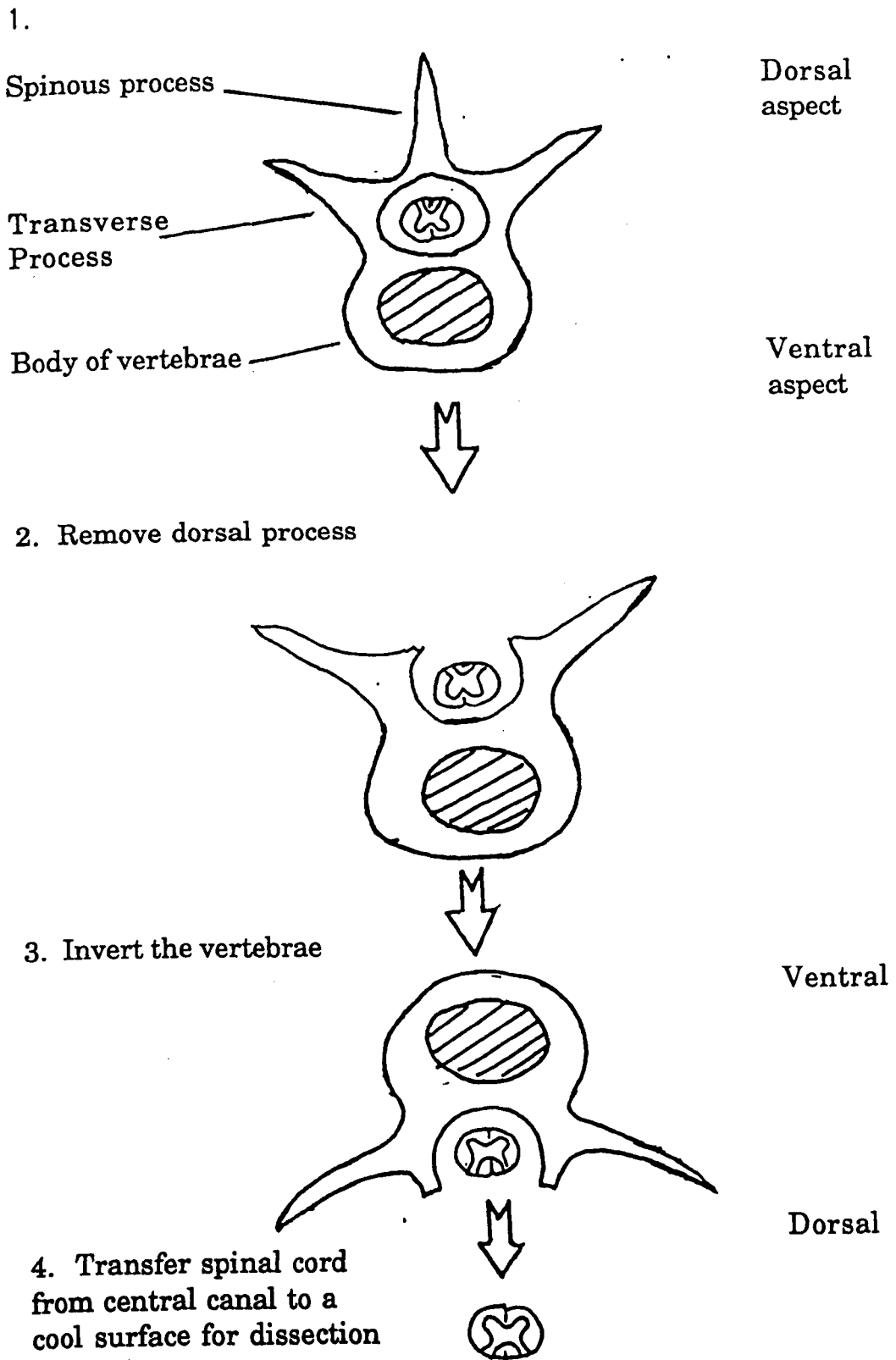
M

Fig. 2.12 Dissection of the rat spinal cord from the vertebrae.

Catecholamines were extracted and concentrated from the samples on alumina. A half ml of Tris-HCL pH 8.6 was shaken with 0.5ml of supernatant and 20mg of alumina (final pH 8.45) for 5 minutes. The alumina was washed twice by shaking in double-distilled water and the catecholamines were eluted in 0.3ml of perchloric acid (0.1M PCA) containing 0.02% sodium metabisulphite (NaMBS). Samples were cleaned on alumina by spinning at 1000g for 2 minutes and filtering (ACRO LC35 filters). Standards were stored on ice until injected onto the column. Comparisons were made of the % recovery after alumina extraction and stability over 7 hours of standards made up in perchloric acid (0.1 M) containing sodium metabisuphite (0.2 g/l i.e. 0.02%) (PCA/NaMBS) and in the selected common extraction medium. The latter was composed of absolute ethanol (450 ml), 0.1 M acetic acid (450 ml) made up to one litre with double distilled water (DDD) and sodium metabisulphite (0.02%) (eth/AA/NaMBS).

After extraction, samples for peptide analysis were dried down in a Buchler vortex evaporator at 70°c and stored at -20°c for RIA.

Extraction Media

Comparisons of the levels and % recoveries of TRH, substance P, 5HT and 5HIAA in rat tissue using different extraction media were made. The media compared were:

    (1)   90% methanol.

    (2)   50% ethanol / 50% 0.1 M acetic acid.

    (3)   0.1 M PCA with NaMBS (0.02%) (PCA / NaMBS).

    (4)   Methanol (450 ml) / 0.1 M acetic acid (450 ml) with NaMBS (0.02%) made up to 1 litre with DDD (meth / AA/NaMBS).

    (5)   Ethanol (450 ml) / 0.1 M acetic acid (450 ml) with NaMBS (0.02%) made up to 1 litre with DDD (eth/AA/NaMBS).

    (6)   90% methanol containing 0.1 M PCA (meth/PCA).

Media 1,2 and 3 are the media used previously for the extraction of TRH (Lighton et. al.,1984) substance P and indoleamines respectively (Bennett et.al., 1986). The other 3 are variations of the former.

Indoleamine and catecholamine determinations

An ACS 110 pump delivered mobile phase at a constant flow rate of 1.0 ml/min to a reverse phase column packed with 5 μm Spherisorb ODS 2. Electrochemical detection was performed using a BAS amperometric detector with a working electrode maintained at a positive potential of 0.65V versus a Ag/AgCl electrode. At this potential the indoleamines and catecholamines to be measured are all oxidized. The detector was set at 1 nA full scale and the output from the detector was recorded on both a pen recorder and a Spectra-Physics 4290 programmable integrator.

Mobile phase was made up with HPLC grade solvents and DDD. After filtering through 0.45μm filters (Millipore), mobile phase was degassed in a sonic water bath for 20 minutes prior to use. Increasing the methanol content of the mobile phase reduces the retention time of all analytes, whereas decreasing pH and increasing ion-pair reagent concentration suppresses their ionization and so selectively increases their retention time. According to these parameters methanol content, pH and ion pair reagent content where appropriate were adjusted until the optimal amine separation was attained.

5HT, 5HIAA and an internal standard 5-hydroxy-N-methyl oxalate (5HNMO) were separated in a mobile phase of 0.1M sodium acetate, 0.1 M citric acid, 10% methanol, pH 4.6 and detected using a carbon paste working electrode.

Noradrenaline, adrenaline, DOPAC, dopamine and the internal standard dihydroxybenzoic acid (DHBA) were separated and detected on a reverse phase ion pair column using a glassy carbon working electrode and a mobile phase of 0.1 M sodium dihydrogen orthophosphate, 1mM EDTA, 1.6 mM 1-octanesulphonic acid sodium salt (ion pair reagent), 9% methanol, pH 3.

Standards and Calculations

Standards of 10^{-7} M catecholamines and indoleamines were prepared in the same medium as tissue samples were

extracted, from stock solutions stored at 10^{-3} M. Twenty μl (2 pmol) were injected onto the column before and after the samples (20 μl of each sample). The amine and metabolite levels in the samples were calculated by comparing the heights and areas of the peaks in the samples with the heights and areas of the peaks in the standard. The levels were related to the protein content of the sample and expressed as pg/mg protein.

Calibration

Standards of indoleamines and catecholamines at a series of known concentrations between $2x10^{-8}$ and $5x10^{-7}$ M were injected onto the systems to determine the linearity of the detector response.

Amine Recovery

An internal standard was used to determine the % loss of amines caused by the extraction process. For the indoleamines and catecholamines 5HNMO (2 pmols) and DHBA (4 pmols) respectively were added to the tissue samples before extraction. The internal standard in the sample was compared to a fresh standard and the ratio used to determine % recovery. The recovery of 5HT and 5HIAA was assumed to be the same as that of 5HNMO.

A cocktail of catecholamine standards including the internal standard DHBA was injected on to the column before and after extraction on alumina. The % recoveries of noradrenaline, adrenaline, DOPAC, DHBA and dopamine were determined and the ratio of each % recovery to that of DHBA calculated. In each subsequent experiment DHBA was added to both the tissue samples and the standard allowing the determination of the % recovery of DHBA and of the catecholamines using the above ratios.

A separate study was made of % recovery of catecholamines after extraction from rat spinal cord without concentration on alumina. The spinal cord was bilaterally dissected and synthetic noradrenaline, adrenaline, DOPAC and dopamine was added at approximately twice the endogenous level to one half (200 pmols.). The tissue was extracted in PCA/NaMBS and processed as above

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but the extracted sample was not concentrated on alumina. Catecholamine levels were determined and the endogenous level subtracted from the levels in the paired spiked sample. Percentage recovery of the added catecholamines was calculated from the ratio of determined level to the known amount added.

Effect of storage at -80°c

One half of each extract of the rat spinal cord spiked with catecholamines as described above was stored at -80°c with a cocktail of catecholamine standards at 10^{-7}M. Fresh samples were analysed by HPLC on the same day that they were extracted whereas frozen samples were analysed after storage at -80°c for 48 hours. The levels of noradrenaline, adrenaline, DOPAC and dopamine were determined as pmols/μl in the paired fresh and frozen extracts by comparison with fresh and frozen standards respectively.

Peptide determinations

Diluting and pipetting procedures for RIA were performed using the programmable Microlab 1000 (Hamilton).

TRH was measured by a modification (Lighton et.al.,1984) of the radioimmunoassay method of Jeffcoate et.al., (1973). Standards were produced by serial dilution of synthetic TRH (0-10 ng/ml). All dilutions were made in RIA buffer of 0.04 M phosphate buffered saline containing 0.1% bovine serum albumin (BSA). Standards (50 μl) and samples resuspended in 0.5-1.0 ml of RIA buffer (50 μl) were incubated with diluted TRH antiserum (50 μl) and diluted tracer, ^{125}I-TRH (50 μl) in quadruplicate. Tubes containing tracer only (50 μl) and tracer (50 μl) and buffer (100 μl) only were used to determine total and non-specific binding respectively.

All tubes were incubated overnight at 4°c after which time tracer bound to antiserum was separated from free tracer by precipitation in 0.75 ml. ice-cold ethanol and centrifugation (4°c, 1000g, 15 minutes). The supernatant was discarded and the bound

tracer content of the pellet determined by counting radio-activity on an LKB gamma counter.

Radio-labelling of TRH

TRH was iodinated with ^{125}I using the Chloramine T method of Bassiri and Utiger (1972). To 1 µg of synthetic TRH 15 µl of 0.4M phosphate buffer pH 7.4 and 1 mCi sodium ^{125}iodide (10 µl) were added. The addition of 10 µl of chloramine T (2.5 mg/ml) initiated the oxidation of sodium ^{125}iodide to ^{125}I which was stopped after exactly 30 seconds by the addition of 250 µl of NaMBS (2mg/ml). Surplus iodine in the mixture was diluted with 100 µl of potassium iodide (10 mg/ml).

A sephadex G10 column (10 cm) in a 10 ml glass pipette was prepared and prewashed prior to the iodination with 5 ml each of 0.04 M phosphate buffer, 0.04 M phosphate buffered saline containing 1% BSA and 0.04 M phosphate buffered saline containing 0.1% BSA. The iodination mixture was placed on the column to separate iodinated TRH and ^{125}I. The latter buffer was placed in a reservoir above the column, allowed to flow through at a rate of 0.5 ml/minute and collected in 1 minute intervals for 20 minutes. The radio-active elution profile was determined by counting the fractions in a well gamma counter (Mini Instruments type 6-20) for 10 seconds. Fractions occurring on the shoulder of the first radio-active peak were tested for their ability to bind TRH antiserum and to be displaced by synthetic TRH in the TRH RIA. These were stored at -20°c in aliquots for use in RIA. Iodinated TRH was diluted prior to use such that 50 µl produced approximately 10,000 counts per minute.

The TRH antiserum was raised in sheep using a peptide conjugate prepared by the method of Bassiri and Utiger (1972) in which TRH is coupled to bovine thyroglobulin using diazotized benzidine. The TRH conjugate was emulsified in the general immuno-potentiator complete Freund's Adjuvant and ewes received the conjugate (300 µg/animal) in one intramuscular and multiple subcutaneous injections. Subsequently booster immunizations were administered subcutaneously at monthly intervals and after 6 months the antibody titre of the ewes was

tested at weekly intervals. The resultant antiserum had a high titre, was used at a 1 in 20,000 dilution and in cross-reactivity studies was found to be specific for TRH, cross-reacting only with peptides containing both the C and N terminal residues of synthetic TRH. The sensitivity of the TRH radioimmunoassay was 28fmols/tube and the intra-assay and inter-assay coefficients of variation were 2.1% and 12% respectively. (Lighton et.al.,1985).

Substance P

Substance P was measured using a modification (Gilbert et.al.,1982) of the method of Powell and co-workers (Powell et.al., 1973). Standard displacement curves were produced as described on page 49 using a final dilution of 1 in 4000 of substance P antiserum 304G. Samples were resuspended in 0.5-2.0 ml of substance P RIA buffer (page 49) and 100 μl were added to 50 μl of diluted antiserum and 50 μl of [^{125}I]-tyr-8-substance P. Tubes containing tracer only (50 μl) and tracer (50 μl) and buffer (150 μl) only were used to determine total and non-specific binding respectively. Samples and standards were incubated overnight and then antiserum bound tracer was separated from free tracer and measured as described (page 49).

The substance P antiserum (304G) was shown to have less than 0.5% cross-reactivity with both C and N terminal fragments of substance P and with certain tachykinins as described in detail in the first part of this chapter (pp. 55-56). Assay sensitivity and reproducibility are given on page 56.

Radio-labelling of substance P: see page 47

Calculations

The substance P-LI or TRH like immunoreactivity (TRH-LI) in the samples was determined by interpolation of their respective standard displacement curves. Levels were expressed as pmol/mg protein.

Peptide recovery

The percentage of the total peptide detectable after the extraction process and RIA was determined for TRH and substance P in rat spinal cord using exogenous peptide.

The spinal cord was bilaterally dissected and synthetic peptide was added at approximately 10 fold the estimated endogenous levels to one half of the tissue samples (~20 ng). Tissue was processed and assayed as above. Peptide levels were determined and the endogenous levels subtracted from the levels in the paired spiked sample. Percentage recovery of added peptide was calculated from the ratio of the determined level to the known amount added. Percentage recovery determined in this way was assumed to be that of peptide recovery in subsequent peptide measurements in both rat and human (chapter 3) tissues.

Statistics

Data were analysed using the Student t-test for paired or unpaired data as appropriate.

Chemicals

5HT, 5HIAA, 5HNMO, noradrenaline, adrenaline, DOPAC, DHBA and dopamine, were purchased from Sigma. Substance P and TRH were obtained from Peninsula Laboratories Europe Ltd. The radio-labelled peptides were produced using ^{125}I obtained from Amersham International.

Results

HPLC with ED

The lower limit of detection for 5HT and 5HIAA was 0.4 pmols/injection (signal: noise ratio, 3:1). The detector response to 5HT and 5HIAA was linear over the range 0-10 pmol. The lower limit of detection for noradrenaline, adrenaline, DOPAC, DHBA and dopamine was 0.09, 0.1, 0.12, 0.14 and 0.15 pmol respectively. The detector response to noradrenaline, dopamine and DOPAC was linear over the range 0-10 pmol. The HPLC separation of 5HT and 5HIAA and of noradrenaline, adrenaline, DOPAC, DHBA and dopamine is shown in Figs.2.13 and 2.14.

Approximately 4 fold lower limits of detection could be obtained using the carbon paste rather than glassy carbon electrode enabling measurement of 0.1 pmol/injection of indoleamines. This level of sensitivity required careful and patient polishing of the electrode to achieve and was not easy to maintain. Detection levels given above are those routinely attained.

TRH Radio-immunoassay

After iodination of TRH the separation of ^{125}I-TRH from ^{125}I produced a radio-activity profile with two peaks (Fig.2.15). The fractions from the trailing shoulder of the first peak, fractions 6,7 and 8 were found to have the greatest binding coefficient, 15-25%, and competed with synthetic TRH for antiserum binding. Displacement of the label was at least 65% as illustrated in Fig.2.16. The fractions were stored in aliquots at -20°c.

Development of a common extractant

A. 90% methanol / 0.1M PCA

An attempt to extract peptides from rat spinal cord in 90% methanol/0.1M PCA failed as the PCA did not evaporate and was oxidized to a concentrated brown liquid in the vortex evaporator.
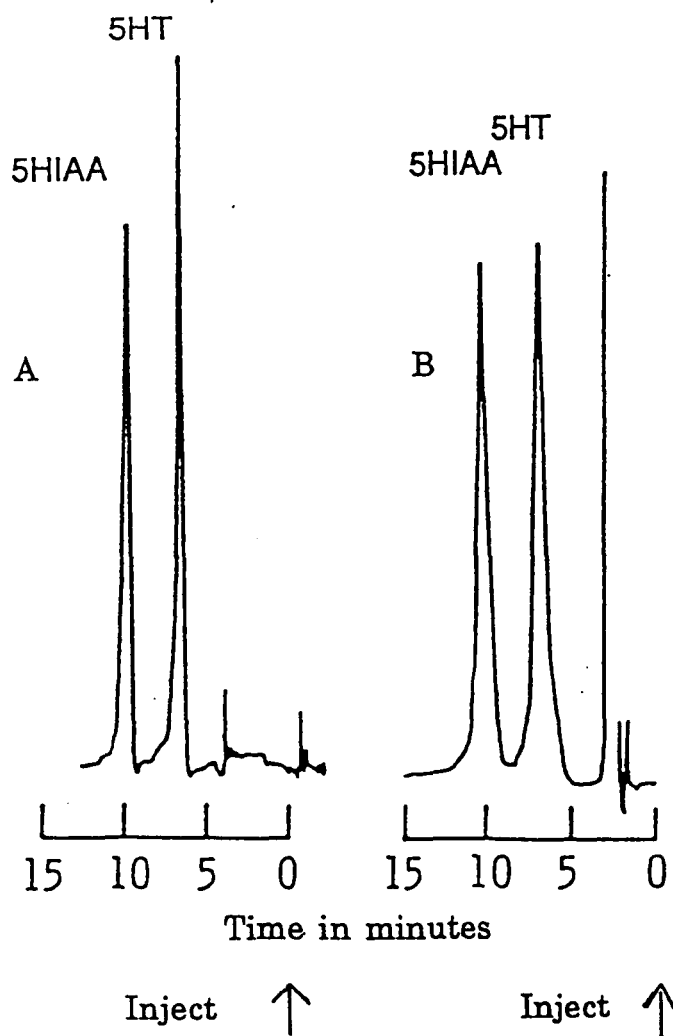
Fig.2.13

Chromatograms of standard 5HT and 5HIAA prepared with two different extraction media: A) perchloric acid (0.1M) plus sodium metabisulphite (0.02%), B) ethanol plus acetic acid (0.1M) plus sodium metabisulphite (0.02%). Two pmols of each standard were injected onto the column with the following mobile phase: 0.1M sodium acetate, 0.1M citric acid, 10% mehtanol, pH 4.6 using Sperisorb 5 ODS column (5μm) and the detector potential set at +0.65V.
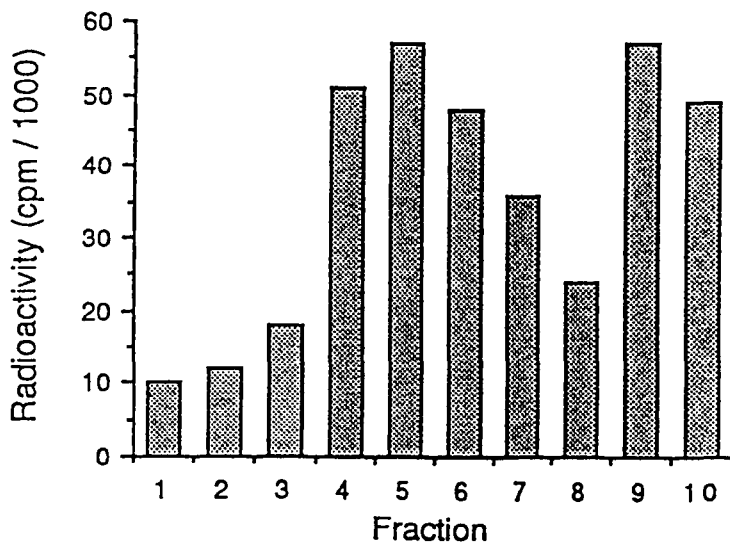
Fig. 2.15 Radioactive fractions collected after TRH
iodination

Fractions collected in one minute intervals from the bottom of the
column on which TRH conjugated- and free ^{125}I were separated.

Fig 2.16 TRH displacement curve

This figure shows a displacement curve from a typical TRH RIA.
Log. TRH concentration is plotted against %Bound. Each point is
determined from the mean of four replicates. [^{125}I] TRH was
incubated with TRH (5 - 500 pg/tube) and TRH antiserum diluted 1
in 20,000.
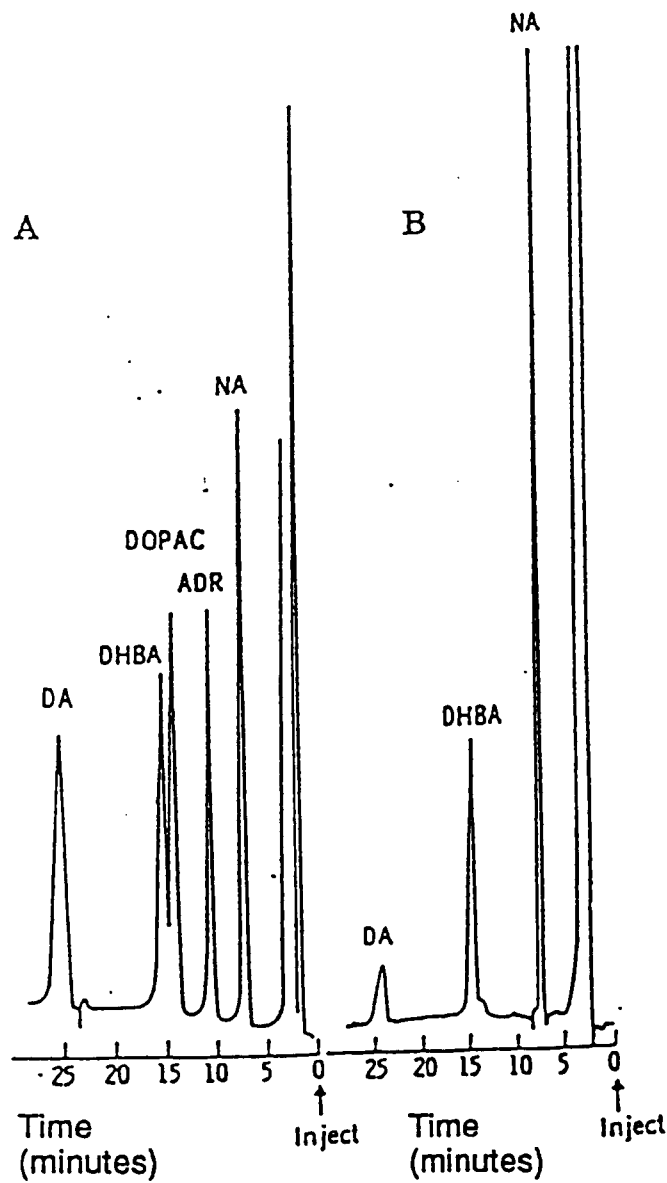
Fig. 2.14

A typical separation of A) a standard mixture of NA, ADR, DOPAC, DHBA (internal standard) and DA; B) a sample of whole rat thoracic cord. The catecholamines were extracted using ethano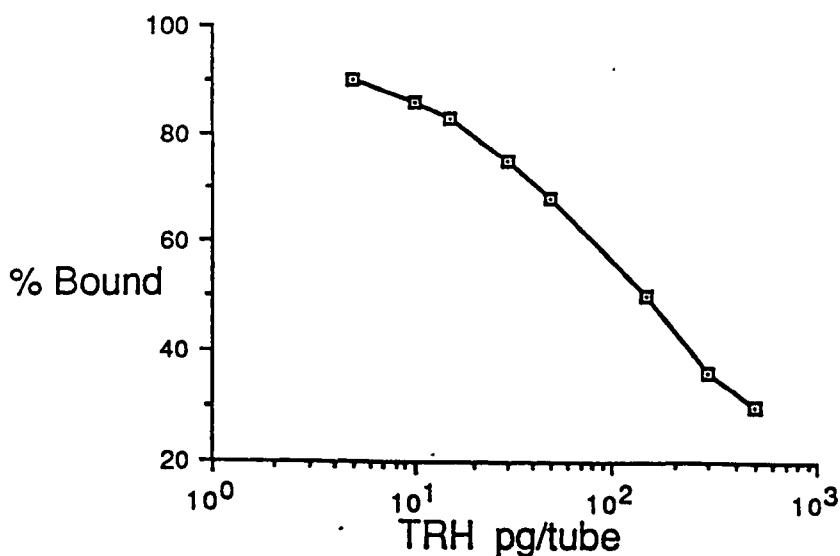l plus acetic acid (0.1M) and sodium metabisulphite (0.02%) followed by alumina extraction as described in the text. The separation was obtained on a Spherisorb 5 ODS 2 column using the following mobile phase: 0.1M sodium dihydrogen orthophosphate, 1mM EDTA, 1.6mM IPR, 9% methanol, pH 3.0. The glassy carbon electrode was held at +0.65V.

NA=noradrenaline; ADR=adrenaline;DA=dopamine

B. Methanol or ethanol / acetic acid / NaMBS

A composition of methanol or ethanol with acetic acid and NaMBS was shown to be suitable for indoleamine extraction but it produced wider peaks on the amine HPLC chromatogram than PCA/NaMBS (Fig.2.13). When ethanol was substituted for methanol it was found that there was no significant difference between the levels of 5HT or 5HIAA extracted from rat tissue using either methanol or ethanol (Fig.2.17). However the variation in the measured levels, determined as the coefficient of variation (standard error/mean), was marginally less using ethanol in the extraction medium (Fig.2.17). In subsequent studies the recovery and tissue levels of the amine and peptides using the common ethanol/acetic acid /NaMBS extraction medium was compared with the previously established individual media (Figs.2.18, 2.19 and 2.20).

C. Indoleamines

The medium of eth/AA/NaMBS was compared with PCA/NaMBS for stability of indoleamine standards, chromatography and indoleamine, including internal standard (5-HNMO) extraction from rat tissue samples. The area of the peaks on the chromatograms were not significantly different though the peaks of standards prepared in eth/AA/NaMBS were slightly lower and wider. The variability in peak area of standards (measured over seven hours) was low (Fig.2.18). There was no difference in the recovery of the internal standard or the 5HT levels measured in rat spinal cord but 5HIAA levels measured were significantly higher in spinal cord samples using eth/AA/NaMBS (Fig.2.18).

D. TRH

Comparison of TRH extraction in eth/AA/NaMBS and the medium generally used, 90% methanol, indicates that there is no significant difference in % recovery or TRH levels measured but

81
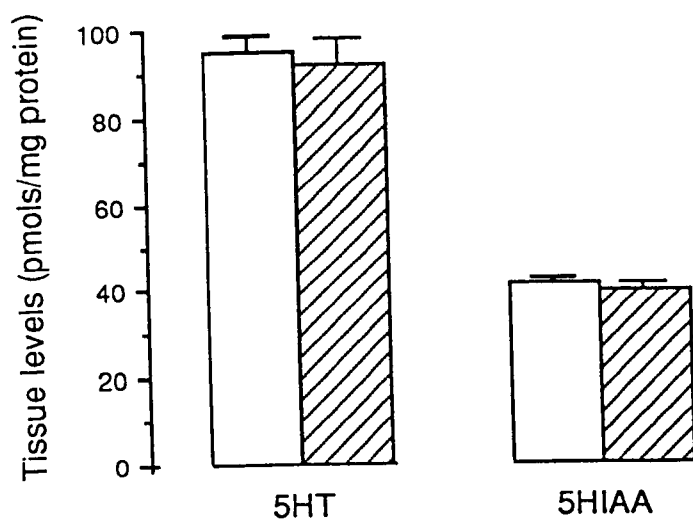
Fig.2.17
Comparison of the levels of 5HT and 5HIAA in rat lumbar spinal
cord measured using two extractants; a mixture of ethanol ☐ or
methanol ▧ with acteic acid and sodium metabisulphite. See text
for exact composition. There was no significant difference
between compared results. n=5 in each case.
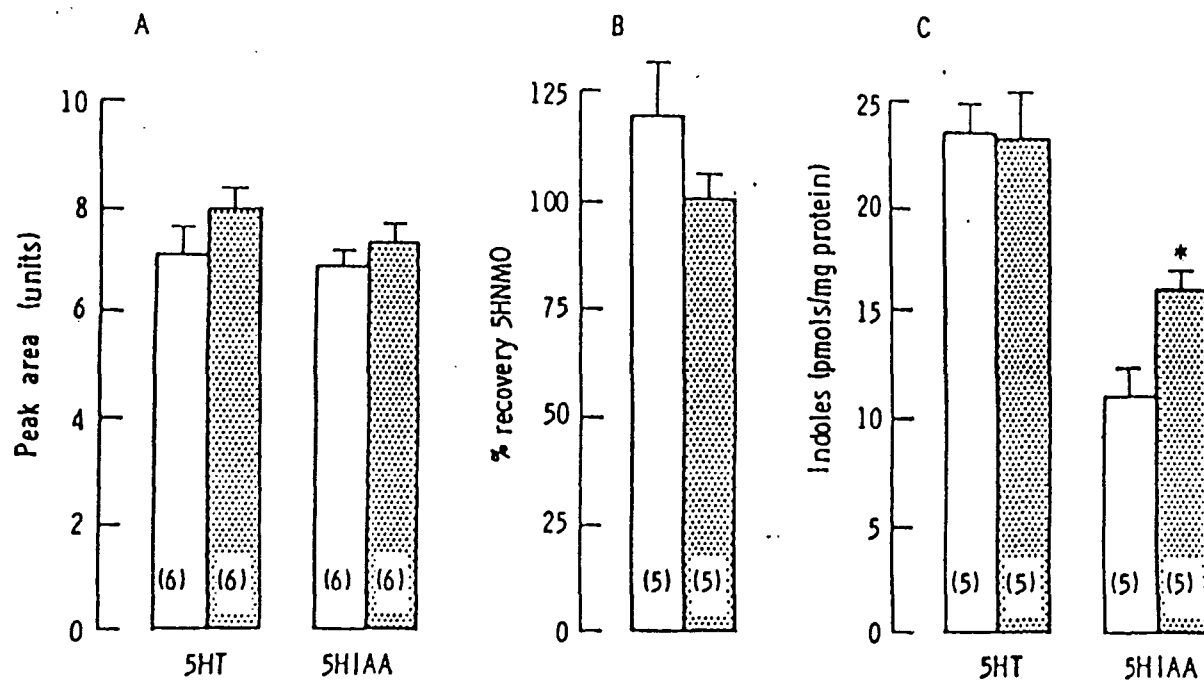
Fig. 2.18

(A) Comparison of peak areas of standards (2 pmols) , (B) % recovery of the internal standard 5-hydroxy-N-methyl oxalate (5HNMO) and (C) rat thoracic cord levels of 5HT and 5HIAA extracted in either 0.1 M perchloric acid plus sodium metabisulphite (0.02%) ☐ or ethanol plus 0.1 M acetic acid and 0.02% sodium metabisulphite ▨ . Figures in brackets refer to number of determinations or animals. *p<0.05.

TRH levels and % recovery tended to be slightly greater (Fig.2.19) using eth/AA/NaMBS extraction medium.

E. Substance P

Comparison of substance P extraction in eth/AA/NaMBS and the medium generally used (50% ethanol/50% 0.1M acetic acid) indicates no significant difference in % recovery or substance P levels measured (Fig.2.20).

F. Catecholamines

(i) Alumina Extraction

In the present study catecholamines were measured in rat spinal cord tissue after concentration on alumina following their initial extraction using eth/AA/NaMBS. Alumina treatment produced a much clearer chromatogram with less baseline interference and removal of peaks other than those of the catecholamines being measured. Catecholamine extraction on alumina was compared using standards made up in either eth/AA/NaMBS or PCA/NaMBS (Table 2.1B&C). In each case % recovery was greatest when standards were made up in PCA/NaMBS ($p<0.05$, n=5), though there was no significant difference for DOPAC standards (Table 2.1B&C). However, consistent recovery of between 53 and 62% were attained for all the catecholamines and DHBA when the standards were made up in eth/AA/NaMBS and eluted off alumina with PCA/NaMBS (Table 2.1C). Catecholamines extracted in the common media and injected onto the column in PCA/NaMBS produced better chromatograms than using eth/AA/NaMBS to elute the catecholamines off the alumina (Fig.2.21).

There was no significant difference ($p<0.05$; n = 5) in percentage recovery of catecholamine standards made up in eth/AA/NaMBS whether the standards were eluted off alumina (into PCA/NaMBS) immediately after alumina extraction or retained on the alumina and eluted just prior to injection onto the HPLC system (Table 2.1C&D).
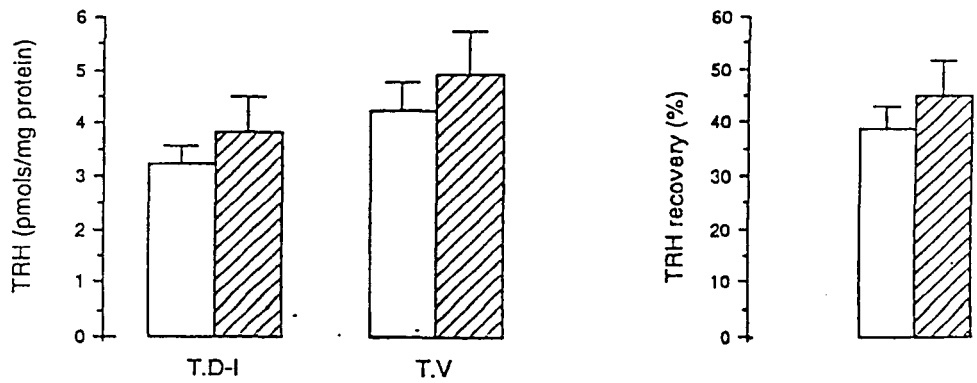
Fig.2.19

(A) Levels of TRH measured in rat thoracic dorso-
intermediolateral (T.D-I) and ventral (T.V) cord using two
different extraction media; 90% methanol ☐ and ethanol, 0.1M
acetic acid and 0.02% sodium metabisulphite ▨ n=12 in each
case. (B) Comparison of the recovery of exogenous TRH added to
bilaterally paired pieces of rat whole thoracic cord. n=5 in each
case. There were no significant differences in any of the
comparisons made.
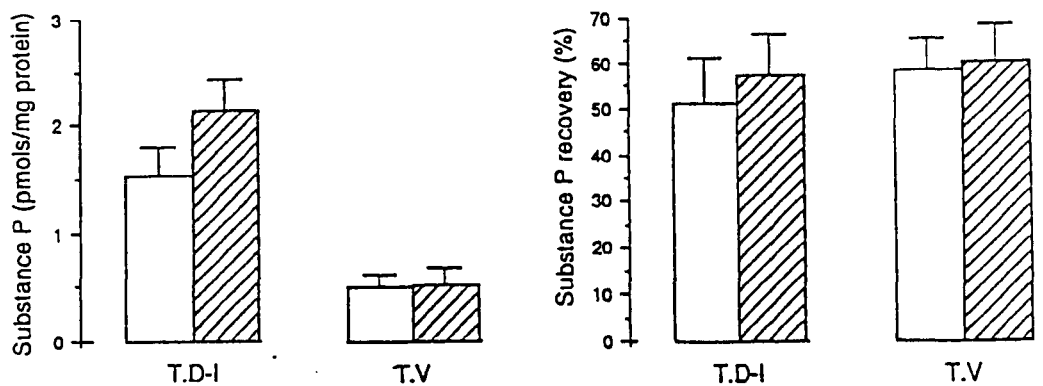
Fig.2.20

(A) Levels of substance P measured in rat thoracic
dorsointermediolateral (T.D-I) and ventral (T.V) cord using two
different extraction media; ethanol, 0.1M acetic acid ☐ and
ethanol, 0.1M acetic acid and 0.02% sodium metabisulphite ▨',
n=11 in each case. (B) Comparison of the recovery of exogenous
substance P added to bilaterally paired pieces of rat thoracic dorso-
intermediolateral and ventral cord, n=12 in each case. There
were no significant differences in any of the comparisons made.
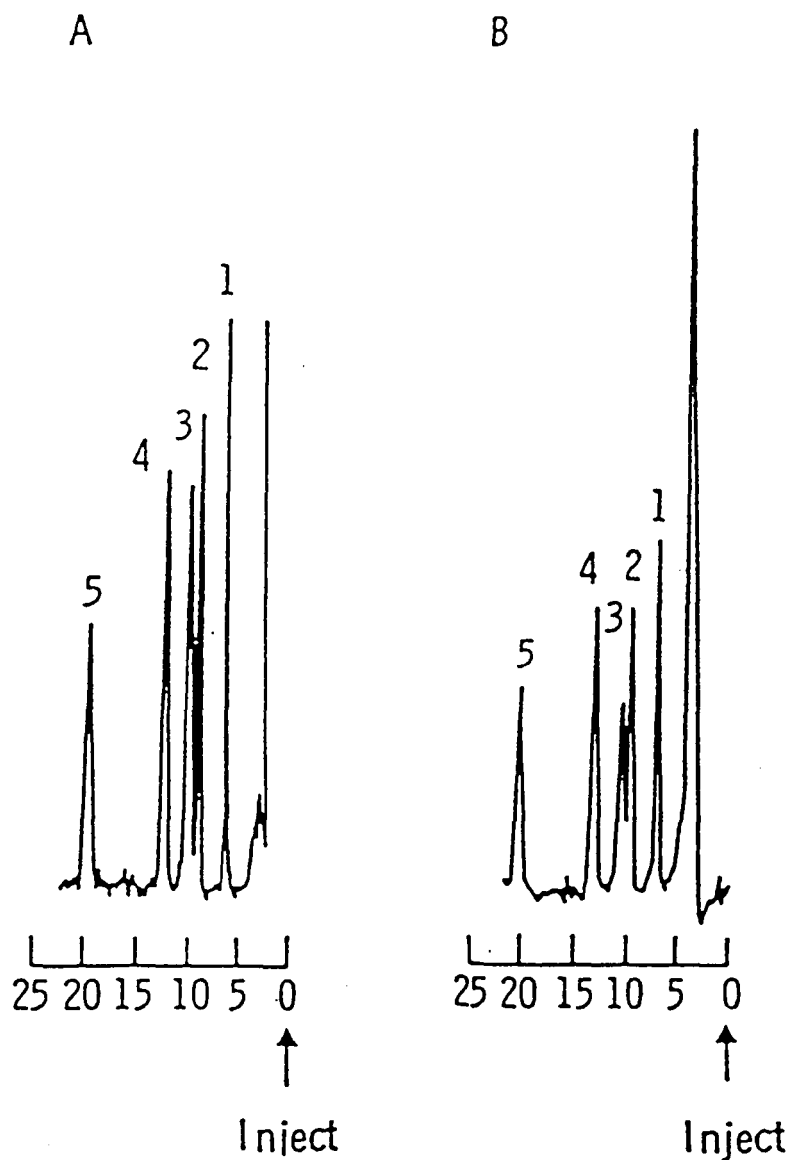
Fig. 2.21

Chromatograms of standard mixtures of NA (1), ADR (2), DOPAC (3), DHBA (4), and DA (5) eluted off alumina into two different extraction media: A) perchloric acid (0.1 M) plus sodium metabisulphite (0.02%), B) ethanol plus acetic acid (0.1 M) plus sodium metabisulphite (0.02%). Two pmols of each standard was injected onto a spherisorb 5 ODS 2 column using the following mobile phase: 0.1 M sodium dihydrogen orthophosphate, 1 mM EDTA, 1.6 mM IPR, 9% methanol, pH 3.0. The glassy carbon electrode was held at 0.65 V.

Table 2.1 Catecholamine extraction

Comparison of % recovery of catecholamines from rat spinal cord sample spiked with catecholamine standards (100pmols) without alumina extraction (A) and standards made up in either PCA/NaMBS (B) or eth/AA/NaMBS (C&D) with elution in PCA/NaMBS. Catecholamines were eluted off alumina immediately after extraction (B&C) or stored on alumina and eluted just prior to HPLC analysis (D). n=5 in each case

	% Recovery			
	A. PCA/NaMBS No extraction	B. PCA/NaMBS Alumina extraction	C. eth/AA/NaMBS Alumina extraction	D. eth/AA/NaMBS stored on alumina
NA	76.7±6.1	74.3±4.2	62.0±3.6	62.9±6.1
ADR	75.6±6.9	74.3±3.8	58.1±3.6	58.1±3.7
DOPAC	91.9±4.8	58.0±1.7	56.2±5.4	58.2±4.2
DHBA	—	74.0±4.3	60.9±4.2	62.3±3.6
DA	84.5±5.7	80.5±5.2	53.0±4.6	54.2±4.2

Percentage recovery of catecholamines measured in rat tissue spiked with synthetic catecholamines and extracted without alumina extraction was not significantly different from recovery after alumina extraction (Table 2.1A&B) except in the case of DOPAC recovery which was significantly reduced after alumina extraction (p<0.01, n=5).

(ii) Effect of frozen storage

There was no significant difference (p<0.05, n = 5) between the levels of noradrenaline, adrenaline, DOPAC and dopamine measured in extracted tissue samples spiked with catecholamines (10^{-7}M) and either analysed on the same day or stored at -80°c for 48 hours before analysis (Fig.2.22). Catecholamine levels were determined by comparison with standards (2 pmols/injection) either made fresh or stored frozen with the samples as appropriate. Peak areas of frozen standards were 5-8% less than those of the fresh standards.

Rat spinal cord

Using eth/AA/NaMBS the levels and % recoveries of substance P, TRH, 5HT, 5HIAA, noradrenaline and dopamine were measured in rat thoracic and lumbar spinal cord and the results are given in Tables 2.2 and 2.3.
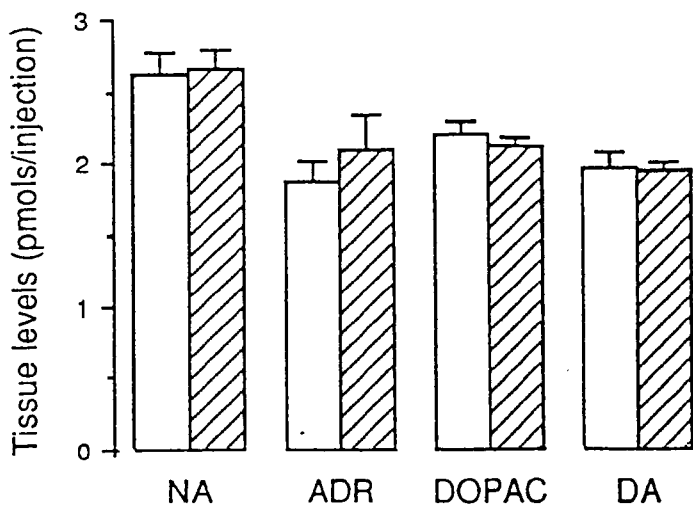
Fig.2.22   Effect of freezing on catecholamine levels

Catecholamine levels measured in the rat spinal cord after spiking the tissue with exogenous catecholamine standards (100nM). There were no significant differences in levels determined by HPLC immediately ☐ or after storing the samples at -80°c for 48 hours ▨ ,n=5 in each case.
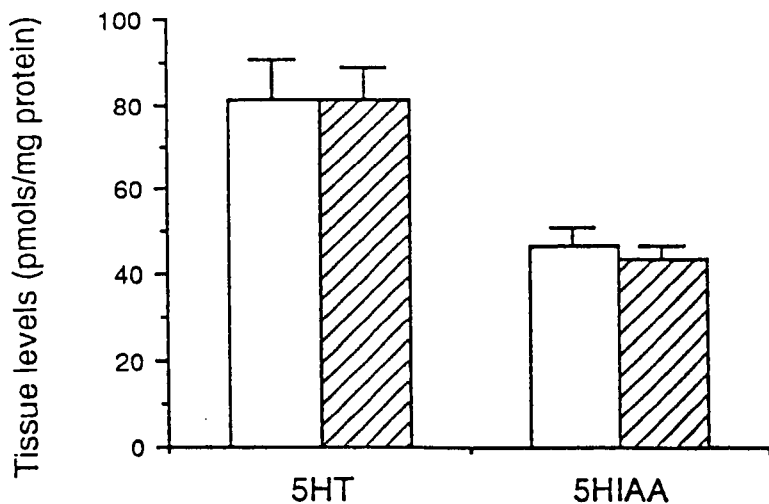
Fig 2.23   Levels of 5HT and 5HIAA in rat spinal cord measured using sonification or homogenisation of the tissue.

Levels of 5HT and 5HIAA in the rat lumbar spinal cord were determined by HPLC after sonification ☐ or homogenisation ▨ of the tissue in the common extractant. There were no significant differences in the levels measured using these methods. n=6 for each value.

Table 2.2 Levels of substance P and TRH in the rat spinal cord measured using an extraction medium of ethanol/ 0.1 M acetic acid/ sodium metabisulphite.

Levels are expressed in pmols/mg protein and are corrected for recovery at the values of % recovery given. Values are the mean ± SEM, n values are given in brackets.

Region	TRH		Substance P	
Thoracic ventral	10.86 ± 1.84	(12)	0.9 ± 0.24	(11)
Thoracic dorso-intermediolateral	8.47 ± 1.5	(12)	3.65 ± 0.51	(12)
Lumbar ventral	13.7 ± 0.75	(6)	1.12 ± 0.09	(12)
Lumbar dorsal	8.85 ± 0.89	(6)	3.41 ± 0.22	(11)
% Recovery	45.2		58.7	

Table 2.3 Levels of indoleamines and catecholamines in the rat spinal cord measured using an extraction medium of ethanol/ 0.1 M acetic acid/ sodium metabisulphite

Levels are expressed in pmols/mg protein and are corrected for recovery at the values of % recovery given. Values are the mean ± SEM, n=6 for indoleamine results and 5 for catecholamine results.

Region	5HT	5HIAA	NA	DA
Thoracic	22.3 ± 2.12	16.1 ± 1.15	25.9 ± 0.7	4.36 ± 0.34
Lumbar	93.2 ± 3.9	40.2 ± 1.5	18.1 ± 1.5	2.58 ± 0.49
% Recovery		101.9	58.0	50.0

Discussion

Consideration of the methods

The HPLC systems were sensitive enough to detect 5HT, 5HIAA, noradrenaline and dopamine in rat spinal cord the levels of which fell within the range over which electro-chemical detection was linearly related to amine concentration. Greatest sensitivity was attained using the carbon paste rather than glassy carbon electrode. However great care was needed when polishing the carbon paste electrode as any irregularities in the surface resulted in high baseline noise. In addition the electrode surface was easily damaged by variations in pump pressure and the passage of air bubbles across it when either remounting the electrode or making changes to the column or mobile phase. The glassy carbon electrode retained its sensitivity for longer (several weeks) and would therefore be the one to use wherever detection limits of 0.1pmol or less are indicated.

Similarly the limits of detection of, and the peptide concentration range covered by, the radioimmunoassays for substance P and TRH enabled the measurement of these peptides in the rat spinal cord. The TRH and substance P antisera were specific for TRH and substance P respectively with low cross-reactivity with other peptides and the assays were reproducible as illustrated by the small inter-assay and intra-assay variability.

The levels of the amines and peptides in the spinal cord were determined after sonication of the samples in extractant. The spinal cord is more difficult to degrade than brain tissue, possibly because of the spinal meningues. Sonification of the spinal cord takes some time (up to 5 minutes) and the sample must be kept cool to avoid breakdown of the amines and peptides. Degradation of the tissue using an homogenizer (Ultra-turax) proved to be quicker though the tissue indoleamine levels and % recoveries measured in rat cord using homogenization or after sonification of samples kept on ice were not significantly different (Fig.2.23). It was decided that sonification would be employed as the methods were

developed for future use with human tissue. Due to the potential
biohazard associated with working with human tissue only
equipment that could be cleaned and decontaminated could be used
and this was easier with the probe of the sonicator than the
grinding parts of the homogenizer.

The levels of catecholamines in extracts of rat tissue
analysed immediately were not significantly different to those
analysed after storing the samples at -80°c when compared with
standards treated in the same way. Although the peak areas of the
frozen standards were 5-8% less than those of the fresh standards
this may have been a result of changes in the sensitivity of the
HPLC system rather than an indication of the effect of freezing.
Frozen samples could have been analysed with freshly extracted
samples to avoid HPLC sensitivity changes but this would prevent
the use of paired samples. A fresh standard should have been run
with the frozen standards though a small variation in standards
made on different days could also be expected. In the light of the
results it was assumed that catecholamines in extracted samples
were stable when stored at -80°c.

Properties required of a common extractant

The extraction media selected for testing in the development
of a common extractant for substance P, TRH, indoleamines and
catecholamines were combinations of media generally used in our
laboratory for the analysis of various compounds. The original
media have been used to measure peptides, indoleamines and
catecholamines for a number of years but a common medium for
the extraction of peptides and amines would be a great advantage.
A common medium has to incorporate several factors. Firstly a
large part of the mixture must be an organic solvent such as
ethanol or methanol which will evaporate completely in the vortex
evaporator and thereby enable sample concentration. Secondly it
must include an acid to facilitate protein precipitation which must
also evaporate. Thirdly NaMBS must be present to protect the
labile amines from oxidation. Fourthly, the common media
should not compromise the HPLC chromatography and the

reproducibility of the determinations should be preserved.
Finally, using the selected common medium peptide and amine
levels and recoveries should not be very different from those
obtained using each of the original media.

PCA, originally used for amine extraction was not volatile
but acetic acid was found to be particularly suitable as the volatile
acid component. Using methanol or ethanol in combination with
acetic acid and NaMBS produced little difference in levels
measured (Fig.2.17) or the chromatography of 5HT and 5HIAA.
Ethanol was chosen for further evaluation as the organic solvent of
a common extractant because it produced marginally better
reproducibility of results.

Comparing the common extractant and other extractants

The ability of the indoleamines and catecholamines to be
easily oxidized, the very property which enables their electro-
chemical detection, means that they are susceptible to ~~metabolism.~~ air oxidation
The variation in peak area of standards injected repeatedly over
several hours during analysis of tissue amine levels was therefore
determined as an indication of their stability.
Although the indoleamine standards prepared in
eth/AA/NaMBS produced slightly smaller and wider peaks on the
chromatogram than those in PCA/NaMBS (Fig.2.13) there was no
significant difference in peak area. The variability in peak area
was also low indicating that the stability of the standards in
eth/AA/NaMBS was at least as good as in PCA/NaMBS (Fig 2.18)
and that the indoleamines were protected from air oxidation.
While there was no significant difference in % recovery of the
internal standard which was approximately 100% using the two
extractants, the levels of 5HT and 5HIAA measured in spinal cord
were the same (5HT) or greater (5HIAA) using eth/AA/NaMBS
demonstrating an advantage over PCA/NaMBS which may cause
some oxidation of 5HIAA.
Using eth/AA/NaMBS, catecholamines extracted from rat
tissue, concentrated on alumina and eluted in PCA/NaMBS
produce clear sharp chromatography (Fig.2.14) with % recoveries

of between 53 and 62% (Table 2.1C). These values were reproducible which is more important than obtaining high % recovery. Variation in peak area of catecholamine standards over time was also low indicating that they were protected from oxidation.

Substance P and TRH levels and recoveries were not significantly different when extracted in eth/AA/NaMBS compared with the original media though both levels and % recoveries were generally slightly greater using eth/AA/NaMBS for both substance P and TRH extraction. It is possible that peptides are metabolised after tissue extraction by membrane bound peptidase enzymes and the common extractant may provide greater protection against metabolism than more generally used media. This extractant has also been used to measure other peptides including calcitonin gene related peptide levels in the rat spinal cord (Fone et.al., 1988b) and it is reasonable to assume that it is appropriate for the extraction of other neuropeptides from tissue samples.

Alumina extraction cleans up the sample and removes everything but the catecholamines. It also allows concentration of the sample which means that the HPLC system can be used at a lower sensitivity which is easier to maintain. There is up to 25% loss of catecholamines during extraction from tissue samples (Table 2.1A) and being able to concentrate the sample compensated for this loss. The degree of loss was always determined using the internal standard DHBA. Either storing the catecholamines on alumina or eluting them immediately into PCA/NaMBS did not affect the % recovery which meant that batches of samples could be extracted together, stored on ice and eluted into PCA/NaMBS when convenient between injecting samples onto the HPLC system. These results suggest that, in combination with alumina extraction of catecholamines, eth/AA/NaMBS can be used as a common medium for the extraction of the catecholamines, indoleamines and certain neuropeptides.

Comparison of absolute levels reported in this paper with previous studies for catecholamines, indoleamines and neuropeptides in rat spinal cord is difficult because there is a great deal of variation in methods used in the regional dissection of cord and in the method of expressing the results (Marsden et. al.,1982; Di Guilio et.al., 1985). Preliminary studies have shown that when measuring peptides in the human spinal cord significantly higher levels were obtained when cores of tissue were taken from the gray matter than when white matter was included (Bennett et.al., 1986). However, 5HT levels reported here (Table 2.3) in lumbar cord are similar to those reported in lumbar ventral cord (Lighton et.al., 1984). TRH levels in lumbar and dorsal cord (Table 2.2) are approximately twice those reported by Lighton et.al., (1985) but the relative distribution between dorsal and ventral cord is similar in that thoracic and lumbar TRH levels are approximately 1.5x that of the dorso-intermediolateral or dorsal regions respectively (Table 2.2). The regional distribution of substance P is also similar to that previously reported (Helke et. al., 1982; Marsden et.al.,1982) in that substance P dorsal levels invariably exceed ventral levels in both lumbar and thoracic cord by 3-4 times.

The levels of catecholamines in rat lumbar cord shown in Table 2.3 are similar to those reported by Mouchet et.al.; (1982) and of the same order of magnitude as reported by Commissiong and Neff (1979) and Fleetwood-Walker and Coote (1981) with noradrenaline levels approximately 7 fold greater than those of dopamine.

As discussed in part 1 (pp. 43-44), care must be exercised in comparing levels of peptides measured in different studies using RIA methods. The values obtained are of peptide-like immunoreactivity and how closely this reflects the actual level of the peptide will depend on the specificity of the antiserum being used which is not always stated in the literature.

Another factor which may affect the level of radio-immunoactivity measured is the quality of the peptides used. Using amino acid analysis, mass spectrometry and HPLC, Brown and co-workers (1986) analysed a number of commercially

available peptides. They reported discrepancies between what the peptides were and what they were supposed to be in three areas; (1) the absolute content could vary as much as 3.6 fold, (2) peptides were supplied with incorrect amino acid sequences and (3) in samples of [^{3}H] substance P from 5-95% of the radio-activity was not associated with authentic substance P. As they suggested, researchers can reduce the possibility of these errors by only accepting peptides accompanied by amino acid, mass spectrometry and HPLC analysis but this information is not always available when comparing published data.

As determined from the recovery of 5HNMO, the recovery of the indoleamines was approaching 100% whereas that of the catecholamines and peptides was nearer 50%. The common extractant obviously offered a higher degree of protection from metabolism for the indoleamines but catecholamine and peptide loss occurred in the process of extraction from tissue samples. The peptide recovery determined in this study was that of exogenous peptide. Endogenous peptide present in vesicles may be more highly protected from peptidase activity than exogenous peptide and therefore the % peptide recovery determined using exogenous peptide may be lower than that for endogenous peptide. All the measured levels given in Tables 2.2 &2.3 have been corrected for % recovery and it is important to note when comparing results whether this factor has been accounted for by other authors.

In conclusion, using the medium eth/AA/NaMBS it was possible to extract the neuropeptides TRH and substance P , 5HT and 5HIAA as well as noradrenaline and dopamine from tissue samples. It should also be possible to measure adrenaline and DOPAC in the rat spinal cord by eluting the alumina extracts into very small volumes and thereby concentrating the samples further. Greater sensitivity of the HPLC system can be achieved if necessary by using a carbon paste rather than a glassy carbon working electrode. This medium maintained the peptides through the extraction and RIA procedure, facilitated protein precipitation and drying down of the supernatant and protected the amines and peptides from breakdown. A common medium for the extraction of these substances reduced considerably both the amount of tissue

required and the assay time. The method described was as effective as the original media in terms of % recovery, although there was some loss in chromatographic clarity with respect to peak sharpness. In the case of catecholamine extraction this problem was overcome by re-elution of the sample from alumina in PCA/NaMBS. From the results obtained in the development of both a specific substance P RIA and a common extractant for indoleamines, catecholamines and peptides it was considered that the methods had been satisfactorily validated for future use with human spinal cord tissue.

Chapter 3   Post-mortem spinal cord levels of catecholamines, indoleamines, thyrotrophin-releasing hormone and substance P in Parkinson's disease

Introduction

In the rat spinal cord two neuropeptides, TRH and substance P are reported to co-exist with 5HT in a large proportion of the serotonergic neurones that descend from the medullary raphe nuclei to the ventral spinal cord (Gilbert et. al.,1982; Johansson et.al.,1981; Helke et.al., 1986). Similarly noradrenergic and dopaminergic neurones descend from brainstem nuclei to innervate the ventral and dorsal horns of the spinal cord (Mouchet et.al., 1982; Westlund et.al., 1983; Nakazato, 1987; Takada et.al., 1988). Evidence indicating the presence of serotonergic-peptidergic, noradrenergic and dopaminergic descending pathways in animals and their role in motor control is discussed fully in the General Introduction. Recent studies have shown that the distribution of 5HT, TRH and substance P in human spinal cord is similar to that in rat (Przewlocki et.al., 1983; Bennett et al., 1986). Furthermore, a possible role for the descending aminergic neurones in motor control in the human is indicated since in a previous report (Scatton et.al., 1986) the levels of NA and 5HT in post-mortem Parkinsonian lumbar spinal cord were lower than the levels obtained in control subjects and the most marked symptoms seen in patients with Parkinson's disease include loss of motor control and co-ordination (Parkinson, 1817).

In the present study catecholamines, 5HT and its metabolite 5HIAA and the neuropeptides TRH and substance P were measured in both thoracic as well as lumbar ventral and dorsal horn tissue of spinal cord obtain from patients who had died with Parkinson's disease or from non-neurological control subjects. These studies were undertaken to extend the previous analysis of amine levels in Parkinsonian spinal cord and to determine whether the neuropeptides, reported to co-exist in animal descending bulbospinal neurones, are also altered in Parkinson's disease. The levels of catecholamines, indoleamines, substance P and TRH measured in the human spinal cord in this study are compared with reported levels in the rat and human brain and spinal cord.

In addition, there are a number of important factors to consider when analysing the results of biochemical measurements made in post-mortem human tissue. These include the effects on the parameters being measured of: the sex of the subject; their age at death; the interval between death and removal of the tissue for storage (PMI); and ante-mortem drug therapy. These factors have been taken into account in this study and a rigorous statistical analysis of the effects of age, sex and PMI on post-mortem amine and peptide levels has been made.

Methods

Donor details

Sections of cervical, thoracic and lumbar spinal cord were obtained post-mortem from patients with Parkinson's disease and from control subjects with no history of neurological or psychiatric illness. Informed consent for the post-mortem removal of tissue for experimental purposes was obtained from subjects before death or from their next-of-kin in all cases. The mean and range of the age and interval between death and autopsy for control and Parkinsonian patients is shown in Table 3.1.

The causes of death of the control subjects were heart failure, pneumonia, septicaemia and one road traffic accident. Causes given for patients having died with Parkinson's disease, apart from the condition itself were heart failure, pneumonia, pulmonary embolism and post-operatative peritonitus. All patients with Parkinson's disease, with one exception, were receiving either Sinemet or Madopar (which contain Levodopa in conjunction with peripheral dopa-decarboxylase inhibitors) or Levodopa itself. Bromocriptine, a dopamine agonist was also prescribed to 2 of these patients and to the one patient not on Levodopa therapy. The time of the last dose of treatment administered varied from 48 to only 6 or 7 hours before death. All patients were confirmed post-mortem as having Parkinson's disease by determination of dopamine loss in the striatum and cell loss in the substantia nigra.

Dissection procedures

Cords, dissected into cervical, thoracic, lumbar and sacral segments were obtained frozen on dry ice from the Cambridge Brain Bank and the Parkinson's Disease Society Brain Bank (Professor C.D. Marsden) and stored at -80ºC until dissection. Other cords were obtained from the mortuary at Nottingham University Hospital immediately after they had been removed from the body.

These fresh cords were dissected into cervical, thoracic and lumbar segments after removing the dura mater by cutting the

Table 3.1 Age, sex and interval between death and autopsy (PMI) for control and Parkinsonian patients

		Control	Parkinsonian
Age	mean	63.5	75.4
(years)	range	37 - 81	61 - 89
PMI	mean	33.0	35.8
(hours)	range	6 - 53	9.5 - 78
n	total	12	9
	females	6	5
	males	6	4

cord between the spinal nerves C8 and T1, T12 and L1 and L5 and S1 with the aid of the diagram shown in Fig. 3.1a. The tissue was 'slow frozen' to prevent it from fragmenting by storing it first at -20°c for 3-5 hours before transferring it to a -80°c freezer for storage in sealed plastic bags to prevent dehydration. Tissue was stored from 6 months to 2 years.

The tissue was dissected on a metal surface over a mixture of ice and dry ice within a microbiological hood in order to maintain a partially frozen condition during dissection. The cords were cut into 5mm slices and 2mm diameter punches were taken from the gray matter in the dorsal (posterior) and ventral (anterior) horn regions of cervical, lumbar and mid-thoracic segments using a stainless steel punch as illustrated in Fig.3.1b. The method used has been described by Bennett and co-workers (1986).

Tissue Extraction

The tissue was sonicated (NSE Soniprobe 150) in 2mls of an extractant developed previously for the concomitant extraction of catecholamines, 5HT, 5HIAA, substance P and TRH from rat and human spinal cord (Chapter 2.part 2). The composition of this extractant is ethanol (450 ml), 0.1M acetic acid (450 ml) with sodium metabisulphite (0.02%) made up to one litre with deionised double distilled water. After centrifugation (1000g, 15 mins, 4°C) the supernatant was divided into 500 µl aliquots and either stored at -80°c or analysed immediately for the measurement of indoleamines and catecholamines by HPLC with electrochemical detection and of substance P and TRH by radio-immunoassay (R.I.A.). The tissue pellets were retained for protein analysis by the method of Lowry et al., (1951, Appendix 1). To determine % recovery of the amines, DHBA (2×10^{-8}M) and 5HNMO (10^{-7}M) were added to tissue samples before sonication as internal standards for the catecholamines and 5HT and 5HIAA respectively.
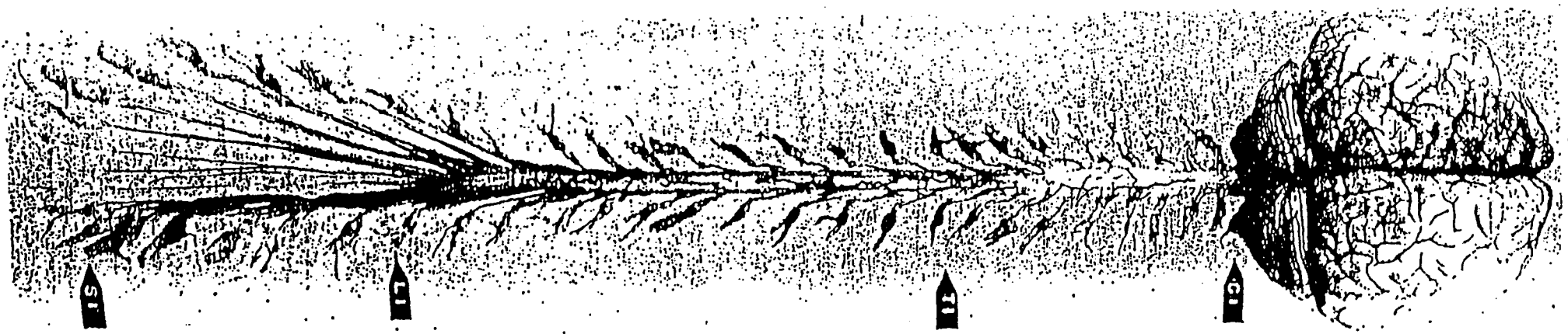
Fig 3.1a (above)   Identification of spinal roots for dissection of
spinal cord into cervical, thoracic, lumbar and sacral regions. This diagram,
taken from Grays Anatomy (Eds. Longmans, 1967), illustrates cervical (C: 1-8),
thoracic (T:1-12), lumbar (L:1-5) and sacral (S: 1-5) nerve roots.
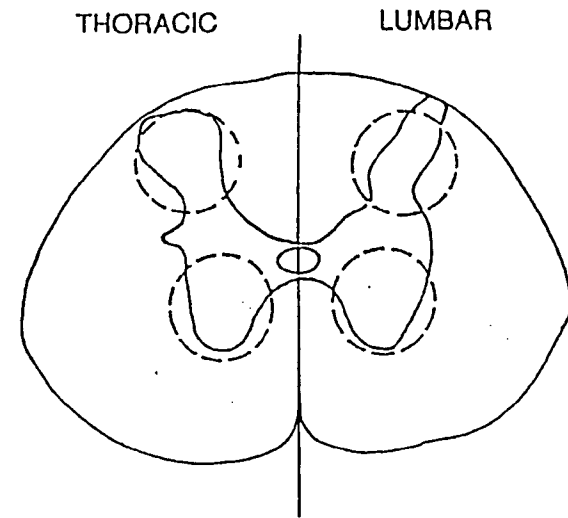
Fig 3.1b (right)   Diagram of the areas dissected from the dorsal and
ventral horns of the grey matter of the spinal cord. The dissection employed a
2mm diameter steel punch.

Measurement of 5HT, 5HIAA and catecholamines.

HPLC with electrochemical detection was performed as described for detection of the levels and % recoveries of indoleamines and catecholamines in rat tissue in chapter 2 (pp. 74-75) with the following modifications:

1. A 100µl injection loop was used in the HPLC system.
2. A glassy carbon electrode was utilised.
3. After extraction on alumina, samples were resuspended in 150µl of PCA/NaMBS (chapter 2, page 73).

An attempt was made to measure indoleamines in the supernatant from the alumina extraction of samples. After the extract had been shaken with alumina and centrifuged (1000g, 2min.) the supernatant was removed, centrifuged again (1000g,2min.), filtered (ACRO LC35 filters) and injected onto the indoleamine HPLC system.

Percentage recovery of the indoleamine internal standard, 5HNMO, was compared in samples run on the day the extracted samples were taken from storage at -80°c and in the same samples stored overnight at 4°c.

Measurement of peptides.

TRH and substance P were measured by R.I.A. as described in chapter 2 (pp.76-79).

Biohazard Procedures

Samples of frozen spinal cord were transferred to the human tissue laboratory on dry ice in a sealed container. Dissection of sections of the cord was carried out in a microbiological safety hood with the extractor fan on in the human tissue laboratory. Dissected tissue was transferred to tubes containing extractant and sonicated within the hood. All tubes were capped before removal from the safety cabinet for centrifugation. Pipetting of the supernatant and all fluid transfers took place within the microbiological hood over a plastic tray with a disposable plastic insert. Disposable apron, gloves and mask were worn during

these procedures. Sterilisation and disposal procedures for the equipment and waste are described in Appendix 2.

During RIA, HPLC and protein determination of the samples any process which may have lead to the production of an aerosol of the sample, such as centrifugation or vortex mixing was carried out in sealed tubes.

Statistics

For comparison of tissue levels of amines and neuropeptides between Parkinsonian and control or treatment and control groups the data were analysed using the Student t-test for unpaired samples.

The data were analysed for evidence of correlations between post-mortem tissue levels of the amines and peptides and (i) age of subject at death (ii) interval between death and autopsy (PMI) and (iii) sex of the subject. Initially, Pearsons correlation coefficient was used to investigate the degree of linear relationship between age and PMI and the levels of 5HT, noradrenaline, dopamine, substance P and TRH. This analysis was then refined by removing potentially biasing data. For example to investigate the relationship between noradrenaline levels and age at death, data from subjects with extreme values for PMI (less than 8 or more than 46 hours) were removed. Data exhibiting evidence of a correlation with either PMI or age were analysed by regression analysis employing the least squares method with t-values indicating the goodness of fit of the data to the line. Points were plotted with distinct symbols for female and male subjects to highlight any gender related bias. Secondly multivariate regression was performed on the data. This test can be used to investigate whether a set of values (e.g. noradrenaline levels) is dependent to some degree on 2 variable factors (e.g. age and PMI). These analyses indicated the positive or negative direction of any correlation. Finally analysis of covariance (ANCOVA) was performed to compare data from control and Parkinsonian subjects considering the effects of PMI, age and sex and to determine whether correlations between transmitter levels and the above factors were statistically significant. This test can be used

to analyse the effect of a treatment when the values are correlated to variable factors. It relies on comparison of the slope and intercept of the regression lines from 2 or more sets of data to determine whether they are from the same population. The contribution of each parameter (age, sex, PMI or Parkinson's disease) to the variation in concentration of each neuroamine and neuropeptide measured was determined as the mean square (MS) where

$$MS = SS/d.f$$

and SS and d.f are the sum of squares from the regression line and the degrees of freedom respectively. Statistical significance for the effect of a variable was determined from the F ratio test where

$$F = MS_p / MS_r$$

and where: MS_p is the mean square of the variance of each parameter; MS_r is the mean square of the residual variance (the variance not accounted for); and F is the F-value.

Analysis of correlation coefficients, simple and multivariate regression were performed on a BBC microcomputer using the Unistat statistical package from University Software. ANCOVA was performed on the University mainframe VAX computer using GLIM from the Numerical Algorithms Group.

Results.

Preliminary studies of the methodology

The effect of overnight storage at 4°c on % recovery of indoleamines in extracted samples was determined by measuring % recovery of 5HNMO in samples of extracts from cervical tissue. Comparisons were made between the results from samples analysed on the same day that they were removed from frozen storage and from the same samples re-analysed on the following day after storage at 4°c. There was no significant difference between the values obtained (92.5±2.7, 94.7±0.9%, n=5; same day and following day analysis respectively).

The levels of 5HT and 5HIAA were compared in extracts of thoracic spinal cord and in the first supernatant produced by alumina processing of the same extracts. The levels of 5HT measured in each case were not significantly different (31.7±5.3, 28.8+4.6 pmols/mg; extract and supernatant respectively, n=4) whereas the level of 5HIAA was significantly lower after alumina extraction than in the original sample (36.3±5.72, 23.1±3.2 pmols/mg; extract and supernatant, n=4, p<0.05).

Percentage recovery of indoleamines measured using 5HNMO was 103±3.8% (n=10).

Indoleamine, catecholamine and peptide levels in human spinal cord

HPLC chromatographs of samples of indoleamines and catecholamines extracted from the human spinal cord are illustrated in Fig.3.2.

Substance P levels increased down the cord from cervical through thoracic and lumbar cord in the ventral horn compared with a similarity in substance P levels in the dorsal horn in cervical and thoracic regions (Table 3.2). Levels of TRH and 5HT were also lowest in cervical spinal cord with little difference between respective thoracic and lumbar levels. In contrast, noradrenaline levels were significantly greater (p <0.05) in lumbar compared with thoracic spinal cord (Table 3.2).

Fig. 3.2 Typical HPLC chromatograms of indoleamine and catecholamine standards and extracts of human thoracic spinal cord.
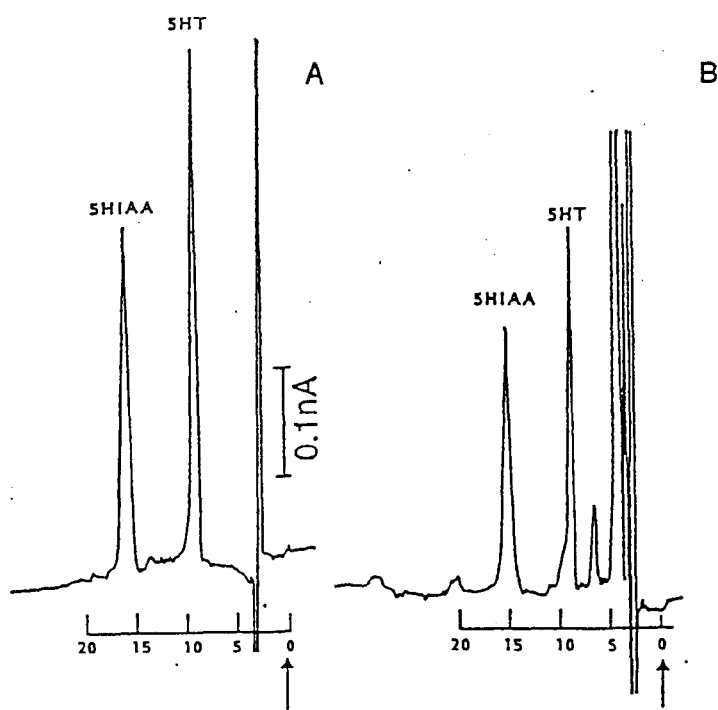
Chromatograms of standard 5HT and 5HIAA, 2 pmols (A) and an extract of human thoracic spinal cord (B). Samples and standard were injected onto the column with the following mobile phase: 0.1 M sodium acetate, 0.1 M citric acid, 10% methanol, pH 4.6 using a Spherisorb 5 ODS column (5 μm) and the detector potential set at +0.65 V. The arrow indicates the time of injection.
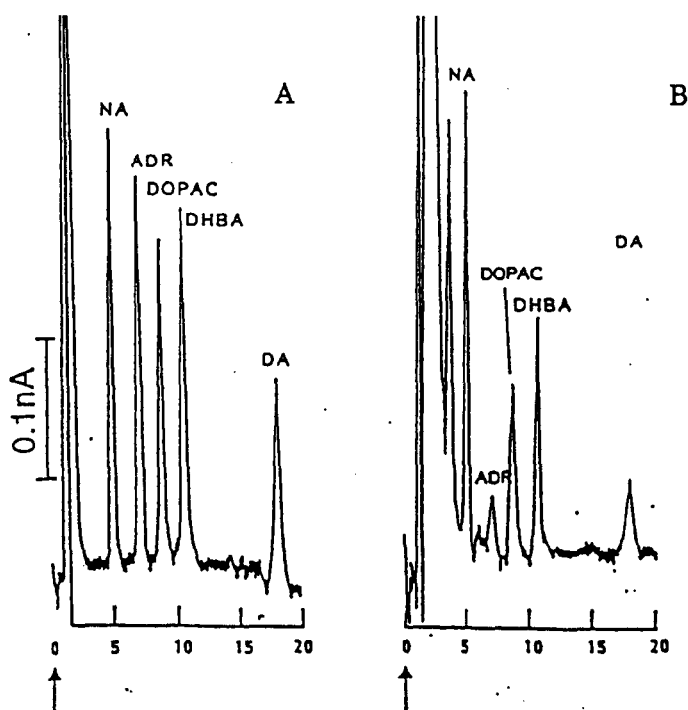
Chromatograms of a standard mixture of 0.4 pmols of NA, ADR, DOPAC DHBA and DA (A) and an extract of human thoracic spinal cord (B) injected onto a Spherisorb 5 ODS 2 column in a mobile phase of 0.1 M sodium dihydrogen orthophosphate, 1mM EDTA, 1.6 mM ion pair reagent, 9% methanol, pH 3. The detector potential was held at +0.65 V. The arrow indicates the time of injection.

NA=noradrenaline; ADR=adrenaline; DA=dopamine; DHBA is the internal standard

Table 3.2 Distribution of Substance P, TRH, Indoleamines and Catecholamines in the Human Spinal Cord

The levels of substance P (S.P), thyrotrophin-releasing hormone (TRH), 5-hydroxytryptamine (5HT), 5-hydroxyindole acetic acid (5HIAA), noradrenaline (NA), adrenaline (ADR), dyhydroxyphenyl acetic acid (DOPAC) and dopamine (DA) measured in the post-mortem spinal cord from control subjects. Values are mean ± SEM and are expressed as pmols/mg protein, n values are given in brackets .

Segment	S.P	TRH	5HT	5HIAA
CERVICAL				
Ventral	2.1±0.5 (4)	2.5±0.3 (4)	24.8±7.8 (4)	
Dorsal	9.2±1.2 (4)	0.9±0.2 (4)	12.8±4.3 (4)	
THORACIC				
Ventral	3.1±0.6 (6)	8.1±0.9 (12)	36.0±4.9 (11)	33.7±3.6 (12)
Dorsal	9.2±2.2 (8)	1.5±0.5 (10)	19.1±3.4 (10)	21.8±2.4 (12)
LUMBAR				
Ventral	18.3±4.0 (5)	7.5±1.0 (5)	30.2±4.6 (5)	40.5±8.8 (5)
Dorsal	---	2.5±0.4 (5)	22.4±2.8 (5)	39.1±6.4 (5)

	NA	ADR	DOPAC	DA
THORACIC				
Ventral	1.73±.31 (11)	0.12±.05 (11)	0.32±·12 (11)	0.11±0.08 (9)
Dorsal	0.70±0.14 (11)	0.56±.03 (11)	0.24±.09 (9)	0.07±.04 (8)
LUMBAR				
Ventral	4.78±0.74 (5)	0.11±0.11 (5)	1.54±1.03 (5)	2.16±1.12 (4)
Dorsal	4.43±1.05 (5)	0.10±0.06 (5)	0.21±0.14 (5)	0.85±0.49 (4)

The following comparisons of amine and peptide levels in Parkinsonian and control subjects were made using the Student t-test.

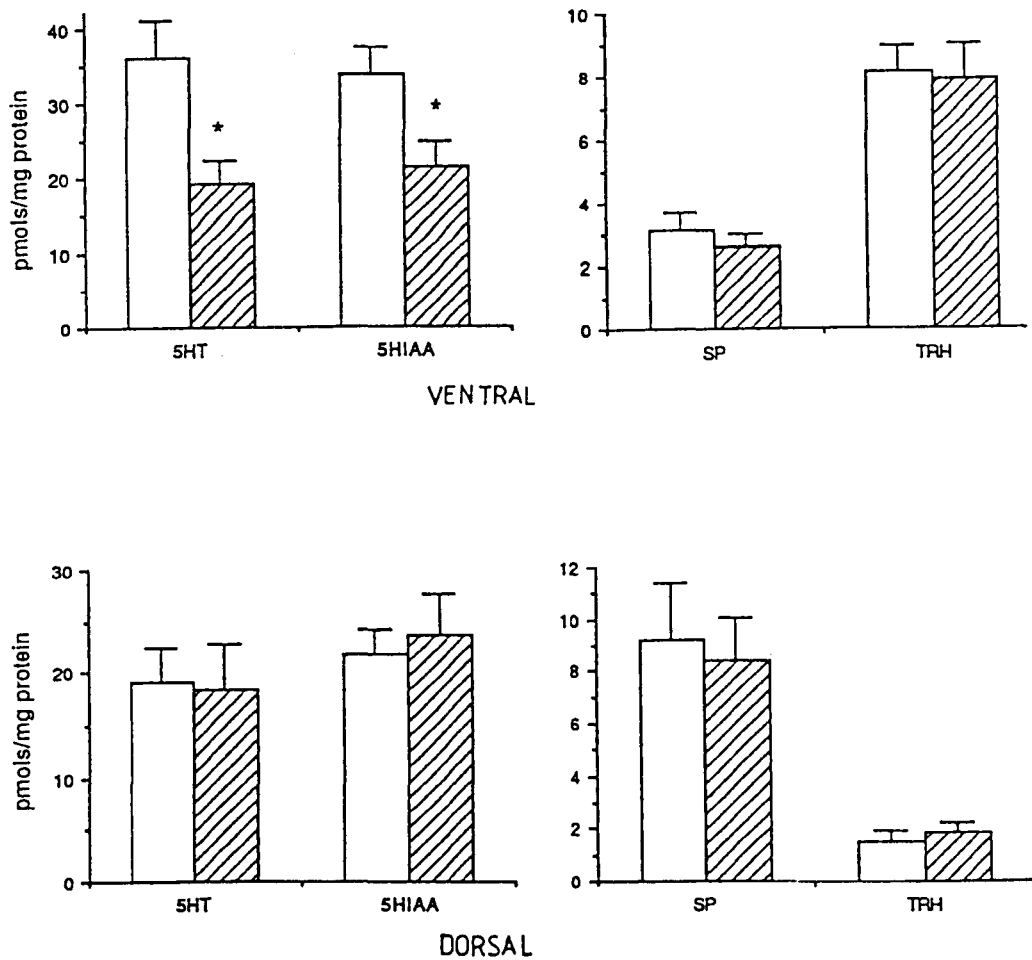## A. Thoracic Spinal Cord.

Indoleamines

In the thoracic spinal cord from control subjects 5HT and 5HIAA levels were greater in the ventral horn (36.0 and 33.7 pmols/mg protein respectively) than in the dorsal horn (19.1 and 21.8 pmols/mg protein respectively) while in the Parkinsonian tissue compared with the control cord both 5HT and 5HIAA were significantly lower in the ventral horn (approx. 40% less, $p<0.05$ in each case, student t-test. See Fig.3.3). In contrast 5HT and 5HIAA levels in dorsal thoracic cord were not significantly different in Parkinsonian compared with control tissues. In addition, there was no apparent change in the rate of metabolism of 5HT in the Parkinsonian ventral cord since the ratio of 5HIAA to 5HT was similar to that in equivalent control tissue (Table 3.3).

Peptides.

The levels of substance P and TRH were between 2 and 10 fold lower than the 5HT and 5HIAA assayed in the same tissue samples. Substance P levels in dorsal cord (9.2 pmols /mg protein) were three fold greater than in ventral cord (3.1 pmols/mg protein). In contrast TRH levels were approximately 4-5 fold greater in ventral (8.1 pmols/mg protein) compared with dorsal cord (1.5 pmols/mg protein). In thoracic spinal cords from Parkinsonian patients the peptide levels were not significantly different from those measured in control samples (Fig.3.3).

Catecholamines.

Noradrenaline was the most abundant catecholamine present in human spinal cord. The levels of noradrenaline in control ventral horn from thoracic spinal cord (1.73 pmols/mg

Fig. 3.3   Levels of 5HT, 5HIAA, substance P (SP) and TRH
measured in control(□) and Parkinsonian(▨) human thoracic
ventral and dorsal spinal cord.
The n values are respectively, for ventral and dorsal horn spinal
cord from Parkinsonian subjects ; 7 & 8 (5HT); 7 & 8 (5HIAA); 9
& 9 (SP); 6 & 8 (TRH). The n values for the control subjects are
given in Table 3.2.
p* <0.05.
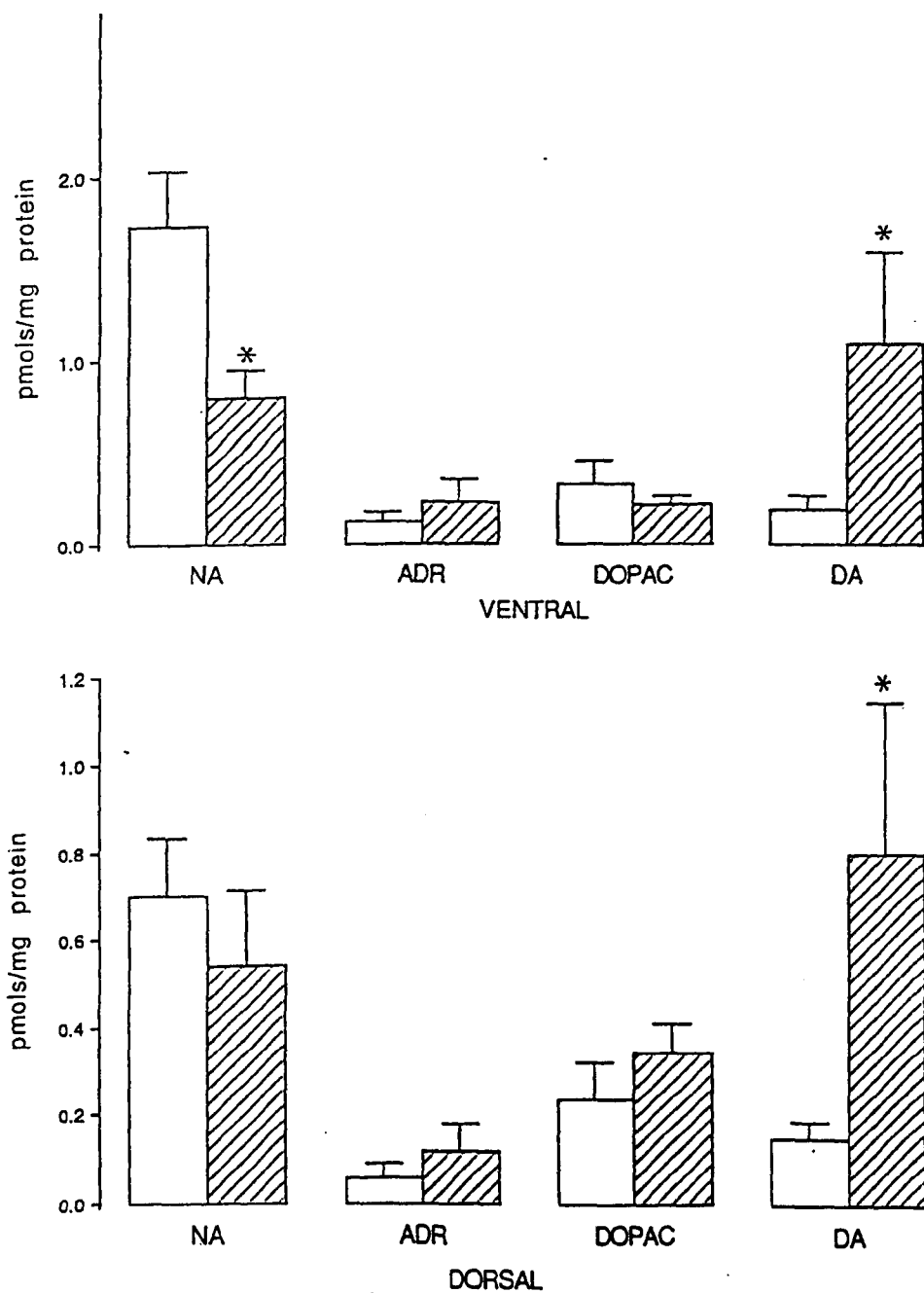
Fig. 3.4   Levels of noradrenaline (NA), adrenaline (ADR)
dihydroxyphenylacetic acid (DOPAC) and dopamine (DA)
measured in control (□) and Parkinsonian (▨) human thoracic
ventral and dorsal spinal cord.
The n values for ventral and dorsal horn spinal cord from
Parkinsonian subjects are; 8 (NA); 8 (ADR); 8 (DOPAC); 6 (DA).
The n values for the control subjects are given in Table 3.2. p*
<0.05.

protein) were approximately twice those in dorsal cord (0.7 pmols/mg protein). These noradrenaline levels were over five fold greater than the levels of adrenaline, dopamine or its metabolite DOPAC in the ventral horn of control spinal cord (Fig.3.4). In the ventral horn tissue from patients with Parkinson's disease the noradrenaline levels were approximately half those present in controls (p<0.05) while the dorsal horn levels of noradrenaline were not significantly different between groups (Fig.3.4). In contrast with noradrenaline distribution the levels of dopamine in the Parkinsonian cord were significantly greater than control values in both the ventral and dorsal gray matter (Fig.3.4). In addition there were no differences in the levels of adrenaline or DOPAC in either dorsal or ventral cord tissue from Parkinsonian and control subjects. Furthermore, there was no significant difference between the % recovery of the catecholamine internal standard DHBA measured in either control or Parkinsonian spinal cord. Percentage recovery was 48.6±4.0 (mean±SEM, n=10) and 45.3±6.2 (mean±SEM, n=8) for control and Parkinsonian tissue respectively.

B. Lumbar Spinal Cord.

Catecholamines and indoleamines

The number of cords used in the study of the lumbar region was low, there being only 5 control and 5 Parkinsonian cords. The differences in amine levels in the ventral horn from lumbar cord between tissue from Parkinsonian and control subjects paralleled those in thoracic cord. Compared with the levels of 5HT and 5HIAA in the ventral horn of control cords (30.2 and 40.5 pmols/mg protein), the tissue from subjects with Parkinson's disease showed lower levels of 5HT and its metabolite though these did not reach significance (Fig.3.5) possibly due to the low numbers used. The noradrenaline levels, however, in Parkinsonian ventral horn were markedly reduced (~80%, Fig.3.6) compared with that in equivalent control tissue (4.78 pmols/mg protein in control cord).

Fig. 3.5   Levels of 5HT, 5HIAA, SP and TRH measured in
control (□) and Parkinsonian (▨) human lumbar ventral and
dorsal spinal cord. The n values are respectively, for ventral and
dorsal horn spinal cord from Parkinsonian subjects ; 3 & 3 (5HT);
3 & 3 (5HIAA); 3 & - (SP); 3 & 3 (TRH). The n values for the
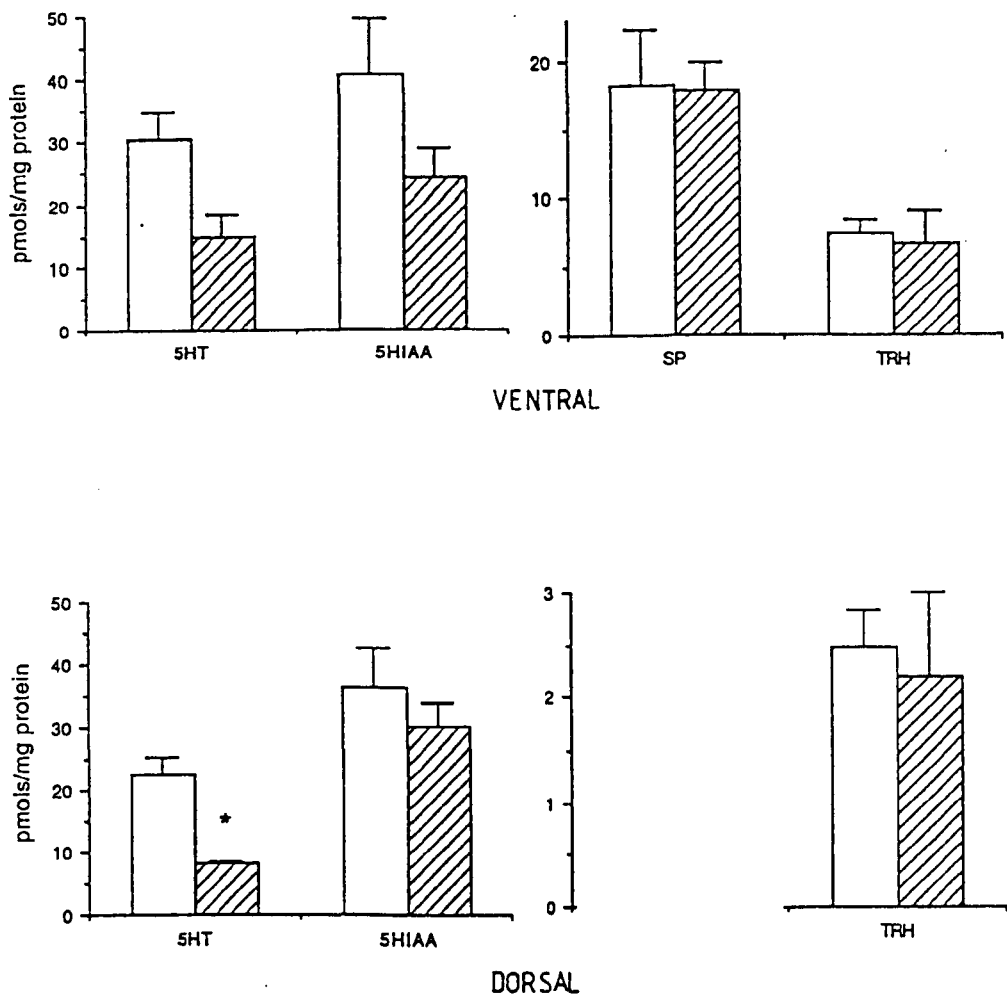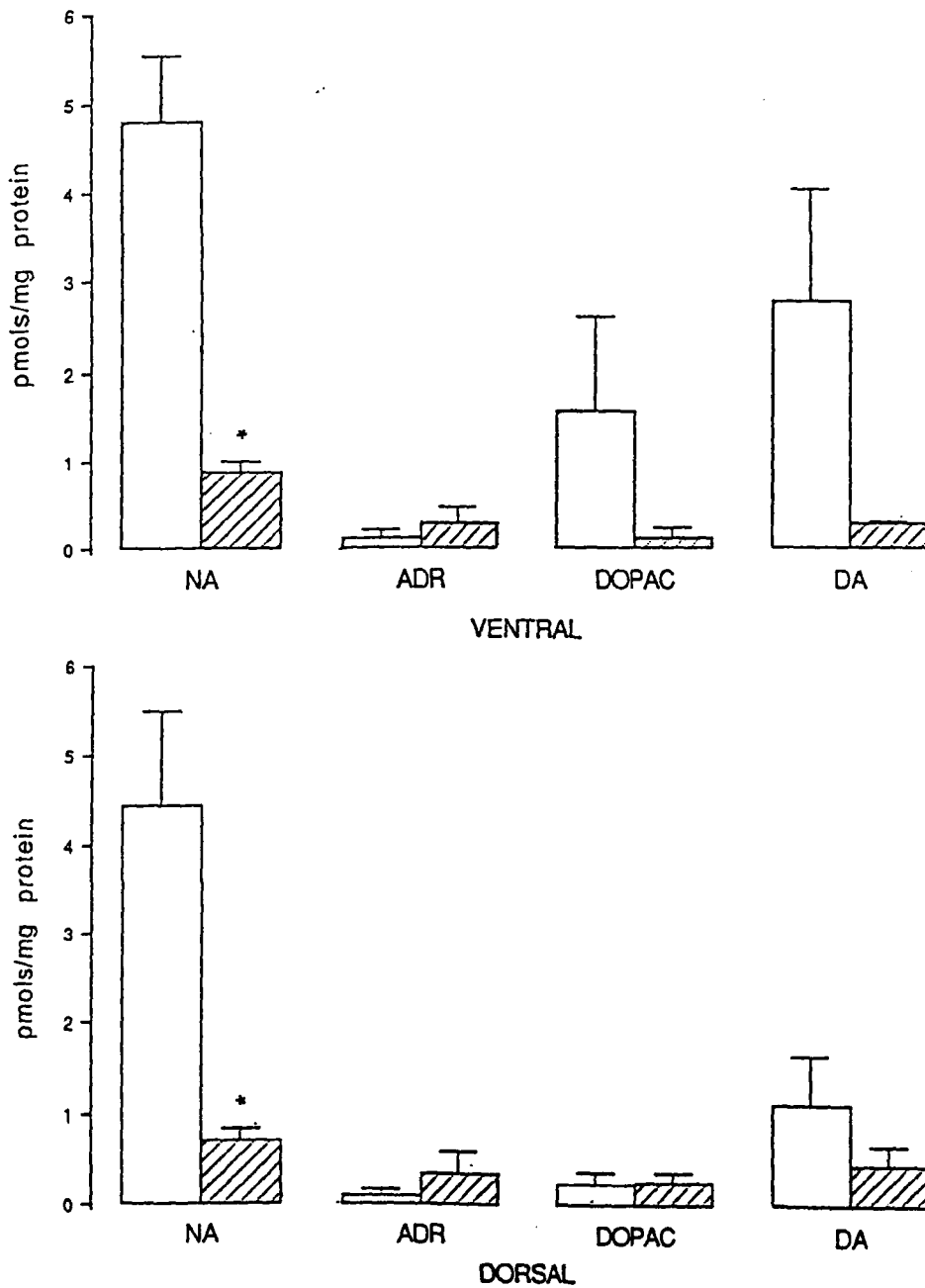control subjects are given in Table 3.2.
p* <0.01.

Fig. 3.6   Levels of NA, ADR, DOPAC and DA measured in
control (□) and Parkinsonian (▨) human lumbar ventral and
dorsal spinal cord. The n values for ventral and dorsal horn
spinal cord from Parkinsonian subjects are; 5 (NA); 5 (ADR); 3
(DOPAC); 4 (DA). The n values for the control subjects are given
in Table 3.2. p* <0.01.

In the dorsal horn of lumbar cord from control subjects 5HT levels were similar to equivalent thoracic tissue (22.4 pmols/mg protein) whereas noradrenaline levels were greater than in equivalent thoracic tissue (4.43 pmols/mg protein). In contrast with the findings in thoracic dorsal tissue there were significantly lower levels of 5HT and noradrenaline in the Parkinsonian lumbar dorsal horn samples compared with controls. 5HT and noradrenaline levels were approximately 3 and 7 fold lower respectively (Fig.3.5 and 3.6). As 5HIAA levels were only moderately (and not significantly) diminished the mean ratio of 5HIAA to 5HT in the Parkinsonian lumbar dorsal cords was over twice that in the equivalent control cords (Table 3.3). The levels of adrenaline and DOPAC in the lumbar cords were approximately 2 -30 % of the noradrenaline levels (Fig.3.6) and there were no significant differences in the levels between control and Parkinsonian lumbar cord in either the dorsal or ventral horns.

Dopamine levels in control lumbar cord were very much greater than those in equivalent regions of the thoracic cord (2.76 and 1.09 pmols/mg protein, ventral and dorsal horn respectively) and in contrast to the findings in the thoracic cord, in the lumbar cord the dopamine levels were lower, but not significantly so, in Parkinsonian compared with control cord in both the ventral and dorsal horns.

Substance P and TRH

Substance P levels were determined only in the ventral horn (18.3 pmols/mg protein, in controls) due to experimental error. The relative distribution of TRH in the lumbar cord of control subjects was similar to that in thoracic cord (7.54 and 2.48 pmols/mg protein, ventral and dorsal horns respectively). Moreover, the substance P and TRH content of the Parkinsonian cord was no different from controls in either dorsal or ventral samples (Fig.3.5).

In summary, the levels of 5HT, 5HIAA and noradrenaline were significantly lower in the ventral horn of thoracic spinal

Table 3.3 Ratio of 5HIAA/ 5HT in spinal cord

Ratio of 5HIAA/ 5HT in different regions of post-mortem spinal cord from Parkinsonian and control subjects. Significant differences (p<0.05) from control are indicated by * and n values are given in brackets.

Region	Parkinson's disease		Control	
Thoracic ventral	1.19 ± 0.21	(7)	1.06 ± 0.14	(11)
Thoracic dorsal	1.68 ± 0.38	(8)	1.23 ± 0.21	(10)
Lumbar ventral	1.69 ± 0.46	(3)	1.51 ± 0.38	(5)
Lumbar dorsal	3.76 ± 0.60*	(3)	1.58 ± 0.10	(5)

cord from Parkinsonian patients compared with control subjects with no difference in the dorsal horn levels (Fig.3.3 and 3.4). In contrast, dopamine levels were significantly greater in thoracic spinal cord from Parkinsonian compared with control subjects in both the ventral and dorsal horns (Fig.3.4). The significant differences in neuroamine content in the lumbar spinal cord from Parkinsonian compared with control subjects were (1) lower levels of noradrenaline in both the ventral and dorsal horns (Fig.3.6) and (2) lower levels of 5HT in the dorsal horn (Fig.3.5) with an increase in the ratio 5HIAA/5HT (Table 3.3).

Effects of age, sex and PMI on amine and peptide levels in the spinal cord

Using Pearson's correlation coefficient analysis there was no correlation between sex and either TRH, substance P, noradrenaline or dopamine levels. Pearson's correlation coefficients (c) and regression coefficients (r) indicated that levels of noradrenaline increased with age (c =0.79, p<0.05) and that 5HT levels decreased with both age (r=-0.84, p<0.01) and PMI (c = -0.81, p<0.05). Multivariate analysis indicated a positive correlation between TRH levels and both PMI and age (r= 0.69, p<0.05) when these factors were considered together but not separately. There was no evidence for correlations between dopamine or substance P with either age or PMI.

Using ANCOVA the effect of each of the factors age, sex, PMI and Parkinson's disease on noradrenaline, 5HT and TRH levels was statistically determined after accounting for the correlations with each of the other factors. Results of factors for which there was evidence of some degree of correlation are given in Table 3.4. There was clearly no parallel between the sex of the subject and neurotransmitter levels in either thoracic or lumbar, dorsal or ventral spinal cord regions.

Effects of age and PMI on noradrenaline levels

In the thoracic and lumbar ventral horns noradrenaline levels tended to increase with age but not to a significant degree

and they were not correlated with PMI (Table 3.4). Furthermore, in the thoracic and lumbar dorsal horns there was no relationship between either age or PMI and noradrenaline levels. Considering these factors there were significant differences in noradrenaline levels in the thoracic and lumbar ventral horn (p<0.05, Table 3.4) and in the lumbar dorsal horn (p<0.01, Table 3.4) between Parkinsonian and control subjects .

Effects of age and PMI on 5HT levels

In thoracic ventral and dorsal horns there was a significant decline in 5HT levels with the age of the subject (p<0.05, Table 3.4). Such was the fall in levels with age that it accounted for the lower 5HT levels in the Parkinsonian thoracic ventral cord and considering this factor there was no significant difference between 5HT levels in Parkinsonian and control cord in the thoracic ventral horn attributable to Parkinson's disease. However in lumbar cord there was no parallel between 5HT levels and age in either the dorsal or ventral horns. 5HT levels were lower in Parkinsonian cord than in control cord in both the ventral and dorsal horns but this difference was significant (p<0.05) only in the dorsal horn (Table 3.4) after accounting for the effects of age and PMI, possibly because of the low numbers used. In addition there was no effect of PMI on 5HT levels in any region measured.

Effects of age and PMI on TRH levels

In the thoracic ventral horn TRH levels increased significantly with age (p<0.01) and PMI (p<0.05) whereas in the lumbar ventral cord the tendency for TRH to increase with age and PMI did not reach a significant level (Table 3.4). In the thoracic and lumbar dorsal horns however there was no correlation between TRH and either age or PMI. In none of these regions were there any differences between TRH levels in Parkinsonian and control cord.

Table 3.4 Correlation between neurotransmitter levels and PMI, age and Parkinson's disease.

ANCOVA details for analysis of correlation between neurotransmitter levels and (A) PMI (B) age and (C) Parkinson's disease (Pd) in regions of the thoracic and lumbar spinal cord. Statistical information given in the table includes mean square (MS), degrees of freedom (d.f), and the residual variance (res.var) from which the F value was calculated. Details are given where there was a statistically significant correlation and for selected data which showed no correlation. Data from analysis of the effect of sex on transmitter levels are not given here as there was no correlation between these factors. Statistical significance is indicated by * ($p < 0.05$) or ** ($p < 0.01$).

A. PMI	region		MS	d.f.	F value
NA	thoracic		0.00	1	0.00
	ventral	res.var	0.83	12	
5HT	thoracic		86	1	0.56
	ventral	res.var	154	11	
TRH	thoracic		37.0	1	7.54*
	ventral	res.var	4.91	12	
TRH	lumbar		11.2	1	2.31
	ventral	res.var	4.84	3	

Table 3.4 continued

B	Age	region		MS	d.f.	F value
NA		thoracic		1.53	1	1.84
		ventral	res.var	0.83	12	
NA		thoracic		0.39	1	1.70
		dorsal	res.var	0.23	12	
NA		lumbar		3.97	1	1.84
		ventral	res.var	2.16	4	
NA		lumbar		3.43	1	1.47
		dorsal	res.var	2.33	3	
5HT		thoracic		1265	1	6.45*
		ventral	res.var	196	12	
5HT		thoracic		687	1	5.73*
		dorsal	res.var	120	11	
5HT		lumbar		717	1	4.29
		ventral	res.var	167	3	
TRH		thoracic		61.1	1	12.4**
		ventral	res.var	4.91	12	
TRH		thoracic		2.69	1	1.49
		dorsal	res.var	1.81	11	
TRH		lumbar		22.7	1	4.69
		ventral	res.var	4.84	3	

Table 3.4 continued

C. Pd	region		MS	d.f.	F value
NA	thoracic		4.54	1	5.47*
	ventral	res.var	0.83	12	
NA	thoracic		0.24	1	1.04
	dorsal	res.var	0.23	12	
NA	lumbar		30.1	1	13.9*
	ventral	res.var	2.16	4	
NA	lumbar		43.8	1	22.1**
	dorsal	res.var	1.98	4	
5HT	thoracic		499	1	2.54
	ventral	res.var	196	12	
5HT	lumbar		648	1	5.18
	ventral	res.var	125	3	
5HT	lumbar		205	1	14.9*
	dorsal	res.var	13.8	3	
TRH	thoracic		11.9	1	2.42
	ventral	res.var	4.91	12	
TRH	thoracic		0.00	1	0.00
	dorsal	res.var	1	11	

In summary, (1) there were no significant effects of either age or PMI on noradrenaline levels but (2) there were significantly lower levels of noradrenaline in the spinal cords from Parkinsonian patients in the thoracic ventral ($p<0.05$) and lumbar ventral ($p<0.05$) and dorsal horns ($p<0.01$). Furthermore, (3) 5HT levels decreased with age in the thoracic ventral and dorsal horns ($p<0.05$ in each case) but not in the lumbar cord and (4) the decrease with age in 5HT levels accounted for the lower levels of 5HT in the thoracic ventral horn of spinal cords from Parkinsonian patients which were therefore not attributable to Parkinson's disease. In addition, (5) in the thoracic ventral horn TRH levels increased significantly with age ($p<0.01$) and PMI ($p<0.05$) and (6) accounting for these correlations there were no differences in the levels of TRH in the spinal cord of Parkinsonian and control subjects. Finally, (7) there were no effects of either age, sex or PMI on substance P or dopamine levels and therefore the effects of Parkinson's disease on substance P and dopamine are as determined by t-test analysis above.

The % of the total intra-subject variation in the levels of noradrenaline, 5HT and TRH due to PMI, sex, age and Parkinson's disease was determined from the residual variance after accounting for all these factors. PMI, sex, age and Parkinson's disease accounted for 56.5±17%, 64.0±12% and 52.6+10% of the intra-subject variation in the levels of noradrenaline, 5HT and TRH respectively (n=4 regions in each case).

Discussion.

In this chapter the levels of substance P and TRH and of 5HT, the catecholamines and their metabolites were studied in post-mortem spinal cord from subjects who had died with Parkinson's disease. This was performed to investigate the proposal that 5HT and noradrenaline levels are reduced in the spinal cord in Parkinson's disease (Scatton et.al., 1986) and to determine whether changes in 5HT levels are accompanied by changes in the levels of TRH and substance P.

Preliminary experiments to improve the methods

Preliminary investigations and modifications were made in order to refine the methods and these will be discussed first. Initially, problems were experienced in obtaining a high enough level of HPLC sensitivity to detect dopamine, DOPAC and adrenaline levels. This was overcome in several ways; a glassy carbon electrode was used with which higher levels of sensitivity were attained; samples were made as concentrated as possible on alumina ; a 100µl injection loop was employed so that greater volumes of the sample could be injected on to the HPLC column; and the catecholamines were extracted in a more specific medium in which the % recovery of catecholamines was significantly greater than in the common extractant (previous chapter, page 82). The % recovery of the internal standard for the catecholamines was within an acceptable limit but the levels of dopamine in the thoracic cord samples were in some cases below the level of system sensitivity assuming a signal to noise ratio of 3:1. Therefore for dopamine only a lower level of precision in measurement was employed (signal to noise ratio of 2:1) in order to measure the low levels. The tissue levels of DOPAC were determined in this study as a metabolite of dopamine. However although DOPAC is the major metabolite of dopamine in the rat brain (Wilk et.al.,1975), in the human CNS current evidence indicates that the major dopamine metabolite is HVA (homovanillic acid) and that HVA is present in concentrations 10-100 fold those of DOPAC (Wilk and Stanley, 1978; Scatton et. al.,1983; Gibson et. al.,1985). It may

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therefore have been more appropriate, and easier in terms of the HPLC system sensitivity required, to determine the levels of HVA instead of DOPAC. A description of the metabolic pathway of the catecholamines is given in the General Introduction (pp. 23-24). Thirdly, the % recovery of the peptides was not determined directly in the human spinal cord because this would necessitate spiking the tissue with exogenous peptide and using a considerable proportion of limited amount of tissue available. Peptide levels were therefore corrected using the % recoveries determined in the rat spinal cord. These values are likely to err on the low side, if at all, as recovery from bisected samples of the whole cord (as used in the rat samples) would be expected to be lower than from punches of tissue as degradation of the myelin sheath surrounding the cord required prolonged sonification.

An attempt was also made to utilize the supernatant from the alumina extraction of samples to measure 5HT and 5HIAA. After shaking a sample of tissue extract with alumina and Tris-HCL the supernatant, usually discarded, contains the indoleamines and other non-catecholamine components. 5HT was detectable in the supernatant after injection onto the HPLC system at a concentration not significantly different from that determined in tissue extract. However, the levels of 5HIAA were significantly lower in the supernatant than in tissue extract indicating its instability in acidic solutions as discussed in the previous chapter. This procedure could not therefore be used to measure indoleamine levels.

Levels of indoleamines, catecholamines, substance P and TRH

The levels of substance P, TRH and of 5HT, noradrenaline, dopamine, adrenaline and their metabolites were determined in the human spinal cord. Levels of 5HT, 5HIAA, peptides and catecholamines measured in control human spinal cord tissues exhibited a similar range, and regional distribution as previously reported in humans considering differences due to variation in the exact regions dissected (Przewlocki et. al.,1983; Bennett et al., 1986; Scatton et al., 1986; Mitsuma et.al., 1984).

There was a widespread and characteristic distribution of substance P in the human spinal cord. Higher levels of substance P in the dorsal compared with the ventral horns corresponds to the distribution described previously in humans (Przwelocki et.al.,1983; Anand et.al., 1983; Helme and White 1983; Bennett et.al.,1986) in rats (Helke et.al., 1982; Marsden et al.,1982; this author, Table 2.2) and in the rabbit (Fone et.al.,1987b). Substance P has also been located in non-human primates in the dorsal and ventral horns of the spinal cord (DiFiglia et.al.,1982; Bowker ,1986). The increase in substance P levels from the cervical through to lumbar spinal segments seen in this study has been reported by others for the human (Anand et.al.,1983; Przewlocki et.al.,1983) and rabbit (Fone et.al.,1987b).

In contrast TRH levels in the human spinal cord were greater in the ventral than in the dorsal horns. This corresponds to the distribution of TRH in the spinal cord of the human (Yates et.al.,1983; Bennett.et.al.,1986; Mitsuma et.al.,1986), the rat (Marsden et.al.,1982; Lighton et.al.,1984; this author, Table 2.2), the rabbit (Fone et.al.,1987b) and the monkey (Lechan et.al.,1984). Thoracic and lumbar TRH levels were similar and were greater than cervical levels.

In this study 5HT levels were 1.3 -2 fold greater in the ventral compared with the dorsal horns of cervical, thoracic and lumbar segments of the human spinal cord which corresponds to the regional distribution of 5HT described in the human (Bennett et.al.,1986; Scatton et.al.,1986), the rat (Gilbert et.al,.1981; Marsden et. al.,1982; Lighton et al.,1984) and the rabbit (Zivon et al.,1975; Fone et.al.,1987b). Furthermore, 5HT levels were similar in equivalent regions of the thoracic and lumbar spinal cord segments in this study as reported in the rabbit (Zivon et.al.,1975; Fone et.al.,1987b) but not the rat (Basbaum et.al.,1987, see General Introduction page 6). The ratio of 5HIAA to 5HT was consistent between dorsal and ventral regions of the thoracic and lumbar spinal cord in the human which contrasts with evidence of a higher ratio in the ventral cord in the human (Bennett et.al.,1986). However, the region in the latter study included white matter in which the relative levels of transmitters can be very different compared to that in gray matter (Bennett et.al.,1986).

Of the catecholamines measured, the levels of noradrenaline were the greatest in the human spinal cord and corresponded to those reported by Scatton and co-workers (1986). However, the levels of noradrenaline were one tenth or less of those measured in the rat or cat spinal cord (Commissiong et.al.,1978a; Mouchet et.al.,1982; Basbaum et.al.,1987; Fleetwood-Walker and Coote,1981). In the thoracic cord noradrenaline levels were approximately 10 times greater than dopamine levels which is consistent with the relative distributions of noradrenaline and dopamine in the spinal cord of the human (Scatton et.al.,1986) and rat (Commissiong et.al.,1978a; Mouchet et.al.,1982; Basbaum et.al.,1987; this thesis, chapter 2, Table 2.3). The distribution of dopamine in the human was not similar to that described in the rat in which dopamine levels are reported to be lower in the ventral than the dorsal horn (Commissiong et.al.,1978a; Commissiong and Neff, 1979; Mouchet et.al.,1982; Basbaum et.al.,1987). In the lumbar cord in this study the relatively larger dopamine levels are due to (i) 2 possibly spuriously large values out of 5 in each region and (ii) the exclusion of low values which were below the level of HPLC system sensitivity. For these reasons the values for dopamine levels in the lumbar cord may not be accurate. Adrenaline levels were very low in the human ventral and dorsal spinal cord being 14 - 47 fold lower than those of noradrenaline. This is in accordance with the reports of sparse adrenergic innervation to these regions in the rat (Coote et.al,.1981; Ross et.al.,1984; Kohno et.al.,1988).

Amine and peptide levels in Parkinson's disease

This study has demonstrated that the content of specific amines in spinal cords from patients having died with Parkinson's disease was significantly different from that from non-neurological control subjects in selected regions. The content of 5HT, 5HIAA and noradrenaline was approximately 50% less in thoracic ventral cord from Parkinsonian patients compared with control subjects with no significant difference in the ratio of 5HIAA to 5HT, while no change in the levels of these amines were observed in the dorsal cord. In contrast the content of dopamine in

the thoracic ventral and dorsal cord was greater in the
Parkinsonian than the control cord. In Parkinsonian lumbar
cord 5HT and 5HIAA levels tended to be lower in ventral horn
tissue and 5HT alone was significantly lower in dorsal horn
tissue thereby resulting in an increase in the ratio of 5HIAA/ 5HT
in the dorsal horn only. However, the most marked reduction was
with respect to noradrenaline in lumbar spinal cord where the
noradrenaline content of both ventral and dorsal regions was less
than 20% of control levels. No significant differences were
demonstrated between Parkinsonian and control tissue in the
content of dopamine in the lumbar spinal cord or of TRH,
substance P, adrenaline or DOPAC in any region.

The lower level of 5HT in Parkinsonian thoracic ventral
and dorsal cord compared with control values was dependent on
the age of the subjects. When the two groups (Parkinsonian and
control) were adjusted for age differences there was no significant
difference between 5HT levels in either thoracic region. In the
lumbar cord, however, the level of 5HT was unaffected by age and
the difference in levels between Parkinsonian and control cords
was attributed to Parkinson's disease. In contrast, noradrenaline
levels tended to increase with age in lumbar and thoracic ventral
spinal cord though not to a significant degree. The difference in
noradrenaline levels between Parkinsonian and control cords
was, therefore, accentuated considering that the mean age of the
Parkinsonian patients was greater than that of the control subjects
by 12 years. Furthermore, TRH levels increased significantly
with both PMI and age in the thoracic ventral cord but there was no
effect on the levels attributable to Parkinson's disease.

The results indicate that the lower levels of indoleamines in
the thoracic and lumbar Parkinsonian cord compared with
equivalent control cord are due to different factors. The reduction
in thoracic cord being due to age and the reduction in lumbar cord
being due to Parkinson's disease itself. In a similar study of
indoleamine levels in lumbar cord Scatton and co-workers (1986)
found 50% loss of 5HT and 5HIAA in lumbar ventral and dorsal
spinal cord which they attributed to the effects of Parkinson's
disease. They reported that patient age, sex and PMI did not affect
5HT levels without, however, providing evidence that they had

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used statistical analysis to make this conclusion and the dangers of this are discussed below. Nevertheless, their Parkinsonian and control subjects were well matched for age, in fact the control subjects were on average older than the Parkinsonian patients. It is likely therefore, that the 5HT reduction in the lumbar cord in their study and this one is due to the effects of Parkinson's disease and the increase in the ratio of 5HIAA to 5HT may indicate a compensatory increase in the rate of 5HT turnover.

Co-existence of substance P, TRH and 5HT in human spinal cord

The reductions in the levels of 5HT and 5HIAA found in the Parkinsonian cords may result from degeneration of bulbospinal neurones. However, in the light of evidence of a concomitant unaltered peptide state it may be that the dysfunction is more complex. The distribution of descending serotonergic and peptidergic neurones in the raphe nuclei of the rat suggest that a large fraction of the raphe-spinal neurones contain serotonin co-existing with substance P and/or TRH in the same cell as discussed previously (pp.9-11). If this were the case in humans, spinal serotonergic depletion may be expected to be accompanied by a loss in spinal peptide content, which has not been demonstrated.

It is not surprising that dorsal horn substance P levels do not parallel loss of 5HT in this region as the major innervation in rat (50-80%) of substance P in the dorsal horn is from the dorsal root ganglion (Jessell et. al.,1979; Ogawa et.al.,1985; Tessler et.al., 1984) or intra-spinal neurones (Hunt et.al., 1981; Tessler et.al., 1984). Whether there is a descending serotonergic/substance P innervation of the dorsal horn is undetermined but at most, it makes a small contribution to dorsal horn substance P in rat (Gilbert et.al.,1981; Marsden et.al.,1982; Helke et.al., 1986). Similarly, dorsal horn TRH is probably largely independent of the bulbo-spinal serotonergic system (Helke, 1986; Harkness and Brownfield,1986). The origins of substance P and TRH in the ventral cord in the rat are mainly supra-spinal including nuclei in the medulla (the raphe magnus, raphe obscurus and raphe pallidus) and the ventrolateral medulla (nucleus reticularis gigantocellularis [NG] and the nucleus interfascicularis

hypoglossi [NIH] also known as nucleus reticularis ventralis pars alpha) (Hokfelt et.al.,1978;Gilbert et.al., 1981; Hokfelt et.al.,1981; Bowker et.al.,1983; Helke et.al.,1982). Part of this innervation is co-existent with serotonin innervation from the descending ventral medullary nuclei and the selective serotonergic neurotoxin 5,7-DHT substantially reduces ventral horn substance P and TRH (Hokfelt et.al., 1978; Helke et.al., 1986; Gilbert et.al.,1982; Harkness and Brownfield,1986). However, there are a number of possible reasons why in this study reductions in peptides levels did not parallel those in 5HT. Firstly, massive destruction of serotonergic neurones using 5,7-DHT and the accompanying loss of peptides occurs within days of toxin administration. The effects of aging or Parkinson's disease on neuronal integrity is most likely to occur over a period of years and may not therefore be comparable with neurotoxin induced serotonergic denervation. It is possible that unaffected peptidergic intra-spinal and supra-spinal innervation of the spinal cord has time to adapt and compensate for long term neuronal changes. Davis and co-workers (1984) found that after mechanical lesions of the cervical cord substance P-LI in the intermediolateral cell column was unchanged or elevated and proposed that intraspinal substance P neurones were capable of compensating for loss of spinal substance P. Furthermore, the present study has demonstrated an increase in ventral spinal cord TRH levels with age which may be evidence of a homeostatic mechanism compensating for loss of serotonergic innervation with age. Secondly, although a large proportion of TRH projection to the ventral horn is co-existent with serotonin this region also receives non-serotonergic TRH innervation from the NIH (Harkness and Brownfield, 1986; Holets et.al.,1987). Changes in TRH metabolism in this non-serotonergic TRH projection to the spinal cord may compensate for TRH loss due to destruction of serotonergic /TRH neurones.

Alternatively, the observation that the neuropeptide levels do not show parallel reductions with the 5HT and 5HIAA levels and further that the 5HT loss is only partial may indicate that any possible spinal degeneration in Parkinson's disease (or aging) may be restricted to a selective sub-population of neurones containing 5HT alone. The heterogeneous distribution of the

transmitters throughout the raphe system in rodents indicates various sub-populations of raphe spinal neurones containing 5HT, substance P and TRH either together, separately or in various combinations (Bowker et al., 1982). In addition there is evidence of a descending serotonergic projection from the locus coeruleus (Leger et.al,1979; Wiklund et.al.,1981; Lai and Barnes, 1985). Thus rather than being indicative of a lack of spinal serotonin-peptide co-existence in humans, the unaltered peptide levels in Parkinsonian cord may result from preservation of 5HT neurones which also contain substance P and/or TRH. Such a proposition is consistent with the evidence that certain peptide containing neurones may be spared in Parkinsonian brain (Studler et al., 1982). It is possible that the peptides TRH and substance P which have been reported to have trophic functions (Jonsson and Hallman, 1982; Schmidt-Achert et al., 1984; Fone et al.,1988a) may help to maintain the integrity of selective descending serotonergic neurones in disease or normal aging.

The different effect on 5HT levels in the thoracic compared to the lumbar cord could be explained by differences in the degree of serotonergic innervation to each cord segment from the various medullary nuclei. There is evidence that neurones from the three raphe neurones send collaterals to different spinal segments and innervate the different segments in varying degrees in the cat (Bowker et.al.,1987). In addition, Di Carlo (1983) reported a possible additional source of serotonergic innervation of the lumbar cord from the dorsal root ganglia. The evidence therefore indicates that projections from the raphe nuclei to the different segments of the cord are not evenly distributed and that some of the lumbar serotonergic fibres do not originate in the raphe.

Catecholamine levels in Parkinson's disease

The dramatic diminution of noradrenaline levels in the Parkinsonian lumbar compared with thoracic cord may indicate noradrenergic neuronal loss which is more progressive down the cord. This difference in degree of noradrenergic depletion of the lumbar and thoracic cord in Parkinson's disease may reflect differences in the nuclei of origin of the thoracic and lumbar

noradrenergic projections. The pontine noradrenergic cell groups, in particular the caudal and ventral locus coeruleus and subcoeruleus are cited as sources of spinal noradrenaline innervation though in immunocytochemical studies in the rat no obvious differences in source nuclei has been seen for different cord segments (Commissiong et.al., 1978; Westlund et.al., 1983; Nakazato, 1987).

Contrary to the effects on the other amines, dopamine levels were significantly greater in the thoracic ventral and dorsal spinal cord in Parkinson's disease compared with controls and these levels were not correlated with age, sex or PMI. It is possible that this result is dependant on pre-morbidity drug treatment. Differences in the interval between the last dose of L-dopa and death may account for the variations demonstrated between Parkinsonian patients in spinal cord dopamine levels. Post-mortem striatal dopamine levels are 9 - 15 times higher in Parkinsonian patients treated with L-dopa than in untreated patients (Lloyd et.al.,1975). Furthermore, the levels of dopamine in a number of brain regions in patients having died of Parkinson's disease have been shown to be related to the interval between the last dose of L-dopa and death, levels being greater in those patients who received medication nearer to the time of death (Lloyd et.al., 1975; Scatton et al., 1983). In addition, it has been demonstrated that L-dopa can be converted to dopamine in the spinal cord in elements other than dopaminergic neurones, probably glia and non-monoaminergic nerve terminals (Commissiong, 1985). It would have been preferable to use tissue only from patients who had not received medication within the 24 hours preceding death but this would not have been practical due to the small sample size. Scatton and co-workers (1986) who were able to work within these limitations reported no differences between dopamine levels in the lumbar spinal cord of Parkinsonian and control subjects. The evidence indicates that exogenous L-dopa is mainly converted to dopamine and not to noradrenaline and that the synthesis and metabolism of noradrenaline in the CNS is unaffected by L-dopa administration (see Commissiong and Neff, 1979 for references; Scatton et.al.,1983). More-over, the effects of L-dopa treatment on spinal

cord motor reflexes are probably mediated through dopamine alone and not noradrenaline (see Commissiong and Neff, 1979). Other sources indicate that synthesis of noradrenaline may be stimulated in the spinal cord and brain by L-dopa but that this reserve is rapidly metabolized or displaced by increased levels of dopamine, maintaining constant levels of neuronal noradrenaline and contributing little if at all to functionally significant releasable stores of noradrenaline (see Commissiong, 1985; Ehrenstrom and Johansson,1984). It is therefore assumed in the present study that the dopamine levels but not noradrenaline levels may be affected by the interval between death and the last dose of L-dopa and that this may disguise the effect of Parkinson's disease on the level of dopamine in the spinal cord. In addition, the results of the dopamine levels in the lumbar cord may be somewhat misleading for technical reasons as discussed above (page 109).

A conclusion arising from the differential effect of Parkinson's disease on spinal cord noradrenaline and dopamine levels is that this supports the evidence out-lined in the General Introduction that the dopaminergic and noradrenergic innervation of the spinal cord are independent of each other.

Effects of age, PMI and sex on neuroamine and peptide levels

There were a number of factors which directly affected the levels of neurochemicals measured in the spinal cord. The following factors are discussed here: sex of the subject; age of subject; and PMI.

Effects of age

The factor which demonstrated the greatest effect on neurotransmitter levels was the age of the subject. 5HT levels declined with age in thoracic dorsal and ventral regions but not in lumbar spinal cord and, in contrast, TRH levels increased with age in the thoracic ventral cord but not the dorsal horn nor in lumbar regions. The failure for the increase in age in the lumbar ventral region to reach a significant level was again probably due

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to the low numbers in the study. However, noradrenaline, dopamine and substance P levels were unaffected by age. The literature is in agreement that substance P levels in human CSF or post-mortem brain tissue are generally reported to be unaffected by age (Helme and White, 1983; Buck et. al.,1981). 5HT levels in selected brain regions are reported to be stable with age (Stanley et. al.,1986; Morgan 1987; Allen et al., 1983; Severson et.al.,1985) whereas serotonergic receptors are reported to be reduced with age (Mann et.al 1986; Marcusson et.al., 1984; Allen et.al., 1983). The literature concerning the catecholamine levels in the human CNS is contradictory. Indicators of catecholamine turnover and / or catecholamine levels are reported to be reduced or unaffected by age (Morgan 1987; see Allen et. al.,1983 for references).

Effects of PMI

The literature covering the effect of PMI on neuroamine and peptide levels is similarly confusing. In this study the only significant effects were of increases in TRH levels in selected spinal cord regions with PMI. Substance P, noradrenaline, 5HT and dopamine levels were unaffected by PMI. With respect to substance P, however, there is a high degree of compatibility with published data as a number of authors report stability in immunoreactivity of substance P and other peptides including neurotensin and somatostatin in post-mortem rat and human brain tissue (Helme and White,1983; see Buck et.al.,1981 for references; Bennett et.al., 1986) and it has been proposed (Buck et.al.,1981) that the important factor affecting post-mortem substance P levels is not the PMI but the time until the body is stored at 4°c. Surprisingly, this stability is not apparent for all peptides as TRH levels are reported to decline (Bennett. et.al.,1986), increase (Emson et. al.,1981a; Bird and Iverson, 1982) and remain stable (Yates et.al., 1983; Mitsuma et.al., 1984) with increasing PMI. Some sense can be made of these conflicting proposals when the actual post-mortem time intervals are taken into account. There is evidence that in the human spinal cord tissue TRH levels initially decrease over the first 24 hours post-mortem and then begin to rise again (Bennett et.al., 1986) which would account for

variations in results depending on the mean and range of the PMI of the sample. However, reports which draw conclusions about the effects of PMI on human CNS transmitter levels from plots of PMI against levels can be misleading. Such a plot from the results of the present study is illustrated in Fig.3.7 and might be taken to indicate that there was no correlation between TRH levels and PMI. The strong correlation is only revealed using ANCOVA statistical analysis by accounting for the effect of the parallel correlation between age and TRH levels. It is important then that proposals of correlations with any variable are substantiated by suitable statistical analysis.

Variation in transmitter levels with PMI may be dependent on enzyme activity occurring after death. Geola and co-workers (1981) demonstrated that synthesis of another peptide, somatostatin, from its precursor continued for some time post-mortem. Presumably, post-mortem enzymatic peptide degradation also occurs. Therefore, in a similar manner, the levels of TRH at different intervals after death may depend on the relative balance between the rates of synthesis and degradation. Furthermore, that the precursor of TRH is processed to produce 5 molecules of TRH (Lechan et.al.,1986) may account for the significant post-mortem increases in the levels of this neuropeptide. It has been proposed that stability of post-mortem peptide levels is due to their location within synaptic vesicles which protects them from degradative enzymes (Emson et.al 1981a). Therefore instability of TRH may reflect a different storage mechanism to that of other peptides.

Effects of sex

In this study there were no differences in the levels of noradrenaline, dopamine, 5HT, substance P or TRH between male and female subjects. Differences could have been expected considering that there are a number of gender related variations which might affect neurotransmitter state including expected life-span, hormonal balance and diet. Furthermore, TRH levels have
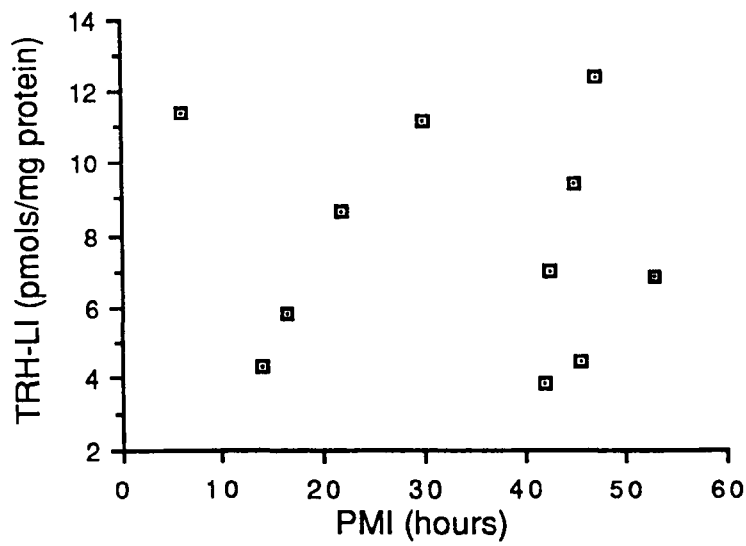
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Fig. 3.7 TRH-LI levels in thoracic ventral spinal cord samples from control subjects plotted against the interval between death and post-mortem (PMI). Each point represents one subject. Note that the positive correlation between the two factors revealed statistically using analysis of variance (see text) is not obvious when the data is plotted in this way.

been reported to be higher in men than women in a number of
discrete brain regions (Edwardson and McDermott, 1982).
However, in the majority of evidence, sex is reported to have no
effect on post-mortem neurotransmitter measurements in the
human. There have been reported to be no gender differences in
the levels of noradrenaline, 5HT, 5HIAA and dopamine
(Gottfries,1980; Scatton et. al.,1986; Severson et.al.,1985) or in
adrenergic or serotonergic binding (Mann et.al.,1986) in the
human brain.

Evidence discussed in the General Introduction (pp. 34-35)
indicates that the effects of both PMI and age on CNS transmitter
levels vary depending on the CNS region studied and this is
supported by the results of this study in which age and PMI affected
very selective regions of the spinal cord. In addition the evidence
(pp.34-35) indicates that the effects on transmitter levels depends
on the exact ranges of age or PMI investigated. The corollary of
this evidence in the light of the present results is that every study of
amine and peptide levels in the human CNS should be
accompanied by an analysis of the relationship between these
transmitters and age and PMI as it is unlikely that this
relationship has been previously investigated within identical
limitations of age, PMI and CNS region, factors which may have
very specific effects on neurochemical levels.

Whether the differences in spinal cord levels of amines and
peptides in both aged and Parkinsonian subjects are due to effects
specific to the spinal cord or secondary to changes in the cell nuclei
in the brain is undetermined. In Parkinson's disease there is loss
of noradrenaline and 5HT content from their brainstem nuclei,
the locus coeruleus and raphe nuclei respectively (Farley and
Hornykiewicz,1976; Greenfield and Bosanquet,1953). Evidence
from rats demonstrates that electro-thermic destruction of the
pontine noradrenergic nuclei, the locus coeruleus, results in
approximately 60% loss of both thoracic and lumbar ventral cord
and 35% loss of thoracic and lumbar dorsal noradrenaline content
(Commissiong and Neff, 1979). However, this study has shown
that in Parkinson's disease the amine losses are not the same in

equivalent regions of the thoracic and lumbar cord which may indicate that they are not solely dependant on brain-stem cell nuclei destruction. Although, the complication of the effects of age on these levels must also be considered. Furthermore the proportional deficiency of noradrenaline and 5HT is much greater in the lumbar spinal cord than in the respective brain stem nuclei.

In summary, the levels of 5HT and 5HIAA in the thoracic ventral horn and of noradrenaline in thoracic and lumbar ventral horns of Parkinsonian spinal cords were significantly lower than in equivalent control cords with no apparent changes in the 5HIAA/5HT ratio. The reduction in 5HT levels in the thoracic ventral cord was however dependent on the difference in age between the Parkinsonian and control subjects and not on Parkinson's disease. Reductions in noradrenaline and 5HT levels were found in lumbar dorsal horn regions in Parkinson's disease but here the 5HIAA/5HT ratio was increased compared with controls. Dopamine levels were significantly higher in thoracic ventral and dorsal Parkinsonian cord compared with controls. This finding was discussed with reference to L-dopa therapy. In contrast to these findings, there were no differences in the levels of either substance P, TRH, adrenaline or DOPAC in any of the spinal cord regions. The lack of changes in the peptide levels was discussed with respect to co-existence of these peptides with 5HT in the human spinal cord.

The results indicate that Parkinson's disease may be associated with reduced levels of two amine neurotransmitters (noradrenaline and 5HT) known to be involved in spinal regulation of motoneurone, and possibly nocioceptive functions. The results also demonstrate that spinal levels of substance P and TRH are unchanged in Parkinson's disease. This may indicate that either (i) these neuropeptides do not co-exist to a great extent with 5HT in the human spinal cord, (ii) that serotonergic-peptidergic neurones are spared in Parkinson's disease or (iii) that compensatory processes maintain the spinal cord peptide levels when 5HT levels are depleted.

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Chapter 4   Serotonergic and adrenergic binding in the human spinal cord: ligand binding and autoradiographic evidence

Introduction

In the previous chapter the levels of indoleamines and catecholamines in human spinal cord tissues from Parkinsonian patients were compared with those from control subjects. Some of the possible reasons for the lower levels of neuro-amines observed in the Parkinsonian cord, including neuronal loss and degeneration, were also discussed. It was decided that a study of the effect of Parkinson's disease on the aminergic receptors may clarify the nature of the changes occurring in this condition at the neuronal level assuming that changes in the numbers of receptors and their affinities for certain ligands may occur as a result of effects such as neuronal loss or variations in transmitter release. In this chapter radio-ligand binding and autoradiography were used to investigate serotonergic and adrenergic binding in human spinal cord tissue. The evaluation of these two methods for use with human spinal cord material is described as there have been very few quantitative reports of serotonergic or adrenergic ligand binding in this tissue and furthermore a number of modifications of the protocols had to be made to adapt them for use with human spinal cord material. The results of ligand binding studies using rat brain tissue (and in some cases human cortical tissue), a comparison of the results with previously published data, the subsequent attempt to use these same methods with control human spinal cord material and the problems arising are detailed here. Determination of the number and affinity of α_2-adrenoceptors in Parkinson's disease is found in the following chapter.

To comprehend the results of the ligand binding studies and understand how receptor affinities and number may be related to the levels of endogenous neurochemicals it is important to know the location of the receptor sites under investigation. The binding studies were therefore performed with ligands for which the affinities for specific subtypes of serotonergic and noradrenergic sites were well documented. Ligands were chosen on the basis of their specificity and on the location of the sites to which they are purported to bind.

Radio-ligand Binding Studies

Serotonergic receptors

Radio-ligand binding is a well established technique for investigating the characteristics of the binding of 5HT and 5HT analogues to CNS tissue membrane preparations (Peroutka and Snyder 1979; Pedigo et. al., 1981;Pazos et.al., 1984a,b; Peroutka and Hamik, 1988). Multiple 5HT binding sites have been demonstrated using radio-ligand binding with selective ligands for these sites. [^{3}H] 5HT binding in rat brain has been divided into $5HT_1$ and $5HT_2$ types on the basis of the different affinities of 5HT and spiroperidol for the sites (Peroutka and Snyder,1979). More recently evidence for a $5HT_3$ receptor in the CNS and specifically in rat cortical membranes has been reported (Kilpatrick et. al., 1987; Hoyer and Neijt, 1987; Peroutka and Hamik, 1988). The $5HT_1$ site has high affinity for 5HT whereas the $5HT_2$ site has a low affinity for 5HT and high affinity for a number of classical 5HT antagonists such as ketanserin (Leysen et.al., 1982; Peroutka and Snyder, 1981; Engel et. al.,1984b). However the binding of [^{3}H] 5HT to $5HT_1$ sites has also been shown to be heterogeneous (Pedigo et. al., 1981) and with the development of selective agonists, the 5HT binding site in CNS tissue from various species has been further classified into $5HT_{1A}$, $5HT_{1B}$, $5HT_{1C}$ and more recently $5HT_{1D}$ subtypes (Middlemiss and Fozard, 1983; Gozlan et. al., 1983; Doods et. al., 1985; Peroutka, 1986; Heuring et. al., 1986; Heuring and Peroutka, 1987).

The functional correlates of these receptor subtypes are as yet unclear however there is evidence that the $5HT_1$ receptor may be associated with the 5HT autoreceptor mediating prejunctional inhibition of neuronal transmitter release (Engel et. al., 1986; Schlicker and Gothert, 1981). The evidence indicates that in rats analogues with selectivity for the $5HT_{1B}$ receptor act at an autoreceptor on nerve terminals (Middlemiss,1985) and in vivo voltammetry studies support this proposal (Martin and Marsden, 1986; Marsden et.al.,1987). Furthermore, the $5HT_{1A}$ receptor in the rat appears to be an autoreceptor located on pre-synaptic 5HT

cell bodies and dendrites involved in regulation of neuronal firing and 5HT release (Sprouse and Aghajanian, 1986; Garrett et.al.,1988). There is also evidence of a post-synaptic $5HT_{1B}$ receptor mediating inhibition of adenylate cyclase stimulation (De Vivo and Maayani, 1986; Sleight et.al.,1988) and of post-synaptic $5HT_{1B}$ and $5HT_{1C}$ receptors mediating hypophagia (Kennett and Curzon,1988). In addition, there is species specificity in receptor subtypes, for instance, the $5HT_{1B}$ autoreceptor is present in rat but not human brain membranes and the autoreceptor in the human cortex may be the $5HT_{1D}$ receptor (Heuring et. al.,1986; Heuring and Peroutka, 1987; Galzin et.al., 1988). In contrast the $5HT_2$ receptor in the rat CNS is located post-synaptically (Hoyer et.al.,1985b) and has been implicated in mediation of behavioural effects including head twitches, fore-paw treading and wet-dog shakes (Leysen, 1989; Fone, 1989a).

On the basis of published data it was proposed to investigate the binding to both $5HT_1$ and $5HT_2$ receptors using ligands which have been well documented as showing specificity for these sites. Firstly, 5HT from the above evidence is known to label all $5HT_1$ receptors whereas 8-hydroxy-2-(di-n-propylamino) tetralin (DPAT) is now well established as a 5HT agonist with high affinity for the $5HT_{1A}$ receptor specifically (Middlemiss and Fozard 1983; Peroutka et. al., 1986). Secondly, $[^3H]$ ketanserin was used to label $5HT_2$ receptors. Ketanserin has been established as a 5HT antagonist with high affinity for the $5HT_2$ receptor and importantly with negligible affinity for the $5HT_1$ receptor or for the α -adrenoceptor when used up to a concentration of 2 nM $[^3H]$ Ketanserin (Leysen et. al., 1982).

Adrenoceptors

In vitro binding studies have identified adrenergic receptor sites in the CNS of humans and rats (Greenberg et. al.,1976; Miach et. al., 1978; Sumners et al., 1983) which were pharmacologically identical to peripheral α_1, α_2, β_1 and β_2-adrenoceptors. The cellular localisation of these receptors has not however been clarified. Alpha1-adrenoceptors seem to be post-synaptic since

their numbers increase after treatment with 6 hydroxy dopamine, a neurotoxin causing lesions in catecholaminergic neurones specifically (Dausse et. al., 1982; Reader and Briere, 1983) whereas the localisation of α_2-adrenoceptors is controversial and there is evidence for both pre- and post-synaptic sites (Langer, 1974; Levin, 1984; U'Prichard et. al.,1980)

[^{3}H] Idazoxan (RX 781094) was selected as the ligand to distinguish α_2-adrenoceptor binding sites as its specificity for this receptor is well documented (Doxey et. al., 1982 ;Langer et. al., 1983; Hamilton et. al., 1988; Howlett et. al., 1982).

[^{125}I] AmiK (7-amino-8-iodo-ketanserin) was also employed in preliminary ligand binding studies to identify both α_1-adrenoceptors and $5HT_2$ receptors for which it is reported to have high affinities (Wouter, 1986; Schotte and Leysen, 1988).

Autoradiographic localisation of serotonergic and adrenergic receptors

In this chapter autoradiographic studies were performed to determine the distribution of serotonergic and adrenergic receptors in the human spinal cord. The binding kinetics and pharmacological profiles of the ligands used had been established previously either in ligand binding studies, in autoradiographic investigations or in both. Briefly, (i) the use of AmiK in autoradiographic studies has provided data on the density and localisation of $5HT_2$ receptors and α_1-adrenoceptors in the rat brain which supports published results from ligand binding studies (Schotte and Leysen,1988; Wouters et.al., 1986) (ii) DPAT has been reported to be an appropriate ligand for labelling $5HT_{1A}$ receptors in both ligand binding studies (see above) and autoradiography (Pazos and Palacios, 1985) whereas (iii) the specificity of Idazoxan for α_2-adrenoceptors has been confirmed in binding studies (see above) but its use in autoradiography has not been reported.

The results using these ligands either in autoradiographic studies or for binding to sections of tissue on glass slides are

compared with results from ligand binding to homogenates of human cord and rat brain.

Methods

Autoradiography

Autoradiography studies were made in rat brain and human spinal cord samples using the radio-ligands [^{125}I] 7-amino-8-iodo-ketanserin (AmiK), [^{3}H] DPAT and [^{3}H] Idazoxan. The various concentrations of the radio-active ligands used were checked by gamma counting (LKB, Riagamma) the diluted solutions before incubation of the sections.

and β scintillation

Tissue preparation

Whole rat brains were dissected from male Wistar rats (250-300g) cut into 5 horizontal or sagittal slices of equal width and stored at -20°c for 4-5 hours before transferal to -80°c to match the storage conditions of the human tissue. Spinal cord tissue was also taken from two human control subjects without histories of neurological illness. The subjects were both females aged 62 and 73 years who had died of respiratory failure. The intervals between death and post-mortem were 11.5 and 19 hours respectively. The spinal cord was dissected into thoracic and lumbar regions and segments from the first subject were stored at -20°c for 4-5 hours before transferal to -80°c. The tissue from the second subject was 'fast frozen' by holding sections, wrapped in tin foil to maintain their shape, in a beaker of liquid nitrogen within a trough of dry ice for 5 - 10 seconds. The segments were then stored at -80°c.

Rat brain tissue was transferred from the freezer on dry ice for cryostat sectioning. On a metal sheet, cooled over a mixture of ice and dry ice, horizontal and sagittal sections were cut into slices of 2-3mm width manually using a razor blade. Cryostat blocks were covered with a layer of tissue paper and the slices were then adhered to the block with Tissue Tek OCT compound (Miles Scientific), a tissue embedding medium. The tissue and block adhered in this manner were then slowly lowered into a glass beaker of isopentane cooled to sub-zero temperature in liquid

nitrogen. The blocks were placed in the cryostat for 2 hours prior to sectioning to equilibrate with the temperature of -15 to -20°c.

Samples of human thoracic and lumbar spinal cord were transferred from storage to the microbiological hood on dry ice within sealed containers. Slices of 2 - 3mm width were cut and mounted on cryostat blocks and precooled as described for the rat brain samples. From experience it was found that to cut the human spinal cord tissue successfully the slices must be completely embedded in the Tissue Tek whereas for the rat brain only enough Tissue Tek to cover the base of the tissue sample was required.

Sectioning

Horizontal and sagittal slices of rat brain and ~~coronal~~ transverse slices of human spinal cord were cut into 20μm sections in a microtome cryostat (Brighton Instrument Company) at -15 to -20°c. The sections were made manually by rotating a handle to sweep the block mounted sample across the cryostat knife blade which was static. Sections were thaw mounted onto glass slides i.e. the section was melted onto the slide by pressing a finger onto the back of the slide. Glass microslides (Chance Propper Ltd) had been prepared either with acid washing and/or gelatin subbing (see below). Sections were collected on separate labelled slides and stored in the same sequence as they were cut. They were left in the cryostat for 1 - 2 hours after which time they were stored at -80°c within sealed plastic bags. Sections were used within 3 weeks of storage and generally within 1 week. Autoradiographic images of the rat brain in horizontal and sagittal section are given in Fig.4.2 demonstrating the regions of the brain revealed.

Experience in cryostat sectioning improved the uniformity of the sections. However under the light microscope small tears could be sometimes be seen in the sections. As this may have been the result of the formation of ice crystals during storage the effect of slow and fast freezing on the condition of the tissue was observed. There was no apparent difference with respect to the occurrence of small tears using either method but great care had to be taken when fast freezing the tissue that it did not fracture and that its shape was preserved. In the studies described below only sections in a good
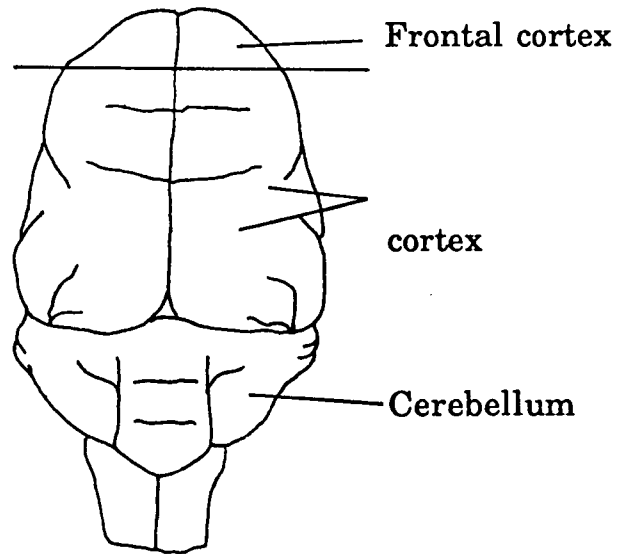
Fig. 4.1 The dorsal aspect of the rat brain showing the regions dissected for ligand binding experiments. Frontal cortex was used in studies of $5HT_1$ receptor binding and the remainder of the cortex was taken for studies of α_2-adrenoceptor binding. Whole cortex was used in $5HT_2$ binding studies.

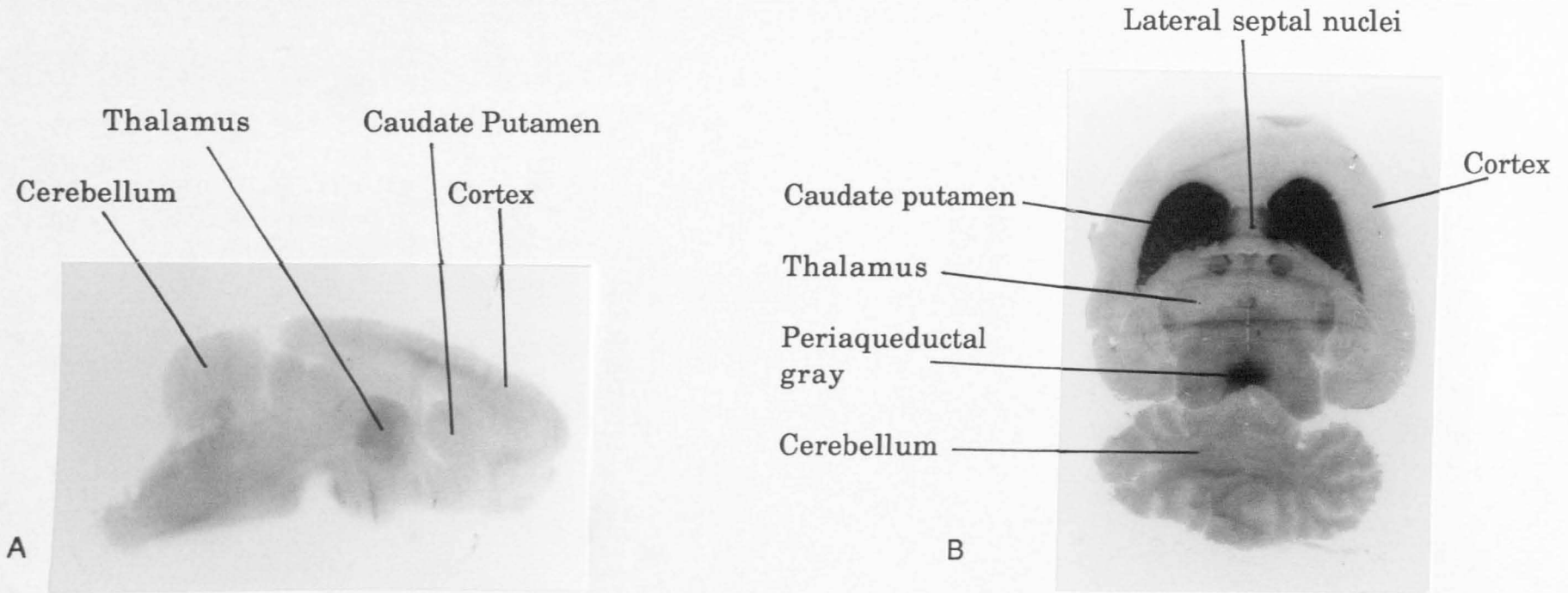Frontal cortex was dissected 4 mm from the optic lobe.

Fig. 4.2 Autoradiographs of (A) sagittal and (B) horizontal section through the rat brain showing the thalamic nuclei, periaqueductal gray-matter, cerebellum, lateral septal nuclei, caudate putamen and cortex. Areas were identified using a rat brain atlas (Paxinos and Watson, 1982). Autoradiography employed 0.5 nM [^{125}I] AmiK. (A) shows non-specific binding

condition were used and there was no difference between the results obtained using slow or fast frozen tissue.

Slide preparation : acid wash

Chromic acid was made by careful addition of 125 mls of sulphuric acid to 5g $K_2Cr_2O_7$ dissolved in 200mls DDD. This procedure was performed within a bucket of ice in a fume cupboard. Glass slides were left in chromic acid over night, washed in continuously flowing water for for 2 hours and finally rinsed in distilled water and allowed to dry in a heated cabinet.

Gelatin subbing: Gelatin (2.5g) was dissolved in boiling water and allowed to cool to 55°c. At this temperature chromic potassium sulphate (0.25g) was dissolved into the solution. The solution was filtered into a large staining jar and the acid washed slides were dipped into the solution and coated in one movement. Slides were left to drain in a warm dry dust-free atmosphere.

Staining slides

Sections were stained and examined under the light microscope and on a light box to determine the physical condition of the sections and to ensure the uniformity of the sections. After testing a number of different fixing and staining techniques the following simple method was found to be suitable for staining sections quickly to a standard adequate for making the required observations.

Solutions   10% Buffered formalin: Add NaH_2PO_4. $2H_2O$ (5.2g) and Na_2HPO_4. $12H_2O$ (5.75g) to 40% formaldehyde solution (55mls) and make up to 500mls with DDD.

40% Formaldehyde solution: Stir 40g paraformaldehyde into 100mls DDD over heat in a fume cupboard. When the temperature of the mixture reaches 60°c add sodium hydroxide pellets until it clears. Allow the solution to cool and filter.

Method   Fix and defat the tissue by placing the sections in 10% formalin for 10 minutes, rinsing sections in DDD, dipping in 70% alcohol (2 seconds) and rinsing again in DDD. Stain

sections with Toluene Blue for 30 seconds. Wash the slides in running water until the water runs clear, dehydrate in Xylene and mount the sections with DPX (80% Xylene, BDH Ltd.). Cover the sections with a coverslip.

Autoradiography using [^{125}I] AmiK

Rat brain and human spinal cord sections were incubated with [^{125}I] 7-amino-8-iodo-ketanserin (AmiK) and a number of displacing drugs specific for different receptor types. AmiK has been reported to bind specifically to four different sites, α_1 adrenoceptors, $5HT_2$ and histamine H_1 receptors and tetrabenazine displaceable sites (Schotte and Leysen, 1987). By using a combination of displacing drugs the specific binding to each receptor type was investigated as described by these authors. The displacing drugs, the concentrations at which they were used and the receptors which they were used to identify are given in Table 4.1. The buffer used was Tris-HCl (50mM, pH 7.4, see page 135 for further details).

Sections were removed from the freezer and allowed to dry in a stream of cold air for 1 hour. The effect of pre-incubating the sections in buffer on the levels of specific and non-specific binding of AmiK to the samples was investigated by comparing (i) pre-incubation of the sections with Tris-HCl containing 0.1% bovine serum albumin (BSA) and (ii) pre-incubation with Tris-HCl containing 1% BSA. During pre-incubation the sections were placed in racks in staining jars of the respective buffers for 10 minutes. Slides were dried again in cold air. Gelatin solution (5%) was then melted in a water bath (37^{0}c) and used to paint a circle around the tissue section on the glass slide to confine the incubation solution. Slides were laid out flat on racks and the Tris-HCl containing [^{125}I] AmiK pipetted carefully onto the tissue section.

Total binding was measured by incubating sections with 300μl (rat brain) or 150μl (human spinal cord) of 0.5nM [^{125}I] AmiK (2000 Ci/mmol) in Tris-HCl. Binding inhibition was measured by adding 0.1μM Prazosin or 1μM of BW 501 (N-[2-(3-chloro-phenyl)propyl]-2-(phenylamino) ethanimidamide sulphate

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Table 4.1 Drugs used to displace [^{125}I] AmiK binding in the human spinal cord.

A. Concentrations of drugs used to displace [^{125}I] AmiK and the binding sites identified by them.

Displacing drug	Incubation concentration	Binding site
Prazosin (PRZ)	$0.1 \mu M$	$\alpha 1$ adrenoceptor
BW 501 (BW)	$1 \mu M$	$5HT_2$
Pyrilamine (PYR)	$1 \mu M$	Histamine H_1
Tetrabenazine (TBZ)	$1 \mu M$	tetrabenazine displaceable site

B. Displacing drug combinations used to identify specific sites

Drug combination	Binding site identified
PYR + TBZ + BW	$\alpha 1$
PYR + TBZ + PRZ	$5HT_2$
TBZ + BW + PRZ	H_1
PYR + BW + PRZ	tetrabenazine displaceable site
PYR + TBZ + BW + PRZ	NSB

(The Wellcome Res. Lab. Beckenham) or the other displacing drugs given in Table 4.1. Binding to specific receptor sites and non-specific binding was determined using displacing drugs in different combinations. A combination of all the displacing drugs was used to distinguish binding which was not specific to α_1-adrenoceptors, 5HT$_2$, H$_1$ or tetrabenazine displaceable sites (NSB). At least three replicates of each of the 6 combinations (Table 4.1B+ total binding) were used. These were incubated with adjacent tissue sections in such a way that the binding to each receptor site could be compared with NSB and total binding in a section that was no more than 100µm distant in the original tissue. An incubation period of either 60 or 30 minutes was used. More Tris-HCl containing the correct drugs was added to the sections if the buffer appeared to be evaporating during this time. After the incubation period the slides were placed in racks and rinsed in ice-cold Tris-HCL and finally rinsed in DDD. The duration of the rinse periods was varied to find the optimal combination for use with human spinal cord tissue samples. Rinse protocols tested are shown in Table 4.2. The sections were allowed to dry. Dry sections were then opposed to photographic [^{125}I] sensitive Hyperfilm (Amersham) within light-tight X-ray cassettes for a range of exposure times between 17 and 65 hours. Each cassette contained at least one replicate of each drug combination used for both rat and human tissue samples. Each film was also exposed to [^{125}I] microscales (Amersham). The microscales are [^{125}I] standards covering a radio-activity range of 0.185 - 23.68 kBq / mg against which the activity in the sections was determined.

Developing the autoradiograph

The X-ray cassette was opened in safelight condition (using light filter 6B) and the film removed. The film was placed in developer (Kodak D19) for 5 minutes then washed for 2-3 minutes in running water. The film was placed in fixer (Ilford Hypam fixer diluted 1 in 5 with distilled water) until the image had cleared and the excess emulsion washed away. This was followed by rinsing the film thoroughly in running water for 20 minutes or more before leaving it to dry.

Table 4.2 Autoradiography protocol modifications for use with human spinal cord sections

Pre-incubation/ incubation periods minutes	Rinse time (minutes) 1st	2nd	3rd	glass slides	tissue condition
10 / 60	15	15	---	acid washed	~50% loss of sections
1 / 30	10	10	---	acid washed	poor
- / 30	6	6	6	acid washed	poor
- / 30	3	3	3	acid washed	poor
1 / 30	5	5	5	gelatin subbed	improved
10 or 30 / 30	10	10	---	gelatin subbed	>90% of sections completely intact

Analysis of the autoradiographic image

A qualitative estimation of the degree of binding of [^{125}I] AmiK to the different receptor sites was made by comparing by eye the density of the images produced. Ligand binding to a specific site was estimated by comparing the binding in sections incubated with the displacing drugs (Table 4.1) to total and non-specific binding. Binding to one specific site was distinguished as the binding remaining in the presence of drugs which displaced the other specific sites minus NSB. As no specific binding to α_1-adrenoceptors or $5HT_2$ receptors was discernible in the human spinal cord sections quantitative analysis was not performed.

Tissue homogenate binding using [^{125}I] AmiK

Ligand binding assays of [^{125}I] AmiK binding to human spinal cord homogenate (prepared as described below) were performed. The protocol was a modification of that described below for [^{3}H] ligands. The total incubation volume used was 500µl comprising 400µl of tissue suspension (10mg/ml), 50µl [^{125}I] AmiK (0.12 nM) and 50µl buffer or drug. Displacing drugs used and their concentrations were the same as those given in Table 4.1. Combinations of these drugs were used so as to identify $5HT_2$ receptors, α_1-adrenoceptors, tetrabenazine displaceable sites and NSB.

[^{3}H] DPAT binding to tissue sections

Preliminary studies were undertaken to measure [^{3}H] DPAT binding in human spinal cord using the autoradiographic methods reported by Pazos and Palacios (1985). Sections on gelatin subbed slides were pre-incubated for 30 minutes in 0.2 M Tris-HCL assay buffer, containing 10µM pargyline, 0.1% ascorbic acid and 4mM CaCl. The incubation was performed using 2 nM [^{3}H] DPAT in Tris assay buffer at room temperature for 60 minutes. After incubation the sections were washed (2 x 5 minutes) in ice-cold buffer. NSB was determined in sections incubated with [^{3}H] DPAT and 100 µM 5HT. The slides were then wiped with filters

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(Whatman GF/B) which were subsequently placed in 10 mls of scintillant. Background activity was measured by wiping the back of a slide with a filter. Activity was counted on the liquid scintillation counter. This protocol was carried out both on tissue which had been sectioned the same day and on stored tissue.

[^{3}H] Idazoxan binding to tissue sections

Similar preliminary studies of [^{3}H] idazoxan binding were also made using a modification of the method employed by Young and Kuhar (1979) to measure α_2-adrenoceptors. Briefly, tissue sections were incubated with 2.5 nM [^{3}H] idazoxan in 50 mmol Tris-HCL buffer pH 7.7 for 60 minutes at room temperature. They were washed twice (10 and 5 minutes) and then wiped with filters which were treated as above. Inhibition of [^{3}H] idazoxan binding was determined using eleven clonidine concentrations within the range 10mM to 1 nM and comparisons were made between sections of human spinal cord and rat cortex treated in the same way.

Calculations

As the half-life of ^{125}I is relatively short (60 days) there can be substantial loss of activity between the dates of supply and use of the label. As ligand concentration is determined from its radio-activity (cpm) and specific activity (Ci/mmol) the actual activity on the day of assay was calculated before ligand concentration could be accurately determined. The activity at the time of assay was determined with computer assistance from the specific activity on the day of supply, the number of days between supply and use and the half-life of ^{125}I AmiK.

Radio ligand binding studies

Ligands

The ligands used and their specific activities were:
[^{3}H] 5HT in the form of 5 hydroxytryptamine creatine sulphate (0.74 - 1.48 TBq/mmol); [^{3}H] Ketanserin (60 Ci/mmol); [^{3}H] DPAT

[8-hydroxy-2-(di-n-propylamino) tetralin, 2.6 - 4.1 TBq/mmol]; and [^{3}H] Idazoxan (manufacturers name: RX 781094, 1.5 - 2.2 TBq/mmol). Ligand binding assays were performed to determine (i) that the ligands were binding to a finite number of sites and that the binding was therefore saturable (ii) that binding was reversible and could be displaced (iii) the affinities of the ligands for the binding site and (iv) the number of receptor sites per unit tissue.

Preparation for studies using tissue homogenate

Rat brain dissection

Male wistar rats (250g) were killed by decapitation after stunning. The skull was cut open and the whole brain removed onto a cooled metal surface. The olfactory bulb was discarded and the frontal cortex dissected as shown in Figure 4.1. The remaining cortex comprising the parietal and striate regions was also removed. The tissue was weighed and stored at -20^{0}c in sealed eppendorf tubes.

Human brain and spinal cord dissection

Brain and spinal cord tissue was obtained from the Cambridge Brain Bank, the Parkinson's Disease Society Brain Bank and Nottingham University Hospital. Tissue was taken from 4 female and 6 male control subjects with no history of neurological illness who had died from either respiratory or cardiac failure with one exception who died after a road traffic accident. The mean age and interval between death and post-mortem were 68.9 $\pm$ 4.4 years and 31.5 $\pm$ 5.7 hours respectively (mean $\pm$ SEM, n=10). Brains from control subjects were removed from the skull at autopsy, transfered to a -20^{0}c freezer for 4 - 5 hours and then stored long term at -80^{0}c. Samples of the frontal cortex were dissected from the brain on a cooled tray, weighed and used in binding studies the same day. The spinal cord was removed, dissected into thoracic and lumbar regions and stored frozen as described in chapter 3 (pp. 94-95). On the day of the binding assay

spinal cord tissue was dissected into 5mm slices and in some cases
into dorsal and ventral regions comprising 2mm punches of the
gray matter as illustrated in Fig 3.1b in the previous chapter.
Tissue was weighed and combined from adjacent sections so that
samples of ~1g were used in binding assays.

Membrane Preparation

The methods used for tissue membrane preparation were
modifications of that described by Hamon et. al. (1984). Tissue
was homogenized in 50 volumes of ice cold buffer using a Polytron
(setting 5, 20 seconds), followed by centrifugation at 4000g for 15
minutes at 4^0c. Supernatant was removed and the pellet was
resuspended in buffer, homogenized and centrifuged twice more
as above. Samples were kept on ice as far as possible. For [3H]
5HT, Ketanserin and DPAT binding the tissue suspension was
incubated at 37^0c for 15 minutes in buffer before the final spin to
remove endogenous 5HT. After the third spin supernatant was
discarded and the pellet was resuspended in 70 (5HT binding) or
100 (adrenoceptor binding) volumes of assay buffer of specific
composition depending on the ligand binding assay. For human
tissue biohazard precautions were taken (Appendix 2), in
particular centrifugation was carried out in sealed tubes and
supernatant was aspirated into chloros solution in the
microbiological hood.

Assay protocols

The assays were based on the general ligand binding
protocol described by Peroutka and Snyder (1979).

(1) [3H] 5HT and [3H] ketanserin

Briefly, this assay was initiated by addition of 850µl of tissue
suspension to tubes containing 50µl [3H] ligand and 100µl of drugs
or assay buffer. Tubes were vortex mixed and incubated for 15
minutes at 37^0c. For the ketanserin binding assay incubation
time was 10 minutes.

Buffers: Membrane preparation: Tris-HCL buffer - 50mM Tris (tris [hydroxymethyl] amino methane) hydrochloride made with DDD, pH 7.5. Assay buffer: 100mls 50mM Tris-HCL buffer, $10\mu M$ pargyline (mono amine oxidaze inhibitor), 0.1% (wt/vol) ascorbic acid (antioxidant) and 4mM $CaCl_2$ adjusted to pH 8.0. The pH of Tris buffer is temperature dependent and a pH of 8.0 obtained at room temperature will give pH 7.5 at 37°c.

(2) [3H] DPAT binding

In this assay the volumes of tissue suspension and drugs were taken from a paper by Middlemiss and Fozard (1983). Tissue homogenate (0.7 ml), [3H] DPAT (0.1 ml) and the competing drug (0.1 ml) made up to a final volume of 1ml with assay buffer were incubated at 37°c for 15 minutes.
Buffers: Membrane preparation: 50 mM Tris-HCL, pH. 7.7.
Assay buffer: Binding was compared using two buffers (i) 50mM Tris-HCL pH. 7.7 and (ii) Tris-SALT - 50mM Tris-HCL pH. 7.7 containing $10\mu M$ pargyline, 0.1% ascorbic acid and 4mM $CaCl_2$.

(3) [3H] Idazoxan binding

Modifications were taken from a report by Giron and co-workers (1985). A total assay volume of 1.1ml was used comprising 1ml of resuspended tissue, $50\mu l$ of [3H] ligand and $50\mu l$ of drug or assay buffer. After addition of the tissue the tubes were vortex mixed and incubated for 30 minutes at 25°c.
Membrane preparation and assay buffer: 50mM Tris-HCL, pH 7.7.
The tissue concentration for all assays was approximately 10mg/ml.

After the incubation period bound and free ligand were separated by filtering the suspension under vacuum through Whatman GB/F filters. The filtration block had 10 wells, in each of which a filter was supported by a metal mesh. Filters were washed with 3 x 3mls ice-cold Tris-HCL buffer. Filters were transferred to scintillation vials containing 10mls of scintillant

(LKB Optiphase 'X') and allowed to soak for at least 5 hours. The amount of bound ligand was measured by scintillation counting on a liquid scintillation counter (LKB 1214 Rackbeta) at ~40% counting efficiency. Radio-activity in standards containing ligand only in the absence of tissue suspension was measured to determine the exact concentrations of ligand used in each assay.

In competition studies one concentration of [^{3}H] ligand was incubated with a range of concentrations of an unlabelled drug which competed for the same binding site. Displacing drug and [^{3}H] ligand concentrations used are given in Table 4.3A. Non-specific binding (NSB) of the ligands was determined in the presence of drugs at the concentrations given in Table 4.3A. Total binding was determined from tubes containing ligand and buffer in the absence of the competing drug.

In saturation studies a range of [^{3}H] ligand concentrations was used. Total and non-specific binding at each ligand concentration were determined using respectively either buffer or the drug used to define non-specific binding at the concentration described above. The concentrations of [^{3}H] ligand and displacing drugs used in saturation studies are given in Table 4.3B. For competition and saturation studies there were 3 replicates of each tube per assay and each assay was performed in triplicate.

Protein content of the tissue was determined by the method of Lowry et. al.,1951 (Appendix 1).

Studies undertaken

Using the protocols outlined above a number of studies of ligand binding to serotonergic sites and α_2-adrenergic sites in rat and human cortex and human spinal cord tissues were made.

(i) Ketanserin binding in fresh and frozen rat cortical tissues. Specific [^{3}H] ketanserin binding was compared in rat brain tissue which had been freshly dissected without storage and in tissue dissected and stored for 24 hours at -20°c.

(ii) Ligand binding to a heterogeneous population of 5HT$_1$ receptors was demonstrated by displacing [^{3}H] 5HT binding in rat cortex with DPAT.

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Table 4.3 Details of [^{3}H] ligands and displacing drugs used in binding assays.

A. Competition assays

[^{3}H] Ligand	5HT	DPAT	Idazoxan	Ketanserin
Assay [ ] (nM)	2	1	4	1
Displacing drug (1)	DPAT	5HT	clonidine	methysergide
Drug [ ] range (M)	10^{-4} - 10^{-10}	10^{-4} - 10^{-10}	10^{-4} - 10^{-10}	10^{-5} - 10^{-8}
Displacing drug (2)			idazoxan	
Drug [ ] range (M)			10^{-4} - 10^{-9}	
NSB: Displacing drug	5HT	5HT	clonidine	methysergide
Drug [ ] M	10^{-6}	10^{-5}	10^{-5}	10^{-6}

B. Saturation assays

[^{3}H] Ligand	DPAT	Idazoxan
[ ] range (nM)	0.2 - 10	0.3 - 20
NSB: Displacing drug	5HT	clonidine
Drug [ ] M	10^{-5}	10^{-5}

[ ] = concentration

(iii) The number of receptor sites for [3H] DPAT and [3H] idazoxan and the affinities of the ligands to their receptor sites in rat cortex were determined from competition and saturation studies and the values obtained were compared with previously published data.

(iv) [3H] Ketanserin, [3H] 5HT, [3H] DPAT and [3H] idazoxan binding were performed in human thoracic spinal cord tissue.

(v) Synaptosomal Preparation: An attempt was made to reduce the level of NSB observed in crude homogenate by using a synaptosomal preparation of the tissue:

Rat cortex (500mg or 1g) and human spinal cord (1g) were homogenized in 3.2M sucrose (10 mls) and centrifuged for 10 minutes (1000g, 4°c). The first supernatant (S1) was collected and the pellet (P1) discarded. S1 was centrifuged at 20,000g for 20 minutes (4°c), S2 was discarded and 10mls of cold DDD added to P2. The pellet was homogenized for 30 seconds (Polytron, setting 6) to disrupt the synaptosomes. The volume was made up to 15 mls with cold double distilled deionised water (DDD) and the suspension spun at 8,000 g for 20 minutes (4°c). Centrifuge tubes were shaken to disperse the buffy coat surrounding P3 and then S3 and the buffy coat were collected and centrifuged at 48,000g (10 minutes, 4°c). After discarding P3 and S4 10mls DDD were added to P4 which was homogenized further (Polytron, 30 seconds). To break the pellets down the volume was made up to 20 mls and they were centrifuged at 48,000g for 10 minutes (4°c). Finally the supernatant (S5) was disposed of and the pellet retained and stored overnight at - 20°c.

(vi) To see whether the NSB of [3H] idazoxan to human spinal cord tissue was dependent on the concentration of displacing drug a saturation binding assay was performed using boiled tissue. Tissue homogenate prepared in the usual way was boiled for 5 minutes, allowed to cool and assayed according to the method generally used.

(vii) To maximize the use of the human spinal cord tissue an investigation was made into the effect on [3H] idazoxan binding of reducing the amount of tissue per assay tube. [3H]

displacement was performed using 5 mg/ml and the standard concentration of 10 mg/ml of rat cortical membranes.

Data analysis

In order to analyse the results of the competition and saturation studies a number of assumptions were made regarding the kinetics of the interactions between the ligands and the receptors sites at which they bind. Reversible ligand receptor interactions were assumed to follow kinetics similar to the well established interactions between enzymes and their substrates as described here:

For reversible ligand-receptor interactions where [R] = concentration of unoccupied receptor sites, [L] = concentration of free ligand and [RL] = concentration of receptor-ligand complex

$$a[R] + b[L] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} c[RL] \tag{1}$$
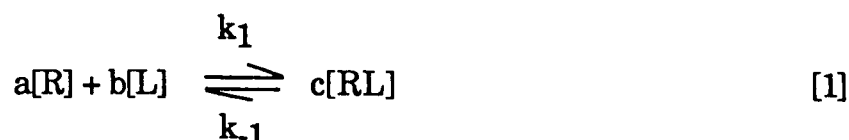
is a description of binding phenomena with a, b and c representing the stoichiometry of the reaction. At equilibrium the rate of the forward reaction equals the rate of the reverse reaction

$$k_1 [R]^a [L]^b = k_{-1} [RL]^c \tag{2}$$

and the equilibrium binding constant can be defined as a dissociation binding constant (K_D)

$$K_D = \frac{k_{-1}}{k_1} = \frac{[R]^a [L]^b}{[RL]^c} \tag{3}$$

or as the association constant (K_A) which is the inverse of K_D.

It was also assumed that there were a finite number of specific sites per unit of tissue for ligand binding and therefore that the binding was saturable. This maximum number of specific receptor sites is generally described as B_{max}.

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$$B_{max} = [RL] + [R] \qquad [4]$$

From equation [3] and [4] and assuming $a = b = c = 1$

$$[RL] = \frac{B_{max} [L]}{[L] + K_D} \qquad [5]$$

which is the law of mass action adapted to receptor-ligand interactions.

By defining RL as bound ligand = B, and L as free ligand = F and after rearrangement of equation [5]

$$B/F = \frac{B_{max} - B}{K_D}$$

which is the Scatchard (Scatchard, 1949) equation. From this equation and knowing the concentration of ligand bound and free at equilibrium both the equilibrium binding constant (K_D) and the maximum number of binding sites (B_{max}) can be determined (see below).

Inhibition curves were constructed from the data obtained from competition studies as follows. Binding at each drug concentration was determined as the ratio of binding in the presence of drug to total binding (in the absence of drug) and expressed as % total binding. These values were plotted against -log. drug concentration as illustrated in Fig 4.3B. Non-specific binding (NSB) was determined as the proportion of total binding remaining at maximum binding inhibition. Specific binding was defined as binding minus NSB (Fig.4.3A) and Bmax was calculated as total binding minus non-specific binding. Where the cold (displacing) and labelled ligand are the same their K_D is defined as the IC_{50} (the concentration of unlabelled ligand at which the maximum binding of labelled ligand is displaced by

50%). Estimations of the affinity of the displacing drug for the site labelled by the radio-active ligand were obtained in the form of the inhibition constant (Ki) from

$$Ki = \frac{IC50}{1 + [L] / K_D} \qquad \text{(Cheng and Prusoff, 1973)}$$

Saturation curves were produced using data obtained in saturation kinetic studies. Specific binding was plotted against ligand concentration as illustrated in Fig.4.4A.

The equilibrium binding constants of the ligand-receptor interactions and the maximum number of binding sites for each ligand were determined using Scatchard and Hill analysis of the data. The analyses were performed using LIGAND which is a computer package developed by Munson and Rodbard (1980) for characterizing the results of ligand binding studies. (This program, its approach, uses and versatility are discussed in the chapter 5, pp.165-166 & 176).

Scatchard analysis

Scatchard plots were constructed by plotting bound ligand (B) against the ratio of bound / free ligand (B/F). Bound ligand was measured directly as described above and free ligand was calculated as the difference between the amount of ligand added (determined from standards) and the amount of ligand bound. A straight line indicates binding to one receptor site. Using LIGAND a weighted least squares estimate of the line of best fit was fitted to the the data and a correlation coefficient for the fit of the data to the line was obtained. A typical Scatchard plot is illustrated in Fig. 4.4B using data obtaining from idazoxan binding in rat cortical membranes. K_D was estimated as the negative reciprocal of the slope of the line. B_{max} was estimated by the abscissa intercept of the line.

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Hill Plots

From Scatchard plots values for K_D and B_{max} were estimated assuming that the ligand-receptor interaction followed the law of mass action i.e that the ligand was specifically binding to one receptor site. Hill plots were then used to evaluate the validity of this assumption.

The Hill equation is

$$\frac{B}{B_{max}} = \frac{[L]^n}{K'_D + [L]^n}$$

where K'_D is a composite constant composed of the dissociation constant and interaction factors that determine the degree to which K_D is altered at each binding step and n is the theoretical number of ligand binding sites per receptor molecule. When n = 1 the ligand binding obeys the law of mass action and the Hill plot is a straight line with a slope of unity . Ligand binding data was fitted to the Hill equation using LIGAND by plotting $\log.B / B_{max} - B$ against $\log.[L]$ where B is specific binding. A plot with a slope of approximately 1 was considered to be representative of a system in which the ligand was specifically bound to one receptor site.

Precautions for the use of biohazard material

Precautions as set out in Appendix 2 were taken while applying the methods described above to human spinal cord tissue.

Collaboration

The autoradiography using $[^{125}I]$ AmiK was carried out in collaboration with Jose Leysen and Alain Schotte at Janssen, Belgium.

Drugs

[^{125}I] 7-amino-8-iodo-ketanserin (AmiK), ketanserin, prazosin, BW 501, pyrilamine and tetrabenazine were obtained from and used at Janssen Pharmaceutical, Belgium. [^{3}H] 5 hydroxytryptamine creatine sulphate, [^{3}H] DPAT [8-hydroxy-2-(di-n-propylamino)tetralin and [^{3}H] Idazoxan (RX 781094) were obtained from Amersham.

Results

Autoradiography

[^{125}I] AmiK studies

Protocol modifications for use with human spinal cord material

Applying the assay conditions used to measure [^{125}I] AmiK binding in rat brain sections (Schotte and Leysen, 1988) to human spinal cord tissue resulted in gross deterioration of the tissue sections. The incubation and pre-incubation periods were therefore reduced from 60 to 30 minutes and from 10 minutes to 1 minute respectively. Even after these adaptations and after cutting the rinse protocol to a minimum, part or all of the the tissue sections were washed off the slides in contrast to the rat brain sections which were 100% intact under the same conditions. However a great improvement in maintaining the condition of the tissue was made by mounting the sections on gelatin subbed slides. Using subbed slides the tissue sections were completely preserved in over 90% of the samples after 30 and 10 minute incubation and pre-incubation periods followed by two rinse intervals of ten minutes each as described in Table 4.2.

Binding in rat brain and spinal cord

In horizontal slices of the rat brain [^{125}I] AmiK binding could be seen in the cortex, caudate putamen, lateral septal nuclei, periaquaductal gray-matter and various thalamic nuclei (Fig. 4.6A). Non-specific binding visualised using μM tetrabenazine (TBZ), μM pyrilamine (PYR), μM BW501 (BW) and 0.1μM prazosine (PRZ) produced a uniform grey coloration with a slightly darker pigmentation in the caudate putamen (Fig.4.6B). Inhibition by TBZ, PYR and PRZ revealed 5HT$_2$ binding sites in the cortex and in the caudate putamen and not in the thalamic nuclei when compared with NSB as shown in figure 4.6F. 5HT$_2$ binding in the cortex is shown clearly in sagittal section of the brain (Fig.4.7A). Inhibition by the other combinations of these drugs (out-lined in Table 4.1B) revealed (a) dense α1-adrenoceptor

binding in the thalamic nuclei and cortex (Fig. 4.6C and 4.7B) and (b) very dense labelling of tetrabenazine displaceable sites in the caudate putamen and periaqueductal gray and distinct but less dense labelling in the lateral septal nuclei (Fig.4.6E).

The distribution and intensity of binding to these sites was unaffected by substituting the pre-incubation protocol by 1 hour incubation in Tris-HCl buffer containing 1% BSA.

[^{125}I] AmiK Binding in human spinal cord

The images of the autoradiographs developed after 24 hour exposure to human spinal cord sections labelled with [^{125}I] AmiK were very weak. The images were a little darker but no clearer after 28 (not shown) or 65 hour (Fig. 4.8A-F) exposure periods. Total [^{125}I] AmiK binding demonstrated the highest density of binding in the gray matter, especially in the ventral horns. There was little or no inhibition of binding in the gray matter by PRZ, PYR or BW (not shown). Using combinations of these drugs the only binding site indicated in the human cord was for a small degree of [^{125}I] AmiK binding to tetrabenazine displaceable sites in the gray matter (Fig. 4.8E). These results were unchanged by using a 1 hour pre-incubation period in Tris-HCl buffer containing 1% BSA.

[^{3}H] DPAT and [3H] idazoxan binding to human spinal cord sections

There was no 5HT displaceable [^{3}H] DPAT binding in sections of human spinal cord incubated with 2nM [^{3}H] DPAT which was detectable by scintillation counting of filters used to wipe the slides. Similarly, there was no consistent displacement of [^{3}H] idazoxan binding by clonidine both for sections incubated with [^{3}H] ligand on the day the sections were cut or after storage of sections at -80°c for a period of less than 3 weeks. On occasions saturable idazoxan binding and NSB of 50% of total binding was demonstrable. However, this was not reproducible and on other occasions under the same conditions there was no measurable specific binding. In contrast, there was a concentration dependent inhibition of [^{3}H] idazoxan binding by clonidine in rat brain

sections processed simultaneously in the same manner as shown in Fig. 4.9.

In summary, autoradiography was performed to determine α_1-adrenoceptor and 5HT2 binding in the human spinal cord using [125I] AmiK. Autoradiography of [125I] AmiK binding in the rat brain was performed simultaneously to validate the protocol. However, α_1-adrenoceptor and 5HT2 binding was demonstrated in the rat brain but not in the human spinal cord. In addition, preliminary studies were made to determine the optimal conditions for autoradiography of [3H] DPAT and [3H] idazoxan in the human spinal cord. As no consistent specific [3H] DPAT or [3H] idazoxan binding could be measured it was decided to continue the investigation of adrenergic and serotonergic binding in the human spinal cord using radio-ligand binding studies.

Radio-ligand binding studies

[3H] Ketanserin: 5HT2 binding

Specific [3H] ketanserin binding displaceable by methysergide was demonstrated in the rat cortex (Table 4.4). There was 16.5% less specific binding measurable in frozen compared with freshly dissected rat frontal cortex although there was no significant difference in %NSB ($p < 0.5$, n = 3) as shown in Table 4.4.

There was also measurable specific [3H] ketanserin binding in frontal cortex tissue from 2 human subjects. Maximum specific binding and NSB were at a similar level to that in the rat (Table 4.4). However there was no measurable methysergide displaceable [3H] ketanserin binding in either ventral or dorsal horn tissue from the thoracic spinal cord of 4 human subjects.

[3H] 5HT: 5HT1 binding in rat cortex

Displacement of [3H] 5HT binding by DPAT in rat frontal cortical tissue was shallow occurring over the range 1nM to 10mM

Table 4.4 [³H] Ketanserin binding in tissue homogenate preparations

Tissue Prep.	n	Bmax (fmols/mg ww)	% NSB
Rat frontal cortex (Fresh)	3	5.56±0.04	53±5
Rat frontal cortex (Frozen)	3	4.64±0.21	55±2
Human frontal cortex	2	4.40±0.30	51±4
Human spinal cord			
dorsal horn	4	---	>90
ventral horn	4	---	>90

NSB was defined by [³H] Ketanserin binding in the presence of µM methysergide. Specific binding (Bmax) was determined as the difference between total binding in the absence of methysergide and non-specific binding and is expressed as wet weight (ww) of tissue.

(Fig. 4.5). DPAT at a concentration of 100nM, reported to produce displacement of the $5HT_{1A}$ binding site (Gozlan et. al.,1983; Middlemiss and Fozard, 1983; Petrouka et. al 1986; Heuring et. al., 1986) displaced 21.7± 3% of binding. Micromolar 5HT produced 59.5±3% inhibition indicating a level of non-specific binding of 40%. Values are expressed as mean ± SEM from three experiments.

[^{3}H] 5HT binding in human cortical and spinal cord tissue

In human frontal cortical tissue DPAT displacement of [^{3}H] 5HT binding closely resembled that seen in tissue from the same region of the rat CNS. Displacement of [^{3}H] 5HT was shallow over the range 1 to 10,000 nM. Binding inhibition with 100nM DPAT was 16±3% and NSB measured in the presence of μM 5HT was 43.5±2% (mean±SEM, n = 2). However, in contrast, in human spinal cord tissue 100nM DPAT produced no inhibition of [^{3}H] 5HT binding and NSB was very high (86.5± 2%, mean±SEM, n = 4).

[^{3}H] DPAT binding in rat cortex: effects of different buffers

There were marked differences in the characteristics of [^{3}H] DPAT binding to membranes of the rat frontal cortex determined using Tris-HCL buffer and Tris-SALT buffer (containing 5.7mM $CaCl_2$, 0.1% ascorbic acid and 10μM pargyline). Using Tris-HCL [^{3}H] DPAT binding was inhibited by 1nM through to 10μM 5HT whereas with Tris-SALT the displacement curve was steeper with 90% displacement of total binding at 0.1μM 5HT. Specific binding was saturable in both cases but in the presence of pargyline, ascorbic acid and $CaCl_2$ both the affinity of the ligand and the total number of binding sites obtained from Scatchard analysis of the data were lower than in Tris-HCL (Table 4.5A). However, the Hill plots of the data obtained in the presence of the added compounds were closer to unity than they were using Tris-HCL alone. K_D, Bmax and the gradients of the Hill plots obtained using the two buffers are given in Table 4.5A

Table 4.5   [^{3}H] DPAT binding in rat cortex

A. Effects of different buffers

[^{3}H] DPAT binding to membranes of the rat frontal cortex determined using Tris-HCL buffer and Tris-SALT buffer (containing 5.7mM $CaCl_2$, 0.1% ascorbic acid and 10µM pargyline).

Buffer	K_D (nM)	Bmax (fmols/mg ww)	Hill slope
Tris-HCL	0.96 + 0.06	75±5	1.15 ± 0.02
Tris-SALT	2.24 + 0.06	40 ± 4.0	0.97 ± 0.02

B. Comparison of results

The K_D (nM) and Bmax (fmols /mg tissue) values for [^{3}H] DPAT binding in rat cortical tissue obtained in this study are compared with previously published data. Specific binding was defined in the presence of 5HT except for * where it was defined in the presence of DPAT. In all cases binding was performed in Tris-HCL containing 4-6 mM $CaCl_2$, 0.1% ascorbic acid with or without 0.1 µM pargyline.

Author	Rat tissue	KD	pKD	Bmax
This study	frontal cortex	2.24±0.06	8.52	40±4
Hoyer et al. 1985	cortex		8.77±0.05	132±18
Peroutka, 1986	frontal cortex	2.2±0.5		*3.3

[^{3}H] DPAT binding in human spinal cord

There were only a very low levels of [^{3}H] DPAT binding in human spinal cord determined using Tris-SALT buffer and this was not inhibited by μM 5HT and was therefore considered to be non-specific binding.

[^{125}I] AmiK binding to tissue homogenate preparation

In a study of [^{125}I] AmiK binding to a homogenate preparation of human spinal cord samples there was no displacement of binding by PRZ, BW or by TBZ or by these drugs used in various combinations to define α_1, 5HT$_2$ or tetrabenazine displaceable sites.
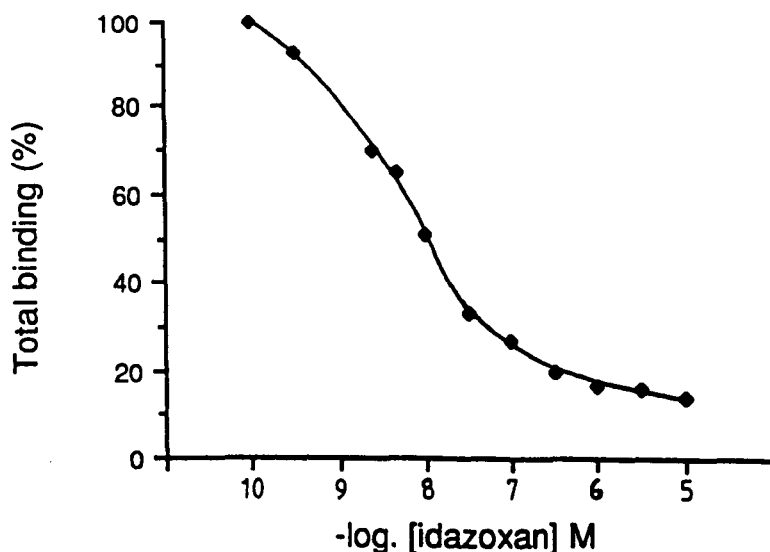
[^{3}H] Idazoxan binding in rat cortex

Unlabelled idazoxan produced approximately 90% inhibition of [^{3}H] idazoxan binding to rat cortical membranes (Fig.4.3B). The K$_D$ of Idazoxan binding in this tissue calculated from the IC50 as described in the methods was 4.5 ± 0.7 nM (n = 4) compared with previously published values of 3.1 ± 0.4 and 4.0 ± 0.8 (Doxey, 1982; Howlett,1982). Specific binding as determined by 10μM clonidine (Fig.4.3A) was 70-75% of total binding which is in agreement with reported data (McLaughlin and Collins, 1986). However the 1C50 and Ki values for clonidine inhibition were 18.0 ± 2, and 11.6 ± 2nM respectively which is rather lower than reported. Langer and co-workers (1983) and McLaughlin and Collins (1986) found IC50 and Ki values for clonidine inhibition of [^{3}H] idazoxan binding in rat cortex of 80nM and 55.9 nM respectively. Typical competition curves for clonidine and idazoxan displacement of [^{3}H] idazoxan binding in rat cortex are given in Fig.s 4.3A &4.3B. [^{3}H] Idazoxan binding was found to be saturable (Fig.4.4A) and of high affinity. The KD and Bmax determined from Scatchard analysis of the saturation study (Fig.4.4B) are given in Table 4.6 with data from other comparable studies using [^{3}H] idazoxan to label α_2-adrenoceptor sites. The

Fig. 4.3 [^{3}H] Idazoxan inhibition curves
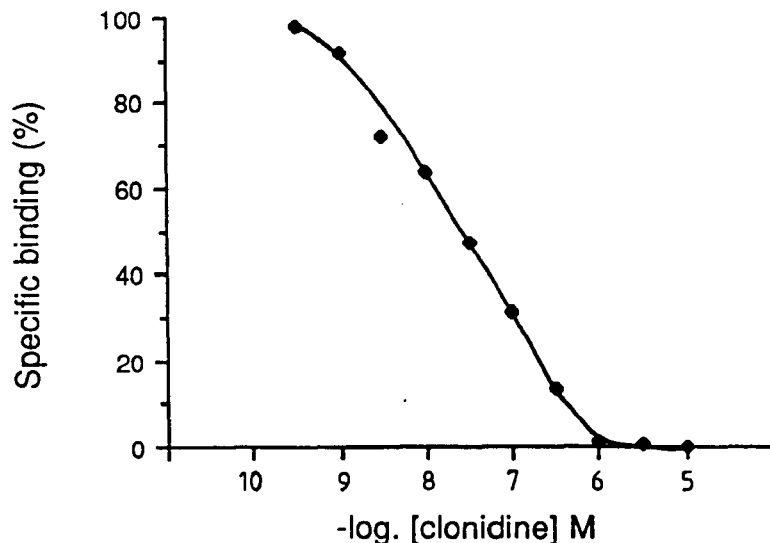
A. Inhibition of specific [^{3}H] Idazoxan binding in rat cortical membranes by clonidine. The data is from a typical experiment. Various concentrations of clonidine were incubated in triplicate with 2.2 nM [^{3}H] Idazoxan. Non-specific binding defined by 10 µM clonidine was 28% of total binding and has been subtracted to determine specific binding. Binding is expressed as a % of maximum binding in the absence of clonidine. The Ki calculated from the IC50 as described by Cheng and Prusoff (1973) was 16.7 nM.

B. Inhibition of total [^{3}H] Idazoxan binding in rat cortical membranes by unlabelled idazoxan. The data is from a typical experiment. Various concentrations of idazoxan were incubated in triplicate with 2nM [^{3}H]idazoxan. Binding is expressed as a % of maximum binding in the absence of Idazoxan and non-specific binding defined by 10 µM Idazoxan was 14% of total binding.
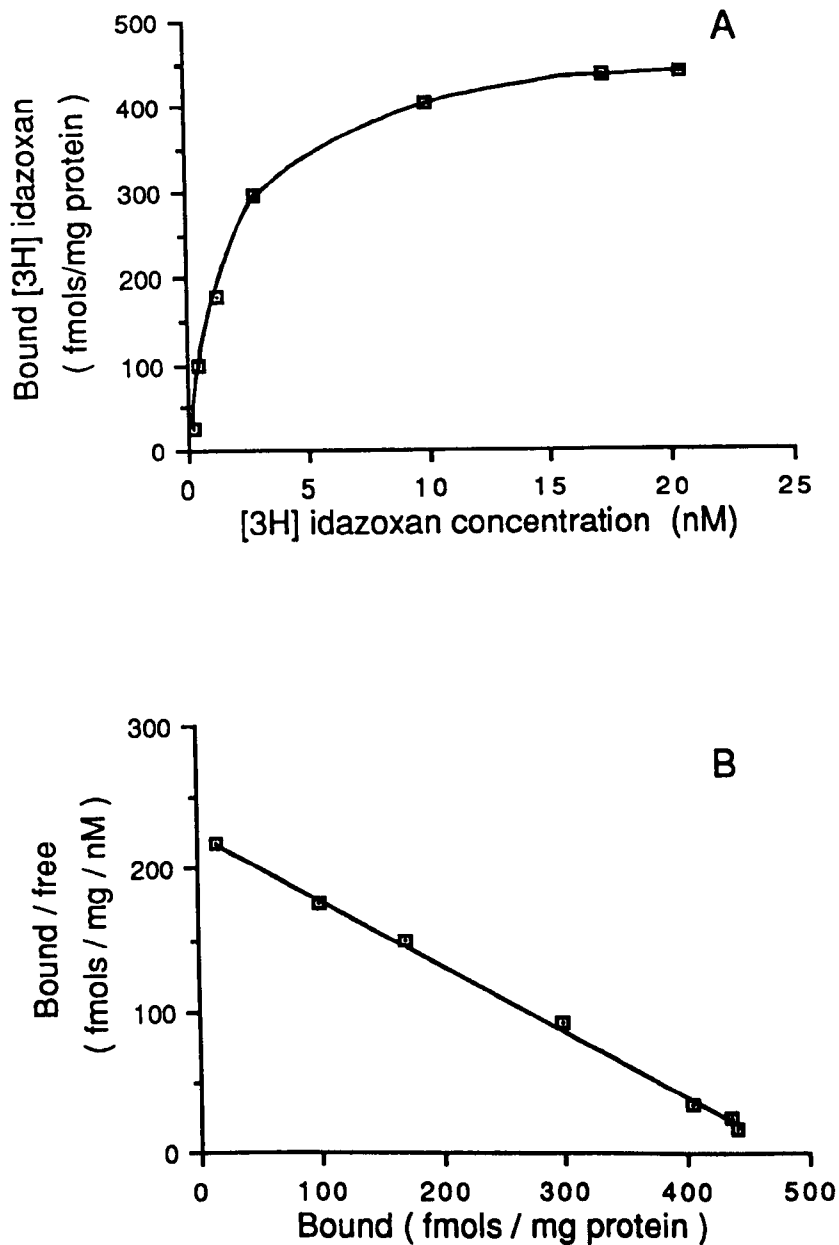
Fig. 4.4 Specific binding of [³H] Idazoxan binding to rat cortical membranes.

The data is from a typical experiment and each point is the mean of a triplicate measurement. NSB was defined by 10 μM clonidine (A) Saturation binding curve. (B) Scatchard plot of the data. The line of the plot is a linear regression determined by a least squares fit (r= -0.999). K_D and B_{max} calculated from the plot were 2.1 nM and 483 fmols/mg protein.
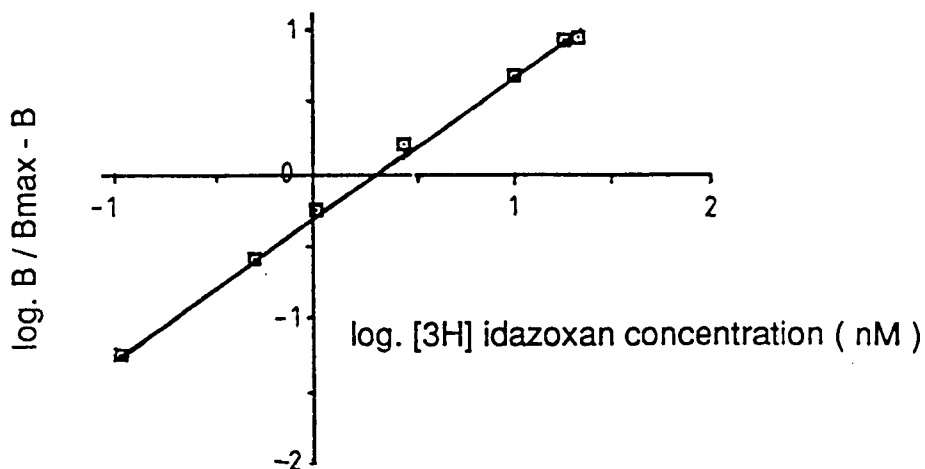
Fig. 4.4 Specific binding of [^{3}H] Idazoxan binding to rat cortical membranes.
(C) A Hill plot of the same data. Log. B / Bmax - B is plotted against log. of Idazoxan concentration where B is the specific binding. The gradient of the line is near unity (+0.98) which is indicative of binding to a single site.

Fig.4.5 [^{3}H] 5HT inhibition curve

Inhibition of total [^{3}H] 5HT binding in membrane homogenate of rat frontal cortex. The data is from a typical experiment. Various concentrations of DPAT were incubated in triplicate with 2nM [^{3}H] 5HT. The shallowness of the displacement is indicative of binding to more than one site. $5HT_{1A}$ binding defined by 100nM DPAT was 25% of total binding.

Table 4.6   [^{3}H] Idazoxan binding in the rat cortex

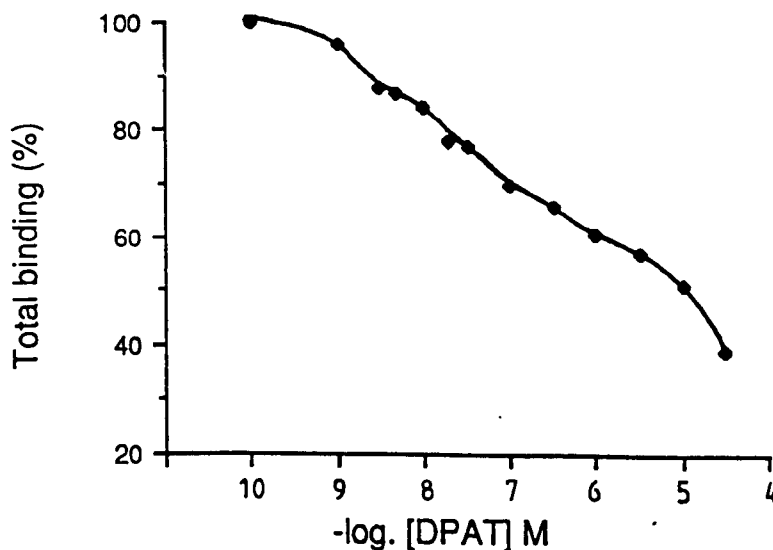[^{3}H] Idazoxan binding in the rat cortex determined in ligand binding studies: a comparison between the results of this and earlier studies. In this study NSB was determined by 10μM clonidine and n=4. KD is expressed in nM and Bmax in fmols / mg protein.

Author	Rat tissue	KD	Bmax
This study	cortex	2.79±0.29	361 ± 42
Mclaughlin and Collins, 1986	olfactory cortex	5.71±0.42	251±8
Langer et.al 1983	cortex	3.9±0.4	190±13
Howlett et. al 1982	brain minus cerebellum	2.8±0.2	230±14

Table 4.7   Effect of reduced tissue concentration on [^{3}H] Idazoxan binding

Specific [^{3}H] idazoxan binding measured in rat cortex using two concentrations (10 and 5 mg/ml) of tissue homogenate. There was a significant difference in both Bmax and non-specific binding (*p<0.05).

Tissue concentration	Bmax (fmols/mg ww)	%NSB
10 mg/ml	5.5 ± 0.3	29 ± 4
5 mg/ml	*3.6 ± 0.2	*52 ± 3

Hill plot of the data was linear (Fig.4.4C) with a gradient near unity (0.97±0.02).

Effect of reduced tissue concentration on [^{3}H] idazoxan binding

Specific [^{3}H] idazoxan binding was measured in rat cortex using two tissue concentrations. Bmax was significantly lower (p <0.05, n=3) when the assay tissue concentration was reduced from 10 to 5 mg/ml (Table 4.7). Moreover, NSB defined by 10µM clonidine was significantly greater (p<0.5, n=3) with the lower tissue concentration (Table 4.7).

[^{3}H] Idazoxan binding in human spinal cord

In initial competition studies of [^{3}H] idazoxan binding to human thoracic spinal cord membranes cold idazoxan produced 80-85% inhibition at µM concentrations comparable with displacement in rat cortex. However, the inhibition by clonidine was markedly less and NSB defined by 10µM clonidine was approximately 60% of total binding. Binding to boiled tissue was independent of the concentration of clonidine which indicated that although the level of NSB was high it was consistent. The KD determined from inhibition curves using unlabelled idazoxan was 5.0±0.3 nM (n = 3). The Ki values for clonidine measured in two human spinal cords were 523 and 230 nM. On the basis of these preliminary results further [^{3}H] idazoxan binding was performed in human spinal cord which is described fully in chapter 5.

Synaptosomal preparation.

Initially the same wet weight of tissue as generally used for the tissue homogenate preparation was used in the protocol for the synaptosomal preparation. The final membrane pellet produced was smaller than the pellet produced after tissue homogenate preparation. More_over, total [^{3}H] idazoxan binding was lower in synaptosomal preparations of both rat cortex and the human spinal cord. In [^{3}H] idazoxan competition binding studies total [^{3}H] idazoxan binding to the synaptosomal preparation was 0.2 - 0.3

fold that to homogenate preparations of equivalent initial weight of tissue. Furthermore, there was very little or no inhibition of [^{3}H] idazoxan binding by clonidine. The inhibition of total binding by unlabelled idazoxan (100 μM) was also reduced from approximately 85% to 60%. By increasing the initial mass of tissue used by approximately 2 fold some inhibition of [^{3}H] idazoxan binding by clonidine was seen (30 - 40%) but this did not attain the level normally reached using the homogenate preparation for either rat or human tissue.

In summary, 5HT$_{1A}$ and 5HT$_2$ receptors have been demonstrated in the rat and human cortex in ligand binding studies using [^{3}H] DPAT and [^{3}H] ketanserin. Furthermore, α2-adrenoceptors have been demonstrated in the rat brain using [^{3}H] idazoxan. Although attempts to measure 5HT$_{1A}$, 5HT$_2$ and α2-adrenoceptor binding in the human spinal cord with [^{3}H] DPAT, [^{3}H] ketanserin and [^{125}I] AmiK were unsuccessful, displaceable, high affinity [^{3}H] idazoxan binding was identified in this tissue. Additionally, the effects of a number of modifications to the ligand binding protocol were investigated.
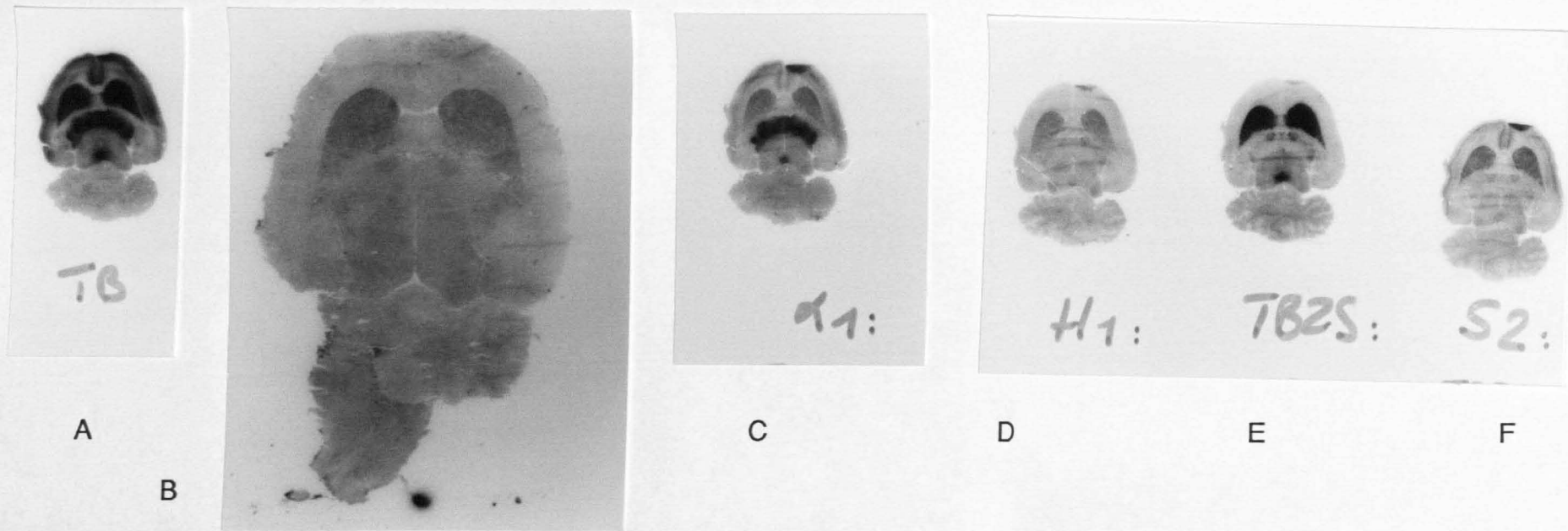
FIG. 4.6 [^{125}I] AmiK binding to rat horizontal brain sections

These autoradiographs show: total binding (A), NSB (B) and binding to α-1adrenoceptors (C), histamine-H$_1$ receptors (D), tetrabenazine displaceable sites (E) and 5HT$_2$ receptors (F).

These sites were distinguished using the following combinations of displacing drugs: 1μM tetrabenazine, 0.1μM prazosine, 1μM BW 501 and 1μM pyrilamine (B),
1μM tetrabenazine, 1μM BW 501 and 1μM pyrilamine (C),
1μM tetrabenazine, 0.1μM prazosine and 1μM BW 501 (D),
0.1μM prazosine, 1μM BW 501 and 1μM pyrilamine (E)
1μM tetrabenazine, 0.1μM prazosine, and 1μM pyrilamine (F).

A

B
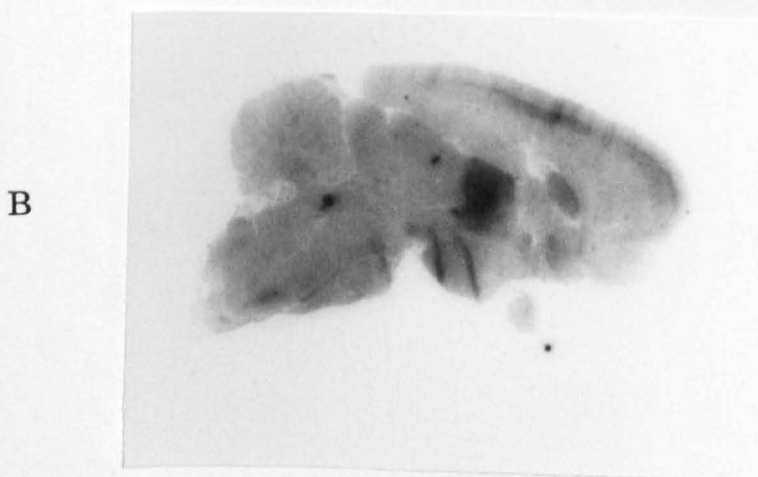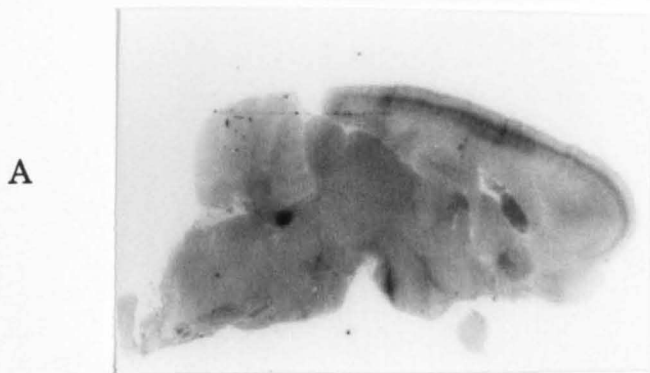
FIG. 4.7 [^{125}I]AmiK binding to rat sagittal brain sections

[^{125}I] AmiK binding to (A) 5HT$_2$ receptor sites and (B) α1-adrenoceptor sites in the cortex is more easily visualised using sagittal sections of the rat brain; 5HT$_2$ receptor sites were demonstrated in the presence of 1μM tetrabenazine, 0.1μM prazosine, and 1μM pyrilamine; α1-adrenoceptor sites were demonstrated in the presence of 1μM tetrabenazine, 1μM BW 501 and 1μM pyrilamine
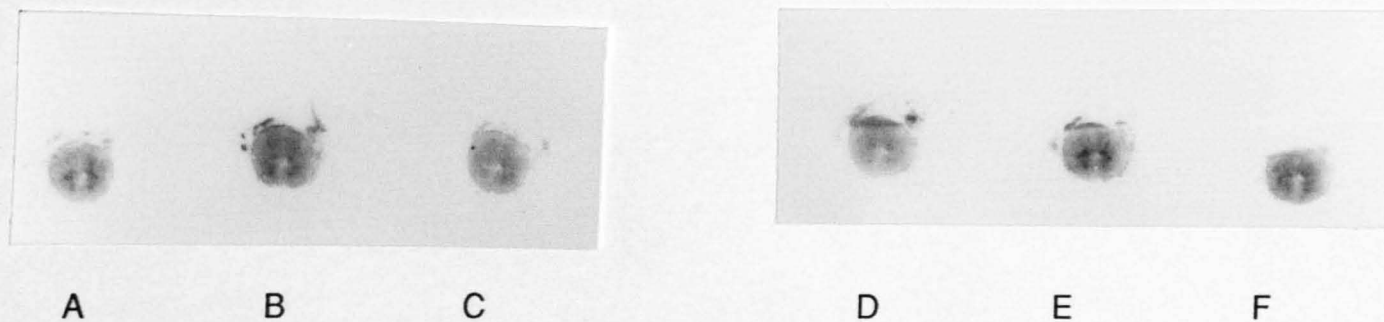
Fig. 4.8 [^{125}I]AmiK binding to sections of human thoracic spinal cord.

[^{125}I]AmiK binding was performed in the presence of the same combinations of displacing drugs as described in the legend of Fig.4.6 to distinguish total binding (A); NSB (B); and binding to 5HT$_2$ receptors (C); histamine H$_1$ receptors (D); tetrabenazine displaceable sites (E) and $\alpha_1$adrenoceptors (F). In this autoradiograph the sections had been exposed to [^{125}I] sensitive film for 65 hours.

Fig. 4.9 [^{3}H] Idazoxan inhibition in sections of rat brain and human spinal cord.

Competition between [^{3}H] Idazoxan and clonidine for receptor sites in sections of human spinal cord (◆) and rat brain (▣) on glass slides. Each point is the mean of triplicate measurements. Sections were incubated with 4 nM [^{3}H] Idazoxan and a range of clonidine concentrations. Bound [^{3}H] Idazoxan was determined by wiping the slides with filter paper and measuring the activity by scintillation counting. The results found using human cord sections were not consistent and in this case there was no measurable specific binding
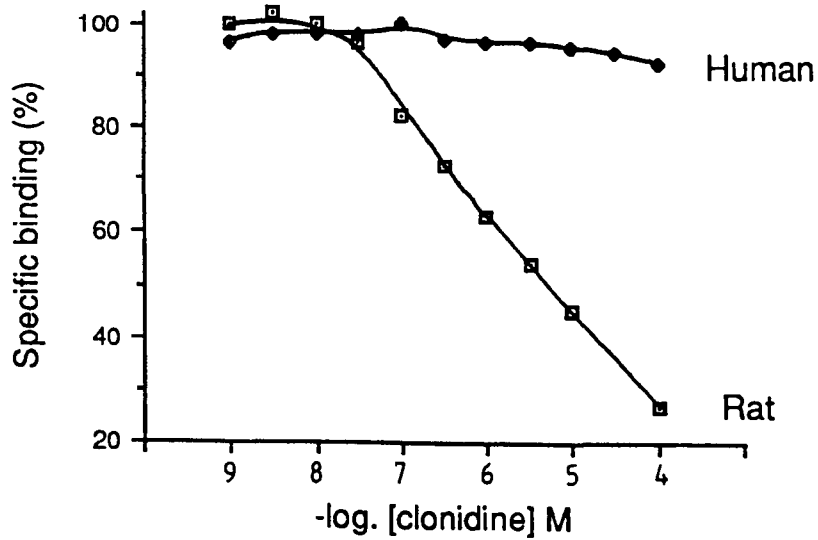
Discussion

This chapter describes in vitro studies of adrenergic and serotonergic binding in post-mortem control human spinal cord tissue. Because of the limited supply of human cord tissue available and in order to prove the reliability of the methods all protocols were initially or simultaneously performed using rat brain tissue. The binding characteristics of the ligands used in these methods have previously been documented by other authors as described in the introduction. In general the results obtained in the rat and human brain with respect to receptor numbers and distribution and binding affinities were in close accordance with earlier reports. However, there were a number of major difficulties in determining the same parameters in the human spinal cord and these will be discussed here.

Autoradiography

Consideration of the protocol

Autoradiography is a useful technique for receptor location. It is very appropriate for investigating the distribution of receptors throughout a number of different regions, for example through the whole of one section of the rat brain. The area of tissue which can be investigated is limited mainly by the practical problems of cutting large sections on the cryostat (of human brain, for instance) and mounting them in one piece on glass slides. It is important not to have holes or tears in the tissue section where ligand may collect as these will form pockets of apparently high density binding. No difference in the quality of the tissue condition was found in this study after freezing the tissue slowly or rapidly and it was found that the condition of each tissue section could be determined easily by holding the slide up to the light. There is controversy over the use of autoradiography for quantitative determination of KD and Bmax values. The primary criticism is that the values are related to the density of binding which is determined from the intensity of the colour of the autoradiographic image. The radio-activity bound is quantified by comparing the images produced by tissue sections with

those produced by standards of known activity. However, the autoradiographic image formed after exposure to standards may not be representative of that produced by tissue sections as the activity is quenched by passage through the tissue. Moreover, the degree of quenching may vary throughout different tissue regions and especially between white and gray matter. The accuracy of the correlation between image density and activity bound in the sample is therefore arguable. However, this problem was not encountered here as the technique was used qualitatively only, to determine the distribution of $5HT_2$ and α_1-adrenergic receptor sites in rat brain and human spinal cord.

Performing autoradiography it was discovered that the assay conditions used to measure [^{125}I] AmiK binding in rat brain sections (Schotte and Leysen, 1988) were not entirely appropriate for use with human spinal cord tissue. Initial attempts to incubate the sections under these conditions resulted in the sections breaking up or being washed off the slides. This problem was solved mainly by mounting the sections on gelatin subbed slides which helped to maintain the integrity of the tissue sections throughout the incubation, pre-incubation and rinse cycles.

The variation between the levels of non-specific binding in spinal cord and brain tissue may depend on differences in the composition of these tissues. The myelin sheath around the spinal cord probably accounts for much of it. Differences in the composition of the brain and spinal cord are especially noticeable when preparing the tissue for autoradiography. In order to successfully cut sections of the spinal cord the tissue blocks must be completely supported in embedding medium. Otherwise the tissue sections produced are wrinkled. This appears to be because the outer layer of the spinal cord is tougher and less penetrable than the core and therefore the force required to cut through the cord will damage the section unless it is firmly supported. In contrast the composition of rat brain tissue appears to be more homogeneous and Tissue Tek is necessary only for adhering the tissue to the block. Furthermore whilst cutting the rat brain sections the knife became greasy much more quickly compared to sectioning human spinal cord and had to be cleaned more regularly. It may be that this fatty characteristic of the brain tissue explains the ready adherence of brain sections to glass slides. The

differences seen were distinct and may account for the high levels of NSB to human spinal cord tissues in the binding studies. High NSB however, is not necessarily a problem. Idazoxan binding to boiled tissue using a constant concentration of idazoxan was found to be unaltered by varying clonidine concentration in ligand binding studies and displacement of 40-50% of total binding is a feasible level to work with.

Lack of $5HT_1$, $5HT_2$ receptors and α_1-adrenoceptors in human spinal cord

[^{125}I] AmiK studies

In studies made in parallel to those using human spinal cord [^{125}I] AmiK binding in the rat brain had the characteristics of $5HT_2$ and α_1-adrenoceptor binding found in other binding studies with homogenates (Leysen et. al., 1982; Cash et. al 1986) and in autoradiography (Schotte and Leysen, 1988; Palacios et. al.,1987; Jones et. al.,1985). The greatest concentration of specific binding to $5HT_2$ receptors and to α_1-adrenoceptors was in the cortex and in the cortex and the thalamus respectively. To identify these sites $1\mu M$ pyrilamine was used to inhibit AmiK binding to histamine-H_1 receptors which has been reported (Schotte and Leysen, 1988) though histamine-H_1 binding in the rat brain is very low relative to $5HT_2$ and α_1-adrenoceptor binding as seen here and as described previously (Taylor and Snyder, 1971). In this study specific [^{125}I] AmiK binding to $5HT_2$ receptors, to α_1-adrenoceptors and to the densely labelled tetrabenazine displaceable binding in the caudate putamen have been distinguished in accordance with the findings of Schotte and Leysen (1988). Furthermore, the total labelling with [^{125}I] AmiK in horizontal sections of rat brain is similar to [^{3}H] Ketanserin binding in rat cortex and caudate putamen (Pazos et al., 1985) but as reported by Schotte and Leysen [^{125}I] AmiK also labels the lateral septal nuclei and various thalamic nuclei.

In contrast, in human spinal cord there was no distinguishable labelling of either histamine-H_1, $5HT_2$ or α_1-adrenoceptor sites with [^{125}I] AmiK. Pre-incubating the sections in 1% BSA, reported to reduce NSB (Joshi, personnel communication)

had no perceivable effect on binding. There was however some indication of the presence of tetrabenazine displaceable sites in the gray matter, particularly in the ventral cord. The labelling of non-serotonergic sites by AmiK which is displaced by tetrabenazine is an interesting phenomenon. Early in vitro binding experiments of AmiK with tissue homogenates showed binding to $5HT_2$ receptors at subnanomolar concentrations and to histamine-H_1 and α_1-adrenoceptors at nanomolar concentrations (Wouters et. al., 1986). Leysen and co-workers (1987) described a further AmiK binding site which was displaceable by tetrabenazine. They characterized the tetrabenazine displaceable [^{3}H] ketanserin binding in rat striatum and proposed that these sites are associated with a DOPAC release system on dopamine and serotonergic nerve endings (amine releasing sites). Schotte and Leysen (1988) subsequently identified tetrabenazine displaceable AmiK binding sites in the rat brain. Reported here is the first description of tetrabenazine displaceable AmiK binding sites in the human spinal cord.

In tissue homogenate studies using [^{125}I] AmiK there were no distinguishable $5HT_2$ receptors or α_1-adrenoceptors in human spinal cord in accordance with the autoradiography results. However, there was also no evidence of tetrabenazine displaceable sites. Lack of discernible tetrabenazine displaceable sites in the homogenate preparation is understandable considering that the binding seen in the sections was low and limited to the gray matter. The binding could therefore have been masked by NSB to the rest of the tissue.

The evidence obtained using [^{125}I] AmiK in autoradiography and homogenate binding studies indicates that there are negligible or very low levels of $5HT_2$ receptors in the human spinal cord. It also indicates negligible levels of histamine -H_1 and α_1-adrenoceptor sites. However, there is evidence of amine releasing sites in the human spinal cord comparable to those described in the rat brain (Schotte and Leysen, 1988).

[^{3}H] DPAT binding to tissue sections

Experiments were performed in which sections of human cord were incubated with [^{3}H] DPAT and the ligand binding measured by wiping the sections with filters and determining the activity by

scintillation counting. This was done as a preliminary step to set up the optimal conditions with respect to incubation and wash times for autoradiography of [^{3}H] DPAT binding in sections of human cord. However due to the results of these preliminary tests no autoradiographs were prepared using the tritiated ligands. No specific [^{3}H] DPAT binding could be measured. This indicated that there are very few or no 5HT$_1$ and in particular 5HT$_{1A}$ receptors in human spinal cord. The method used was that described by Pazos and Palacios (1985) for autoradiography of 5HT$_1$ receptors in the rat CNS.

Consideration of α_2-adrenoceptors in the human spinal cord

The results obtained after incubating [^{3}H] idazoxan with spinal cord sections in numerous preliminary experiments designed to obtain optimal conditions for autoradiography were neither consistent nor conclusive. On all occasions the NSB was high, exceeding 60%, but in some cases specific, saturable binding was apparently measurable and in a similar number of other cases there was no specific binding. Factors which may have been affecting the binding were investigated including (i) the effect of refreezing; sections were incubated on the day of sectioning without refreezing and up to 3 weeks after sectioning with frozen storage (ii) preventing the solutions evaporating during incubation by performing the experiment in humid boxes (iii) ensuring that the binding had come to equilibrium by varying the incubation period from 5 to 60 minutes and (iv) attempting to obtain the lowest possible ratio of specific to non-specific binding by testing a large range of wash protocols of 1-3 rinses for 1 - 15 minutes each. None of these manipulations resulted in satisfactory reproducible results. It was decided therefore not to set up autoradiographs which would take 2-3 months to develop but to continue the line of research using ligand binding with which reproducible results could be rapidly obtained.

The reasons for the failure to obtain satisfactory conditions are not clear but it is likely that the protocol used was not suitable. As no autoradiography using [^{3}H] idazoxan has been reported the method was developed on the basis of reports of general protocols for autoradiography and those used with other α_2-adrenoceptor antagonists (Edwards and Hendrickson, 1982; Young and Kuhar

1979; Stephenson and Summers, 1985). The buffer was taken from the ligand binding protocol for [^{3}H] idazoxan (Giron et. al., 1985). It may have been better to pre-incubate the tissue with Krebs-phosphate buffer containing Na$^+$-GTP which is reported to increase α_2-antagonist binding possibly by removing endogenous noradrenaline and adrenaline which compete for the sites (Unnerstall et. al., 1984). Furthermore McLaughlin and Collins (1986) report that in studies of [^{3}H] idazoxan binding to rat cortex specific binding was considerably less when the experiments were performed in Tris-HCL compared with physiological salt solution though KD and the Hill coefficients were unaltered. Other authors measuring α_2-adrenoceptor binding with idazoxan or other α_2-adrenoceptor specific ligands report using a number of different buffers including Tris-HCL buffer alone or in conjunction with chloride salts, phosphate buffers, physiological salt solutions and glycylglycine buffer (Doxey et. al., 1982; Giron et. al., 1985; Petrash and Bylund, 1986; Simmons and Jones, 1988). In the light of the differences reported by Mclaughlin and Collins and those discussed below concerning serotonergic binding studies it would be useful to compare α_2-adrenoceptor binding with autoradiography using different buffers. From such a study it may be possible to select a more appropriate buffer for autoradiography with idazoxan. In the small number of investigations made using rat brain sections idazoxan binding appeared to be reproducibly displaceable which would indicate that the problems arose due to the tissue rather than the methodology. It is however possible that in a larger study similar variability would have occurred. On the other hand this may be further evidence of the idiosyncratic nature of the spinal cord tissue which may require tailored conditions.

Radio-ligand binding studies

Evidence of little or no serotonergic binding in the human spinal cord

Consideration of the protocol

There is controversy over the use of additives to Tris-HCL buffer in the binding assays for serotonergic receptors. The compounds commonly added to the buffer are $CaCl_2$, pargyline, a mono-amine oxidase inhibitor to prevent the breakdown of 5HT by endogenous enzymes and ascorbic acid an anti-oxidant which protects 5HT from air oxidization. However the presence of ascorbic acid in the buffer has been reported to be deleterious for several neurotransmitter receptors including those for serotonin due to its proposed destruction of receptor sites (Leslie et. al., 1980; Weiner et. al., 1982; Hall et. al., 1985). Furthermore, the inclusion of physiological concentrations of electrolytes and / or 0.1% ascorbic acid and 1µM pargyline was reported to reduce the B_{max} value of [^3H] ketanserin binding to $5HT_2$ sites by 30% and its binding affinity by up to 3 fold (Leysen et. al., 1982). It was proposed by these authors that this was due to the effect of the additives on the surface phenomena inherent in interaction between ligands in solution and membrane micelles. However, more recently, there has been evidence indicating that inclusion of ascorbic acid in serotonergic ligand binding assays is imperative. Firstly, Hamblin and co-workers (1987) reported that in the absence of ascorbic acid [^3H] 5HT decomposes and that the breakdown products mimic specific ligand/receptor binding though the binding is irreversible. Secondly, Demopoulus and Peroutka (1987) provided thorough evidence of [^3H] DPAT binding to glass fibre filter paper which appeared to saturate and could therefore be misinterpreted as specific binding. This binding was prevented in the presence of 0.1% ascorbic acid. In the present study the affinity of [^3H] DPAT binding to rat cortical tissues and the B_{max} value were approximately 50% lower when determined in the presence of 0.1% ascorbic acid, 0.1µM pargyline and 5.7mM $CaCl_2$. However although the Scatchard plots of the data using both buffers were straight lines the displacement curves and Hill plots

indicated that binding in the absence of these additives may not be to one specific site only. With the inclusion of additives all specific binding was inhibited with 0.1μM 5HT whereas in their absence NSB was defined by a 100 fold greater 5HT concentration. More-over, the slope of the Hill plot in the absence of additives was less close to one (Table 4.5). [This final point indicates the importance of the Hill plot which can indicate that the ligand is binding to more than one specific site even when binding is apparently saturable and the Scatchard plot may be linear with a good correlation coefficient.] These results therefore support the proposal that in the absence of $CaCl_2$, pargyline and ascorbic acid there is a component of [3H] DPAT binding which may be misinterpreted as specific binding. In view of the previously published reports this component may be partly or wholly due to saturable binding to glass fibre filters. Studies of [3H] DPAT, [3H] 5HT and [3H] ketanserin binding in rat brain and human spinal cord were therefore performed in Tris-HCL containing 0.1% ascorbic acid, 0.1μM pargyline and 5.7mM $CaCl_2$.

5HT$_2$ receptors

[3H] Ketanserin binding to 5HT$_2$ receptors in the rat frontal cortex was comparable with that reported by Leysen and co-workers in 1983. The relatively low level in this study (5.56±.04 compared with 11.5±1 fmols/mg tissue) can be accounted for by the differences in protocol. In particular Leysen reports that inclusion of salts in the buffer (as used in this study) can reduce specific binding by as much as 90% (Leysen et. al., 1982). Furthermore the level of specific binding and the %NSB to 5HT$_2$ receptors measured in human frontal cortex using [3H] ketanserin were in accordance with earlier reports of 5HT$_2$ binding in human frontal cortex using [3H] ketanserin (Reynolds et. al., 1984) and other 5HT analogues (Stanley et. al., 1986; Marcusson et. al., 1984).

In contrast, there was negligible specific binding of [3H] ketanserin in the human spinal cord indicating that the numbers of 5HT$_2$ receptors in the human spinal cord are below levels detectable by these methods. This supported the evidence from autoradiography and homogenate binding studies using [125I] AmiK.

Binding studies using [^{3}H] 5HT indicated that this ligand reveals more than one binding site in rat brain as reported previously (Peroutka, 1986; Hamon et. al., 1984) and the % of total binding accounted for by 5HT$_{1A}$ receptors as defined by inhibition by 100 nM DPAT was also close to the value reported by Peroutka (1986). Similarly, inhibition of [^{3}H] 5HT binding by DPAT in human frontal cortex indicated the presence of multiple 5HT receptor sites in this tissue which has also recently been reported in a study using [^{3}H] 5HT with DPAT and a number of other 5HT analogues (Todd and Ciaranello, 1987).

[^{3}H] DPAT binding in the rat frontal cortex was shown to reveal a single binding site which, from previous evidence, was the 5HT$_{1A}$ receptor. The binding was saturable, reversible and the KD value corresponded closely to that reported by other authors using a similar buffer composition (Table 4.5B).

However, in contrast, when the above studies were performed using human spinal cord negligible specific binding of [^{3}H] 5HT or [^{3}H] DPAT was measurable. Where there was substantial total ligand binding 85-100% of this was accounted for by non-specific binding. These results probably indicate that the 5HT$_1$ receptors in the human spinal cord in the lumbar region are present in numbers that are difficult to measure with the methods used. The evidence obtained in ligand binding studies supports the results obtained using [^{3}H] DPAT binding to sections on slides indicating that there are negligible or very low levels of 5HT$_1$, and in particular, 5HT$_{1A}$ receptors in the human spinal cord.

The few reports of the number or distribution of serotonergic receptors in spinal cord tissue which have been published confirm these findings. Low levels of 5HT$_2$ receptors are reported in the rat, guinea-pig and rabbit spinal cord compared with concentrations in a number of cortical regions, the striatum and nucleus accumbens (Leysen et. al., 1982). Levels of 5HT$_2$ binding in the rat spinal cord revealed by autoradiography by Pazos and Palacios (1985) were described as 'very low' (0 and 6.2 - 6.4 fmols /mg protein using [^{3}H] mesulergine and [^{3}H] ketanserin respectively). In a similar study

they described low levels of [^{3}H] 5HT binding in the rat cervical cord which they attributed to 5HT$_{1A}$ and 5HT$_{1B}$ sites. There are apparently no data published concerning serotonergic receptor sites in the human spinal cord. In future attempts it may be possible to measure lower levels of receptors using the membrane preparation employed by Leysen and co-workers (1982) from which nuclei and cell debris were removed by homogenising and spinning the tissue in 0.25 M sucrose. However this would require more tissue (original wet weight) for each assay as discussed below.

Although there are apparently no reports of serotonergic receptors in the human spinal cord Pazos and Palacios (1985) reported finding low or very low concentrations of 5HT$_1$ receptors in the rat spinal cord and low (1-2 fmols/mg protein) but distinct levels of 5HT$_2$ binding have been described in the rat spinal cord in in vitro binding studies (Leysen et al., 1983). The lack of evidence for serotonergic receptors in this study may result from inappropriate methodology and the assays may not be sensitive enough to measure the level of receptors present in the spinal cord. Although the methods described in this chapter have been used to provide reproducible results in rat brain tissue a better preparation of the spinal cord tissue may be necessary if serotonergic receptor binding in the human spinal cord is to be determined. Also, considering the effect of freezing on receptor number (below), where receptor numbers are low it is important to use fresh tissue. Bearing in mind the differences between brain and spinal cord tissue a greater effort to attain the optimal conditions of incubation and rinse protocol, the incubation temperature and the ligand concentration may maximize the ratio of specific to non-specific binding and facilitate the measurement of low levels of binding.

[^{3}H] Idazoxan binding

The results of [^{3}H] idazoxan binding in rat cortex revealed high affinity of the ligand for a single receptor site with saturable, displaceable binding. From the data KD and Bmax values were calculated which were very close to the published results of a number of other authors (Table 4.6). Displaceable, high affinity [^{3}H] idazoxan binding was also demonstrated in human spinal cord. It

was therefore assumed that the α_2-adrenoceptor binding method was satisfactory. However, a number of possible refinements were explored as adaptations to factors which were relevant to using human spinal cord tissue.

Modifications of the binding protocol

Initially, as the availability of human tissue was limited the possibility of reducing the tissue concentration per incubation tube used was investigated. [^{3}H] Idazoxan binding was performed using half the tissue concentration stated in the methods. This modification was found to significantly reduce specific binding and increase NSB and so was not incorporated into the protocol. Published data supports this finding, Leysen and co-workers (1982) reported that in rat cortical membranes specific [^{3}H] ketanserin binding per mg tissue decreased marginally and % NSB increased with decreasing tissue concentration.

The effect of freezing tissue on specific binding and % NSB was also investigated as all human tissue was stored at -80°c before analysis. Freezing was found to produce a significant reduction of approximately 16% in specific [^{3}H] ketanserin binding with no effect on % NSB. It was decided that frozen storage of the tissue would be continued despite the loss in receptor number for a number of reasons. Firstly as the tissue could arrive at any time of day performing the binding assay immediately on its delivery would be practically inconvenient. Secondly, as the methods were developed for a comparative study between Parkinsonian and control subjects it was less essential to obtain absolute values whereas the facility to store the tissue and assay paired samples concomitantly was important. Thirdly the majority of studies on human tissue are performed with frozen samples and therefore the results would be directly comparable with published data. For completion the effect of freezing on binding in the human spinal cord tissue with each ligand used should be investigated as there may be differences due to variation in species, CNS region and the ligand used.

As NSB binding in the human spinal cord was high (approximately 60% of total binding) relative to that in rat brain an attempt to increase the ratio of specific to non-specific binding was

made by producing a synaptosomal preparation rather than the crude membrane homogenate. However, as a result, specific binding was in fact reduced using the synaptosomal preparation probably because of the loss of tissue mass which occurs with this method. In the light of this evidence binding experiments were continued with the homogenate preparation and the human spinal cord was treated in the same way as the rat brain except for application of the biohazard procedures laid out in Appendix 2.

In conclusion, ligand binding and autoradiography were used to determine levels and / or distributions of $5HT_{1A}$ and $5HT_2$ receptors and α_1- and α_2-adrenoceptors in the rat brain which corresponded to previously published results. However, using the same techniques, no $5HT_{1A}$ or $5HT_2$ receptors or α_1-adrenoceptors were identified in the human spinal cord. Nevertheless it was possible to determine high affinity, reversible binding of $[^3H]$ idazoxan, a selective α_2-adrenoceptor antagonist, in the human thoracic spinal cord and a number of modifications of the protocol were investigated. It is proposed that success in future attempts to determine serotonergic binding in the human spinal cord may be obtained using synaptosomal preparations of fresh tissue.

Chapter 5   Alpha 2-adrenoceptors in the spinal cord in Parkinson's disease

Introduction

Alpha 2-adrenoceptor binding has been determined in the rat spinal cord (Simmons and Jones,1988; Giron et.al.,1985; Unnerstall et.al.,1984) but although α_2-adrenoceptor binding has been demonstrated in the human spinal cord (Unnerstall et.al.,1984) it has not been defined in terms of number of receptors or receptor affinity. In the previous chapter experiments were performed to obtain a ligand binding protocol suitable for measuring [^{3}H] idazoxan binding in this tissue. The use of this method to define α_2-adrenoceptor binding in the human spinal cord is described here. Binding is defined in terms of the affinity of [^{3}H] idazoxan to α_2-adrenoceptors and the number of receptor sites.

The CNS location of α_2-adrenoceptors has been investigated in the rat using chemical and surgical lesions of noradrenergic neurones (U'Pritchard et.al.,1980; Dausse et.al., 1982). The Parkinsonian spinal cord was shown in chapter 3 to have up to 80% depletion of noradrenaline in selected regions. It is therefore used in this study as a model of noradrenergic depletion to investigate α_2-adrenoceptor binding in the human. There is evidence that sensory (Howe et.al.,1983; Fleetwood-Walker et.al.,1985; Sawynok and Reid, 1986) and motor (Anden et.al.,1970; Kitazawa et.al., 1985) function is mediated by spinal cord α-adrenoceptors in the rat. In addition, there is evidence for interaction between spinal cord serotonergic and noradrenergic systems (Archer et.al.,1985; Clatworthy et.al.,1988; Gorea and Adrien, 1988). In the human these functions may be important in the symptoms of Parkinson's disease which include motor dysfunction and pain. This study was therefore performed to determine α_2-adrenoceptor binding in the post-mortem spinal cord from Parkinsonian subjects.

In the light of earlier results which demonstrated significant effects of age and PMI on transmitter levels serious consideration was given to the problems associated with the use of

human tissue and ligand binding data was analysed for correlation with subject age, sex and the PMI using ANCOVA.

The analysis of ligand binding data has been made easier and less time consuming with the advent of computer packages which are capable of performing multiple data transformations very rapidly. Use of a computer package also enables a more accurate determination of KD and Bmax values from lines fitted to the data by least squares non-linear regression analysis instead of by guesswork. The ligand binding data described in this chapter and the previous one has been analysed by the computer package LIGAND (Munson and Rodbard, 1980) the use of which will be reported here.

Methods

Spinal cord samples

Spinal cord tissue was obtained from the Cambridge Brain Bank, the Parkinson's Disease Society Brain Bank and Nottingham University Hospital. Thoracic and/or lumbar tissue was taken from 2 female and 6 male control subjects with no history of neurological disorder who had died from cardiac failure, bronchopneumonia or septicaemia. Thoracic and / or lumbar spinal cord tissue was taken from 4 female and 2 male subjects who were confirmed as Parkinsonian by post-mortem neurochemical analysis. The details of subject age, sex and the PMI are given in Table 5.1. Tissue was dissected and stored as described in chapter 3, pp.94-95.

[^{3}H] Idazoxan binding

Adjacent 5mm slices of spinal cord segments were combined so that samples of ~1g were used in binding assays. Membrane homogenate preparation and [^{3}H] idazoxan binding were performed using the methods and buffers described in the previous chapter (pp.134-136). An assay tissue concentration of ~10mg/ml was used. Eight [^{3}H] idazoxan concentrations within the range 0.2-16 nM were employed and NSB was determined with 10μM clonidine.

Analysis of data binding using LIGAND

Results from the ligand binding studies were analysed to define the binding in terms of the affinity (1/KD) of [^{3}H] idazoxan for its binding sites and the number of binding sites (Bmax). Initially, data obtained using thoracic spinal cord tissue from control subjects were plotted as saturation curves to ensure that binding site saturation was attained within the [^{3}H] idazoxan concentration range used. The computer program LIGAND produced by Munson and Rodbard (1980) and adapted for use on microcomputers (McPherson, 1985) was used for analysis of the

Table 5. 1   Age, sex, and interval between death and autopsy (PMI) of control and Parkinsonian subjects.

		Control	Parkinsonian
Age (years)	mean	64.4	80.5
	range	39-81	73-89
PMI (hours)	mean	36.6	35.8
	range	6-53	9.5-78
n	total	8	6
	females	2	4
	males	6	2

ligand binding data. The program employs non-linear least squares curve fitting to estimate values for the KD, Bmax and NSB. The program was used to obtain Scatchard transformation of the data using linear regression in the first instance to find estimates of KD and Bmax. Hill plot transformations were also produced using linear regression. These initial estimates were then applied to a one site binding model and iteration continued until the lowest residual variance for the fit of the line to the data was achieved. If the iteration failed then the initial estimates were adjusted and the iteration begun again. In the event of a successful fit the sum of squares (of variance from the line) and mean square were displayed. Also displayed were the results of the runs test which indicated whether the points were randomly distributed about the line. A graphic display of the data and the fitted line was then viewed. Whether the fit was considered to be appropriate was dependent on there being a small residual variance (i.e.~1000 or less) and a random distribution of points about the line. The latter was determined from the runs test and the graphic display. The final estimates of KD, Bmax and NSB were given with the approximate SEM of each parameter. The SEM were also used to estimate the goodness of fit of the line to the data. A number of successful fits were obtained for the data from each spinal cord using different initial parameter estimates. The F- ratio test (page 98) was used to determine whether there was any statistically significant difference between the various fits. The final estimates of KD and Bmax were then taken from the best fit.

Saturation curves for selected samples which did not fit to a one site model using all the data points were plotted manually to determine whether the binding sites were saturated

Statistics

Two distinct groups of subjects were identified. The data
from one group represented specific, saturable [^{3}H] idazoxan
binding and the data from the other group did not. Analysis of
covariance (ANCOVA, pp.97-98) was used to investigate whether
differences in age or PMI were associated with the two groups. In
addition, the Chi square Exact test was used to determine whether
either group was associated with Parkinson's disease.

Specific [^{3}H] idazoxan binding

The data corresponding to specific, saturable binding was
analysed further. ANCOVA was employed to determine whether
there were any differences in KD and Bmax values of the thoracic
and lumbar segments. Results obtained from thoracic and lumbar
spinal cord were combined and analysed to determined whether
there were any correlations between the KD and Bmax values and
the age, sex or PMI of the Parkinsonian and control subjects. Data
from Parkinsonian and control subjects were identified as being
from different groups for purposes of analysis although there were
only 2 Parkinsonian subjects from whom the data represented
specific binding.

Results

[³H] Idazoxan binding in the human spinal cord

Specific, saturable [³H] idazoxan binding was demonstrated in the spinal cord from 5 out of 8 control subjects and 2 out of 6 Parkinsonian subjects (illustrated in Fig. 5.1). The binding data from these subjects were fitted to a one site model in which the data points were randomly distributed about the Scatchard plot regression lines (with residual variances less than 1000) and the Hill coefficients were close to unity An example of the results for two different fits generated by LIGAND for the data from one spinal cord sample is given in Table 5.3. In addition, typical graphical displays of Scatchard plots generated from the different fits are shown in Fig. 5.3. The following results relate to data representing specific [³H] idazoxan binding.

KD and Bmax values of [³H] idazoxan binding in the human thoracic and lumbar spinal cord from control subjects were 6.62 ± 2 nM, 342 ± 189 fmols/mg protein (thoracic cord, n=3) and 7.89 ± 3 nM, 315 ± 166 fmols/ mg protein (lumbar cord, n=3). Statistical analysis using ANCOVA revealed that there was no significant difference in the values of KD and Bmax between thoracic and lumbar spinal segments (Table 5.2). Values from the different segments were therefore combined as the numbers of each were small. Where samples from both segments were taken from one subject the mean value of each was used (and for this reason total number of samples is not simply the sum of the numbers of lumbar and thoracic segments).

The KD of [³H] idazoxan in the control spinal cord was 7.1 ± 2.1 nM and number of binding sites (Bmax) was 341 ± 134 fmol/mg protein (n=5 in each case) (Table 5.4 and Figs. 5.6 & 5.7). NSB was 30 -60% of total [³H] idazoxan binding.
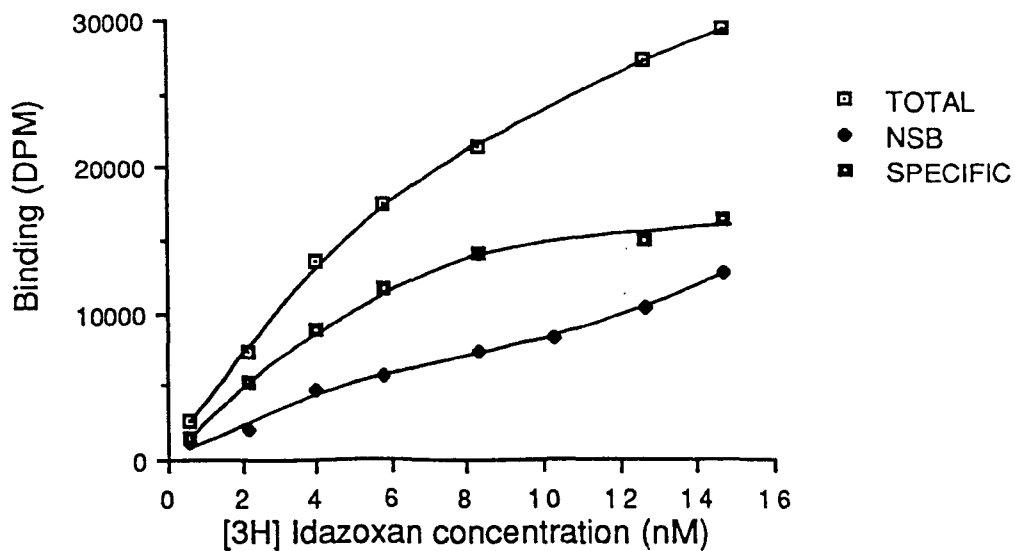
Fig. 5.1 Total, specific and non-specific binding (dpm) of [³H] idazoxan binding (0.2 - 15 nM) to homogenate of thoracic spinal cord from a control subject. Each point is the mean of 3 replicates. Scatchard transformation of this data was fitted to a single binding model. This plot is typical of a number of other control and Parkinsonian subjects for which the data represented binding to a single specific receptor site.
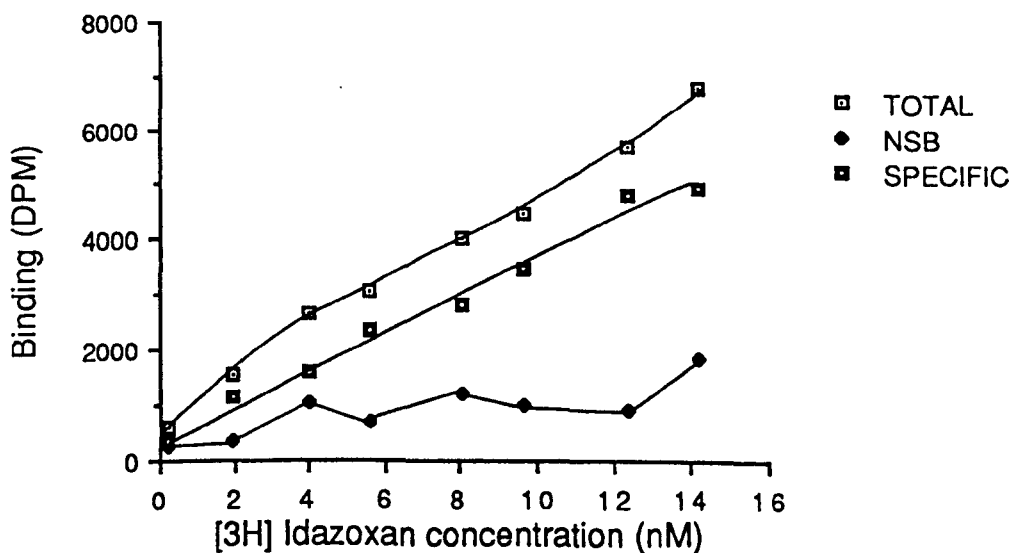
Fig. 5.2 Total, specific and non-specific binding (dpm) of [³H] idazoxan binding (0.2 - 15 nM) to homogenate of lumbar spinal cord from a Parkinsonian patient. Each point is the mean of 3 replicates. From this data specific [³H] idazoxan binding could not be determined. Scatchard transformation of this data could not be fitted to a binding model of either one or more sites using LIGAND.
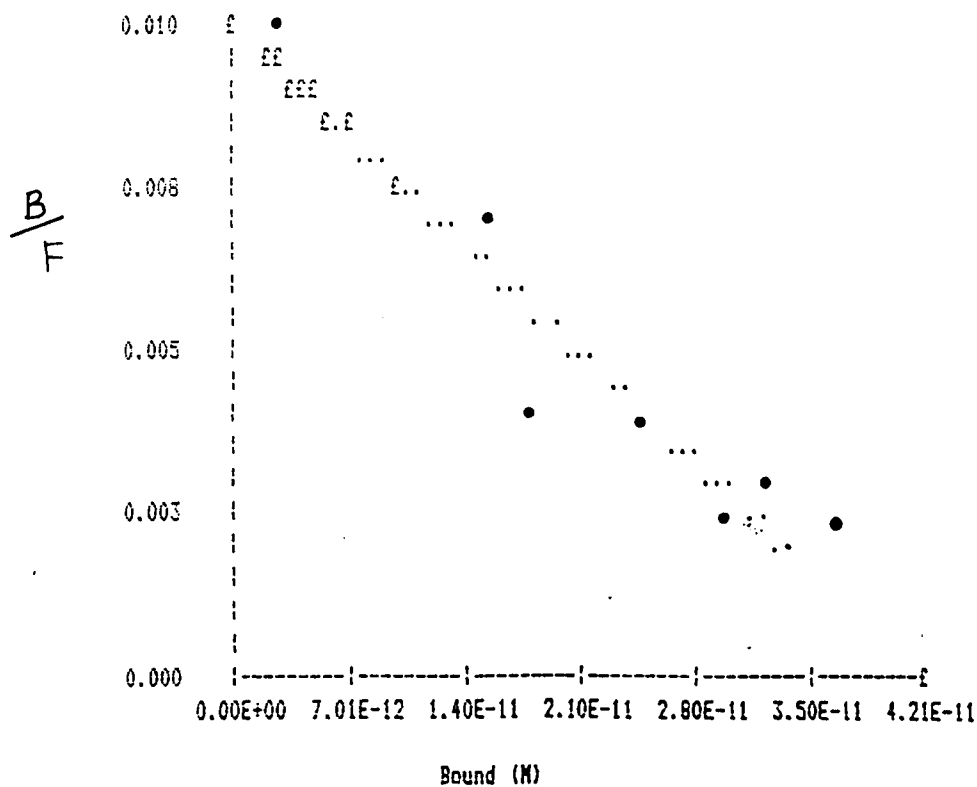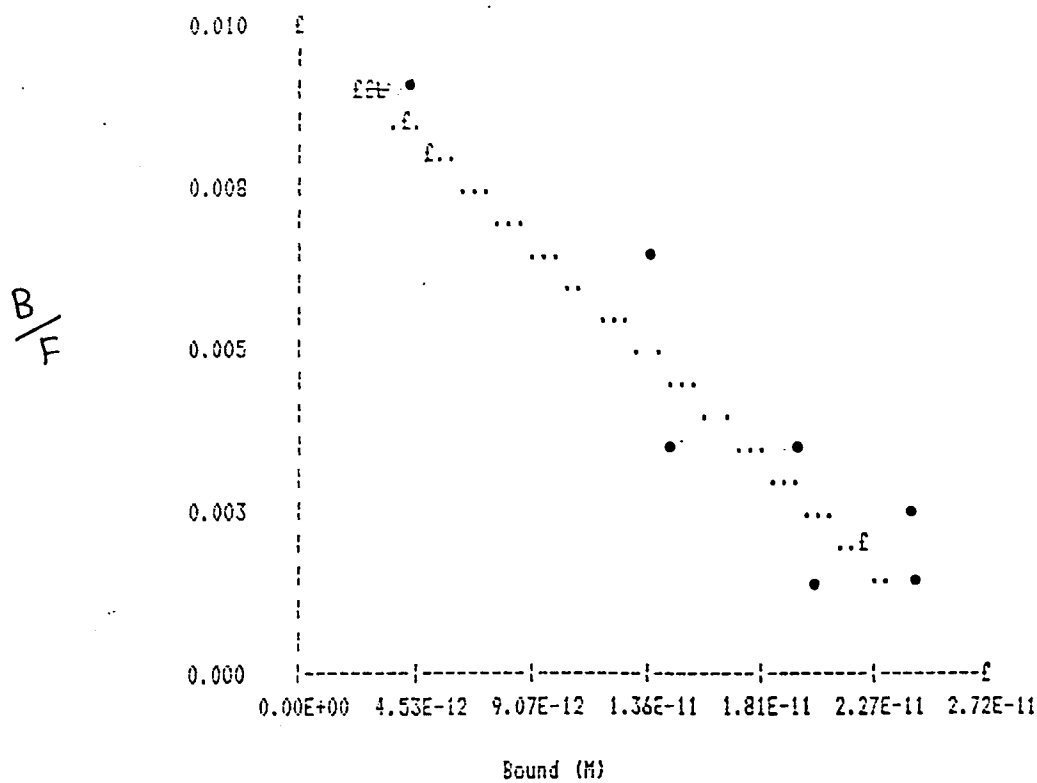
Fig 5.3   Graphic display of Scatchard plots of the same data
fitted to two different lines by the computer package LIGAND.
Data was generated from saturation studies of [^{3}H] Idazoxan
binding in the thoracic spinal cord from a control subject.
Scatchard plots were used in conjuntion with data such as that
shown in Table 5.3 to determine the best fit of the data.

Table 5.2   ANCOVA details for analysis of α_2-adrenoceptor binding in the human spinal cord.

Correlations between (A) KD and (B) Bmax and age, PMI, Parkinson's disease (Pd), sex and segment (SEG: lumbar or thoracic). (C) Correlation between age and PMI and specific, saturable binding. MS=mean square ; d.f=degrees of freedom ; res.var=residual variance . The results indicate that KD and Bmax are correlated with age, PMI and Pd and that specific saturable binding depended on subject age.

	Factor	MS	d.f	Res.var	d.f	F value	$p<0.05*/p<0.01**$
A.	AGE	50.8	1	.115	1	442	*
	PMI	22.8	1	.115	1	198	*
	Pd	71.0	1	.115	1	617	*
	SEX	0.26	1	.115	1	2.26	
	SEG	0.88	1	.115	1	7.65	
B.	AGE	256260	1	7750	2	66.1	*
	PMI	124280	1	7750	2	32.1	*
	Pd	212660	1	7750	2	54.9	*
	SEX	1080	1	7750	2	0.14	
	SEG	372	1	7750	2	0.05	
C.	AGE	723	1	847	12	10.2	* *
	PMI	37.6	1	6571	10	0.06	

FIT	1/KD	SEM	KD	Bmax	SEM	MS	RESIDUALS +	RESIDUALS -	RUNS p<0.05*	F p<0.05*
1	3.8E+8	1.4E+8	2.6nM	2.7E-11	8.6E-12	91	4	3	6	- - -
2	2.5E+8	3.8E+7	4.0nM	4.2E-11	3.4E-12	98	4	3	5	1.4

Table 5.3   Typical output from LIGAND used to obtain values of KD and Bmax.

This table shows the results generated for two different fits of ligand binding data. The results were obtained from [^{3}H] idazoxan binding in the spinal cord of a control subject. Values are given for 1/KD and Bmax with their approximate standard errors. These values have not been adjusted for assay volume or protein content. The residuals are the number of points above and below the fitted line and these are expressed as the number of runs of points either above or below the line. The runs test does not reach a significant level indicating that the points are randomly distributed about the line. MS is the mean square of the residual variance from which the F ratio is calculated. There is no significant difference between the two fits and the second fit was selected as the best because the SEM values were smaller and a better fit was indicated by the graphic display (Fig.5.3).

	KD	Bmax	Hill
Parkinson's disease	1.03	56.1	0.99
	2.47	140	0.99
n	2	2	
Control	7.1±2.1	341±134	0.98 ±0.01
n	5	5	

Table 5.4

Alpha 2-adrenoceptor binding in the human spinal cord of
Parkinsonian and control subjects. Binding determined by [^{3}H]
idazoxan is expressed in terms of KD (nM), Bmax (fmols/mg
protein) and the Hill coefficient. Control values are expressed as
mean ± SEM.

Effects of age, sex, PMI and Parkinson's disease on [^{3}H] idazoxan binding

KD and Bmax values were found to increase significantly with both age and PMI (p<0.05 in each case) as shown in Table 5.2 and Fig.5.8. For each subject KD was significantly correlated with Bmax (r=0.96, p<0.001, Fig.5.5).

KD and Bmax values were determined for only two Parkinsonian subjects. These values given in Table 5.4 and Figs. 5.6 & 5.7 were significantly lower than in the control subjects (Table 5.2).

In addition, there were no differences in either KD or Bmax between females and males (Table 5.2).

The percentages of the total intra-subject variation accounted for by differences in age and PMI, determined from the residual variance of ANCOVA analysis, was; 16% and 45% (age) and 17% and 26% (PMI) for KD and Bmax values respectively.

Non-saturable binding

The data from the remaining 7 subjects were not representative of specific saturable binding and did not fit to a one or two site binding model using LIGAND. An example of total, specific and non-specific binding data which could not be fitted to any binding model is given in Fig.5.2. It appears that the 'specific binding' (determined from the difference between total and non-specific binding) did not saturate and this was typical of the results for which the data did not fit a binding model. Whether the data represented saturable binding or not was dependent on the age of the subject (p<0.01, Table 5.2 and Fig.5.4) i.e. the non-saturable data was from older subjects and was not correlated with either PMI (Table 5.2) or Parkinson's disease.
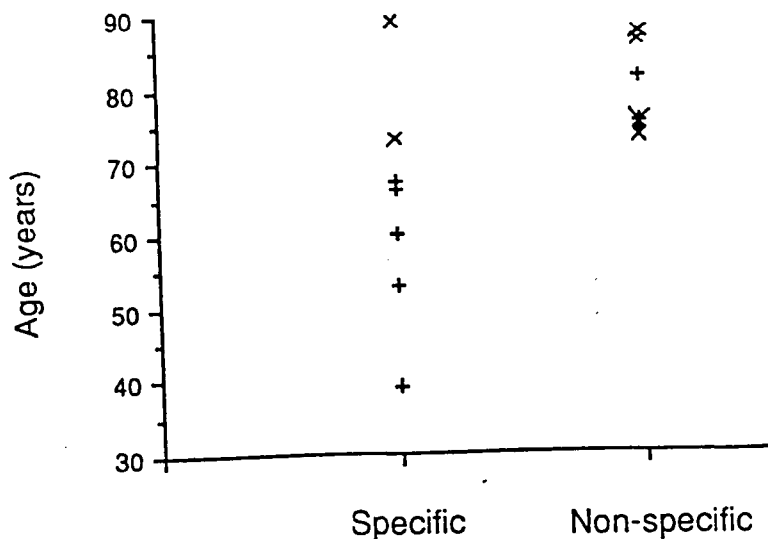
Fig 5.4 Age and specific [^{3}H] idazoxan binding

Ages of the subjects from whom tissue was taken to determine [^{3}H] idazoxan binding in the spinal cord. Subjects are Parkinsonian **x** or control **+** and are divided into those for whom the binding data did and did not exhibit   specific binding. This figure illustrates that data obtained from many of the older subjects did not correspond to [^{3}H] idazoxan binding to a single.specific site
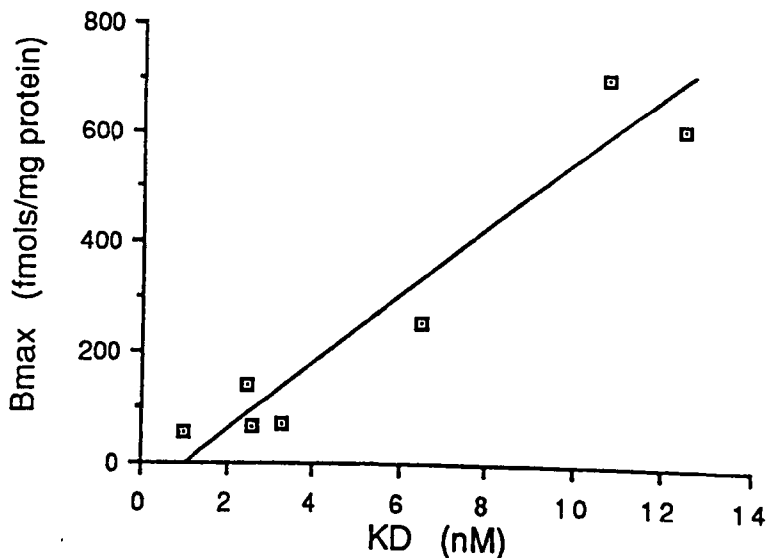
Fig 5.5   Correlation between KD and Bmax.
This figure illustrates the correlation between α_2-adrenoceptor affinity and receptor number (Bmax) in the human spinal cord determined using [^{3}H] idazoxan. Each point represents KD and Bmax values determined in the spinal cord of one subject. Low receptor affinity (1/ KD) was correlated with high receptor number. r= 0·96
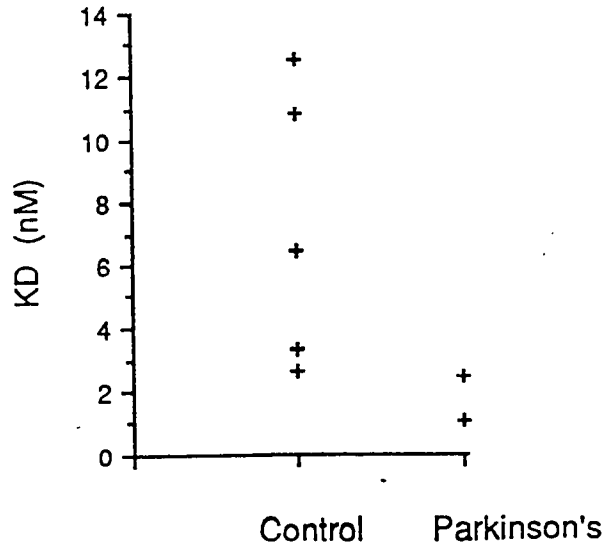
Fig 5.6   The affinity of [3H] Idazoxan for binding sites in human spinal cord. The affinity for sites in spinal cords from Parkinsonian and control subjects determined using ligand concentrations of 0.2-15 nM are shown. Each point represents the result from one subject.
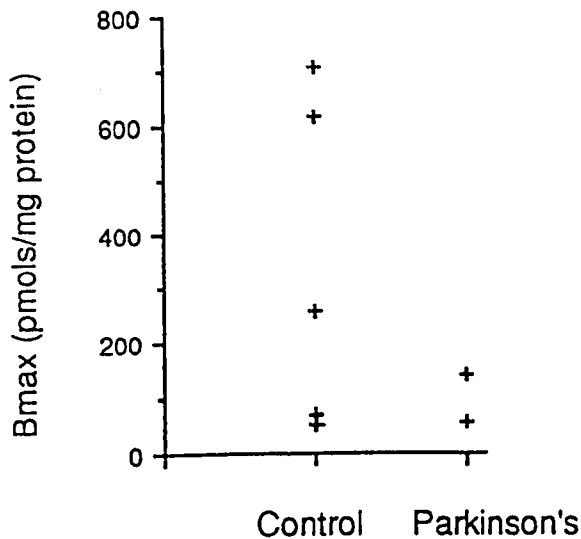
Fig 5.7   [3H] Idazoxan binding in the human spinal cord. The number of binding sites (Bmax) in spinal cords from Parkinsonian and control subjects determined using [3H] Idazoxan concentrations of (0.2 -15 nM) are shown. Each point represents the result from one subject.
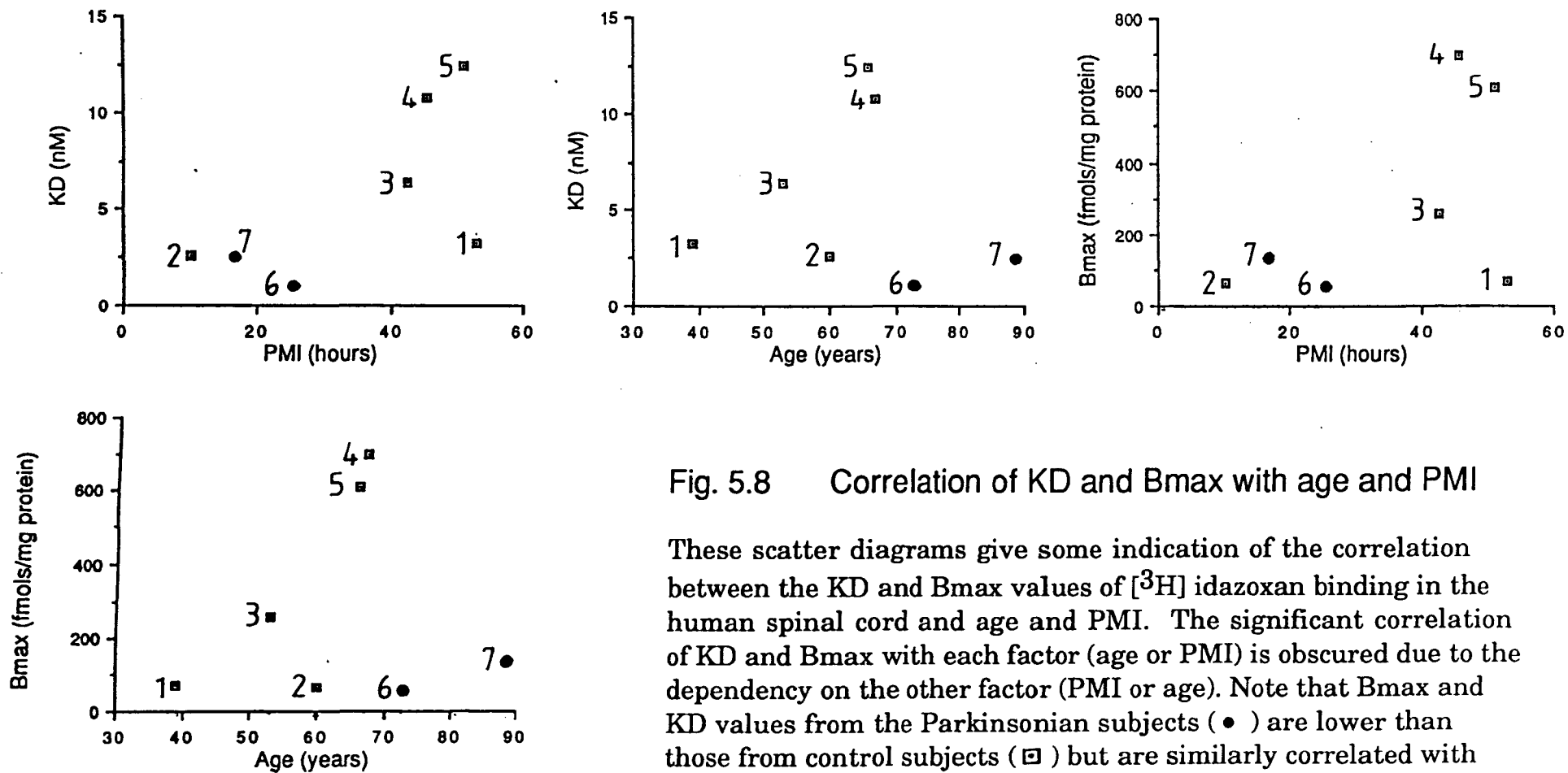
Fig. 5.8   Correlation of KD and Bmax with age and PMI

These scatter diagrams give some indication of the correlation between the KD and Bmax values of [^{3}H] idazoxan binding in the human spinal cord and age and PMI. The significant correlation of KD and Bmax with each factor (age or PMI) is obscured due to the dependency on the other factor (PMI or age). Note that Bmax and KD values from the Parkinsonian subjects (•) are lower than those from control subjects (▣) but are similarly correlated with age and PMI. Individual subjects are numbered for cross-reference.

Discussion

Alpha 2-adrenoceptor binding sites in the human spinal cord

There were problems in defining [^{3}H] idazoxan binding in the human spinal cord as the data from a number of subjects did not appear to represent binding to a specific saturable site. However, data from half of the subjects represented saturable, displaceable [^{3}H] idazoxan binding and this data will be considered first.

There is growing evidence for heterogeneity of the α_2-adrenoceptor (Cheung et.al.,1982; Lanier et.al.,1988) and Petrash and Bylund (1986) have proposed that there are subtypes of α_2-adrenoceptor in specific areas of the human brain. However, in this study [^{3}H] idazoxan binding appeared to be binding to a specific site. Specific [^{3}H] idazoxan binding to α_2-adrenoceptors was demonstrated in the human spinal cord with an affinity of approximately 7nM. In addition, the number of binding sites has been determined as approximately 360 fmols/mg protein. Due to the wide variation in the results these values are considered to be initial estimates in preliminary experiments and suggestions for more accurate determinations are discussed below. The results of this study are comparable with previously published work. Autoradiography employing [^{3}H] para-aminoclonidine (PAC) has been used to locate α_2-adrenoceptors in the human thoracic spinal cord (Unnerstall et.al., 1984). High levels of α_2-adrenoceptors were described in the dorsal horn substantia gelatinosa and in the intermediolateral cell column whilst lower levels were distributed in the ventral horn in association with motor nuclei. The distribution in the human cord paralleled that in the rat spinal cord, However, binding in the rat tissue was quantified in terms of KD and Bmax whereas binding in the human cord was not. Reasons for this were not given but it is possible that difficulty in distinguishing specific binding in the human cord may have been due to high levels of non-specific binding. High and variable levels of non-specific binding in rat spinal cord have reported (Howe and Yaksh, 1984; Janns et.al.,1987; Simmons and Jones

1988) which is consistent with findings in human cord in the present study. Moreover, no other reports of quantitative analysis of α_2-adrenoceptors in the human spinal have been published. However, α_2-adrenoceptors have been located in the rat spinal cord (Jones and McEnna, 1980) and the α_2-adrenoceptor is described as a homogeneous binding site distinct from α_1-adrenoceptors present in ventral and dorsal regions of cervical, thoracic and lumbar segments (Giron et.al.,1985; Simmons and Jones, 1988). Furthermore, α_2-adrenoceptor binding to a single site is reported in the cat spinal cord (Howe and Yaksh,1984). Bmax and KD values for α_2-adrenoceptors in the human cord described here using [^{3}H] idazoxan are greater than those in the whole spinal cord of the rat (21 and 70 fmols/mg protein, 1.7 and 1.39 nM) using [^{3}H] PAC (Giron et.al.,1985; Simmons and Jones, 1988). Alpha2-adrenoceptor binding in the human brain however, is similar to that in the spinal cord. Bmax values of 375-940 fmols/mg protein and KD values of 4.6 -18.4 nM are described for various regions of the brain (Shimohama et.al.,1986). This study therefore indicates that in the human consistent with other species α_2-adrenoceptor binding in the spinal cord is homogeneous and is of a similar order of affinity and number to that in the human brain.

Non-saturable binding data

There were difficulties in fitting to either a one or two site binding model certain ligand binding data obtained using spinal cord both from patients who had died with Parkinson's disease and control subjects. This binding data was assumed to represent non-specific, non-saturable binding and lack of specific sites or specific sites which could not be distinguished from relatively high and variable levels of non-specific binding. The non-saturable binding was dependent on age of the subjects at death and not Parkinson's disease. Data from those subjects older than 74 years of age did not represent specific binding to a single site in all but one subject whereas specific [^{3}H] idazoxan binding sites were identified in all 5 subjects below the age of 70. However, the Parkinsonian subjects were on average older than the control subjects. In addition, in the Parkinsonian patients duration and

degree of illness is also likely to increase with age. There may, therefore, be parallels between severity of Parkinson's disease and loss of α_2-adrenoceptors

Effect of Parkinson's disease, age, sex and PMI on adrenoceptors

The results may indicate higher affinity and lower numbers of α_2-adrenoceptor binding sites in the Parkinsonian spinal cord compared with controls. This can only be considered as a possible indication because of the low numbers and wide variation in both values. The variation in KD and Bmax was partly accounted for by their significant correlation with age and PMI (see below). Considering the intra-group variation and the low sample number it is concluded that a much larger sample size is required to determine the effects of Parkinson's disease on the α_2-adrenoceptors in the human spinal cord. Whether the changes in α_2-adrenoceptors seen in the older subjects are dependent to any degree on Parkinson's disease could be investigated further in age matched subjects.

This study has demonstrated that there was an increase in number and decrease in affinity of α_2-adrenoceptors with PMI. This may be explained by structural alterations occurring in the synaptic membranes after death resulting in hidden receptors being revealed.

Similarly there was a decrease in the affinity of [3H] idazoxan for the α_2-adrenoceptor and an increase in the number of binding sites in the human spinal cord with age. For each individual subject low binding affinity was correlated with high Bmax. [It must be noted that the Parkinsonian subjects were considered in this analysis to be from a different population from the control subjects (with a lower KD and Bmax) in which the KD and Bmax values similarly increased with age.]   A conformational change in the adrenoceptor with age resulting in a lower affinity for endogenous noradrenaline and a compensatory increase in receptor number is concordant with these results. Progression of this alteration in binding site conformation to the

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extent that the site is no longer capable of binding [^{3}H] idazoxan may account for the lack of specific receptors in the older subjects. Alternatively, it is possible that the lack of measurable receptors is due to particular susceptibility in the older subjects to the reduced affinity to [^{3}H] idazoxan with increasing PMI.

The increase in adrenoceptor number with age may be dependent to some degree on alterations in the spinal serotonergic system. In chapter 3 (page 115) a reduction in 5HT content of the spinal cord with age was described and there is evidence for the interaction of noradrenergic and serotonergic systems both at the level of the brain stem (Gorea and Adrien, 1988) and the spinal cord, especially regarding the modulation of nocioception (Jensen and Smith, 1983; Clatworthy et. al., 1988; Archer et.al.,1985). Moreover, spinal depletion of 5HT has been shown to induce a drastic potentiation of noradrenaline-induced analgesia (Archer et.al.,1986) and the authors concluded that the spinal noradrenergic system has an important role in modulation of descending serotonergic function. It may be that spinal noradrenergic and serotonergic systems interact in the modulation of spinal cord function and that depletion of the serotonergic system results in adaptation in the noradrenergic system. This proposal is concordant with the results of the present study.

It is interesting that, in accordance with factors affecting the levels of neuroamine levels in post-mortem spinal cord, the characteristics of α_2-adrenoceptor binding are dependent primarily on subject age and the PMI, and are independent of the sex of the subject.

Location of the α_2-adrenoceptor

The exact location of α_2-adrenoceptors in the CNS is unclear. There is evidence of a heterogeneous distribution on both pre- and post-synaptic membranes though the number of pre-synaptic sites is probably minor compared with post-synaptic sites (U'Pritchard et.al.,1980; Dausse et.al., 1982; Levin, 1984). As

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the loss of noradrenaline in the spinal cord of Parkinsonian patients was up to 80% of control values (pp.109-110) the Parkinsonian spinal cord was used as a potential model of adrenergic neuronal depletion in humans to investigate adrenoceptor location. Unfortunately, the results of this study do not help to resolve this question due to the low numbers of Parkinsonian subjects for which specific binding was demonstrated. However, the general indications for higher affinity and lower numbers of α_2-adrenoceptors in Parkinson's disease is not in accordance with comparable studies. It has been reported previously that neurotoxin induced lesion of the locus coeruleus did not significantly modify α_2-adrenoceptor number in several brain regions (Cash et.al.,1986). Similarly, neurotoxic depletion of spinal cord noradrenaline did not affect α_2-adrenoceptor binding in the cat lumbar spinal cord (Howe, Yaksh and Tyce, 1987). The authors concluded that α_2-adrenoceptors may be situated on neuronal elements unrelated to noradrenergic input. However, consideration must be given to factors which complicate the interpretation of the results from human models of neurological denervation. Firstly the results of this study illustrate that limitations on subject age and the PMI are imperative as there are significant changes in α_2-adrenoceptor binding due to these factors. In addition, the time scale of the illness compared to neurotoxically induced onset of neuronal degeneration in experimental animals poses problems. For instance, it has been reported recently that in the brain of patients with Parkinson's disease loss of nigro-striatal dopaminergic neurones is accompanied by atrophy in length and number of the dendritic arbor of post-synaptic striatal neurones (McNeill et.al,.1988). This may result from the reduced innervation of these neurones and means that receptor loss accompanying long term pre-synaptic neuronal degeneration can not necessarily be located to the pre-synaptic terminal. Furthermore, slowly progressing pre-synaptic denervation occurring over a number of years may result in both pre- and post-synaptic compensation in receptor affinity and number. In conclusion, any possible changes in KD and Bmax indicated in the Parkinsonian cord are likely to be a result of both degeneration of pre-synaptic noradrenergic

pathways and the processes associated with long-term deficit in noradrenergic transmission and would not be expected to be exactly similar to changes induced by neurotoxic or surgical lesions.

Future studies

1. [^{3}H] Idazoxan was selected as the ligand to determine α_2-adrenoceptor binding for a number of reasons including (i) its specificity for this receptor which is well documented (Doxey et. al., 1982 ; Langer et. al., 1983 ; Hamilton et. al., 1988; Howlett et. al., 1982) (ii) the classic α_2-adrenoceptor specific agonists clonidine and p-amino clonidine are imidazoline structures which may have actions unrelated to noradrenaline-mediated effects (Diamant et.al.,1987; Bousquet et.al.,1986; Hicks et.al.,1985) and (iii) it was considered that the hydrophilic nature of idazoxan may reduce the non-specific ligand binding to the spinal cord which tends to be high. However, the variability in the KD and Bmax values observed in this study may result from rapid dissociation of [^{3}H] idazoxan from its binding sites. If during rinsing the bound [^{3}H] idazoxan dissociates then the binding affinity determined would be lower and receptor numbers higher than the true values. In addition, both values would be very dependent on variations in rinse time. The rate of dissociation may be reduced by conducting the ligand binding at 0°c and this could be performed to investigate the effect on the KD and Bmax values so determined.

2. In addition, the maximum ligand concentration used in the ligand binding studies was only 2 fold greater than the mean KD of [^{3}H] idazoxan binding in the human spinal cord. These studies should therefore ideally be repeated with a maximum ligand concentration of 5 fold the KD value to ensure that saturation of the binding sites is achieved and to determine the KD more accurately.

3. To fully characterize α_2-adrenoceptor binding in the human cord ligand binding studies performed using a number of

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different specific α_2-adrenoceptor ligands should produce comparable results.

4. More data points per saturation curve would enable more accurate determination of KD and Bmax values.

5. Furthermore, in light of the results, future studies of α_2-adrenoceptor binding in the human cord should utilize high numbers of subjects to overcome intra-subject variation and subjects should be carefully matched for age and PMI in comparisons between control and disease states.

Rationalization for the use of the computer package LIGAND

Analysis of ligand binding data using the computer package LIGAND was easier and quicker than performing the multiple transformations of the data manually. Using the package also enabled a more accurate estimation of the values of KD and Bmax. Firstly, manual determination of Scatchard plots relies on guess-work whereas using LIGAND the line is determined by least squares estimate. Secondly, the best of a number of fits generated from different initial parameter estimates can be obtained and the goodness of fit is tested statistically. LIGAND also calculates standard errors for each parameter which are an indication of the precision of the estimates. In addition, in LIGAND there is the facility to restrict analysis to certain data points which means that in event of binding to more than one site estimates can be obtained for KD and Bmax values for potential high and low affinity sites. These estimates can then be used in non-linear curve fitting to an exact mathematical model to calculate KD and Bmax values for one or more binding sites. Furthermore, non-linear least squares analysis is a better method than using linear regression because in the Scatchard plot there are non-uniform errors in both of the co-ordinates. In LIGAND the total ligand concentration is used as this variable is precisely known. In this study LIGAND was useful for differentiating between data which did and did not correspond to a specific, saturable binding site.

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In summary, the computer package LIGAND was used to determine that [^{3}H] idazoxan binds to a homogeneous population of α_2-adrenoceptors in the human spinal cord. Further investigation is required to determine whether there is any difference between α_2-adrenoceptor affinity and number in spinal cords from Parkinsonian and control subjects as the variation in these values was large and dependent on the age of the subject and PMI. Furthermore, in a number of cords there was no distinguishable specific [^{3}H] idazoxan binding and further studies are needed to determine whether this was due to age, Parkinson's disease or to other factors such as freezing or storage of the tissue.

Chapter 6   General discussion

Summary of the thesis

This study has addressed the projected aims which were (i) to investigate of the levels of indoleamines, catecholamines, substance P and TRH in the human spinal cord (ii) to compare these levels in the spinal cord of Parkinsonian and control subjects (iii) to use the Parkinsonian spinal cord, in the light of co-existence of amines and peptides in the rat, to investigate whether depletion of spinal cord 5HT is paralleled by depletion of substance P and TRH in the human and (iv) to study serotonergic and noradrenergic binding in the spinal cord in Parkinson's disease. In order to carry out these aims a number of existing methods were validated in rat spinal cord or brain tissue or human cortical tissue producing closely comparable results to previously published work. These and other established methods were developed and adapted for use with human spinal cord material including (i) development of a sensitive reproducible and specific RIA for the detection of substance P which does not detect other tachykinins reported to be present in the spinal cord (ii) development of a method for the concomitant extraction of indoleamines, catecholamines and neuropeptides from CNS tissue which enables maximum use of a limited amount of tissue (iii) the concentration of catecholamines on alumina as these were found to be present in low levels in the human spinal cord and (iv) adaptation of autoradiography and ligand binding methods for detection of adrenergic and serotonergic ligand binding in the spinal cord.

The work described here compliments the post-mortem investigations of other authors into the distribution of neurotransmitters and neuropeptides in the human CNS which has been mainly restricted to the brain. The spinal cord has previously been largely neglected possibly because of the greater difficulty incurred in the dissection of the spinal cord from the body. The study also incorporates the novel use of some of these methods, in particular [^{125}I] AmiK and [^{3}H] idazoxan binding, to human spinal cord.

The use of human post-mortem tissue

A recurring phenomenon throughout this study was the greater difficulty incurred in making measurements in the human cord compared with the rat brain. This was dependent both on the tissue being from humans and apparent differences between the spinal cord and the brain. It is important to note that measurement of transmitter and neuropeptide levels and receptor binding made using the rat brain were reproducible and consistent with previously published work indicating that difficulties which arose were specifically associated with human post-mortem spinal cord tissue. Some considerations arising from the use of post-mortem human tissue are discussed here.

There were difficulties due to low levels of both catecholamines and serotonergic receptors in the spinal cord. There were also problems arising from the nature of the cord including maintaining tissue sections on slides for autoradiography and difficulties arising from high non-specific binding in ligand binding studies.

A large influence on the protocol throughout the study was the limited availability of the spinal cord material. The standard problems of obtaining post-mortem tissue, such as waiting for suitable subjects and relying on the good will of the pathologist to dissect the tissue, were further compounded by the difficulty of dissecting the cord from the vertebrae. In the experience of this author, direct contact with the morticians who carry out the dissections increases the likelihood of difficult requests being serviced. However, where all the requests are channelled through a Brain Bank it appears that tissue which is more problematic to dissect receives a lower priority and more over that requests are ranked according to the professional status of the researcher. This would be an important consideration in future work using post-mortem human tissue. Furthermore, the limit on the supply of spinal cord tissue restricted the matching of Parkinsonian and control subjects with respect to post-mortem interval (PMI), age and sex. The statistical evaluation of the effects of these factors on transmitter and receptor levels was therefore very important and revealed significant effects on both measurements. This

indicates that all studies of post-mortem human tissue should include appropriate statistical analysis of these factors.

The cause of death and terminal factors such as coma may affect the concentrations of neurochemicals and receptor levels in the CNS. For example, glutamate decarboxylase, an enzyme marker for GABA-containing neurones is reported to be reduced in the brains of patients dying in coma or prolonged terminal illness (McGeer et.al.,1971; Spokes et al.,1979). In contrast, neither a violent death nor death by hypoxia affects serotonergic or ß-adrenergic receptors in the post-mortem human brain (Mann et. al.,1986). In this study the causes of death reported apart from Parkinson's disease itself were similar for Parkinsonian and control patients and were generally cardiac or respiratory failure and pneumonia. However, the different causes of death and duration and degree of confinement amongst the subjects may have contributed to the intra-group variation.

Ante-mortem medication is an important factor to account for. In this study the effects of ante-mortem L-dopa therapy on post-mortem catecholamine levels has been considered (page 114). L-dopa may also affect serotonergic systems through competition for transport systems and displacement of 5HT by dopamine synthesized in serotonergic neurones (see Reynolds and Reiderer, in press) though it is widely accepted that the predominant effects of L-dopa are mediated via conversion to dopamine in the CNS (Lloyd et.al.,1975; Bunney et.al.,1973; Hefti and Melamed, 1980). However, recent evidence indicates that the effects of L-dopa may be wider than previously believed. L-dopa may directly induce the release of endogenous noradrenaline and dopamine by regulatory mechanisms via effects on pre-synaptic receptors (Goshima et.al.,1986). Further exploration into the effects of L-dopa on CNS transmitter levels in animals would be useful in elucidating the obviously complex nature of the action of L-dopa therapy and deciphering the post-mortem transmitter and receptor changes in Parkinson's disease which are not related to medication .

In this study comparison between Parkinsonian and control subjects was made assuming that effects of PMI and age are the same in this pathological condition as they are in the control subjects, however this may not be so. For example, there are differences in post-mortem stability of ChAT activity and cholecystokinin levels in cerebral cortex from subjects with Alzheimer's disease compared with controls (Davies and Terry,1981; Perry et.al.,1981). This and other evidence discussed in this work indicates that generalizations should not be made about the post-mortem effects on neurotransmitter levels and if post-mortem levels are to be used as an indication of changes found in pathology a clear understanding of these effects, and therefore further investigation, is necessary.

These various factors arising from the use of human tissue have been taken into account where possible. It is these factors and numerous others such as differences in diet, environment and level of fitness during the subjects life that result in the intra-group variation in transmitter and receptor levels which was generally larger in the human studies than in the corresponding studies using laboratory bred rats. It may be that this variation masks some of the effects of Parkinson's disease on transmitter and receptor levels but this problem has been overcome as far as possible by using larger numbers of subjects than in the rat. Furthermore, more than 30% of the variation in transmitter levels and the number and affinity of α_2-adrenoceptor binding sites was accounted for statistically by differences in PMI and age.

It is interesting that although the levels of the indoleamines measured were high compared to the levels of the catecholamines, adrenergic but not serotonergic receptor binding could be determined in the spinal cord. It should be noted that the phenomenon of low levels of receptors in areas of high levels of the corresponding neurotransmitter is widely reported and is the rule rather than the exception. Some reasons given to explain this mismatch include (i) expression of transmitter or receptor molecules not only at their contact site but throughout the neurone (ii) occupied receptors and unrecognized low affinity receptors and (iii) sites of action of

transmitters released from a distance (See Herkenham, 1987).
Mismatch between levels of receptors and transmitters may also be
dependent on the different effects of PMI on transmitter and receptor
levels as this study revealed a significant reduction in affinity and
an increase in number of α_2-adrenoceptors with PMI but no
significant effect of PMI on noradrenaline levels. In addition, in
post-mortem tissue down regulation of receptors may occur due to
spontaneous release of transmitters and peptides at death.

The symptoms of Parkinson's disease

Noradrenaline and 5HT in the spinal cord have previously
been shown to be involved in central generation of locomotion
(Grillner et al., 1975) and evidence for their roles in spinal motor
function has been described (pp.14-16 and 24-25). It is therefore
possible that human spinal deficiency of noradrenaline and 5HT
in Parkinson's disease contributes to the symptoms of motor
dysfunction associated with this disease.. Furthermore, the
changes in the indoleamine levels and adrenergic receptor state
with age may be related to symptoms of motor dysfunction
developed in aging which resemble those in Parkinson's disease.
It has also been demonstrated that antagonism of certain types
of pain is mediated through spinal cord serotonergic and
noradrenergic systems and through α_2-adrenoceptors in particular
(Howe et.al.,1983; Yaksh et.al.,1981; Kuraishi et al.,1985; Tasker and
Melzack,1989). Furthermore, it is now generally accepted that
sensory symptoms including numbness, tingling and pain are
common in Parkinson's disease (Snider et.al,1976; Yahr,1982;
Koller,1984; Quinn et.al.,1986). The CNS is a likely candidate for the
origin of painful symptoms as peripheral nerve disease is uncommon
in Parkinson's disease and evidence indicates that the peripheral
nerves are intact (Koller,1984). It has been proposed that pain may
result from an imbalance between noradrenergic, serotonergic and
dopaminergic systems in Parkinson's disease and, moreover, that
truncal and limb pain which is predominant may result from altered
spinal cord function (Quinn et.al.,1986). The loss of spinal cord 5HT
and NA and the interaction between serotonergic and adrenergic
systems described in this study may therefore be important to the

symptoms of sensory deficit present in Parkinson's disease. In addition, the results are in accordance with an imbalance between spinal cord noradrenergic, serotonergic and dopaminergic systems in Parkinson's disease.

Future studies

It was hoped that this report of transmitter levels in the CNS would be accompanied by a report of transmitter levels in the brains of the same Parkinsonian and control subjects. This would have enabled comparisons to be drawn between the effects of Parkinson's disease on amine and peptide levels in the brainstem nuclei and spinal cord of the same patients. Such evidence would provide an indication of the degree of dependency of spinal cord changes on changes in the brain stem nuclei. For instance, if neurotransmitter depletion were greater in the spinal cord than in the nuclei from which the spinal projections originate in each individual subject this may indicate effects specific to the spinal cord. Unfortunately, this information could not be obtained and it is therefore difficult to ascertain the importance of the changes in the spinal cord to the symptoms of Parkinson's disease. Such a comparison would be worth considering for future study and supplies of tissue from the appropriate regions could be collected from the beginning.

In studies performed in this thesis using human spinal cord tissue the variation in transmitter and receptor levels determined was relatively large for reasons discussed above and it would be worthwhile extending these studies using a larger number of subjects especially regarding results of transmitter levels in the lumbar spinal cord and α_2-adrenoceptor binding to back-up the current findings. In addition, interpretation of data with large intra-group variation may be improved using t-test analysis of log transformed data.

There is a total lack of evidence concerning the effect of long term frozen storage of tissue at $-80^{\circ}c$ on its neurochemical content or receptor stability and authors generally do not mention this

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parameter. This probably arises from the assumption that at -80°c physiological processes including enzymatic degradation and chemical transport systems are inactivated. There is nevertheless value in investigating the effects of this factor in future studies.

Recent developments in dialysis techniques enable in vivo determination of transmitter levels. Such techniques have been used to measure the endogenous levels of 5HT and TRH released in the lumbar spinal cord of the rat (Fone et.al.,1988c). Practical problems arising from the size of the rat spinal cord have inhibited the use of stimulated release of 5HT in the investigation of co-release of TRH and 5HT (Fone, personal communication). However, in larger animals such as the cat, rabbit or primate in vivo dialysis of stimulation-released amines and peptides could be exploited to elucidate the interactions between 5HT, substance P and TRH.

Co-existence in the spinal cord has been based mainly on evidence of 5HT and peptide distribution in the rat spinal cord in control and lesion studies (described fully in the General Introduction). There is evidence however that there may be distinct species variation in the descending bulbospinal system. In the rat 40-50 % of serotonergic descending pathways are reported to contain substance P (Johansson et.al.,1981; Marsden et.al.,1982; Bowker et.al.,1983) whereas in the primate virtually all the spinal cord substance P was found within serotonergic fibres (Bowker, 1986) and in addition there is a descending serotonergic pathway from the locus coeruleus in the cat which has not been identified in the rat (Leger et.al,1979; Wiklund et.al.,1981; Lai and Barnes, 1985). Therefore, in pursuit of understanding of human CNS function more information concerning the pathways and mechanisms of transmitter systems in this species itself is required. Certain measurements in the living human subject such the levels of transmitters and their metabolites in plasma samples and CSF are not necessarily representative of CNS levels. However, investigation of CNS systems in freshly dissected (biopsy) tissue from living subjects is possible and has been used to investigate receptor-mediated second messenger activity

(Kendall and Firth, 1988) and K+ stimulated histamine release (Arrang et.al.,1988) in slices of human cerebral cortex. Such studies have their difficulties. The co-operation of clinicians performing surgical procedures is essential and the researcher must organize the experiment in advance and await the arrival of the tissue. Nevertheless, the success of these methods indicates that they could be used to study indoleamine and peptide release in human CNS tissue to gain an understanding of the mechanisms involved.

Conclusive evidence of co-existence of neurotransmitters and peptides in the human is dependent on immunohistochemical visualization of amines and peptides within the same neurones. Obtaining high quality sections would be difficult as unlike animal tissue it is not possible to fix human tissue at death. However, the use of fresh post-mortem tissue sectioned after fixing and embedding in wax would avoid damage caused to tissue sections by freezing and may be suitable for double labelling immuno-histochemical staining techniques. By this method it may be possible to visualize co-existent neuro-amines and peptides in the human spinal cord.

Appendix 1   Protein Determination with Folin Phenol Reagent   (Lowry et. al.,1951)

Solutions

Stock solution A	2% Na_2CO_3 in 0.1M NaOH
Solution B- make fresh	0.5% $CuSO_4$:$5H_2O$ in 1% potassium sodium tartrate solution
Solution C - make fresh	Mix 50mls. solution A with 1ml. solution B
Solution D	Folin phenol reagent diluted 1:1
Standard albumin	5mg BSA in 10mls double distilled water
Standard curve	Range of BSA concentrations from 0-0.25mg

Method

Resuspend tissue samples in 1ml distilled water.

Take 0.5ml tissue suspension diluted to a concentration within the standard range.

To 0.5ml of each sample and each standard add 0.5ml Solution C. Wait 10 minutes. Add 0.5ml Solution D. Leave 20 minutes in dark.

Measure the optical density of samples and standards at 500nm wavelength using a spectrophotometer.

Calculate protein content of the standards by extrapolation of the standard curve.

Appendix 2   Conditions for work with human tissue

Protocol for:

    (a)   Dissection of frozen human tissue

    (b)   Incubation of sections of tissue with [^{3}H] ligand on glass slides

    (c)   Homogenate ligand binding assay of tissue

All human tissue to be transported in and out of Human Tissue Laboratory (E62) in sealed packaging on dry ice or in capped tubes within a closed container.

1)   Dissection of sections of frozen spinal cord to be carried out in a microbiological safety hood with extractor fan on in E62. Tissue will be dissected on a piece of metal cooled on a trough of ice. Disposable apron, gloves and mask to be worn during this procedure. Single edged razor blades used for dissection will be disposed of in "sharps safe" after immersion in phenolic disinfectant (eg 2% stericol). Trough of ice will be sterilised overnight by immersion in hypochlorite (>2.5% chloros).

2)   The tissue will be transferred to a sealable bag and placed in an insulated closed container with dry ice for transport to the department of Human Morphology for cryostat sectioning.

3)   After sectioning the brain sections will be returned to E62 within a sealed container and incubated with a tritiated ligand inside a second microbiological hood by pipetting the ligand onto the slides. The sections will be dried using a fan which will remain in the hood. The slides will then be either a) wiped with filters (2.5 cm) wetted with buffer solution which will be placed into vials containing 10% perchloric acid within the hood or b) removed from the hood in a dry condition and taken out of E62 for exposure to a [^{3}H] sensitive film in a dark room.

4)   Waste sections on glass slides and slides from procedure 3a will be disinfected and decontaminated by overnight immersion in hypochlorite (>2.5% chloros), and then disposed of through the normal glass disposal route.

After development of the film the slides of 3b will be either disposed of in the same way or stored in a sealed, well labelled box in E62.

5)   For homogenate binding tissue will be dissected as in (1) and homogenized in buffer using a homogenizer permanently housed within the hood. Homogenate will be transferred to a clean tube, capped and centrifuged. Supernatant will be aspirated from the pellet within the hood by means of a water vacuum pump into a trap containing hypochlorite (>2.5% chloros).

6)   The pellet will be resuspended in buffer and procedures in 5) repeated twice. All procedures to be carried out within the hood except for centrifugation which will take place in capped tubes.

7)   The final supernatant will be transferred to a second microbiological hood and added (1 ml approx) to radio-active substrates using a pipette with disposable tips. Supernatant and radioactive substrate will be incubated together in capped tubes within a water bath adjacent to the hood.

8)   The incubation medium will be passed through filters (2.5 cm) under vacuum in a filter block and the filters washed with buffer dispensed from a glass container within the hood. Filters then will be transferred to vials containing 1 ml of perchloric acid (10%). All procedures in 7 and 8 (excluding incubation) to occur within the hood.

9)   All glassware and instruments including the filter block used in E62 will be sterilised by immersion in hypochlorite (>2.5% chloros) or phenolic disinfectant (2% stericol).

10)   All procedures involving open tubes will take place within the microbiological hood. Disposable apron and gloves to be worn

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during these procedures. The automatic pipette bodies will be permanently housed within the hood. Water baths will contain centrimide solution (10%). All fluid transfers inside the microbiological hood will take place over a plastic tray with a disposable plastic insert. This will be sterlised with chloros (>2.5%) after each experiment.

11)   Plastic incubation tubes and all other disposables including small amounts of solid human tissue and protective clothing will be placed in a plastic bag for incineration.

12)   All chloros solutions containing radioisotopes will be disposed of via a designated radioactive sink in E62. The microbiological safety cabinet will be monitored for radioactivity at monthly intervals by swabbing area with a filter paper (2.5 cm) soaked in methanol. This will be added to 1 ml of 10% perchloric acid in a plastic scintillation vial.

13)   An appropriate scintillant will be added to filters from monitoring procedures and experiments half-an-hour after immersion in perchloric acid. After scintillation counting the plastic vials and contents will be disposed of by incineration.

14)   Any spillage will be wiped up with 10% chloros.

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